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Natural Active Ingredients for Diabetes and Metabolism Disorders Treatment

Guest Editors: Hilal Zaid, Abbas A. Mahdi, Akhilesh K. Tamrakar, Bashar Saad, Mohammed S. Razzaque, and Amitava Dasgupta

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Editorial

Natural Active Ingredients for Diabetes and Metabolism Disorders Treatment

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Diabetes mellitus is a common chronic global disease affecting children and adolescents in developed as well as developing countries. Diabetes type I and type II are the major types of diabetes mellitus. The former is an autoimmune disorder, leading to pancreatic β-cell dysfunction and thus inadequate production of insulin. The latter arises from reduced sensitivity to insulin in the target tissues (i.e., skeletal muscle, liver, and adipose tissue) and later on insufficient insulin secretion. In both cases, the common result is sustained hyperglycemia. Uncontrolled hyperglycemia over time leads to deteriorating blood vessels supplying the body organs, leading to heart, eyes, kidneys, and nerves system damage. For instance, macrovascular (atherosclerotic) and microvascular (retinopathy and nephropathy) disorders are the leading causes of morbidity and mortality in diabetic patients [1]. We can appreciate then that diabetes is not a single disease but rather a combination of metabolic disorders [2].

According to the World Health Organization (WHO), more than 220 million people worldwide were diabetic in 2010 and this number will be doubled in 2040. The prevalence of diabetes is the highest in the Middle East, where the number of diabetic subjects reached 15.2 million in 2000 and it will almost be tripled within 30 years (from 15.2 million in 2000 to about and 42.6 million in 2030) [3].

Despite the great progress in synthetic drugs, herbal medicine has continued to be often utilized by people in most developed and developing nations. Furthermore, the popularity of herbal medicines preparations has increased worldwide in the past few decades, probably because of the sustainability of this medicine over the years. Moreover, herbal medicines are relatively inexpensive and are believed to be safer than synthetic drugs. Yet, care should be taken before the administration of any herbal medicine treatment [4].

This special issue provides a comprehensive overview on traditional herbal medicine including state-of-the-art description of traditional antidiabetic herbal medicine, their chemical composition, and activity. For this special issue, the editorial office received 22 papers and after rigorous peer review process 10 papers were accepted for publication. Out of 10 accepted papers, four articles are very informative high class review articles. All published article focused on the theme of this special issue which is utility of herbal medicines to act as complementary and alternative therapy for patients with diabetes and metabolic disorders. Four publications use *in vivo* rat model for their studies while in two publications the authors used *in vitro* model utilizing HepG2 cells. Toxicities of herbal supplements in humans have been reported, especially liver toxicity due to prolonged use of Kava, herbal sleep aid, and antidepressants.

There are four high class review articles published in this special issue. The review paper entitled "Natural Products Improving Hyperuricemia with Hepatorenal Dual Effects"

by S. Hao et al. focuses on hyperuricemia (a risk factor for incident type II diabetes mellitus) treatment by natural products. Notably, classical medications used for treating high ureic acid such as allopurinol are highly toxic. The review article "*Euonymus alatus*: A Review on Its Phytochemistry and Antidiabetic Activity" by X. Zhai et al. summarized utility of an Asian plant *Euonymus alatus* in treating hyperglycemia, diabetic complications, and cancer. More than 100 chemicals including flavonoids, terpenoids, steroids, lignan, cardenolide, phenolic acid, and alkaloids are present in this plant extract. The review article "Antidiabetic Properties, Bioactive Constituents, and Other Therapeutic Effects of *Scoparia dulcis*" by G. Pamunuwa et al. discusses antidiabetic activities as well as antioxidant and anti-inflammatory properties of this herb. The review article "Applicability of Isolates and Fractions of Plant Extracts in Murine Models in Type II Diabetes: A Systematic Review" by G. Coelho et al. discusses both animal and human data available in effects of various herbs in lowering blood glucose level. All four excellent review articles are of valuable addition to current literature on utility of herbs in treating diabetes.

The original paper "The Hypoglycemic and Antioxidant Activity of Cress Seed and Cinnamon on Streptozotocin Induced Diabetes in Male Rats" by S. Qusti et al. demonstrates stimulation of pancreas in streptozotocin induced diabetic rats using 20% (w/w) garden cress seed *(Lepidium sativum)* and cinnamon methanol extracts. In another original paper "Evaluation of Hypoglycemic and Genotoxic Effect of Polyphenolic Bark Extract from *Quercus sideroxyla*" by M. Soto-Garcia et al., the authors evaluated potential hypoglycemic activity of bark of *Quercus sideroxyla* using rats where diabetes was induced by streptozotocin. Another *in vivo* study also using rat model "In Vivo Interrelationship between Insulin Resistance and Interferon Gamma Production: Protective and Therapeutic Effect of Berberine" by M. Mahmoud et al. is also very interesting as other publications have shown beneficial effect of berberine in insulin resistance. *Panax ginseng* antidiabetic activity was discussed in the original paper "Protective Effects of *Panax notoginseng* Saponins against High Glucose-Induced Oxidative Injury in Rat Retinal Capillary Endothelial Cells" by Y. Fan et al. The antioxidant property of another ginseng species is also discussed in this paper.

In two papers the authors used HepG2 cell as *in vitro* model for oxidative stress. "Effect of *Betula pendula* Leaf Extract on α-Glucosidase and Glutathione Level in Glucose-Induced Oxidative Stress" by K. Bljajic et al. demonstrates antioxidant effect of this herbal leaf. The authors used HepG2 cells to demonstrate *in vitro* the antioxidant effect of this leaf extract. "Diosgenin and 5-Methoxypsoralen Ameliorate Insulin Resistance through ER-α/PI3 K/Akt-Signaling Pathways in HepG2 Cells" by K. Fang et al. suggests a new strategy for diabetes type II treatment by targeting ER-mediated Akt signaling pathway.

Acknowledgments

We are thankful to all contributors of this special issue for their valuable research papers. We are grateful to the reviewers for their constructive criticism and timely response that made this special issue possible. Our sincere thanks and gratitude go to the Editorial Board of eCAM for inviting us to edit this special issue. The editorial board hopes that readers will enjoy this special issue.

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Research Article

Evaluation of Hypoglycemic and Genotoxic Effect of Polyphenolic Bark Extract from *Quercus sideroxyla*

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Quercus sideroxyla is a wood species whose bark has phenolic compound and should be considered to be bioactive; the hypoglycemic and genotoxic properties of *Q. sideroxyla* bark were evaluated in this study. Total phenolic compound was determined in crude extract (CE) and organic extract (OE). The OE has the highest amount of phenols (724.1 ± 12.0 GAE/g). Besides, both CE and OE demonstrated effect over the inhibition of α-amylase *in vitro*. Hypoglycemic activity was assessed by glucose tolerance curve and the area under curve (UAC); OE showed the highest hypoglycemic activity. In addition, diabetes was induced by streptozotocin (65 mg/kg) and the extracts (50 mg/kg) were administered for 10 days; OE showed hypoglycemic effect compared with diabetic control and decreased hepatic lipid peroxidation. Acute toxicity and genotoxicity were evaluated in CE; results of acute toxicity did not show any mortality. Besides, the comet assay showed that CE at a dose of 100 mg/kg did not show any genotoxic effect when evaluated at 24 h, whereas it induced slight damage at 200 mg/kg, with the formation of type 1 comets.

1. Introduction

Quercus species have been used in Mexican traditional medicine [1]; specifically *Q. sideroxyla* has antioxidant compounds present in leaves [2] which decrease the levels of inflammatory markers such as COX-2 and IL-8 by modulating the expression of NF-kB [3]. Ten different polyphenols had been reported in the bark of this species [4], which are bioactive phytochemicals [5], found in green tea and found to possess hypoglycemic properties [6, 7].

Polyphenols are the most abundant dietary antioxidants and are common constituents of many plant food sources, including fruits, vegetables, seeds, chocolate, wine, coffee, and tea; thus they have acquired significant interest [8].

Recent studies have shown promising results for these compounds in various pathological conditions such as diabetes, cancer, atherosclerosis, cardiovascular, and neurological disorders [9–11].

The efficacy of polyphenols on carbohydrate metabolism and glucose homeostasis has been investigated *in vitro*, in animal models and clinical trials [12]. The polyphenols regulate the postprandial hyperglycemia through inhibiting carbohydrate-hydrolyzing enzymes such as alpha-amylase and alpha-glucosidase [13].

The origin of these bioactive compounds makes them safe for human consumption; however, some investigations reported toxic effects caused by plants [14–16]. Therefore, it is important to carry out toxicological and genotoxic assays [17], to assess the risk/benefit of its therapeutic use in humans.

Therefore, the aim of this research was to evaluate the hypoglycemic and genotoxic properties of polyphenolic extracts from *Q. sideroxyla* bark in a diabetic murine model.

2. Materials and Methods

2.1. Chemicals. Ethanol was purchased from Merck KGaA (Darmstadt, Germany), and gallic acid, Folin-Ciocalteu, streptozotocin, α-amylase enzyme, and acarbose were acquired from Sigma Chemical Co. All reagents were of analytical grade.

2.2. Plant Material. The bark was collected at Pueblo Nuevo, Durango, México. The samples were identified and botanical specimens were deposited at the Herbarium CIIDIR-Instituto Politécnico Nacional, Durango, with voucher numbers 42842, 428443, and 42844. The bark was mixed, to make a unique sample, and then it was dried at room temperature (24°C), milled (mesh 40), and finally stored in paper bags under refrigeration until further use.

2.3. Extract Preparation and Purification. The bark powder (10 g) was twice soaked with 50% ethanol (ethanol/water 50 : 50 v/v), (2 × 300 mL) at room temperature for 24 h, with stirring, followed by filtration through Whatman no. 1 filter paper. The extracts were combined, filtered, and then evaporated under vacuum at 40°C until ethanol was removed. A portion of the remaining aqueous extract was taken to dryness and identified as crude extract (CE), while another portion was subjected to liquid partition with ethyl acetate (3 × 100 mL). The organic phase was evaporated to dryness under vacuum at 40°C and identified as the organic extract (OE).

2.4. Evaluation of the Phenolic Compounds. The concentration of the total phenolic (TP) was determined by the Folin-Ciocalteu colorimetric method with slight modifications [11]. Measurements were carried out in duplicate and calculations based on a calibration curve were obtained with gallic acid. The total phenolic concentration was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract.

2.5. α-Amylase Assay. The inhibition on alpha-amylase enzyme was determined by Kwon et al. [18] method with some modifications. The IC_{50} values were determinate by linear regression analysis using four different concentrations for acarbose (1000, 2000, 4000, and 5000 μg/mL), CE, and OE (500, 1000, 1500, and 2000 μg/mL) in triplicate. Acarbose was used like drug reference and control, respectively. The reaction was stopped with 1.0 mL of color reagent (contains the mixture of dinitrosalicylic acid 96 M and sodium potassium tartrate 0.005 M); results show mean of the data.

2.6. Animals. Healthy Wistar rats were obtained from the Biorepository of the National School of Biological Sciences (National Polytechnic Institute, México) and ICR mice were obtained from PROPECIA S.A., México. All experiments were approved by the Laboratory Animal Care Committee of the National School of Biological Sciences (National Polytechnic Institute, México) and were conducted in compliance with the Mexican Official Standard (NOM—062-200-1999) technical specifications for the production, care, and use of laboratory animals. The animals were group-housed in polycarbonate cages in a controlled environment at a constant temperature (21 ± 2°C) with 12 h light/dark cycles and access to food and fresh water *ad libitum*.

2.7. Evaluation of Hypoglycemic Properties in Healthy Rats. The hypoglycemic activity was evaluated using a glucose tolerance test in male Wistar rats weighing 300 ± 10 g. The animals were fasted for 12 h before testing and basal blood samples were obtained from the tip of the tail. Three groups of 5 animals each received CE by gavage. One group received 50 mg/kg body weight (CE50), another group 100 mg/kg body weight (CE100), and the last group received 200 mg/kg body weight crude extract (CE200); in the same way two groups of animals (n = 5/group) received OE at doses of 25 (OE25) and 50 mg/kg body weight (OE50) at time zero. Doses were selected according to the content of phenols. After fifteen minutes, all animals received an oral dose of glucose (3 g/kg body weight) using a 35% solution. An additional control group of 6 animals was given only glucose. At 0, 30, 60, 90, and 120 min after glucose administration, blood glucose was measured using glucose test strips in glucometer (Accu-Check Performa with Softclix, Roche) and then the area under the curve was calculated to estimate the glucose tolerance.

2.8. Hypoglycemic Effect in Diabetic Rats. The hypoglycemic effect of the extracts was tested in male Wistar rats (5 animals/dose of extract) with previously induced type I diabetes by intraperitoneal streptozotocin injection (STZ, 65 mg/kg of body weight, in citrate buffer, pH 4.4). Diabetes was confirmed by measuring the glucose levels in blood samples obtained of the tip of the tail, 24 hours after. Five days later, animals received 50 mg/kg body weight CE and OE of *Q. sideroxyla* for 10 days; doses were selected by their hypoglycemic action that showed before section. Blood glucose was determined with glucose test strips (Accu-Check Performa with Softclix, Roche) and compared with a non-diabetic control group and untreated diabetic control group. After the animals were euthanized, fasted liver samples were taken for lipid peroxidation assays.

2.9. Hepatic Lipid Peroxidation Assay. Oxidative stress levels were evaluated by the concentration of malondialdehyde (MDA) in the liver according to Rivera-Ramírez et al. [19]. The MDA concentration was calculated using the molar extinction coefficient of $1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$. The results were expressed in mmol MDA/g tissue.

2.10. Acute Toxicity Study in Healthy Mice. Evaluation of the toxicity of *Q. sideroxyla* bark extracts was made according to Lorke [20]. Four groups containing healthy male ICR mice (n = 3/group) received CE, at doses of 1000, 2000, 3000, and 5000 mg/kg body weight. The toxicological effects were expressed in terms of mortality expressed as LD_{50}. Special attention was directed to the observations of convulsions, diarrhea, lethargy, and piloerection. The number of animals dying during a period was recorded.

2.11. Genotoxic Assay. Thirty-six male mice weighing 25–30 g were divided into six experimental groups of six animals each.

Table 1: Concentration of phenolic compound and IC_{50} of α-amylase of CE of *Q. sideroxyla* bark extracts.

Extract/standard	Total phenolic compound (GAE/g)	IC_{50} (μg/mL)
CE	551.0 ± 8.1^{a}	1979.3 ± 1.2^{a}
OE	724.1 ± 12.0^{b}	1703.3 ± 21.6^{b}
Acarbose	—	4030.1 ± 12.2^{c}

Each value represents the mean of $n = 4 \pm$ standard deviation. Different letters between groups in each column indicate a significant difference ($p \leq 0.05$) assessed using the Fisher-LSD test.

The crude extracts were suspended in 1% Tween-80 aqueous solution and it was administered by gavage at doses of 100 and 200 mg/kg body weight. The negative group received 1% Tween-80 aqueous solution, and the positive control group received an intraperitoneal injection of cyclophosphamide (CPA) at 50 mg/kg body weight. The evaluation of DNA damage was done by comet assay according to Almonte-Flores et al. [21] at 4 and 24 hours, where it was evaluated by examining 100 randomly selected cells (50 cells per coded slide) per animal. These cells were scored visually according to tail size and grouped into the following four classes: class 0, no tail; class 1, tail shorter than the diameter of the head (nucleus); class 2, tail length 1-2 times the diameter of the head; and class 3, tail length more than twice the diameter of the head. The total comet score was calculated by the following equation:

$$\begin{aligned}\text{Total comet} = [\![&(\%\text{ of cells in class } 0 * 0)\\ &+ (\%\text{ of cells in class } 1 * 1)\\ &+ (\%\text{ of cells in class } 2 * 2)\\ &+ (\%\text{ cell in class } 3 * 3)]\!]\,.\end{aligned} \tag{1}$$

2.12. Statistical Analyses. The results are expressed as the mean and the standard deviation. One-way analyses of variance (ANOVA) were performed followed by multiple comparisons of the means with the Fisher-LSD test at a significance level of $\alpha < 0.5$. The statistical analysis software package (Statistica 7) was used for these analyses.

3. Results

The evaluation of phenols compounds of CE and OE from *Q. sideroxyla* bark revealed that OE has a significant higher concentration of these metabolites than CE ($p \leq 0.05$) (Table 1). In the same table, the antidiabetic activity of CE and OE extracts was demonstrated through their higher inhibition (IC_{50} 1979.3 and 1703.3, resp.) of α-amylase activity compared to acarbose (IC_{50} 4030.1).

In regard to the glucose tolerance test in rats, a similar behavior is observed by the organic extracts (Figure 1(a)). The extracts OE50, OE25, and CE50 significantly decreased blood glucose levels (165.3 ± 15.4 mg/dL min; 154.1 ± 11.8 mg/dL min; and 189.1±14.9 mg/dL min, resp.) compared with the control group (233.7 ± 43.0 g/L min) (Figure 1(b)). Both OE50 and OE25 showed a high antihyperglycemic activity, and the former was chosen to be compared with CE50; this is in order to observe the action of all compounds in extract, as well as the effect of the extract with the selectivity of components, using a solvent semipurification.

The effect of repeated oral administration of CE50 and OE50 on blood glucose levels in STZ-diabetic rats is present in Table 2. The OE50 showed a lowering effect on glucose in diabetic rats, compared to diabetic control ($p \leq 0.05$), and was more effective in reducing blood glucose than the CE50 ($p \leq 0.05$).

The evaluation of hepatic lipid peroxidation revealed that the concentration of malondialdehyde (MDA) was significantly higher in diabetic control (Figure 2). On the other hand, the diabetic rats that received CE50 and OE50 have reduced the concentration of MDA, in the case of OE50 even to lower level than the healthy control ($p \leq 0.05$).

The administration in healthy rats of 5000 mg/kg body weight of CE produced no mortality, so $LD_{50} \geq 5000$ mg/kg. The animals did not manifest any sign of convulsions, diarrhea, lethargy, or piloerection.

The results of the genotoxicity assay are shown in Table 3 and Figure 3. The test showed that the CPA group has the highest levels of damage, according to comet class detected (comet classes 2 and 3), while the negative control presented a predominance of comet zero class.

In regard to genotoxicity, the evaluation of CE100 after 24 h revealed no significant differences compared to the negative control ($p \leq 0.05$); nevertheless CE200 after 4 h and CE200 after 24 h showed light damage with the formation of comets of type 1 and showed significant differences with the negative control ($p \leq 0.05$).

4. Discussion

Total phenolic content in extracts of *Q. sideroxyla* bark (CE and OE) differed and is significantly highest in OE (724.1 ± 12 GAE/g), which suggests that the solvent has a concentrating effect of these metabolites. Phenolic compounds as gallic acid, catechin, epicatechin, gallocatechin, dimeric, and dimeric procyanidins have been identified in *Q. sideroxyla* bark [4]. Those polyphenols are bioactive phytochemicals with antioxidant properties. Antioxidants are important in preventing pathologies like diabetes, so low levels of plasma antioxidants imply a risk factor for the development of the disease [22].

This research demonstrates the inhibitory activity of the CE and OE in α-amylase enzyme. We suggest that the effect is due to the synergy of the phenolic compounds present in the extracts, because the inhibitory activity of acarbose on the enzyme was lower. Moreover, antioxidants have been reported because of their capacity to contribute to the prevention of type 2 diabetes through their anti-inflammatory properties [23]; therefore both mechanisms antioxidant effect and α-amylase inhibition could be summarized to produce antihyperglycemic effect.

Inhibiting glucose uptake in the intestines may help to control the blood glucose, since it is known that the α-amylase is one of the main products of secretion from

Table 2: Blood glucose levels during the study period in Wistar rats.

Group	Blood glucose (mg/dL)			
	Day 1	Day 3	Day 6	Day 10
Control	89.6 ± 5.1	92 ± 8.6	100.4 ± 7.5	91 ± 22.9
Diabetic	419.6 ± 49.6[a]	442.2 ± 36.7[a]	500 ± 3.1[a]	500 ± 0[a]
Diabetic + CE50	462 ± 33.9[a]	397.2 ± 61.2[a]	465.5 ± 33.3[a]	500 ± 0[a]
Diabetic + OE50	397 ± 10.4[ab]	337.8 ± 34.6[ab]	372.6 ± 7.5[ab]	414.2 ± 62.5[ab]

Data are expressed as mean ± standard deviation; $n = 5$. a: significant difference versus control group; b: versus diabetic group ($p \leq 0.05$) in Fisher-LSD test.
CE50: crude extract at dose 50 mg/kg; OE50: organic extract at dose 50 mg/kg.

Table 3: DNA damage according to the comet assay for the assessment of the genotoxicity of polyphenolic extract from *Q. sideroxyla* bark.

Treatment	Comet class				
	Total	0	1	2	3
Blood sample (4 h)					
Negative control	8 ± 4.8	92.5 ± 4.1	7 ± 3.5	0.5 ± 0.8	0 ± 0
CPA (50 mg/kg)	67.3 ± 6.9[a]	66 ± 3.1[a]	11.8 ± 3.3[a]	11 ± 2.4[a]	11.1 ± 1.0[a]
CE100	16.8 ± 6.3[ab]	85.8 ± 4.9[b]	12.1 ± 3.5[a]	1.33 ± 0.8[b]	0.66 ± 0.8
CE200	39.5 ± 6.2[abc]	69.8 ± 5.1[a]	22.5 ± 6.0[ab]	6 ± 2.09[abc]	1.6 ± 0.8[b]
Blood sample (24 h)					
Negative control	18.5 ± 6.9	88.6 ± 3.1	7.6 ± 3.3	3.1 ± 2.4	1.5 ± 1.04
CPA (50 mg/kg)	140.3 ± 21.2[a]	37.5 ± 5.5[a]	11 ± 6.2[a]	25.2 ± 8.4[a]	26.3 ± 6.3[a]
CE100	21.3 ± 6.1[b]	85.5 ± 4.0[b]	9.1 ± 0.7[a]	3.3 ± 1.6[b]	1.8 ± 1.4[b]
CE200	60 ± 14.3[ab]	55.3 ± 5.9[ab]	33.3 ± 3.4[ab]	7.3 ± 4.0[b]	4 ± 2.6[b]

Each value represents the mean of $n = 6$ observations ± standard deviation. a: significant difference versus negative control group, b: versus CPA 50 mg/kg, and c: versus CE100; ($p \leq 0.05$) LSD, comparing the treatments separately at 4 and 24 hours.
CPA: group treated with cyclophosphamide; CE100: group treated with crude extract at dose of 100 mg/kg; CE200: group treated with crude extract at dose of 200 mg/kg.

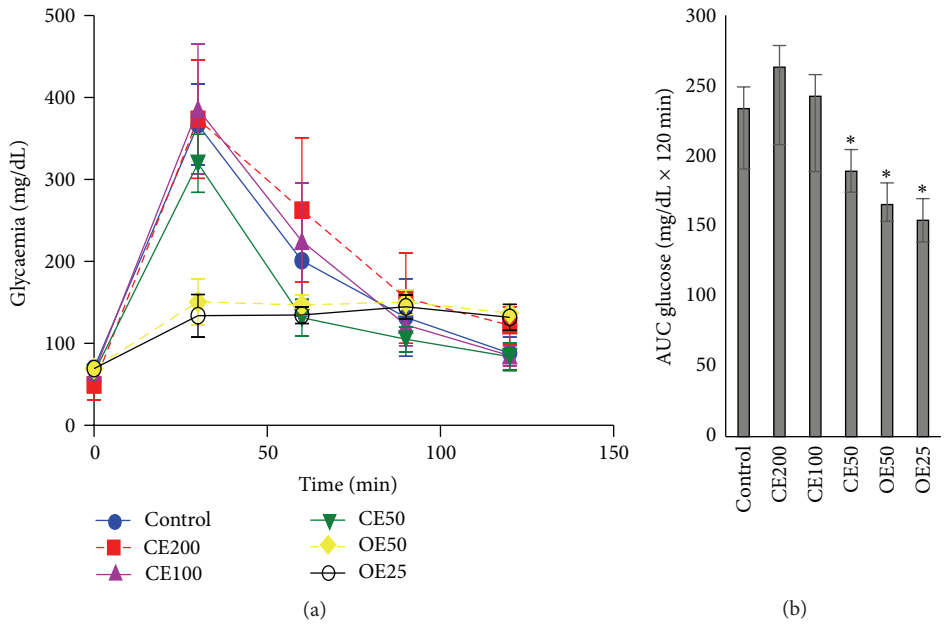


Figure 1: Glucose tolerance test in rats (a). Area under the glucose curve (b). $^{*}p \leq 0.05$, significant difference compared with the control group. CE: crude extract at doses 200, 100, and 50 mg/kg; OE: organic extract at doses 50 and 25 mg/kg.

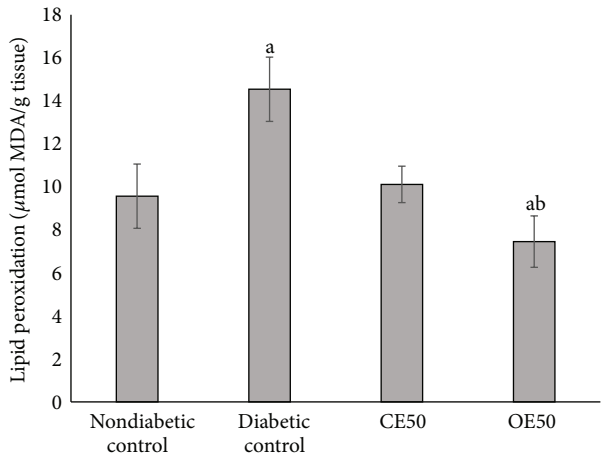
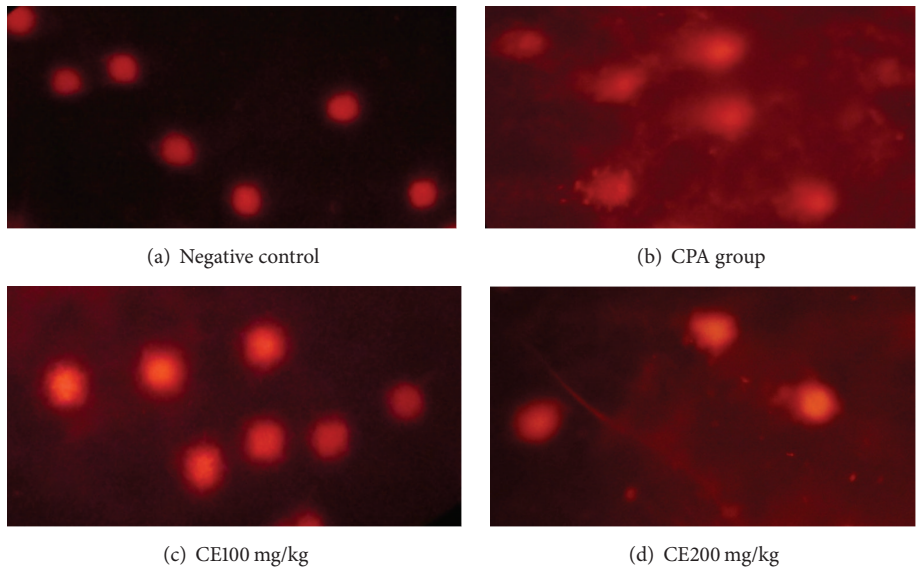


Figure 2: Hepatic lipid peroxidation assay in diabetic rats treated with CE50 and OE50 extracts during 10 days. a: significant difference versus control nondiabetic group; b: versus diabetic control group ($p \leq 0.05$), Fisher-LSD test. CE: crude extract 50 mg/kg; OE: organic extract 50 mg/kg.



(a) Negative control

(b) CPA group

(c) CE100 mg/kg

(d) CE200 mg/kg

Figure 3: Comet assay images in cell blood of mice (400x). (a) Comet type 0, (b) comet type 3, (c) comet type 1, and (d) comet types 1 and 2.

glands in the pancreas and salivary glands, which play an important role in digestion, by participating in the intestinal absorption; it is believed that inhibition of these enzymes can significantly reduce the postprandial increase in blood glucose level, which is important in the treatment of diabetes [24, 25]. For this reason, the bark of *Q. sideroxyla* could be used as an effective treatment for the management of postprandial hyperglycemia and to limit complications of diabetes.

The antihyperglycemic activity of CE (50 mg/kg b.w.) and OE (50 mg/kg/b.w.) in diabetic rats shows significant differences in respect to the diabetic group. Besides that OE can eliminate hyperglycemic peaks so as to maintain the glucose levels to more stables levels. Despite the fact that CE and OE showed a hypoglycemic effect at the beginning of the treatment, this was lost in the case of CE as the treatment continues. Thus, it is possible that inhibition of α-amylase activity by phenolic compounds and the antioxidant effect by compounds like catechin are mechanisms associated with the pharmacologic properties from bioactive compounds of *Q. sideroxyla* bark. The higher hypoglycemic effect shown by OE could be explained by the higher concentration of phenols compared to CE. It is necessary to do more experiments to demonstrate that CE and OE extracts inhibit carbohydrate absorption in gut.

Several studies have demonstrated that the administration of plant extracts containing phenols decreased glucose levels by the presence of flavonoids such as quercetin and catechin which promote a major hepatic glycogen storage [26, 27]. Other possible mechanisms involve the activity

of procyanidin that inhibits the activity of carbohydrate-hydrolyzing enzymes (found in results of α-amylase assay), epicatechin that induces pancreatic β cell regeneration, and catechin and phenolics acids that inhibit intestinal glucose absorption mediated by GLUT 2 and SGLT1 [28, 29].

In diabetes, tissue damage is mediated by free radicals by attacking membranes through lipid peroxidation. The concentration of MDA reflects the degree of lipid peroxidation, and the increase of MDA production plays a key factor in the progression of diabetic pancreas damage [22, 30]. Therefore, a decrease in the peroxidation of fatty acids and improved antioxidant status could contribute to prevention of diabetic complications. In our study, the MDA levels were higher in the diabetic control, in comparison with the diabetic rats treated with CE and OE which decreased the lipid peroxidation ($p \leq 0.05$). This can be explained by the antioxidant effect of polyphenols present in both extracts, which has been observed in other studies from plants [31, 32].

The toxicity results showed that CE100 was not considered a genotoxic agent in this study; however a low level of genotoxicity was observed with CE200. Some studies showed a dose-dependent relationship and even talk about the possible activation of different cellular pathways depending on the tested dose [21, 33]. It has been shown that flavonoids have effects against DNA damage induced by various genotoxic agents, that is, principally by the ability to protect against ROS produced and by the modulation of enzymes responsible for bioactivation and detoxification of genotoxic agents. A relationship has been reported between structure and activity from flavonoids in the protection of genetic material [34].

This research provides the first report on hypoglycemic and genotoxic effect of the extract from *Q. sideroxyla* bark. Polyphenols may explain this activity; however further studies focusing on the mechanism of action are required; in addition the toxicological analysis of OE is necessary.

5. Conclusion

We conclude that extracts of *Quercus sideroxyla* bark at doses of 50 mg/kg may be effective in reducing postprandial hyperglycemia. However, CE extract at doses of 200 mg/kg produces a low level DNA damage. It is necessary to do more studies to evaluate its potential usefulness as an adjuvant for diabetes treatment.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Review Article

Natural Products Improving Hyperuricemia with Hepatorenal Dual Effects

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This review aims to put forth an overview of natural products reducing uric acid level with hepatorenal dual effects. The prevalence of hyperuricemia increased rapidly in recent years and has closely interdependent relationship with other metabolic disorders. Current therapeutically used drugs including a few uricostatic and uricosuric chemical drugs are proved efficient to control serum uric acid level. However, their side effects as well as contraindication in some cases with liver, kidney injury, or other conditions frequently limit their clinic application. More attention thus has been paid to natural products as an alternative means in treating hyperuricemia. Many natural products have been proved efficient in downregulating uric acid level, among which some can improve hyperuricemia with hepatorenal dual effects. It means these natural products can regulate both the production and the excretion of uric acid by targeting the key metabolic enzymes mainly in liver or uric acid transporters in kidneys. Thus, these natural products could have stronger efficacy and broader application, which may be developed for the treatment of hyperuricemia in clinic.

1. Introduction

Uric acid is the end item of purine metabolism in human body, originating from hypoxanthine after double enzyme catalysis by xanthine oxidase (XOD) [1]. Normally it has multiple physiological effects, including modulation of immune responses, regulation of blood pressure, and controlling anti-/prooxidative balance [2]. Either overgeneration of uric acid or a reduction in its excretion can lead to hyperuricemia. The risk factors of hyperuricemia include age, gender, race, genetic factors, environmental factors, and dietary habits. National Health and Nutrition Examination Survey 2007-2008 in USA and Taiwan Nutrition survey demonstrated that the serum uric acid (SUA) level increases with more intake of meat, seafood, and alcohol, especially beer correspondingly [3, 4]. In the past decades, with the change in diet, the prevalence of hyperuricemia has increased worldwide. The incidence of hyperuricemia is 21.2% and 21.6% among men and women, respectively, in USA [5] and 42.1% and 27.4% among men and women in Taiwan [6]. Hyperuricemia is predicted to be the second popular metabolic disease after type 2 diabetes in the future [7].

Uric acid is the well known primary risk factor for developing symptomatic gout [8]. Recently, high level of uric acid has been identified closely related to all the metabolic diseases, such as obesity, hypertension, type 2 diabetes, nonalcoholic fatty liver disease, coronary artery disease, and stroke, in addition to being involved in the pathogenesis of gout and chronic nephropathy [9–13]. It was reported that the prevalence of metabolic syndrome (MS) was high among patients with gout. Even, in those without gout, the prevalence of MS was more than 10-fold higher in those with uric acid levels of 10 mg/dL or greater compared with uric acid levels less than 6 mg/dL. Thus, higher uric acid levels are related to MS, and the prevalence of MS also increased significantly with uric acid levels [14].

2. The Mechanism Contributing to Hyperuricemia and Current Target Medicines

The production of uric acid is regulated by the endogenous (nucleotides originating from cellular metabolism) and

exogenous (dietary) precursors transported to the liver, and the excretion is controlled by the kidneys through renal plasma flow, glomerular filtration, and proximal tubular exchange. Adenine nucleoside and guanine nucleoside can be catalyzed to generate hypoxanthine and guanine, respectively. Through the function of enzyme XOD or guaninase, hypoxanthine and guanine are converted to xanthine, which is subsequently catalyzed into the final product uric acid by XOD. The inhibitors of XOD are proved effective in patients who overproduce uric acid [29, 30]. XOD can be detected using high sensitive method of radioimmunoassay in many kinds of human tissues, but its activity in other tissues was only 1/10 to 1/1000 compared with that in liver tissue [31, 32].

It was reported that less excretion is the pivotal factor of primary hyperuricemia, accounting for about 90% of the cases [29]. Approximately two-thirds of the uric acid load is eliminated through kidneys, while the gastrointestinal tract eliminates one-third [33]. Thus, the kidney is another important organ for regulating uric acid level. Almost all uric acid is filtered from glomeruli, while postglomerular reabsorption and secretion regulate the amount of uric acid excretion. The proximal tubule is responsible for the reabsorption and secretion of uric acid, and approximately 90% is reabsorbed into blood [34]. Therefore, urate transport system of renal proximal tubules plays a vital role in the determination of serum urate levels [35]. It has been reported that urate anion exchanger 1 (URAT1/SLC22A12) and glucose transporter 9 (GLUT9/SLC2A9) play important roles in uric acid reabsorption. Similar to the reabsorption, uric acid secretion in human proximal tubules is performed mainly by exchangers organic anion transporter 1 (OAT1/SLC22A6) and/or OAT3 (SLC22A8) [36]. Figure 1 demonstrated the processes of uric acid secretion and reabsorption in renal proximal tubules as well as the transporters. Consequently, either overgeneration of uric acid (mainly in liver), as in MS or having diets rich in fructose and purines, or a reduction in its excretion (mainly in kidneys), as in acute renal failure or consequent to some drugs, can lead to high serum uric acid levels.

Nonpharmacological therapy including dietary poor in purine-rich food, sugars, alcohol, and rich in vegetables and water intake is necessary for hyperuricemia. However, it is not enough for patients with higher uric acid level. Pharmacological therapy is necessarily required. Since urate homeostasis depends on the balance mainly between production in liver, secretion, and reabsorption in kidney tubule and excretion in intestine, uric-acid-lowering drugs act on inhibiting generation, reducing absorption, and increasing secretion. Currently, the long-term treatment of hyperuricemia is aimed at modulating the activity of key enzymes involved in the metabolism and excretion of uric acid, like XOD and URAT1. Medicines are divided into two main classes: uricostatic drugs (e.g., allopurinol) and uricosuric drugs (e.g., sulphinpyrazone, probenecid, and benzbromarone) [37]. The target of uricostatic drugs focused primarily on XOD, for example, allopurinol, which inhibits XOD since it is an analogue of hypoxanthine. Allopurinol is the classical therapeutically used uricostatic drug and oxypurinol, tisorurinol, febuxostat, topiroxostat, and so forth have the same uricostatic effects. Since kidneys reabsorbed around 90% of filtered urate, inhibiting absorption is especially important in uricosuric drugs. The intestine is responsible for 30% of total body uric acid excretion. By far, the drug research for inhibiting intestinal excretion has been rare. The uricostatic and uricosuric drugs are proved efficient to control serum uric acid level; however, the side effects frequently restricted their clinic application. Among the patients treated by allopurinol, about 2% of patients appeared to have skin rash, 0.4% patients have kidney failure, or concomitant thiazide diuretic therapy may experience a severe idiosyncratic reaction, known as allopurinol hypersensitivity syndrome [38]. Uricosuric agents such as sulphinpyrazone and probenecid are relatively contraindicated in patients with kidney stone. Benzbromarone can be used in patients with chronic kidney disease but may cause incidental risk of hepatotoxicity [39].

3. Natural Products for Improving Hyperuricemia

During the past decades, more and more attention has been paid to natural products as alternative methods in treating hyperuricemia. Natural products reducing uric acid are also divided into two main classes: uricostatic and uricosuric drugs. There are a large number of Chinese medicines or the extracted compounds proved to be able to inhibit XOD activity to attenuate production of uric acid. Glabrous greenbrier rhizome, radix puerariae, mangiferin, celery, turmeric, motherwort, berberine, and so forth have been evaluated as active in inhibiting the enzyme XOD, from a total of 122 traditional Chinese medicines selected according to the clinical efficacy and prescription frequency for the treatment of gout and other hyperuricemia related disorders [40]. Relatively less studies reported natural medicines with effects on the renal urate transport system. Quercetin, at the dose of 50 and 100 mg/kg, could effectively upregulate OAT1 and downregulate GLUT9 and URAT1 in the kidneys of hyperuricemic mice [41]. Esculetin and esculin were found to improve hyperuricemia and renal dysfunction through upregulating OAT1. Through inhibiting GLUT9 or URAT1 in kidneys of hyperuricemic mice, fraxetin and fraxin could enhance urate excretion to some extent. Cortex fraxini coumarines were reported to be partly contributing to their functions of lower SUA level by regulating ABCG2 [42].

Among the reported drugs for treating hyperuricemia, we found some drugs playing the role through hepatorenal dual regulation, by targeting both XOD and excretion concurrently, which may enhance their function and broaden their application condition. The majority of these medicines belong to traditional Chinese medicine and their components. In this section, we focused on the natural products which have hepatorenal dual effects on hyperuricemia. To search for these natural products, we first used the following key words in PubMed: "uric acid" or "hyperuricemia" or "gout" and "medicinal plant" or "herb" or "herbal medicine" or "natural products" or "phytomedicine" or "phytotherapy" and "xanthine oxidase" or "XOD" or "XDH". On this basis, we searched for renal urate transports separately, using words

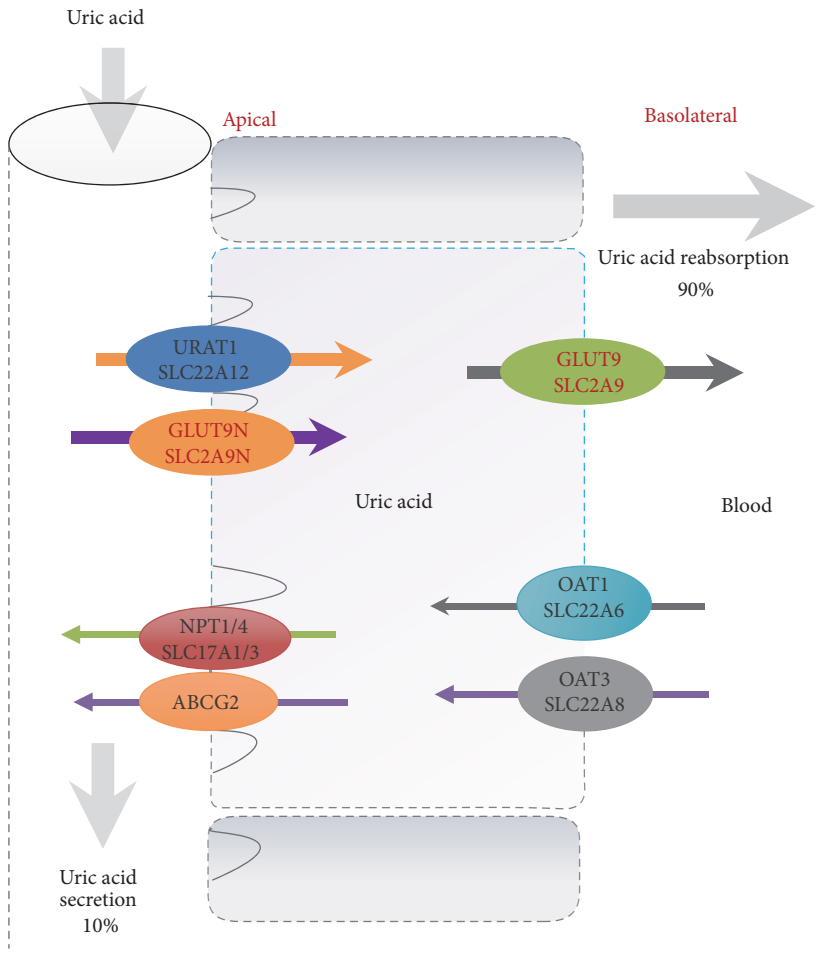

Figure 1: The processes of uric acid excretion and reabsorption and the main transporters in renal tubular epithelial cell.

such as "uricosuric" or "URAT1" or "GLUT9" or "OAT1" or "OAT3" or "OAT4" or "ABCG2" or "NPT1" or "NPT4" or "MRP4" or "PDZK1". In addition, we also searched for the aforementioned key words in Chinese from CNKI (China National Knowledge Infrastructure) and WANFANG DATA knowledge service platform. The results are listed in Table 1.

3.1. Natural Products with Effects of Inhibiting Generation and Absorption of Uric Acid (Target URAT1, GLUT9, and XOD). In the kidney, the absorption of urate can be divided into two stages: urate is first absorbed from renal tubular cavity to renal tubular epithelial cells (mainly mediated by URAT1), subsequently, absorbed into blood across the tubular epithelial basement membrane (mainly mediated by GLUT9) [43]. The kidney specific urate transporter URAT1 (SLC22A12) was first identified by Enomoto et al. It was demonstrated that this molecule is the drug target that alters serum uric acid levels and causes idiopathic renal hypouricemia [44]. The URAT1 protein is specifically localized in the brush border membrane of the proximal tubule. It participates in the apical (luminal) uptake of urate from the primary urine to the proximal tubule cell, thus affecting reabsorption. The *in vivo* experiments found that URAT1 was a biological target of some uricosuric drugs, including probenecid, indomethacin, 6-hydroxybenzbromarone, and salicylate [45]. Li et al. first reported that GLUT9 (SLC2A9) is a gene correlated with SUA level [46]. Subsequently similar results have been reported by several other studies [47–52]. Human GLUT9 has two isoforms (GLUT9 and GLUT9N) depending on the splicing of the intracellular part of the N-terminal region [47]. When artificially expressed in polarized MDCK cells, GLUT9 (or long form) is expressed at the basal side, and GLUT9N (or short form) is expressed at the apical side. GLUT9N transports uric acid from renal tubular cavity into epithelial cells, whereas GLUT9 transits uric acid from epithelial cells into tubulointerstitium or blood [44]. Studies showed that GLUT9 plays a more important role than URAT1 in the absorption of uric acid [53].

In the *in vitro* research, Hou et al. showed that dried longan seed extract (LSE) and its active ingredients inhibited XOD in a dose dependent manner [15]. In the *in vivo* experiment, LSE was discovered to able to reduce serum XOD activity and SUA level in hyperuricemic rats. In addition, LSE increased GLUT1 but decreased GLUT9 protein level in kidney, respectively. These results showed that longan seeds were effective against hyperuricemia and indicated that its

Table 1: The hepatorenal dual actions of natural products on hyperuricemia.

Natural products	Inhibition of the generation	Inhibition of the absorption	Promote the secretion	Refs.
Longan seed extract (LSE)	Inhibit activity of XOD/ADA	Decrease GLUT9 but increase GLUT1	None	[15]
Plantago asiatica L. herbs extracts (PAHEs)	Inhibit activity of XOD	Downregulate renal URAT1	None	[16]
Bergenin	Inhibit activity of XOD	Downregulate URAT1 and GLUT9	None	[17] [18]
Sea cucumber saponin	Inhibit activity of XOD/ADA	Downregulate GLUT9	None	[19]
Sea cucumber polysaccharides	Inhibit activity of XOD/ADA Downregulate XOD/ADA	Downregulate GLUT9	None	[19]
Puerarin	Inhibit activity of XOD	Unidentified	Unidentified	[20]
Total saponins from Rhizoma Dioscoreae Nipponicae	Inhibit activity of XOD/ADA	None	Upregulate OAT1	[21]
Sea cucumber saponin-EA	Inhibit activity of XOD/ADA	None	Upregulate OAT1	[19]
Jasminoidin	Inhibit activity of XOD	Regulate expressions of renal urate transporters	None	[22]
Green tea polyphenols	Reduce XOD expression	Reduce URAT1	Upregulate OAT1 and OAT3	[23]
Smilax riparia A. D. C	Inhibit activity of XOD	Downregulate renal URAT1 and GLUT9	Upregulate OAT1	[24]
Modified Simiao Decoction	Inhibit activity of XOD	Downregulate URAT1	Upregulate OAT1	[25]
Total saponins of *Dioscorea*	Inhibit activity of XOD	Downregulate URAT1	Upregulate OAT1 and OAT3	[26]
Lagotis brevituba Maxim. (LBM) extracts	Inhibit activity of XOD	Downregulate renal URAT1 and GLUT9	Upregulate OAT1	[27]
P. sibircum Laxm. alcohol extract 2.0/4.0/8.0 g/kg	Inhibit activity of XOD	Reduce GLUT9	Upregulate OAT1	[28]
P. sibircum Laxm. alcohol extract 8.0 g/kg	Inhibit activity of XOD	Reduce URAT1 and GLUT9	Upregulate OAT1	[28]

effect depended on inhibiting XOD and modulating urate transporters. Zeng et al. studied the effect of *Plantago asiatica* L. herbs extracts (PAHEs) on serum levels of uric acid level in hyperuricemia mice. The results demonstrated that PAHEs could obviously improve hyperuricemia. The mechanisms include downregulating hepatic ADA and XOD to reduce production of uric acid and enhancing urate excretion and decreasing urate reabsorption by suppressing renal URAT1 [16]. Bergenin belongs to isocoumarin compounds, which can protect liver, shrink ulcer, and improve immune functions [17], and has the inhibitory activity for XOD [54]. Zhou and Chen used bergenin to treat hyperuricemia model mice induced by potassium oxonate [18]. The results showed that bergenin significantly reduced SUA and creatinine level, and urea nitrogen in model mice improved the 24 h excretion of uric acid and creatinine. Bergenin obviously lowered the expression of URAT1 and GLUT9 in kidneys of model mice. The experiments of Xu showed that sea cucumber saponin and polysaccharides from *Pearsonothuria graeffei* (Pg), *Apostichopus japonicus* (Aj), *Cucumaria frondosa* (Cf), and *Isostichopus badionotus* (Ib) could significantly lower SUA level in hyperuricemia mice induced by feeding yeast extract powder for 14 days, and all of the natural products could inhibit hepatic XOD and adenylate deaminase (ADA) activities. Furthermore, the sea cucumber saponin and polysaccharides were found to downregulate the mRNA levels of hepatic ADA and XOD and renal GLUT9, which is the important transporter in the process of the reabsorption of uric acid [19]. Shi et al. explored the effect of puerarin on SUA content and pathways in hyperuricemic rats. This research monitored indicators of hyperuricemic rats intervened with puerarin, such as SUA, oxidase activity of XOD, and uric acid excretion. The results showed that puerarin at reasonable dosage was beneficial to improve SUA levels via inhibiting activity of XOD and promoting uric acid excretion [20].

3.2. Natural Products with Effects of Inhibiting Generation and Promoting Secretion of Uric Acid (Target OAT1, OAT3, and XOD). The organic anion and urate transporters OAT1 (SLC22A6) and OAT3 (SLC22A8) can act as urate/dicarboxylate exchangers [55–57] and are found on the basolateral side of the same cells that express OAT4 [58]. Gene knockout studies *in vivo* found that absence of OAT1

or OAT3 slightly decreased uricosuria, suggesting that their principal function is in urate excretion [59].

Zhou et al. studied the effect of saponins from Rhizoma Dioscoreae Nipponicae in hyperuricemia models and verify the anti-inflammatory effect of these saponins *in vitro*. In the hyperuricemia model mice, it was found that total saponins from Rhizoma Dioscoreae Nipponicae at different doses (600, 300, and 30 mg/kg) could significantly reduce uric acid level through inhibiting the activities of both ADA and XOD. Meanwhile, these saponins could upregulate the expression of OAT1 [21]. The study of Xu showed that the antihyperuricemia effect of saponin and nonsaponin residue was related to the inhibition of hepatic XOD and ADA activities. Dietary sea cucumber saponin-EA also could decrease serum uric acid level significantly by 18.1%. EA was found to increase the secretion of uric acid and upregulate the mRNA levels of renal organic anion transporter 1 (OAT1). Moreover, the expression and activity of hepatic XOD and ADA were elevated by EA [19].

3.3. Natural Products with Effects of Inhibiting Generation, Absorption, and Promoting Secretion of Uric Acid (Target OAT, GLUT9, and XOD). Green tea polyphenols (GTP) are generally well known as the major active component with multiple pharmacological functions in green tea. In one study to investigate the effect of GTP on SUA level, it was found that GTP significantly decreased SUA levels in a dose dependent manner in potassium oxonate-induced hyperuricemic mice. Furthermore, GTP reduced XOD expression in liver and reduced URAT1 expression and increased OAT1 and OAT3 expressions in kidneys, suggesting that GTP might attenuate SUA level through decreasing production and increasing excretion of uric acid [22]. Hu et al. found Jasminoidin significantly lowered the SUA levels and increased the uric acid excretion. This compound from Chinese medicine remarkably inhibited the hepatic XOD activities and regulated the expressions of renal urate transporters in mice [23]. *Smilax riparia* is a botanical widely grown in southern and central region of China. The roots and rhizomes of *Smilax riparia* in traditional Chinese medicine (TCM) have been used to treat the symptoms of gout and hyperuricemia related conditions, including inflammation and some malignancies [60, 61]. Studies by Hou et al. indicated that the synergistic effects of allopurinol combined with pallidifloside D were associated with the inhibition of both serum and hepatic XOD, upregulation of mOAT1, and downregulation of renal GLUT9 and URAT1 [24]. Zhu and Chen studied and found that the total saponins of *Dioscorea* can significantly decrease the level of SUA in hyperuricemia rats caused by adenine and ethambutol. The mechanism may be related to inhibiting XOD activity, as well as increasing the excretion of uric acid through the downregulation of high expression of URAT1 and upregulation of OAT1 and OAT3 [25]. Zeng et al. researched the influence of *Lagotis brevituba* maxim extracts (LBMs) on the level of uric acid in hyperuricemia mice [26]. The results demonstrated that LBMs possessed antigout effect. The main mechanism included inhibiting the activity of hepatic XOD, downregulating renal URAT1 and GLUT9, and upregulating OAT1 expression in hyperuricemiamice. Wang et al. found that *P. sibircum* Laxm. alcohol extract could distinctly lower the SUA level, and the mechanism is related to the inhibition of hepatic XOD activities and the regulation of renal urate transporters. In addition, alcohol extract of *P. sibircum* Laxm. significantly reduced the mRNA expression level of renal GLUT9 in mice and upregulated OAT1 mRNA expression. At the dose of 8.0 g/kg, *P. sibircum* Laxm. alcohol extract also significantly reduced URAT1 in mice [27].

Modified Simiao Decoction (MSD), a complex recipe of Chinese medicine, has been used in recent decades and proven to be efficient in treating gout and hyperuricemia. Hua et al. investigated the effects of MSD in hyperuricemic mice. MSD could decrease SUA levels, serum creatinine, and BUN and restrain XOD activities in liver and serum. It also upregulated OAT1 and downregulated URAT1 protein expressions in the renal tissues of hyperuricemic mice in dose dependent manner [28].

4. Conclusion

A lot of natural products have been proved efficient in downregulating uric acid level, among which some target both the production and the excretion of uric acid. In this case, the function is more powerful and the application condition of patients will have less limitation. Therefore, these natural products for treating hyperuricemia should be given more attention in the future. However, clinical studies for the therapeutic efficacy of these natural products and the underlying mechanism are still required.

Competing Interests

The authors declared no conflict of interests.

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Review Article

Applicability of Isolates and Fractions of Plant Extracts in Murine Models in Type II Diabetes: A Systematic Review

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Type II diabetes mellitus is one of the most common public health problems worldwide. Its increasing prevalence in several countries and the difficult metabolic control of individuals with the disease justify studying strategies for primary prevention. The population has sought alternative and cheaper ways to treat the disease, including the use of plants considered medicinal by the population. In this study, we carried out a systematic review on the applicability of isolates and fractions of plant extracts in animal models in type II diabetes. A literature search was performed in MEDLINE/PubMed and Scopus databases. Studies using other experimental animals (horses, rabbits, and monkeys) and humans as well as articles in Chinese, German, and Russian were excluded. We assessed the quality of the studies included by using the criteria described in the ARRIVE guidelines. In general, the animals that received fractions or isolates presented reduced blood glucose levels, normalization of body weight and plasma insulin levels, and reduced total triglycerides and cholesterol. In addition, we observed wide variation among the analyzed parameters, which hindered comparison between the studies found. In further studies, standardized reports and experimental design would help to establish comparable study groups and advance the overall knowledge, thus facilitating translatability from animal data to human clinical conditions.

1. Introduction

Diabetes mellitus (DM) is a syndrome caused by changes in the metabolism of carbohydrates, lipids, and proteins and may occur in two different forms: type I (10%) and type II (90%) [1]. Type I diabetes results from body inability to produce insulin [2], while type II may be caused by a failure in the production and secretion of insulin by the pancreas, due to insufficient production or a problem in the beta-cell receptors, thus decreasing the sensitivity of the target tissue to the metabolic effect of this hormone. This decreasing sensitivity is known as insulin resistance [3]. Patients with this disease present polydipsia (excessive thirst), polyuria (urine production over 2.5 liters/day), polyphagia (excessive avidity for food), and delayed wound healing [4]. Besides, poor glucose metabolism, reduced insulin signaling, excessive release of free fatty acids, and interleukin-6 are changes also considered important for analysis in clinical and preclinical studies [5].

According to the World Health Organization (2012), diabetes mellitus accounts for 3.5% of the noncommunicable diseases in the world. The International Diabetes Federation (IDF) [6] estimates that, by 2013, more than 382 million people around the world had been affected by the disease, and the figures are increasing in all countries. Portugal, for example, is the second country in Europe with the highest prevalence of diabetes mellitus: 12.7 people with DM per 100 inhabitants in 2011 [7].

In Brazil, according to estimates, there are more than 12 million people with type II diabetes mellitus (DM2) [8],

mainly people who are over 40 years of age and obese. However, recent studies have shown a considerable increase in the number of children and teenagers with the disease, which may be associated with bad eating habits and lack of physical activity. This leads to increasing rates of obesity, which is considered a risk condition for the development of type II diabetes [9].

The population has sought alternative and cheaper treatments for the disease, including the use of plants considered medicinal by the population. In several countries, such as Japan, China, and India, the use of medicinal plants and their derivatives is increasing, since they are considered simple, cheap, and effective treatment alternatives [10, 11]. There are some reports in the literature about the benefits of different herbal treatments on several metabolic changes caused by type II diabetes. The studies generally show positive effects of plant extracts on the normalization of body weight and decrease of glucose levels, total cholesterol, and triglycerides [12].

Therefore, the use of phytotherapy has opened a new perspective for the management and treatment of metabolic diseases, such type II diabetes, since it is an affordable treatment [13]. However, the use of most of these plants has not been investigated [14, 15]. Research on plant extracts should be conducted to ensure that they are effective and safe for the population [16]. Due to these factors and the high number of type II diabetes carriers, the demand for less expensive therapies may significantly benefit population health [11].

In vivo and *in vitro* studies are commonly used in biomonitoring research of plant extracts, aiming to identify their biological activity. The fractions are metabolites obtained by fractioning plant extracts, which provides a more specific analysis of the plant active principle [17]. These studies are important for the treatment of chronic diseases, including diabetes, since the treatment or control of these diseases is expensive and the number of affected people has increased considerably. Besides the fractions, the isolates obtained from plants have also played an important role in the treatment of metabolic disorders. After isolating a particular component of the crude extract, it is possible to ensure that the effects on the tissue are caused only by a specific constituent of the extract. This makes the molecule more attractive to the drug market, which aims to develop drugs from plants or other materials.

A systematic review is based on predetermined criteria and consistent scientific evidence. It aims to collaborate with research selection and/or tools for the development of products based on original information [18], with well-defined criteria selection, to ensure the quality of the summarized studies and their reproducibility. Moreover, a conclusion providing new information based on filtered content is necessary [19]. Generally, due to its rigorous methods to identify, select, collect, and analyze data, this kind of study provides the highest level of scientific evidence. Therefore, our study aimed to make a descriptive and critical analysis of studies on the activity of plant fractions and isolates in the treatment of type II diabetes in animal models.

2. Material and Methods

2.1. Selection of Papers. The papers analyzed in this review were selected from two electronic databases, PubMed and Scopus, accessed on September 3, 2015, using the search filters: "animal model", "plant extract", and "diabetes mellitus type II". These filters have been developed for the search on PubMed, according to the Medical Subject Headings (MeSH terms), used for a more efficient indexing of publications on the subject under study [20]. In order to expand the search, MeSH terms were combined with the title and abstract (TIAB). A standard filter was used [21] to identify all studies with animals in PubMed. The terms used to search on PubMed were adapted for the selection of Scopus publications, and the "animal model" filter was provided by the site itself (Supplemental Data 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3537163).

The Prism guideline was used to develop this review [22]. After the papers were collected from the two electronic databases, the duplicates were excluded by comparing the title, author, year, and country. A screening was performed for title and abstract, guided by the eligibility criteria: *in vivo* experimental studies; studies using rats or diabetic mice; use of fractions or isolates of noncommercial plants; treatment of the main symptoms of type II diabetes; studies written in English or Portuguese.

Next, all selected papers were obtained in full for a second screening, when all of them were examined to select those that met the criteria for the inclusion in the systematic review. Those unavailable on the internet were requested from their respective authors. When they did not respond, the studies were excluded. The entire search process, exclusion, and the number of selected papers were described in detail in the PRISMA Guideline (Figure 1).

2.2. Qualitative Characteristics of Publications. After screening, the papers were reviewed. Table 2 shows the description of the main characteristics of the studies. The following parameters were assessed: (1) publication features: author, year, and country; (2) experimental features: animal model, species, sample number, sex, weight, age, type of caging used, number of animals per cage, number of experimental groups and number of animals in each group, if randomization was made, and control groups; (3) treatment features: plant species used, name of the fraction or isolate, dose, route of administration, and treatment duration; (4) diabetes induction: drug used, dose, route of administration, and testing to prove diabetes occurrence (Table 1).

2.3. ARRIVE (Bias Analyses). The detailed reports of experiments are crucial in the review process, so that they can be validated and used as a source of information for further research. However, many studies do not bring relevant or concise information, which leads to the realization of redundant and duplicated experiments [23]. Therefore, guidelines were developed for animal research reports, such as the ARRIVE guideline, based on the CONSORT Statement. The ARRIVE guidance is a list of 20 items that describe

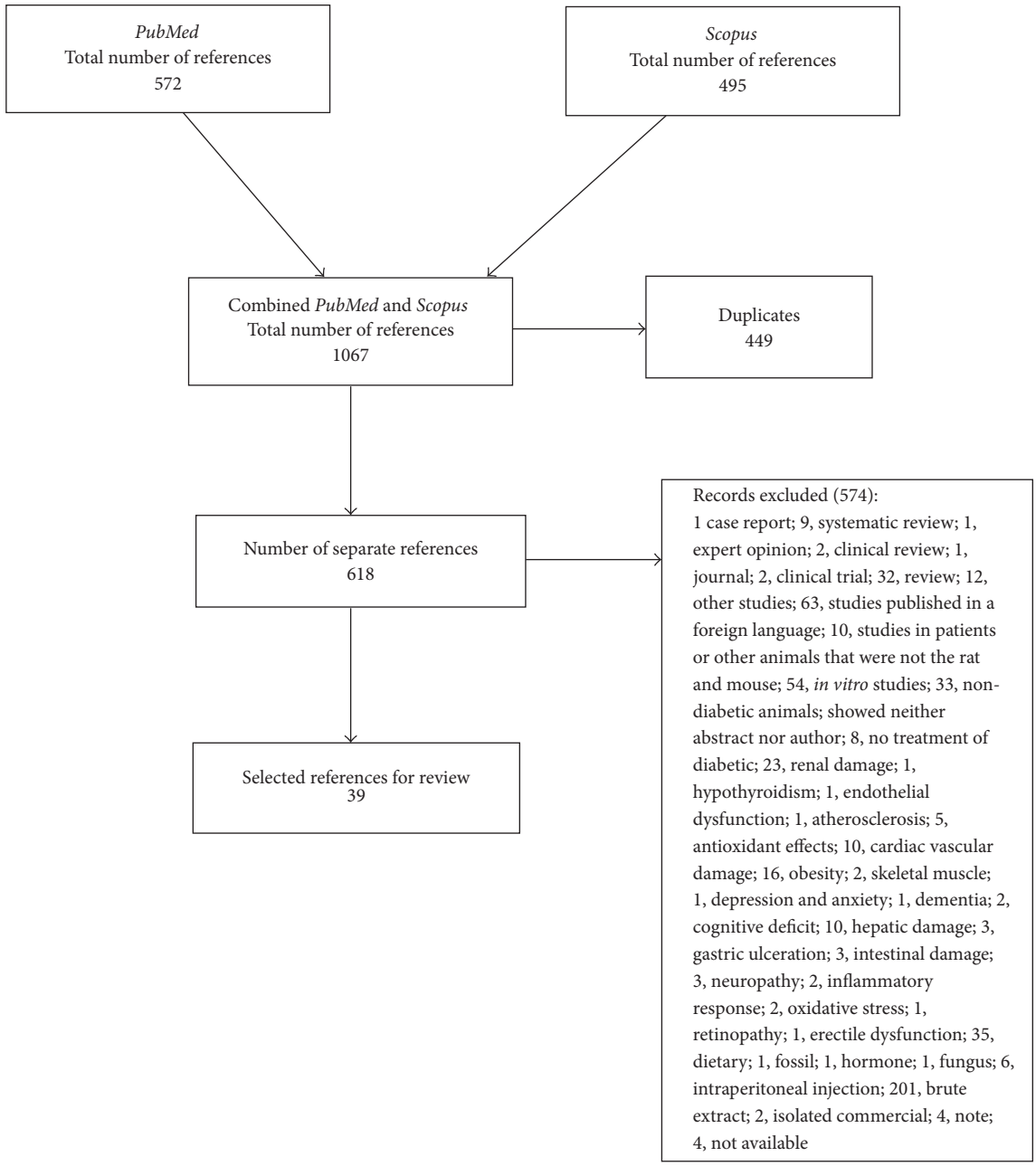

Figure 1: Results obtained after the advanced search in the databases. After literature review, 39 papers were selected from Moher D, Liberati A, Tetzlaff J, Altman DG, and the PRISMA Group (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA Statement. PLoS Med 6(6): e1000097. doi: 10.1371/journal.pmed1000097. For more information, visit www.prisma-statement.org. Prism: systematic review.

the minimum information that all scientific publications reporting research using animals must include, aiming at high quality reports and critical and accurate review of what was performed and found [24]. Thus, based on these fundamentals and the objective of this study, a table displaying the most relevant and applicable items from the ARRIVE was developed for a critical evaluation of the studies included in this review (Table 2). The authors assessed the quality, integrity, and transparency of each publication. Divergent opinions were resolved by consensus.

3. Results

3.1. Prism. The search conducted in this study found a total of 1,067 papers, out of which 571 were found in PubMed and 495 in Scopus. Out of this total, 449 papers were duplicates; thus 618 studies remained. Then, a title and abstract screening was performed, guided by the eligibility criteria listed above. In this respect, 574 studies were excluded due to inadequate research topic. Among the excluded studies, we can highlight those on the crude extract of the plant (200), studies in

Table 1: Table of qualitative descriptors of the studies of the effects of fractions and isolates from the plants on the treatment of the type II diabetes.

(a)

	Title	Author/publication year	Country
Isolates	[25] A Polysaccharide Extract of Mulberry Leaf Ameliorates Hepatic Glucose Metabolism and Insulin Signaling In Rats with Type 2 Diabetes Induced by High Fat-Diet and Streptozotocin	Ren et al. (2015)	China
	[26] Antidiabetic Activity of a Xanthone Compound, Mangiferin	Miura et al. (2001)	Japan
	[27] Anti-Diabetic Effect of a Novel N-Trisaccharide Isolated from *Cucumis prophetarum* on Streptozotocin-Nicotinamide Induced Type 2 Diabetic Rats	Kavishankar and Lakshmidevi (2014)	India
	[28] Antidiabetic Effects of the *Cimicifuga racemosa* Extract Ze 450 *In Vitro* and *In Vivo* in ob/ob Mice	Moser et al. (2014)	Switzerland
	[29] Borapetoside C from *Tinospora crispa* Improves Insulin Sensitivity in Diabetic Mice	Ruan et al. (2012)	Taiwan
	[30] Dehydrotrametenolic Acid Induces Preadipocyte Differentiation and Sensitizes Animal Models of Noninsulin-Dependent Diabetes Mellitus to Insulin	Sato et al. (2002)	Japan
	[31] Effects of Ingested Fruiting Bodies, Submerged Culture Biomass, and Acidic Polysaccharide Glucuronoxylomannan of *Tremella mesenterica* Retz.:Fr. on Glycemic Responses in Normal and Diabetic Rats	Lo et al. (2006)	Taiwan
	[32] Inhibition of Glycogen Synthase Kinase-3β by Falcarindiol Isolated from Japanese Parsley (*Oenanthe javanica*)	Yoshida et al. (2013)	Japan
	[33] Isolation and Antihyperglycemic Activity of Bakuchiol from *Otholobium pubescens* (Fabaceae), a Peruvian Medicinal Pant Used for the Treatment of Diabetes	Krenisky et al. (1999)	USA
	[34] LBP-4a Improves Insulin Resistance via Translocation and Activation of GLUT4 in OLETF Rats	Zhao et al. (2014)	China
	[35] Meliacinolin: A Potent A-Glucosidase and A-Amylase Inhibitor Isolated from *Azadirachta indica* Leaves and In Vivo Antidiabetic Property in Streptozotocin-Nicotinamide-Induced Type 2 Diabetes in Mice	Perez-Gutierrez and Damian-Guzman (2012)	Mexico
	[36] Novel Terpenoid-Type Quinones Isolated from *Pycnanthus angolensis* of Potential Utility in the Treatment of Type 2 Diabetes	Luo et al. (1999)	USA
	[37] Platyconic Acid, A Saponin from *Platycodi radix*, Improves Glucose Homeostasis by Enhancing Insulin Sensitivity *In Vitro* and *In Vivo*	Kwon et al. (2012)	South Korea
	[38] Rhaponticin from rhubarb Rhizomes Alleviates Liver Steatosis and Improves Blood Glucose and Lipid Profiles in KK/Ay Diabetic Mice	Chen et al. (2009)	China
	[39] Rutin Potentiates Insulin Receptor Kinase to Enhance Insulin-Dependent Glucose Transporter 4 Translocation	Hsu et al. (2014)	Taiwan
	[40] Type 2 Antidiabetic Activity of Bergenin from the Roots of *Caesalpinia digyna* Rottler	Kumar et al. (2012)	India

(a) Continued.

	Title	Author/publication year	Country
Fractions	[41] A Study on Hypoglycaemic Health Care Function of *Stigma maydis* Polysaccharides	Zhang et al. (2013)	China
	[1] Antidiabetic Activities of Extract from *Malva verticillata* Seed via the Activation of AMP-Activated Protein Kinase	Jeong and Song (2011)	Korea
	[42] Antidiabetic Activity of Alkaloids of *Aerva lanata* Roots on Streptozotocin-Nicotinamide Induced Type-II Diabetes in Rats	Agrawal et al. (2013)	India
	[43] Antidiabetic Activity of *Pterospermum acerifolium* Flowers and Glucose Uptake Potential of Bioactive Fraction in L6 Muscle Cell Lines with Its HPLC Fingerprint	Paramaguru et al. (2014)	India
	[44] Antidiabetic Activity of *Caesalpinia bonducella* F. in Chronic Type 2 Diabetic Model in Long-Evans Rats and Evaluation of Insulin Secretagogue Property of Its Fractions on Isolated Islets	Chakrabarti et al. (2005)	India
	[45] Antidiabetic Effect of an Acidic Polysaccharide (TAP) from *Tremella aurantia* and Its Degradation Product (TAP-H)	Kiho et al. (2001)	Japan
	[46] Antidiabetic Effect of Orally Administered Conophylline-Containing Plant Extract on Streptozotocin-Treated and Goto-Kakizaki Rats	Fujii et al. (2009)	Japan
	[47] Antidiabetic Effect of Total Flavonoids from *Sanguis d raxonis* in Type 2 Diabetic Rats	Chen et al. (2013)	China
	[48] Antidiabetic Effects of Bitter Gourd Extracts in Insulin-Resistant db/db Mice	Klomann et al. (2010)	Germany
	[49] Anti-Diabetic Effects of *Centratherum anthelminticum* Seeds Methanolic Fraction on Pancreatic Cells, B-TC6 and Its Alleviating Role in Type 2 Diabetic Rats	Arya et al. (2012)	Malaysia
	[17] Anti-Diabetic Effects of Polysaccharides from *Talinum triangulare* in Streptozotocin (STZ)-Induced Type 2 Diabetic Male Mice	Xu et al. (2015)	China
	[50] Anti-Diabetic Effects of the Acetone Fraction of *Senna singueana* Stem Bark in a Type 2 Diabetes Rat Model	Ibrahim and Islam (2014)	South Africa
	[51] Antidiabetic Potential of Polysaccharides from the White Oyster Culinary-Medicinal Mushroom *Pleurotus florida* (Higher Basidiomycetes)	Ganeshpurkar et al. (2014)	India
	[52] Antihyperglycemic Effects of Total Flavonoids from *Polygonatum odoratum* in STZ and Alloxan-Induced Diabetic Rats	Shu et al. (2009)	China
	[53] Antioxidant and Anti-Inflammatory Effects of a Hypoglycemic Fraction from *Cucurbita ficifolia* Bouché in Streptozotocin-Induced Diabetic Mice	Roman-Ramos et al. (2012)	Mexico
	[54] Effect of *Lycium barbarum* Polysaccharide on the Improvement of Insulin Resistance in NIDDM Rats	Zhao et al. (2005)	China
	[55] Effects of Grape Seed Extract and Its Ethylacetate/Ethanol Fraction on Blood Glucose Levels in a Model of Type 2 Diabetes	Hwang et al. (2009)	South Korea
	[56] Hypoglycemic Effect of *Astragaluspolysaccharide* and Its Effect on PTP1B1	Wu et al. (2005)	China
	[57] Hypoglycemic Effects of MDG-1, a Polysaccharide Derived from *Ophiopogon japonicus*, in the ob/ob Mouse Model of Type 2 Diabetes Mellitus	Xu et al. (2011)	China
	[58] Isolation and Pharmacological Activities of the *Tecoma stans* Alkaloids	Costantino et al. (2003)	Italy
	[58] Potent Effects of the Total Saponins From *Dioscorea nipponica* Makino against Streptozotocin-Induced Type 2 Diabetes Mellitus in Rats	Yu et al. (2015)	China
	[59] Tocopherol from Seeds of *Cucurbita pepo* against Diabetes: Validation by In Vivo Experiments Supported by Computational Docking	Bharti et al. (2013)	India
	[60] *Trapa natans* L. Root Extract Suppresses Hyperglycemic and Hepatotoxic Effects in STZ-Induced Diabetic Rat Model	Kharbanda et al. (2014)	India

(b)

	Title	Animal model/strain	Number of animals	Sex	Age	Weight	Housing of animals	Animals per cage	Groups and number of animals in each group	Randomization
Isolates	[25] A Polysaccharide Extract of Mulberry Leaf Ameliorates Hepatic Glucose Metabolism and Insulin Signaling in Rats with Type 2 Diabetes Induced by High Fat-Diet and Streptozotocin	Wistar rats	18	M	7 w	200 g	Cages	5	3 groups (6 in each group)	Yes
	[26] Antidiabetic Activity of a Xanthone Compound, Mangiferin	Mice	?	M	12 w	?	?	?	?	?
	[27] Anti-Diabetic Effect of a Novel N-Trisaccharide Isolated from *Cucumis prophetarum* on Streptozotocin-Nicotinamide Induced Type 2 Diabetic Rats	Wistar rats	36	M&F	?	150–180 g	Cages	?	6 groups (6 animals in each)	?
	[28] Antidiabetic Effects of the *Cimicifuga racemosa* Extract Ze 450 *In Vitro* and *In Vivo* in ob/ob Mice	ob/ob mice	68	M	7-8 w	?	Cages	1	8 groups (8 animals in each)	?
	[29] Borapetoside C from *Tinospora crispa* Improves Insulin Sensitivity in Diabetic Mice	Mice	?	M	8–10 w	?	?	?	?	?
	[30] Dehydrotrametenolic Acid Induces Preadipocyte Differentiation and Sensitizes Animal Models of Noninsulin-Dependent Diabetes Mellitus to Insulin	C57BLK mice	?	M	7 w	?	?	?	?	?
	[31] Effects of Ingested Fruiting Bodies, Submerged Culture Biomass, and Acidic Polysaccharide Glucuronoxylomannan of *Tremella mesenterica* Retz.:Fr. on Glycemic Responses in Normal and Diabetic Rats	Wistar rats	102	M	?	200 g	Cages	1	6 groups (12 animals in each group) and 3 groups (10 animals in each group)	Yes
	[32] Inhibition of Glycogen Synthase Kinase-3β by Falcarindiol Isolated from Japanese Parsley (*Oenanthe javanica*)	GK rats	12	M	?	?	Cages	1	2 groups (6 animals in each group)	?
	[33] Isolation and Antihyperglycemic Activity of Bakuchiol from *Otholobium pubescens* (Fabaceae), a Peruvian Medicinal Plant Used for the Treatment of Diabetes	C57BL/K mice & Sprague-Dawley rats	?	M	8 w	?	?	4	? Groups (8 animals in each group)	?
	[34] LBP-4a Improves Insulin Resistance via Translocation and Activation of GLUT4 in OLETF Rats	Otsuka Long-Evans Tokushima Fatty (OLETF) rats	18	M	26 w	?	?	?	3 groups (6 animals in each group)	Yes
	[35] Meliacinolin: A Potent A-Glucosidase and A-Amylase Inhibitor Isolated from *Azadirachta indica* Leaves and In Vivo Antidiabetic Property in Streptozotocin-Nicotinamide-Induced Type 2 Diabetes in Mice	?/mice	42	M	?	20–25 g	Cages	2	7 groups (6 animals in each group)	?
	[36] Novel Terpenoid-Type Quinones Isolated from *Pycnanthus angolensis* of Potential Utility in the Treatment of Type 2 Diabetes	C57BLK mice	?	M	7-8 w	?	Cages	4	? groups (5–8 animals in each group)	?
	[37] Platyconic Acid, a Saponin from *Platycodi radix*, Improves Glucose Homeostasis by Enhancing Insulin Sensitivity In Vitro and In Vivo	C57BLK mice	90	M	8–10 w	?	?	1	6 groups (15 animals in each group)	Yes
	[38] Rhaponticin from rhubarb Rhizomes Alleviates Liver Steatosis and Improves Blood Glucose and Lipid Profiles in KK/Ay Diabetic Mice	Mice	30	M&F	8–12 w	?	Cages	1	3 groups (10 animals in each group)	Yes
	[39] Rutin Potentiates Insulin Receptor Kinase to Enhance Insulin-Dependent Glucose Transporter 4 Translocation	Mice	?	M	8 w	?	?	?	?	?
	[40] Type 2 Antidiabetic Activity of Bergenin from the Roots of *Caesalpinia digyna* Rottler	Charles Foster Albino rats	30	M	?	?	?	?	5 groups (6 animals in each group)	?

(b) Continued.

	Title	Animal model/strain	Number of animals	Sex	Age	Weight	Housing of animals	Animals per cage	Groups and number of animals in each group	Randomization
Fractions	[41] A Study on Hypoglycaemic Health Care Function of *Stigma maydis* Polysaccharides	SPF km mice	?	M	?	20 ± 2 g	?	?	7 groups (animals in each group?)	Yes
	[1] Antidiabetic Activities of Extract from *Malva verticillata* Seed via the Activation of AMP-Activated Protein Kinase.	C57BLK mice	?	?	5 w	?	Cages	5	5 groups (animals in each group?)	?
	[42] Antidiabetic Activity of Alkaloids of *Aerva lanata* Roots on Streptozotocin-Nicotinamide Induced Type-II Diabetes in Rats	Wistar rats	?	M	?	200–250 g	?	?	?	?
	[43] Antidiabetic Activity of *Pterospermum acerifolium* Flowers and Glucose Uptake Potential of Bioactive Fraction in L6 Muscle Cell Lines with Its HPLC Fingerprint	Albino Wistar rats	66	M	?	180–200 g	Cages	?	11 groups (6 animals in each group)	?
	[44] Antidiabetic Activity of *Caesalpinia bonducella* F. in Chronic Type 2 Diabetic Model in Long-Evans Rats and Evaluation of Insulin Secretagogue Property of Its Fractions on Isolated Islets	Long-Evans rats	?	M&F	12–14 w	150 g	?	?	5 groups (6–9 animals in each group)	?
	[45] Antidiabetic Effect of an Acidic Polysaccharide (TAP) from *Tremella aurantia* and Its Degradation Product (TAP-H)	KK- Ay-TA mice	?	M	5 w	25–30 g	?	1	3 groups (animals in each group?)	?
	[46] Antidiabetic Effect of Orally Administered Conophylline-Containing Plant Extract on Streptozotocin-Treated and Goto-Kakizaki Rats	Goto-Kakizaki rats	14	M	5 w	?	?	?	3 groups (animals in each group?)	Yes
	[47] Antidiabetic Effect of Total Flavonoids from *Sanguis draxonis* in Type 2 Diabetic Rats	Sprague-Dawley rats	72	M	?	180–220 g	?	?	6 groups (6 animals in each group) for SD; 6 groups (6 animals in each group) for SDF	?
	[48] Antidiabetic Effects of Bitter Gourd Extracts in Insulin-Resistant db/db Mice	db/db mice	45	M	5 w	?	Cages	1	5 groups (9 animals in each group)	?
	[49] Anti-Diabetic Effects of *Centratherum anthelminticum* Seeds Methanolic Fraction on Pancreatic Cells, B-TC6 and Its Alleviating Role in Type 2 Diabetic Rats	Sprague-Dawley rats	72	M&F	?	180–200 g	?	?	12 groups (6 animals in each group)	?

(b) Continued.

Title	Animal model/strain	Number of animals	Sex	Age	Weight	Housing of animals	Animals per cage	Groups and number of animals in each group	Randomization
[17] Anti-Diabetic Effects of Polysaccharides from *Talinum triangulare* in Streptozotocin (STZ)-Induced Type 2 Diabetic Male Mice	SPF km mice	50	M	?	20 ± 2 g	?	?	5 groups (10 animals in each group)	Yes
[50] Anti-Diabetic Effects of the Acetone Fraction of *Senna singueana* Stem Bark in a Type 2 Diabetes Rat Model	Sprague-Dawley rats	48	M	6 w	207.60 ± 4.27 g	Cage	2	6 groups (8 animals in each group)	Yes
[51] Antidiabetic Potential of Polysaccharides from the White Oyster Culinary-Medicinal Mushroom *Pleurotus florida* (Higher Basidiomycetes)	Wistar rats	20	M	?	150–200 g	?	?	4 groups (5 animals in each group)	?
[52] Antihyperglycemic Effects of Total Flavonoids from *Polygonatum odoratum* in STZ and Alloxan-Induced Diabetic Rats	Sprague-Dawley rats	?	?	?	220 ± 4.5 g	Special animal house	?	?	?
[53] Antioxidant and Anti-Inflammatory Effects of a Hypoglycemic Fraction from *Cucurbita ficifolia* Bouché in Streptozotocin-Induced Diabetic Mice	CD-1 mice	?	M	?	30–35 g	?	?	?	?
[54] Effect of *Lycium barbarum* Polysaccharide on the Improvement of Insulin Resistance in NIDDM Rats	Wistar rats	40	M	?	230–250 g	Cages	?	?	Yes
[55] Effects of Grape Seed Extract and Its Ethylacetate/Ethanol Fraction on Blood Glucose Levels in a Model of Type 2 Diabetes	C57BL/KsJ-leprdb/leprdb mice	98	M	3 w	9.7–14.2 g	Conventional state	?	7 groups (14 in each group)	?
[56] Hypoglycemic Effect of *Astragaluspolysaccharide* and Its Effect on PTP1B1	Sprague-Dawley rat	34	M	8 w	200 g	?	5	4 groups (2 groups with 10 animals and 2 groups with 12 animals in each)	?
[57] Hypoglycemic Effects of MDG-1, a Polysaccharide Derived from *Ophiopogon j aponicus*, in the ob/ob Mouse Model of Type 2 Diabetes Mellitus	ob/ob mice	?		6-7 w	?	?	?	4 groups (8 animals in each group)	?
[58] Isolation and Pharmacological Activities of the *Tecoma stans* Alkaloids	C57BL/KsJ db/db mice	?	M	8 w	?	?	?	?	?
[61] Potent Effects of the Total Saponins from *Dioscorea nipponica* Makino against Streptozotocin-Induced Type 2 Diabetes Mellitus in Rats	Wistar rats	70	M	?	190–200 g	Cages	1	7 groups (8 animals in each group)	Yes
[59] Tocopherol from Seeds of *Cucurbita pepo* against Diabetes: Validation by In Vivo Experiments Supported by Computational Docking	Albino Wistar rats	24	M	?	150–160 g	?	?	4 groups (6 animals in each group)	?
[60] *Trapa natans* L. Root Extract Suppresses Hyperglycemic and Hepatotoxic Effects in STZ-Induced Diabetic Rat Model	Albino Wistar rats	90	?	?	150–200 g	Cages	?	15 groups (6 animals in each group)	?

(c)

	Title	Plant species	Isolate/fraction	Administration	Doses used	Duration of treatment
Isolates	[25] A Polysaccharide Extract of Mulberry Leaf Ameliorates Hepatic Glucose Metabolism and Insulin Signaling in Rats with Type 2 Diabetes Induced by High Fat-Diet and Streptozotocin	Mulberry	Mulberry leaf polysaccharide	Gavage	200 mg/kg	6 days of treatment
	[26] Antidiabetic Activity of a Xanthone Compound, Mangiferin	*Anemarrhena asphodeloides* Bunge	Mangiferin	Orally	30 mg/kg	?
	[27] Anti-Diabetic Effect of a Novel N-Trisaccharide Isolated from *Cucumis prophetarum* on Streptozotocin-Nicotinamide Induced Type 2 Diabetic Rats	*Cucumis prophetarum*	N-Trisaccharide	Gavage	50, 5 mg/kg	28 days of treatment
	[28] Antidiabetic Effects of the *Cimicifuga racemosa* Extract Ze 450 *In Vitro* and *In Vivo* in ob/ob Mice	*Cimicifuga racemosa*	Ze 450	Gavage	10, 30, 90 mg/kg	7 days of treatment
	[29] Borapetoside C from *Tinospora crispa* Improves Insulin Sensitivity in Diabetic Mice	*Tinospora crispa*	Borapetoside C	Orally	5 mg/kg	4 weeks of treatment
	[30] Dehydrotrametenolic Acid Induces Preadipocyte Differentiation and Sensitizes Animal Models of Noninsulin-Dependent Diabetes Mellitus to Insulin	*Poria cocos* Wolf	Dehydrotrametenolic acid	Gavage	110 mg/kg	14 days of treatment
	[31] Effects of Ingested Fruiting Bodies, Submerged Culture Biomass, and Acidic Polysaccharide Glucuronoxylomannan of *Tremella mesenterica* Retz.:Fr. on Glycemic Responses in Normal and Diabetic Rats	*Tremella mesenterica*	Acidic polysaccharide glucuronoxylomannan (GXM)	Gavage	1 g/kg	15 days of treatment
	[32] Inhibition of Glycogen Synthase Kinase-3β by Falcarindiol Isolated from Japanese Parsley (*Oenanthe javanica*)	*Oenanthe javanica*	falcarindiol	Orally	15 mg/kg	?
	[33] Isolation and Antihyperglycemic Activity of Bakuchiol from *Otholobium pubescens* (Fabaceae), a Peruvian Medicinal Plant Used for the Treatment of Diabetes	*Otholobium pubescens*	Bakuchiol	Gavage	1, 150, 250 mg/kg	2 weeks of treatment
	[34] LBP-4a Improves Insulin Resistance via Translocation and Activation of GLUT4 in OLETF Rats	*Lycium barbarum*	*Lycium barbarum* polysaccharide (LBP-4a)	Orally	10 mg/kg	4 weeks of treatment
	[35] Meliacinolin: A Potent A-Glucosidase and A-Amylase Inhibitor Isolated from *Azadirachta indica* Leaves and In Vivo Antidiabetic Property in Streptozotocin-Nicotinamide-Induced Type 2 Diabetes in Mice	*Azadirachta indica*	Meliacinolin	Orally by gastric intubations	20 mg/kg	28 days of treatment
	[36] Novel Terpenoid-Type Quinones Isolated from *Pycnanthus angolensis* of Potential Utility in the Treatment of Type 2 Diabetes	*Pycnanthus angolensis*	Novel terpenoid-type quinones (SP-18904 and SP-18905)	Gavage	100 mg/kg	4 days of treatment
	[37] Platyconic Acid, a Saponin from *Platycodi radix*, Improves Glucose Homeostasis by Enhancing Insulin Sensitivity In Vitro and In Vivo	*Platycodi radix*	Platyconic acid (PA), platycodin D (PD), platycoside E (PE), and saponin with low activity (DPE)	Gavage	20 mg/kg	8 weeks of treatment
	[38] Rhaponticin from rhubarb Rhizomes Alleviates Liver Steatosis and Improves Blood Glucose and Lipid Profiles in KK/Ay Diabetic Mice	*Rheum franzenbachii* Munt	Rhaponticin	Orally	125 mg/kg	4 weeks of treatment
	[39] Rutin Potentiates Insulin Receptor Kinase to Enhance Insulin-Dependent Glucose Transporter 4 Translocation	*Toona sinensis*	Flavonoid rutin	Gavage	25 mg/kg	?
	[40] Type 2 Antidiabetic Activity of Bergenin from the Roots of *Caesalpinia digyna* Rottler	*Caesalpinia digyna* Rottler	Bergenin	Orally	2.5, 5, and 10 mg/kg	14 days of treatment

(c) Continued.

	Title	Plant species	Isolate/fraction	Administration	Doses used	Duration of treatment
	[41] A Study on Hypoglycaemic Health Care Function of *Stigma maydis* Polysaccharides	*Stigma maydis*	*Stigma maydis* polysaccharides	?	20 mg/kg	4 weeks of treatment
	[1] Antidiabetic Activities of Extract from *Malva verticillata* Seed via the Activation of AMP-Activated Protein Kinase	*Malva verticillata*	Ethanol extract of *M. verticillata* and N-hexane (MVE-H)	Orally	3 different concentrations of MVE-H (10, 20, or 40 mg/kg)	4 weeks of treatment
	[42] Antidiabetic Activity of Alkaloids of *Aerva lanata* Roots on Streptozotocin-Nicotinamide Induced Type-II Diabetes in Rats	*Aerva lanata* Linn.	The partially purified alkaloid basified toluene fraction (PPABTF)	Orally	10, 20 mg/kg	2 weeks of treatment
	[43] Antidiabetic Activity of *Pterospermum acerifolium* Flowers and Glucose Uptake Potential of Bioactive Fraction in L6 Muscle Cell Lines with Its HPLC Fingerprint	*Pterospermum acerifolium*	Ethyl acetate fraction (PAFEF) and subfractions PAFE1, PAFE2, and e PAFE3	Intragastric tube	200, 400 mg/kg, and 15, 30 mg/kg	30 days of treatment
Fractions	[44] Antidiabetic Activity of *Caesalpinia bonducella* F. in Chronic Type 2 Diabetic Model in Long-Evans Rats and Evaluation of Insulin Secretagogue Property of Its Fractions on Isolated Islets	*Caesalpinia bonducella* F.	*Caesalpinia bonducella* aqueous and alcoholic extracts (BM-170 and BM-171)	Orally	250 mg/kg	28 days of treatment
	[45] Antidiabetic Effect of an Acidic Polysaccharide (TAP) from *Tremella aurantia* and Its Degradation Product (TAP-H)	*Tremella aurantia*	Acidic polysaccharide (TAP) and the degradation product (TAP-H)	Orally	0.5 g/L, 1.5 g/L	10 weeks of treatment
	[46] Antidiabetic Effect of Orally Administered Conophylline-Containing Plant Extract on Streptozotocin-Treated and Goto-Kakizaki Rats	*Tabernaemontana divaricata*	The Crude Conophylline Preparation I (CCP-I)	Orally	200, 50 g/kg	15 days of treatment
	[47] Antidiabetic Effect of Total Flavonoids from *Sanguis draxonis* in Type 2 Diabetic Rats	*Dracaena cochinchinensis*	*Sanguis draxonis*(SD) and total flavonoids from SD (SDF)	Gavage	?	21 days of treatment
	[48] Antidiabetic Effects of Bitter Gourd Extracts in Insulin-Resistant db/db Mice	*Momordica charantia*	The lipid fraction, the saponin fraction, or the hydrophilic residue of bitter gourd	Orally	150 mg/kg	5 weeks of treatment

(c) Continued.

Title	Plant species	Isolate/fraction	Administration	Doses used	Duration of treatment
[49] Anti-Diabetic Effects of *Centratherum anthelminticum* Seeds Methanolic Fraction on Pancreatic Cells, B-TC6 and Its Alleviating Role in Type 2 Diabetic Rats	*Centratherum anthelminticum*	Crude methanolic fraction (CAMF)	Injected intraperi-toneally (IP) or orally	50 and 100 mg/kg	4 weeks of treatment
[17] Anti-Diabetic Effects of Polysaccharides from *Talinum triangulare* in Streptozotocin (STZ)-Induced Type 2 Diabetic Male Mice	*Talinum triangulare*	Polysaccharides obtained from *Talinum triangulare* (TTP)	Orally	150 and 300 mg/kg	2 weeks of treatment
[50] Anti-Diabetic Effects of the Acetone Fraction of *Senna singueana* Stem Bark in a Type 2 Diabetes Rat Model	*Senna singueana*	*Senna singueana* acetone fraction (SSAF)	Orally	150 mg/kg and 300 mg/kg	4 weeks of treatment
[51] Antidiabetic Potential of Polysaccharides from the White Oyster Culinary-Medicinal Mushroom *Pleurotus florida* (Higher Basidiomycetes)	*Pleurotus florida*	*P. florida* polysaccharides (PFPs)	Orally	200 and 400 mg/kg	21 days of treatment
[52] Antihyperglycemic Effects of Total Flavonoids from *Polygonatum odoratum* in STZ and Alloxan-Induced Diabetic Rats	*Polygonatum odoratum*	Total flavonoids of *Polygonatum* (P) *odoratum* (TFP)	Orally	50, 100, and 200 mg/kg	30 days of treatment
[53] Antioxidant and Anti-Inflammatory Effects of a Hypoglycemic Fraction from *Cucurbita ficifolia* Bouché in Streptozotocin-Induced Diabetic Mice	*Cucurbita ficifolia*	Aqueous-precipitate fraction (AP-fraction)	Gavage	200 mg/kg	15 days of treatment
[54] Effect of *Lycium barbarum* Polysaccharide on the Improvement of Insulin Resistance in NIDDM Rats	*Lycium barbarum*	*Lycium barbarum* polysaccharide (LBP)	Orally	10 mg/kg	3 weeks of treatment
[55] Effects of Grape Seed Extract and Its Ethylacetate/Ethanol Fraction on Blood Glucose Levels in a Model of Type 2 Diabetes	*Vitis vinifera* grape	Grape seed extract (GSE), ethylacetate (e), and ethylacetate/ethanol (ee)	Orally	50 and 30 mg/kg	8 weeks of treatment
[56] Hypoglycemic Effect of *Astragaluspolysaccharide* and Its Effect on PTP1B1	*Astragalus membranaceus*	*Astragalus polysaccharide* (APS)	Orally	400 mg/kg	5 weeks of treatment
[57] Hypoglycemic Effects of MDG-1, a Polysaccharide Derived from *Ophiopogon japonicus*, in the ob/ob Mouse Model of Type 2 Diabetes Mellitus	*Ophiopogon japonicus*	Water-soluble β-d-fructan (MDG-1)	Intragastrically	150 and 300 mg/kg	23 days of treatment
[58] Isolation and Pharmacological Activities of the *Tecoma stans* Alkaloids	*Tecoma stans*	*Tecoma stans* alkaloids	Gavage	50 mg/kg and 63.4 mg/kg	7 days of treatment
[61] Potent Effects of the Total Saponins from *Dioscorea nipponica* Makino against Streptozotocin-Induced Type 2 Diabetes Mellitus in Rats	*Dioscorea nipponica*	Saponins from *D. nipponica* Makino (TSDN)	Orally	200, 100, and 50 mg/kg	12 weeks of treatment
[59] Tocopherol from Seeds of *Cucurbita pepo* against Diabetes: Validation by In Vivo Experiments Supported by Computational Docking	*Cucurbita pepo*	Tocopherol	?	2, 5 g/kg	6 weeks of treatment
[60] *Trapa natans* L. Root Extract Suppresses Hyperglycemic and Hepatotoxic Effects in STZ-Induced Diabetic Rat Model	*Trapa natans* L.	Methanol fraction, chloroform fraction, and petroleum ether fraction	Orally	50, 100, and 200 mg/kg	15 days of treatment

(d)

	Title	Drug for diabetes induction	Route of induction	Hyperglycemia	Control of glycemia	Insulin tolerance test
Isolates	[25] A Polysaccharide Extract of Mulberry Leaf Ameliorates Hepatic Glucose Metabolism and Insulin Signaling in Rats with Type 2 Diabetes Induced by High Fat-Diet and Streptozotocin	Diet with 41.2% fat and a low-dose STZ (35 mg/kg body weight)	Intraperitoneal	Glucose ≥ 7.8 mmol/L	?	Yes
	[26] Antidiabetic Activity of a Xanthone Compound, Mangiferin	Genetically modified	—	—	?	Yes
	[27] Anti-Diabetic Effect of a Novel N-Trisaccharide Isolated from *Cucumis prophetarum* on Streptozotocin-Nicotinamide Induced Type 2 Diabetic Rats	Nicotinamide (NA) at 230 mg/kg and STZ at 65 mg/kg	Intraperitoneal	Glucose ≥ 250 mg/dL	Yes	Yes
	[28] Antidiabetic Effects of the *Cimicifuga racemosa* Extract Ze 450 *In Vitro* and *In Vivo* in ob/ob Mice	Genetically modified	—	—	?	?
	[29] Borapetoside C from *Tinospora crispa* Improves Insulin Sensitivity in Diabetic Mice	STZ 150 mg/kg	Intraperitoneal	Glucose ≥ 150 mg/dL	Yes	Yes
	[30] Dehydrotrametenolic Acid Induces Preadipocyte Differentiation and Sensitizes Animal Models of Noninsulin-Dependent Diabetes Mellitus to Insulin	Genetically modified	—	—	?	?
	[31] Effects of Ingested Fruiting Bodies, Submerged Culture Biomass, and Acidic Polysaccharide Glucuronoxylomannan of *Tremella mesenterica* Retz.:Fr. on Glycemic Responses in Normal and Diabetic Rats	STZ (65 mg/kg) and nicotinamide (200 mg/kg)	Intraperitoneal	Glucose > 250 mg/100 mL	Yes	Yes
	[32] Inhibition of Glycogen Synthase Kinase-3β by Falcarindiol Isolated from Japanese Parsley (*Oenanthe javanica*)	Genetically modified	—	—	?	Yes
	[33] Isolation and Antihyperglycemic Activity of Bakuchiol from *Otholobium pubescens* (Fabaceae), a Peruvian Medicinal Plant Used for the Treatment of Diabetes	STZ 50 mg/kg	Intravenous	Glucose 300–600 mg/dL	Yes	?
	[34] LBP-4a Improves Insulin Resistance via Translocation and Activation of GLUT4 in OLETF Rats	Genetically modified	—	—	?	Yes
	[35] Meliacinolin: A Potent A-Glucosidase and A-Amylase Inhibitor Isolated from *Azadirachta indica* Leaves and In Vivo Antidiabetic Property in Streptozotocin-Nicotinamide-Induced Type 2 Diabetes in Mice	Nicotinamide (120 mg/kg) and STZ (60 mg/kg)	Intraperitoneal	Glucose > 250 mg/dL	Yes	?
	[36] Novel Terpenoid-Type Quinones Isolated from *Pycnanthus angolensis* of Potential Utility in the Treatment of Type 2 Diabetes	STZ 150 mg/kg	Intravenous	Glucose 300–600 mg/dL	Yes	Yes
	[37] Platyconic Acid, a Saponin from *Platycodi radix*, Improves Glucose Homeostasis by Enhancing Insulin Sensitivity *In Vitro* and *In Vivo*	STZ 20 mg/kg	?	?	Yes	Yes
	[38] Rhaponticin from rhubarb Rhizomes Alleviates Liver Steatosis and Improves Blood Glucose and Lipid Profiles in KK/Ay Diabetic Mice	STZ/?	?	?	Yes	?
	[39] Rutin Potentiates Insulin Receptor Kinase to Enhance Insulin-Dependent Glucose Transporter 4 Translocation	Insulin receptor antagonist S960 (50 nmol/kg)	Intravenous injection	?	Yes	?
	[40] Type 2 Antidiabetic Activity of Bergenin from the Roots of *Caesalpinia digyna* Rottler	STZ (65 mg/kg) and nicotinamide (110 mg/kg)	Intraperitoneal injection	Glucose 200 mg/dL	Yes	Yes

(d) Continued.

	Title	Drug for diabetes induction	Route of induction	Hyperglycemia	Control of glycemia	Insulin tolerance test
Fractions	[41] A Study on Hypoglycaemic Health Care Function of *Stigma maydis* Polysaccharides	High fat-diet and low-dose alloxan (90 mg/kg)	Intraperitoneal	Glucose ≥ 10 mmol/L	Yes	?
	[1] Antidiabetic Activities of Extract from *Malva verticillata* Seed via the Activation of AMP-Activated Protein Kinase	Genetically modified	—	—	Yes	?
	[42] Antidiabetic Activity of Alkaloids of *Aerva lanata* Roots on Streptozotocin-Nicotinamide Induced Type-II Diabetes in Rats	STZ and nicotinamide	Intraperitoneal	?	Yes	?
	[43] Antidiabetic Activity of *Pterospermum acerifolium* Flowers and Glucose Uptake Potential of Bioactive Fraction in L6 Muscle Cell Lines with Its HPLC Fingerprint	STZ (60 mg/kg) and nicotinamide (120 mg/kg)	Intraperitoneal	Glucose > 250 mg/dL	Yes	?
	[44] Antidiabetic Activity of *Caesalpinia bonducella* F. in Chronic Type 2 Diabetic Model in Long-Evans Rats and Evaluation of Insulin Secretagogue Property of Its Fractions on Isolated Islets	STZ 90 mg/kg	Intraperitoneal injection	Glucose 7–12 mmol/L	Yes	Yes
	[45] Antidiabetic Effect of an Acidic Polysaccharide (TAP) from *Tremella aurantia* and Its Degradation Product (TAP-H)	Genetically modified	—	—	?	Yes
	[46] Antidiabetic Effect of Orally Administered Conophylline-Containing Plant Extract on Streptozotocin-Treated and Goto-Kakizaki Rats	Streptozotocin 60 mg/kg	Intraperitoneal	Glucose > 250 mg/dL	Yes	?
	[47] Antidiabetic Effect of Total Flavonoids from *Sanguis draxonis* in Type 2 Diabetic Rats	High fat-diet and a singular injection of streptozotocin (STZ) (35 mg/kg)	Intraperitoneal	Glucose ≥ 11.1 mmol/L	Yes	Yes
	[48] Antidiabetic Effects of Bitter Gourd Extracts in Insulin-Resistant db/db Mice	Genetically modified	—	—	?	?
	[49] Anti-Diabetic Effects of *Centratherum anthelminticum* Seeds Methanolic Fraction on Pancreatic Cells, B-TC6 and Its Alleviating Role in Type 2 Diabetic Rats	Streptozotocin (STZ) (65 mg/kg)	Intraperitoneal	Glucose 22–26 mmol/L	Yes	?
	[17] Anti-Diabetic Effects of Polysaccharides from *Talinum triangulare* in Streptozotocin (STZ)-Induced Type 2 Diabetic Male Mice	Streptozotocin (STZ) (70 mg/kg)	Intraperitoneal	?	Yes	?
	[50] Anti-Diabetic Effects of the Acetone Fraction of *Senna singueana* Stem Bark in a Type 2 Diabetes Rat Model	STZ 40 mg/kg	Intraperitoneal injection	Glucose > 18 mmol/L	Yes	?
	[51] Antidiabetic Potential of Polysaccharides from the White Oyster Culinary-Medicinal Mushroom *Pleurotus florida* (Higher Basidiomycetes)	STZ 50 mg/kg	Intraperitoneal	Glucose > 200 mg/100 mL	Yes	?
	[52] Antihyperglycemic Effects of Total Flavonoids from *Polygonatum odoratum* in STZ and Alloxan-Induced Diabetic Rats	Alloxan (100 and 120 mg/kg)	Intraperitoneal	Glucose > 11.0 mmol/L	Yes	?
	[53] Antioxidant and Anti-Inflammatory Effects of a Hypoglycemic Fraction from *Cucurbita ficifolia*Bouché in Streptozotocin-Induced Diabetic Mice	STZ 137 mg/kg	Intraperitoneal	?	?	?
	[54] Effect of *Lycium barbarum* Polysaccharide on the Improvement of Insulin Resistance in NIDDM Rats	STZ 50 mg/kg	Intraperitoneal	Glucose > 16 mmol/L	Yes	Yes
	[55] Effects of Grape Seed Extract and Its Ethylacetate/Ethanol Fraction on Blood Glucose Levels in a Model of Type 2 Diabetes	Genetically modified	—	—	Yes	?
	[56] Hypoglycemic Effect of *Astragalus* Polysaccharide and Its Effect on PTP1B1	STZ 30 mg/kg	Intravenous injection	Glucose > 6.7 mmol/L	Yes	Yes
	[57] Hypoglycemic Effects of MDG-1, a Polysaccharide Derived from *Ophiopogon japonicus*, in the ob/ob Mouse Model of Type 2 Diabetes Mellitus	Genetically diabetic model	—	—	Yes	Yes

(d) Continued.

Title	Drug for diabetes induction	Route of induction	Hyperglycemia	Control of glycemia	Insulin tolerance test
[58] Isolation and Pharmacological Activities of the *Tecoma stans* Alkaloids	Genetically diabetic model	—	—	?	Yes
[61] Potent Effects of the Total Saponins from *Dioscorea nipponica* Makino against Streptozotocin-Induced Type 2 Diabetes Mellitus in Rats	STZ 30 mg/kg	Intraperitoneal	Glucose > 16.7 mmol/L	Yes	Yes
[59] Tocopherol from Seeds of *Cucurbita pepo* against Diabetes: Validation by In Vivo Experiments Supported by Computational Docking	PX-407 solution (10 mg/kg)	?	Blood glucose level of 200 mg/dL or higher	Yes	Yes
[60] *Trapa natans* L. Root Extract Suppresses Hyperglycemic and Hepatotoxic Effects in STZ-Induced Diabetic Rat Model	STZ (45 mg/kg)	Intraperitoneal	Glucose > 200 mg/dL	Yes	?

TABLE 2: Biases analyses (ARRIVE) of the studies of the effects of fractions and isolates from the plants in the treatment of the type II diabetes.

									Isolates																				Fractions												
References	Miura, et al., 2001 [26]	Kavishankar and Lakshmidevi, 2014 [27]	Moser et al., 2014 [28]	Chen et al., 2009 [38]	Ruan et al., 2012 [29]	Sato et al., 2002 [30]	Lo et al., 2006 [31]	Yoshida et al., 2013 [32]	Zhao et al., 2014 [34]	Kumar et al., 2012 [40]	Luo et al., 1999 [36]	Perez-Gutierrez and Damian-Guzman, 2012 [35]	Kwon et al., 2012 [37]	Krenisky et al., 1999 [33]	Hsu et al., 2014 [39]	Ren et al., 2015 [25]	Agrawal et al., 2013 [42]	Zhang et al., 2013 [41]	Jeong and Song, 2011 [1]	Chakrabarti et al., 2005 [44]	Paramaguru et al., 2014 [43]	Kiho et al., 2001 [45]	Fujii et al., 2009 [46]	Chen et al., 2013 [47]	Arya et al., 2012 [49]	Klomann et al., 2010 [48]	Arya et al., 2012 [49]	Xu et al., 2015 [17]	Ibrahim and Islam, 2014 [50]	Ganeshpurkar et al., 2014 [51]	Shu et al., 2009 [52]	Zhao et al., 2005 [54]	Hwang et al., 2009 [55]	Wu et al., 2005 [56]	Xu et al., 2011 [57]	Costantino et al., 2003 [58]	Yu et al., 2015 [61]	Kharbanda et al., 2014 [60]	Roman-Ramos et al., 2012 [53]		
Accurate and concise description of the content of the article		X	X	X	X	X	X	X	X	X	X		X	X		X	X			X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	33	84.62%
Abstract																																									
Summary of the background, research objectives, methods, main findings, and conclusions	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	36	92.30%
Introduction																																									
Sufficient scientific background	X	X	X	X	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	37	94.87%
Explanation of the experimental approach and rationale		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	37	94.87%
Objectives																																									
Clear primary and second objectives	X	X	X	X			X	X	X	X	X		X	X	X	X	X	X		X	X	X	X			X	X	X	X	X	X	X	X	X		X	X	X	X	32	82.05%
Material and methods																																									
Ethical statement																																									
Nature of the ethical review permissions and institutional guidelines for the care and use of animals		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	36	92.3%
Study design																																									
Number of animals per group		X	X	X			X	X	X	X	X	X	X	X		X				X	X			X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	29	74.35%
Information on whether the experiment was performed as a blind controlled study					X			X															X				X						X				X			6	15.38%
Experimental procedures																																									
Treatment	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	39	100.00%
Dosage of treatment	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	37	94.87%
Route of administration	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	38	97.44%
Duration of treatment	X	X	X	X		X	X			X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	35	89.74%
Time of day for treatment administration		X										X						X	X	X			X	X							X					X				9	23.07%
Location used for administration of treatment																												X												1	2.56%
Rationale for choice of specific dosage					X		X		X			X	X		X					X	X		X										X			X			X	12	30.76%
Rationale for choice of specific route of administration					X		X		X																											X		X	X	6	15.38%

TABLE 2: Continued.

References	Isolates																	Fractions																							
	Miura, et al., 2001 [26]	Kavishankar and Lakshmidevi, 2014 [27]	Moser et al., 2014 [28]	Chen et al., 2009 [38]	Ruan et al., 2012 [29]	Sato et al., 2002 [30]	Lo et al., 2006 [31]	Yoshida et al., 2013 [32]	Zhao et al., 2014 [34]	Kumar et al., 2012 [40]	Luo et al., 1999 [36]	Perez-Gutierrez and Damian-Guzman, 2012 [35]	Kwon et al., 2012 [37]	Krenisky et al., 1999 [33]	Hsu et al., 2014 [39]	Ren et al., 2015 [25]	Agrawal et al., 2013 [42]	Zhang et al., 2013 [41]	Jeong and Song, 2011 [1]	Chakrabarti et al., 2005 [44]	Paramaguru et al., 2014 [43]	Kiho et al., 2001 [45]	Fujii et al., 2009 [46]	Chen et al., 2013 [47]	Arya et al., 2012 [49]	Klomann et al., 2010 [48]	Arya et al., 2012 [49]	Xu et al., 2015 [17]	Ibrahim and Islam, 2014 [50]	Ganeshpurkar et al., 2014 [51]	Shu et al., 2009 [52]	Zhao et al., 2005 [54]	Hwang et al., 2009 [55]	Wu et al., 2005 [56]	Xu et al., 2011 [57]	Costantino et al., 2003 [58]	Yu et al., 2015 [61]	Kharbanda et al., 2014 [60]	Roman-Ramos et al., 2012 [53]		
Experimental animals																																									
Information on animal species	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X		35	89.74%
Strain of the animals		X			X	X	X	X	X	X	X		X			X	X	X	X	X	X	X	X	X	X	X			X	X	X	X	X	X			X	X	X	29	74.36%
Sex of the animals	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X		X	X	X		X	X	X	X	36	92.30%
Animals weight range		X					X					X		X		X	X	X		X	X	X		X	X		X	X	X		X	X	X	X			X	X	X	22	56.41%
Age of the animals	X		X	X	X	X			X		X		X	X	X	X			X	X		X	X		X	X			X				X	X	X		X			22	56.41%
Description of genetic modification status (knock-out, transgenic, and SPF)	X		X	X					X	X	X				X			X	X			X	X					X					X	X		X	X	X	X	18	46.15%
Information related to previous procedures performed on the animals		X	X	X	X	X	X	X			X	X	X	X	X			X	X	X	X	X	X	X	X		X							X			X	X	X	25	64.10%
Housing and husbandry																																									
Housing of experimental animals (type of facility, type of cage or housing, material, and number of cage companions)			X				X	X			X	X	X			X			X		X					X	X	X	X								X			14	35.89%
Husbandry conditions (breeding programme, light/dark cycle, and temperature of water)		X	X	X	X		X	X		X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X		X	33	84.62%
Welfare-related assessments and interventions that were carried out before, during, or after the experiment							X													X	X		X														X			5	12.82%
Sample size																																									
Total number of animals used in each experiment and the number of animals in each experimental group		X	X	X			X	X	X	X		X	X			X					X		X	X	X	X	X	X	X	X		X			X		X			22	56.41%
Explanation regarding the decision of the number of animals and details of sample size calculation							X																										X							2	5.12%
Allocation of animals into experimental groups																																									
Full details of how animals were allocated to experimental groups (including randomization or matching)				X			X		X				X					X					X		X			X	X			X					X			11	28.20%
Order in which the animals in the different experimental groups were treated and assessed	X	X	X	X	X		X	X	X		X	X						X	X	X	X	X	X	X	X	X	X	X		X			X	X	X		X	X	X	28	71.79%

TABLE 2: Continued.

	Isolates																	Fractions																							
References	Miura, et al., 2001 [26]	Kavishankar and Lakshmidevi, 2014 [27]	Moser et al., 2014 [28]	Chen et al., 2009 [38]	Ruan et al., 2012 [29]	Sato et al., 2002 [30]	Lo et al., 2006 [31]	Yoshida et al., 2013 [32]	Zhao et al., 2014 [34]	Kumar et al., 2012 [40]	Luo et al., 1999 [36]	Perez-Gutierrez and Damian-Guzman, 2012 [35]	Kwon et al., 2012 [37]	Krenisky et al., 1999 [33]	Hsu et al., 2014 [39]	Ren et al., 2015 [25]	Agrawal et al., 2013 [42]	Zhang et al., 2013 [41]	Jeong and Song, 2011 [1]	Chakrabarti et al., 2005 [44]	Paramaguru et al., 2014 [43]	Kiho et al., 2001 [45]	Fujii et al., 2009 [46]	Chen et al., 2013 [47]	Arya et al., 2012 [49]	Klomann et al., 2010 [48]	Arya et al., 2012 [49]	Xu et al., 2015 [17]	Ibrahim and Islam, 2014 [50]	Ganeshpurkar et al., 2014 [51]	Shu et al., 2009 [52]	Zhao et al., 2005 [54]	Hwang et al., 2009 [55]	Wu et al., 2005 [56]	Xu et al., 2011 [57]	Costantino et al., 2003 [58]	Yu et al., 2015 [61]	Kharbanda et al., 2014 [60]	Roman-Ramos et al., 2012 [53]		
Experimental outcomes																																									
Clear experimental outcomes assessed		X	X	X	X		X	X	X	X	X	X		X							X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X	28	71.79%
Statistical methods																																									
Statistical methods used for each analysis	X	X	X	X	X		X	X	X	X			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X		X	X	X	34	87.18%
Specification of the unit of analysis for each dataset				X	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	33	84.61%
Methods used to assess whether the data met the assumptions of the statistical approach	X	X	X	X	X		X	X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X	34	87.18%
Results																																									
Baseline data																																									
Description of the animals health status, for each experimental group, before treatment	X	X	X	X					X	X								X	X	X	X	X	X	X			X		X			X		X			X			18	46.15%
Number analyzed																																									
Number or animals in each group included in each analysis (absolute numbers)							X	X		X						X										X	X		X			X	X	X			X		X	12	30.76%
Animals or data not included in the analysis (and explanation for the exclusion)																																		X						1	2.56%
Outcomes and estimation																																									
Information (mean = standard deviation)	X	X	X	X	X							X	X					X	X	X	X	X	X	X	X								X	X			X	X	X	20	51.28%
Information on quantification of inflammatory cells (mean = standard deviation)																								X																1	2.56%
Adverse events																																									
Information on mortality of experimental animal (mean = standard deviation)			X																		X											X								3	7.69%
Modifications to the experimental protocols made to reduce adverse events			X																																		X			2	5.12%
Discussion																																									
Interpretation/scientific implications																																									
Interpretation of the results, taking into account the study objectives and hypotheses	X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			X	X	X	35	89.74%
Comments on the study limitations (sources of bias and imprecision associated with the results)		X	X			X	X		X	X										X													X	X			X	X	X	12	30.76%

Table 2: Continued.

References	Isolates																			Fractions																	
	Miura, et al., 2001 [26]	Kavishankar and Lakshmidevi, 2014 [27]	Moser et al., 2014 [28]	Chen et al., 2009 [38]	Ruan et al., 2012 [29]	Sato et al., 2002 [30]	Lo et al., 2006 [31]	Yoshida et al., 2013 [32]	Zhao et al., 2014 [34]	Kumar et al., 2012 [40]	Luo et al., 1999 [36]	Perez-Gutierrez and Damian-Guzman, 2012 [35]	Kwon et al., 2012 [37]	Kremisky et al., 1999 [33]	Hsu et al., 2014 [39]	Ren et al., 2015 [25]	Agrawal et al., 2013 [42]	Zhang et al., 2013 [41]	Jeong and Song, 2011 [1]	Chakrabarti et al., 2005 [44]	Paramaguru et al., 2014 [43]	Kiho et al., 2001 [45]	Fujii et al., 2009 [46]	Chen et al., 2013 [47]	Arya et al., 2012 [49]	Klomann et al., 2010 [48]	Arya et al., 2012 [49]	Xu et al., 2015 [17]	Ibrahim and Islam, 2014 [50]	Ganeshpurkar et al., 2014 [51]	Shu et al., 2009 [52]	Zhao et al., 2005 [54]	Hwang et al., 2009 [55]	Wu et al., 2005 [56]	Xu et al., 2011 [57]	Costantino et al., 2003 [58]	Yu et al., 2015 [61]	Kharbanda et al., 2014 [60]	Roman-Ramos et al., 2012 [53]		
Generalizability /translation																																					
Comments on how the findings are likely to translate to other species or systems (relevance to human biology)		X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X					X	X	X	X	X									
Funding																																					
List of funding sources and the role of the funder(s) in the study	X	X	X	X						X			X	X	X	X	X																				
Results	17	28	31	29	24	15	33	26	28	27	23	25	27	23	20	24	21	24	22	27	29	25	32	27	27	27	29	27	28	22	21	25	32	32	16	17	38	26	30	22	28	
																																									56.41%	71.79%

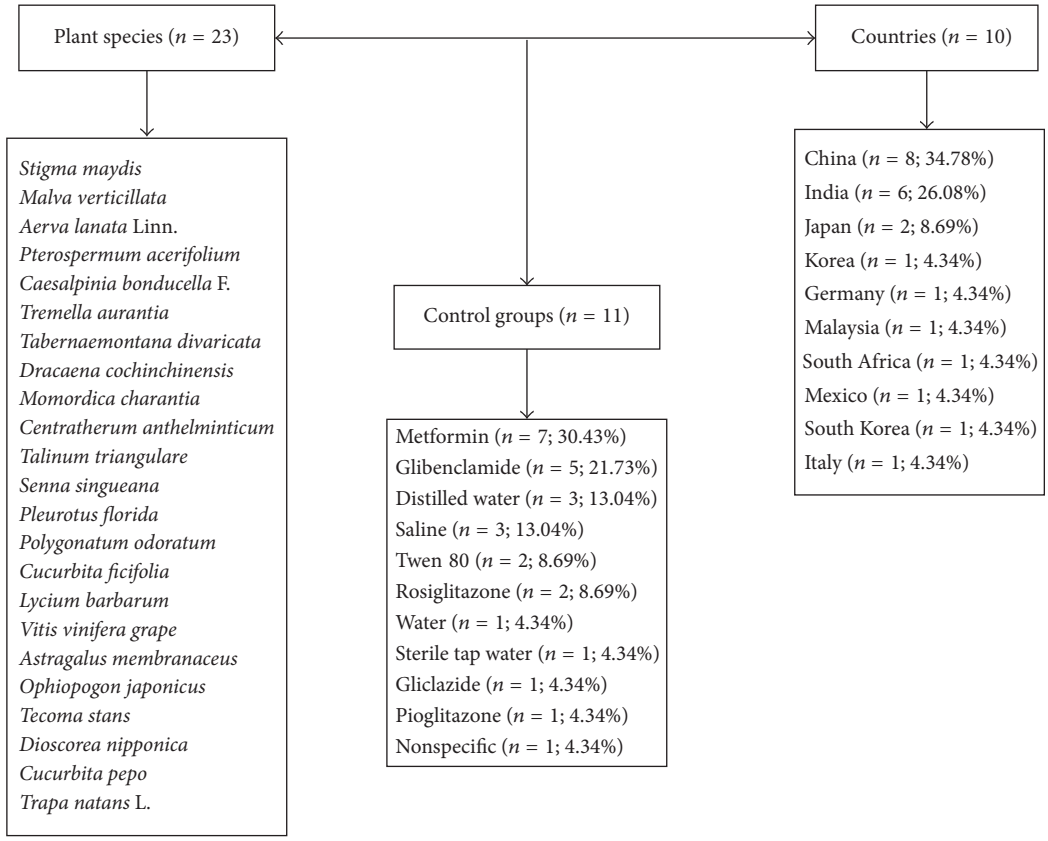

Figure 2: Summary of the articles describing the main fractions of plants, their species, families, used control groups, and the main countries where researches on this topic have been developed. Data obtained from the qualitative and ethnobotanic analysis. Flowchart fractions.

languages other than English and Portuguese (63), secondary studies, literature reviews, editorials, comments (60), *in vitro* studies (54), and studies in which alcohol was administered in the diet (35). Next, 39 studies were selected and their reference lists were screened to identify additional relevant studies missed in the initial search strategy. Thus, all the studies that met the eligibility criteria were included in the review, taking into account the use of fractions and isolates from noncommercial plants in the treatment of type II diabetes in animal models of rats and mice. All search process is shown in Figure 1.

3.2. Qualitative Results. With respect to papers reporting treatments with plant fractions ($n = 23$), the years of publication ranged from 2001 to 2015. Most studies used rats (60.9%) and mice (39.1%). The sample size varied greatly; some studies used 14 animals and others, 98 animals, while 36.4% of the publications did not report such data. Most studies used male animals, but 2 papers reported the use of both sexes, and 21.7% of the studies did not provide this information. The age of the animals ranged from 3 to 14 weeks and 56.5% of the studies did not report these data. The weight of the animals was not reported in 21.74% of the studies. Only 26.1% of the papers reported if randomization was applied in the experimental groups. Fractions of the extracts were administered orally in 91.29% of the studies and the treatment duration ranged from 7 days to 10 weeks. Regarding the drug used to induce type II diabetes, 60.86% of the studies used streptozotocin; 8.69%, alloxan; and 4.35%, PX-407 (Table 1). China (34.9%) and India (26%) are the countries with the largest number of publications on this subject. Around 47.8% of the studies used a control group. Metformin (30.5%) and glibenclamide (21.7%) were the most commonly used drugs (Figure 2).

The studies that used plant isolates in the treatment of diabetes ($n = 16$) were carried out from 1998 to 2014. Mice (56.25%), rats (37.50%), and both (6.25%) were the species used in the experiments. The sample size ranged from 12 to 102 animals, and 37.50% of the studies did not report such information. Most studies used male animals (87.50%) and 12.50% used both sexes. The age of the animals ranged from 7 to 26 weeks. The weight of the animals was not reported in 75% of the papers and the strains used were not provided in 31.25% of the studies, while 62.50% of the papers did not report animal randomization. The treatment was administered orally in all analyzed studies (100%). Diabetes was induced with the use of streptozotocin (56.3% of the studies), PEG300 (6.25%), and insulin solution (12.5%) (Table 2). Japan (23.5%) stands out among the countries that have developed studies in the area, followed by India and Taiwan (17.64%) (Figure 3).

The main results for plant fractions and isolates in the treatment of type II diabetes are shown in Figure 4. The main findings were (A) reduced blood glucose levels in isolate

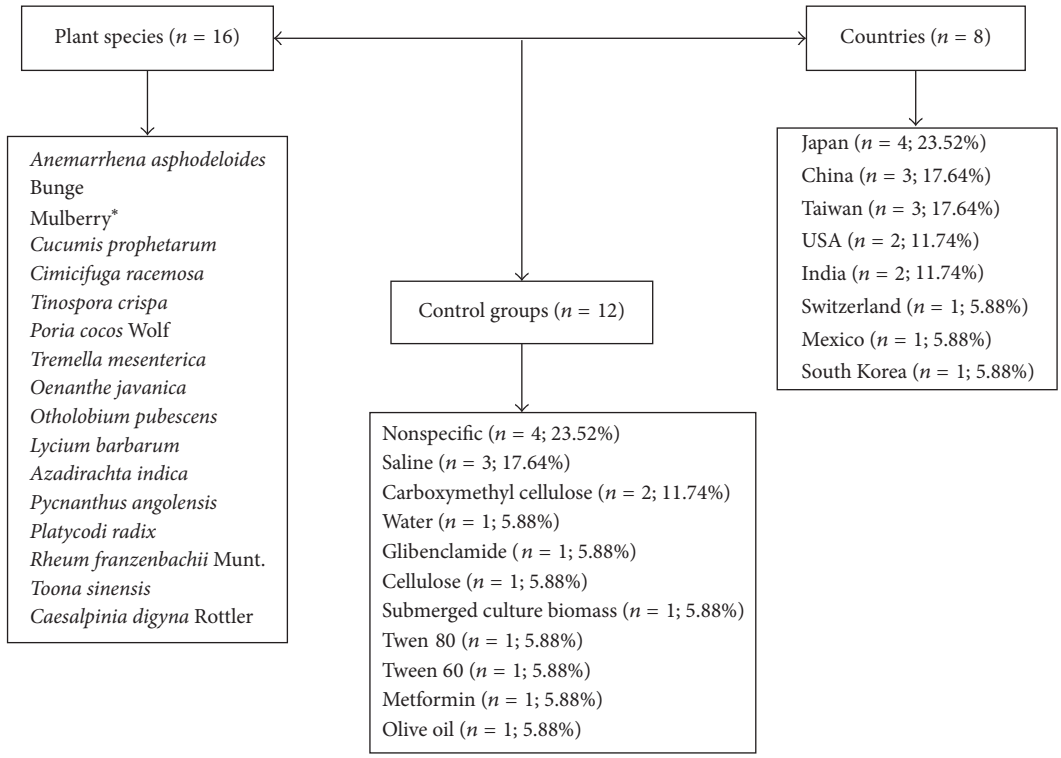

Figure 3: Summary of the articles describing the main isolates of plants, their species, families, used control groups, and the main countries where a research on this topic has been developed. Data obtained from the qualitative and ethnobotanic analysis. Flowchart isolates. *The popular name of the plant because the scientific name was not found.

treatments (25, 27, 30, 31, 32, 33, 34, 35, 36, 37, 39, and 40) and fractions (17, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, and 62); (B) normalization of body weight in studies using fractions (17, 43, 44, 49, 50, 52, 55, 57, and 59) while only two studies evaluated this parameter in isolates (27 and 34); (C) normalization of plasma insulin levels during treatment with fractions (17, 45, 47, 48, 49, 50, 51, 53, 55, 58, 60, and 61) and treatment with isolates (25, 26, 27, 28, 29, 30, 34, 36, and 38); (D) reduced total triglycerides and cholesterol in studies using fractions (17, 42, 45, 44, 46, 48, 52, 54, and 59) and isolates (31, 35, 38, and 40); (E) increased glycogen synthesis in studies using fractions (45, 51, and 60) and isolates (27, 29, and 35). Decreased water and food intake has been described in only one isolate (35) and four fractions (41, 51, 54, and 60). The normalization of glycosylated hemoglobin was observed in seven fractions (44, 49, 52, 54, 56, 60, and 61) and one isolate (27) (Figure 4).

3.3. ARRIVE (Bias Analysis). The ARRIVE guidelines were used to assess the quality of the papers under analysis (Table 1). After reading and performing critical analyses, the researchers observed that 84.61% of the studies had exact title and concise description. Abstracts describing the purpose, methods, main results, and conclusions were found in 92.30% of the studies. Primary and secondary objectives were clearly stated by 82.05% of the studies, while 92.30% reported in the methodology description that they had obtained permission from the ethics committee for performing the research; on the other hand, experimental information about controlled or blind study was observed in only 15.38%. The animal species were cited in 89.74% of the papers, while weight and sex were described in only 56.41% of the studies. It was observed that 46.15% of the publications reported genetic changes in animals, while lodgment and environmental conditions (light/dark cycle, temperature, and water) were reported in 35.89% and 84.61% of the studies, respectively. Regarding the sample size, 56,41% reported the total number of used animals, but only 5.12% explained the reason for choosing such numbers, and 28.20% of the authors reported the use of randomization. It was observed that 87.17% of the studies specified each statistical analysis method. Only 7.69% of the papers reported the occurrence of animal mortality during the experiment. Among the evaluated discussions, 89.74% interpreted the results taking into account the objectives and hypotheses of the study, current theory, and relevant publications. Only 30.76% commented about the limitations of the studies. Comments on the importance of applying the results to human biology were found in 56.41% of the studies.

4. Discussion

This review aimed to describe the main findings in literature on the effects of fractions and isolates obtained from plant extracts on the treatment of type II diabetes in murine models. We believe that the information obtained may help and provide guidance to researchers about the best animal

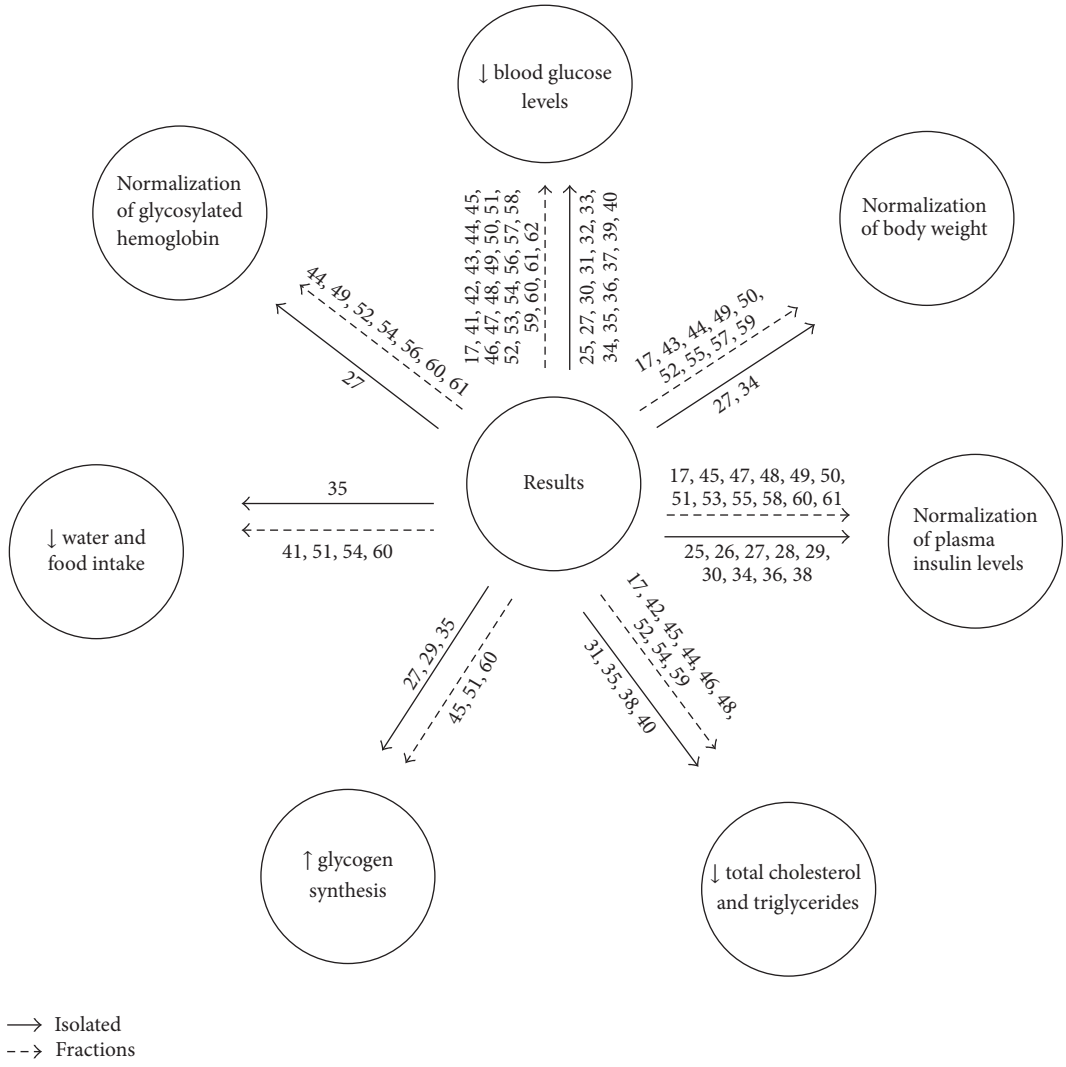

Figure 4: Main results demonstrating the action of fractions and isolates from plants on the treatment of type II diabetes. Flowchart: isolates and fractions.

models, drugs, and most used doses in disease induction. Besides, it will guide further research on the most common and important parameters to describe the best results for controlling the metabolic changes caused by the disease. Studies that tested crude plant extracts were not included in this review, due to their wide variability. Studies that obtained fractions and isolates commercially were also excluded. Although species differences prevent the direct extrapolation to clinical applications in humans, the current findings strongly point to the need for a more controlled preclinical research in animals and then in humans, mainly in relation to the doses of fractions and isolates and the most used plant species.

The present review showed that isolates or fractions of plants had positive effects on diabetes treatment and reduced various animal blood and tissue parameters that had been changed by the disease. This study also highlights important issues related to the quality of the models and protocols, drugs and doses used in the study for inducing the disease, the most commonly used administration routes, and main tests used for disorder confirmation. Systematic review studies are focused on the assessment of the quality of the reviewed studies, using acknowledged scales and protocols. Although these scales have not been formally developed for experimental model studies, the assessment of the quality of the reviewed studies considered the items normally included in scales for randomized clinical studies. Therefore, we used the ARRIVE platform for work quality analysis and observed that most studies did not provide many details about the materials and methods used, which prevents the replication of some studies. There were no reports of the number of animals used, age, weight, and even the presence of randomization to reduce bias in the selection of the animals and assessment of the results in many studies. These findings corroborate the need for guidelines to describe the required information for all scientific publications that use animals as experimental models [24]. In this study, out of the 618 articles analyzed, 39 were selected according to the eligibility criteria and the

proposed objective. The PRISMA recommendations were used to guide the development of this systematic review and improve the visualization of the steps of an effective search [22].

Most animals studied were male, since males suffer less hormonal fluctuation and hence less change in behavior compared to females [62]. The number of studies with rats ($n = 20$) and mice ($n = 19$) was very close. However, is it possible to detect the increasing use of mice in preclinical experiments, due to the genetic similarities between this species and humans. According to Machado and Zatti [63], about 99% of human genes have been mapped in mouse, which allows the association between them. Moreover, it must be taken into account that the small size of these animals reduces the costs of the experiment and makes it easy to handle and perform a great number of procedures. There was wide variation in the age of the animals. The youngest animals were 3 weeks old and the oldest, 26 weeks. In addition, many studies did not provide such information ($n = 18$). The weight of the animals ranged on average from 25.5 g in mice to 61 g in rats. The authors attribute this great variability to the discrepancies in the age of the animals. Besides, the variable weight was not reported in 38.4% of the studies. The number of studies that did not describe variables such as age and weight is worrying, since these characteristics are important for further replication of the studies and elaboration of extensive reports on the procedures adopted [64].

Streptozotocin, either combined or not with another drug, was the main drug selected for type II diabetes induction in animals. Streptozotocin is a large spectrum antibiotic, used as a diabetogenic agent in experimental animals [65]. This action is mediated by the destruction of beta cells in the pancreas, which leads to insulin deficiency and also occurs in human type II diabetes in relation to metabolic characteristics [66]. Wide variation was observed in the streptozotocin dose used to induce diabetes, from 20 mg/kg to 137 mg/kg. The analysis of works with drug-induced diabetes mainly requires the establishment of the most appropriate dose and the correct administration time, since these variables may reduce the time and costs of the experiment. In this review, after the analysis of the work, no consensus was found for the best dose and timing for drug application. Metformin was the main drug selected for the control group, due to its relevant clinical use, favorable toxicity profile, and safety. Besides, it is well tolerated during treatment [67].

Although many drugs have been used for diabetes treatment, some of them are expensive and inefficient and cause severe side effects. Thus, there is a growing interest from researchers and pharmaceutical companies in the development of alternative drugs, such as medicinal plants, for diabetes treatment [68–70]. However, further studies should be conducted, since some plants associated with diabetes mellitus treatment are considered toxic and may cause various tissue lesions [71]. The present study reports several plant species that have been used to obtain both fractions and isolates. Most studies were carried out in China, India, and Japan. These countries have shown great interest in the development of drugs from plant extracts, due to their great flora diversity. Japan has always had an interest in the development of new technologies. Phytotherapy is promising for health care in many ways. The analysis of the results of the studies revealed that most authors reported decreased blood glucose in treated diabetic animals ($n = 33$) and normalized plasma insulin levels ($n = 21$). Postprandial hyperglycemia is a common pathogenesis of type II diabetes induced by insulin resistance, as well as the partial destruction of pancreas β cells [72–75]. The effective control of blood glucose and insulin level is a key step in preventing or reversing diabetic complications and improving the life quality of patients [76]. Hyperlipidemia is another complication caused by diabetes, characterized by high cholesterol and triglyceride levels and lipoprotein composition changes [77]. These data were analyzed in this study, due to their relevance. Thirteen studies reported decreased total cholesterol and triglyceride levels in animals treated with plant derived medicine.

Besides, polydipsia, polyphagia, and changes in weight (weight loss or gain) are common occurrences in patients with diabetes [78]. Weight loss is usually observed when the disease is acquired and can be associated with dehydration and catabolism of fat tissue or protein degradation and consequent muscle mass loss. According to this review, many studies reported increased animal water and food intake, as well as weight normalization. These data can be justified by the increased glucose and insulin uptake and decreased secretion of blood glucose, which indicate improved animal glycemic control [49]. These improved results in the body weight of diabetic animals are consistent and were reported by some studies using medicinal plants with potential antidiabetic effects [79, 80]. The increased glycogen synthesis was also analyzed, since the liver metabolism of this substance regulates glucose blood level [50]. In addition, some studies reported the normalization of glycated hemoglobin levels, which is important to assess diabetes control levels, since its dosage directly reflects the average blood glucose levels, from two to three months prior to the collection of the biological material [81].

5. Conclusion

The results of this study demonstrate that plant fractions and isolates improve the main physiological and morphological changes caused by type II diabetes and decrease food and water intake, total cholesterol, triglycerides, and glucose, thus normalizing body weight and blood insulin levels. However, serious methodological problems were found in many studies, including errors in the details of the procedures performed, which prevents the understanding of some studies and hinders the use of the data found in animals for studies on human clinical condition. Therefore, the improvements in research reports on preclinical studies require a collective effort from authors, journal editors, reviewers, and funding agencies to ensure that the papers will allow other researchers to reproduce the study.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Research Article

Effect of *Betula pendula* Leaf Extract on α-Glucosidase and Glutathione Level in Glucose-Induced Oxidative Stress

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B. pendula leaf is a common ingredient in traditional herbal combinations for treatment of diabetes in southeastern Europe. Present study investigated *B. pendula* ethanolic and aqueous extract as inhibitors of carbohydrate hydrolyzing enzymes, as well as their ability to restore glutathione concentration in Hep G2 cells subjected to glucose-induced oxidative stress. Phytochemical analysis revealed presence of rutin and other quercetin derivatives, as well as chlorogenic acid. In general, ethanolic extract was richer in phenolic substances than the aqueous extract. Furthermore, a comprehensive analysis of antioxidant activity of two extracts (determined by DPPH and ABTS radical scavenging activity, total antioxidant activity, and chelating activity as well as ferric-reducing antioxidant power) has shown that ethanolic extract was better radical scavenger and metal ion reductant. In addition, ethanolic extract effectively increased cellular glutathione levels caused by hyperglycemia and inhibited α-glucosidase with the activity comparable to that of acarbose. Therefore, *in vitro* research using *B. pendula* plant extracts has confirmed their antidiabetic properties.

1. Introduction

Chronic hyperglycemia, which may arise as a consequence of diabetes or metabolic syndrome, induces oxidative stress in sensitive tissues because glucose in high concentrations forms reactive oxygen species (ROS). Elevated glucose concentrations, and thus induced oxidative damage, may adversely affect pancreatic islet β cells, leading to disturbances in insulin production and further aggravating hyperglycemic status. The harm caused by ROS causes damage and impairment of function of the classical secondary targets of diabetes, such as blood vessels, kidneys, nerves, and eyes [1], leading to cardiovascular diseases, as well as microvascular diabetic complications, including nephropathy, retinopathy, and neuropathy [2]. In addition to that, recent research has provided evidence that insulin resistance and impaired insulin signaling, typical for type 2 diabetes, may be a contributory factor to the progression of dementia and other neurological disorders [3].

Endo- or exogenous antioxidants play an important role in alleviating oxidative stress and its consequences. One of the most important cellular nonprotein antioxidants is glutathione (GSH). GSH protects cells by scavenging oxygen and nitrogen radicals and by reducing H_2O_2. GSH is also important in maintaining the concentration of other nonenzymatic antioxidants. For example, the oxidized vitamin C can be restored to the reduced form by enzymatic reaction which uses GSH as substrate [4]. Besides contributing to protection of cells against oxidative damage, GSH detoxifies xenobiotics and regulates the functions of redox-sensitive proteins [5]. However, in states of increased oxidants, production levels of glutathione in cells can deplete. Therefore, in such conditions, constant and rapid replenishment of GSH is required, which is accomplished through both the reduction of oxidized GSH and its *de novo* synthesis. However, high concentration of glucose leads to glycation of glutamate-cysteine ligase, the first enzyme of the glutathione biosynthetic pathway, thus leading to further decrease of GSH levels [6]. There is *in vitro* and clinical evidence that abnormally low levels of glutathione in cells may lead to β-cell dysfunction and the pathogenesis of long-term complications of diabetes. As a

consequence, interest has been developed in the potential for therapeutic modification of glutathione status in the treatment of diabetes. For example, changing the GSH status can be achieved by using natural antioxidants such as lipoic acid [7], curcumin, or sulforaphane [4]. Such approach could be used for development of nutraceuticals with potential in the treatment of metabolic disorder and diabetes [6].

Besides their influences on GSH content, natural substances can exert other biological activities which can be beneficial in treatment of diabetes and its complications. For example, due to their antioxidant activity, directly or via their influence on endogenous antioxidants, they can protect cellular targets and consequently the tissues which are most susceptible to diabetic complications [8]. Besides that, they can influence the enzymes which participate in carbohydrate metabolism, such as α-amylase and α-glucosidase, thus retarding the postprandial increase of glucose concentration. Alpha-amylase is an enzyme secreted in saliva and pancreatic juice which catalyzes the hydrolysis of starch to a mixture of smaller oligosaccharides, which are then degraded to glucose by α-glucosidase, enzyme located in the mucosal brush border of the small intestine. Alpha-amylase and α-glucosidase inhibitors can thus be helpful in the development of compounds for the treatment of diabetes, obesity, and hyperlipemia. Medicinal plants may constitute a good source of α-amylase and α-glucosidase inhibitors [9, 10].

Betula pendula, Roth (Betulaceae) commonly known as silver birch, is a tree native to Europe and Asia. In traditional medicine of Eastern Europe, it is used as diuretic, especially in cases of cystitis, as well as treatment for rheumatism and arthritic diseases. Recent studies have shown that its diuretic potential may stem from its endopeptidases-inhibiting properties, while xanthine oxidase inhibitory properties may be responsible for its use in gout treatment [11, 12]. Furthermore, *B. pendula* leaf extract can inhibit growth and cell division of inflammatory lymphocytes [12], as well as inhibiting tyrosinase, the enzyme that catalyzes the first stages of melanin biosynthesis. Birch leaf extract also displayed antioxidant and metal chelating properties [13].

Besides its well known use as diuretic and anti-inflammatory drug, *B. pendula* leaf is often used as part of herbal mixtures for treatment of diabetes in traditional medicine of southeastern Europe. The aim of this work was to evaluate suitability of such use by studying the inhibitory effects of *B. pendula* extracts against α-amylase and α-glucosidase, as well as to evaluate their antioxidant and GSH protecting activities in hyperglycemic conditions. An additional aim was to determine the best solvent for extraction of active principles of *B. pendula* leaf. To the best of our knowledge, this is the first time that potential use of birch extract as supplementary treatment for diabetes has been investigated.

2. Materials and Methods

2.1. Plant Materials and Chemicals. *B. pendula* leaves were bought in herbalist office in market in Gornje Kolibe, Bosnia, and Herzegovina. The specimens were identified and the voucher is deposited in the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia. Flavonoid and phenolic acid standards were purchased from Sigma-Aldrich (US). Their purity was 97% or higher. Methanol was of HPLC grade. Other reagents and chemicals were of analytical grade. Measurements were performed using Stat Fax 3200 (Awareness Technologies, USA) microplate reader and T70+ UV/Vis spectrometer (PG Instruments Ltd., GB).

2.2. Preparation of Extracts. Prior to the extraction, the dried leaves of *B. pendula* were milled and passed through a sieve of 850 μm mesh size. Powdered plant material (2 g) was suspended with 20 mL of the appropriate solvent (80% ethanol or water) in a 50 mL Erlenmeyer flask. The extraction was performed in an ultrasonic bath (Bandelin SONOREX® Digital 10 P DK 156 BP, Germany) at ultrasonication power of 720 W and frequency of 35 Hz for 30 min at 80°C. The contents of the flasks were centrifuged (30 min at 3400 rpm). The supernatant was collected and evaporated at 30°C in rotavapor (ethanolic extract) or freeze-dried (aqueous extract).

2.3. Spectrophotometric Determinations of Total Phenols, Flavonoids, and Phenolic Acids. Total phenol (TP) content in the extracts was determined by the modified Folin-Ciocalteu colorimetric method [14], while the total flavonoid (TF) content was assessed by chelation of aluminum chloride [15]. Total phenolic acids (TPA) were determined using nitrite molybdate reagent [16]. For all the determinations, the modifications were used as described previously [4]. The contents of the analyzed substances in the extracts were expressed as mg/mL from calibration curves recorded for the standards and expressed as standard equivalents. Namely, TP, TF, and TPA were expressed as gallic acid, quercetin, and caffeic acid equivalents, respectively (Table 2).

2.4. HPLC Analysis of Phenolic Acids and Flavonoids. For determination of phenolic composition, phenolic acids and flavonoids were prepared in concentration of 0.2 mg/mL in methanol while the extracts were prepared in concentration of 2 mg/mL. For hydrolysis, in 1 mL of the corresponding extract solution 400 μL 6 M HCl was added. The obtained mixtures were heated for 2 hours in water bath and then filtered to 5 mL volumetric flask. The precipitate on filter paper was washed with methanol and added to the flask contents to the volume. Phenolic acids and flavonoids were quantified using an HPLC instrument (Agilent 1200 series, Agilent Technologies, USA) equipped with an autosampler and DAD detector. Zorbax Eclipse XDB-C18 column (5 μm, 12.5 mm × 4.6 mm, Agilent, USA) and Zorbax Eclipse XDB-C18 guard column were used for separation. Before the injections, the solutions of the standards and the extracts were filtered through a 0.45 μm PTFE syringe filter. Mixture of water, methanol, and formic acid in proportions 93 : 5 : 2 (v : v : v) and 3 : 95 : 2 (v : v : v) were used as solvents A and B, respectively. Separation was performed at 40°C using following protocol: 0 min 20% B, 10 min 40% B, and 35 min 50% B. The flow rate was 1.0 mL/min. Applied volume was 10 μL or 80 μL for nonhydrolyzed or hydrolyzed samples, respectively. The peak assignment and identification were

Table 1: Calibration curve equation, limit of detection (LOD), and limit of quantification (LOQ) for the standards observed in chromatograms.

Standard	Calibration curve equation	r^2	LOD (μg/mL)	LOQ (μg/mL)
Chlorogenic acid	$y = 2587.3x + 73.42$	0.9996	0.036	0.110
Ellagic acid	$y = 5194.5x + 101.67$	0.9997	0.005	0.031
Myricetin	$y = 2245.3x + 26.53$	0.9999	0.112	0.340
Protocatechuic acid	$y = 2633.3x + 17.58$	0.9999	0.005	0.016
Quercetin	$y = 2200.2x - 36.75$	0.9998	0.027	0.083
Rutin	$y = 1255.1x + 25.37$	0.9998	0.013	0.039

y: area under curve (AUC, arbitrary units); x: concentration of the standard (μg/mL).

Table 2: Total flavonoids (TF), phenolic acids (TPA), and phenols (TP) in *B. pendula* ethanolic (BP-E) and aqueous (BP-W) leaf extract.

Extract	TF (mg/g)	TPA (mg/g)	TP (mg/g)
BP-E	45.0 ± 2.8^{A}	22.3 ± 0.5^{A}	160.4 ± 10.6^{A}
BP-W	20.1 ± 1.6^{B}	35.9 ± 1.4^{B}	80.6 ± 12.1^{B}

$^{A\text{-}B}$Differences within column (samples connected by the same capital letter are statistically different at $P < 0.05$).

based on comparison of retention times of peaks in sample chromatogram and UV spectra with those of the standards. Components were quantified according to their respective standard calibration curve at 270 nm (rutin, myricetin, ellagic acid, and protocatechuic acid) or 290 nm (chlorogenic acid). Limit of detection (LOD) and limit of quantification (LOQ) were determined according to [17] (Table 1). The content of individual phenolic compounds is presented in (Table 3).

2.5. DPPH and ABTS Radical Scavenging Activity. DPPH radical scavenging activity (DPPH RSA) and ABTS radical scavenging capacity (ABTS RSA) were evaluated as described in [18] and [19], respectively. The reactions were performed at room temperature. To the free radical solution of appropriate concentration, extract solution was added. After incubation, absorbance was read at 545 nm or 734 nm for DPPH or ABTS free radical, respectively. RSA was calculated according to the equation $\text{RSA} = (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$, where A_{control} is the absorbance of the negative control (free solution without extract) and A_{sample} is the absorbance of the free radical solution containing extract. Concentration of the extract which scavenges 50% of of free radicals present in the solution (IC_{50} DPPH RSA and IC_{50} ABTS RSA) was calculated using regression analysis. BHA and Trolox were used as standard antioxidant for DPPH RSA and ABTS RSA, respectively.

2.6. Fe^{2+} Chelating Activity. Chelating activity (ChA) of the investigated substances toward ferrous ions was studied as described in [18]. To an aliquot of the methanolic extract solution (150 μL), 0.25 mM $FeCl_2$ solution (50 μL) was added. After 5 min, the reaction was initiated by adding 1.0 mM ferrozine solution (100 μL). Absorbance at 545 nm was recorded after 10 min of incubation at room temperature. A reaction mixture containing methanol (150 μL) instead of extract solution served as a control. EDTA was used as the chelating standard. ChA was calculated using A_{control} (absorbance of the negative control, e.g., blank solution without test compound) and A_{sample} (absorbance of the substance solution). Using regression analysis, chelating activity was calculated as IC_{50} ChA, the concentration that chelates 50% of Fe^{2+} ions.

2.7. Total Antioxidant Activity. Total antioxidant activity (TAA) of extracts was determined using a spectrophotometer according to [20]. Briefly, an aliquot of 0.1 mL of sample solution was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm. Antioxidant activity was calculated based on the calibration curve of ascorbic acid and expressed as mg ascorbic acid equivalent (AAE) per g of dry weight.

2.8. Ferric-Reducing Antioxidant Power. Ferric-reducing antioxidant power (FRAP) was evaluated according to [21]. Fresh FRAP working solution was prepared by mixing 25 mL acetate buffer (300 mM), 2.5 mL of 2,4,6-tripyridyl-2-triazine solution (10 mM in 40 mM HCl), and 2.5 mL ferric chloride solution (20 mM). A mixture of 0.1 mL of extract solution was added to 0.9 mL of the FRAP solution and left in the dark at room temperature for 30 minutes. Absorbance was read spectrophotometrically at 593 nm. FRAP was calculated based on calibration curves of Trolox and expressed as mg Trolox equivalent (TE) per g of dry weight.

2.9. Determination of α-Glucosidase Inhibiting Activity. Inhibition of α-glucosidase was determined as reported earlier [22] with slight modification. In brief, 100 μL of test samples dissolved in 10% DMSO (4, 2, 1, and 0.5 mg/mL solution) was incubated with 50 μL of α-glucosidase from *Saccharomyces cerevisiae* Type I (Sigma-Aldrich, US) (1.0 U/mL dissolved in 0.1 M phosphate buffer, pH 6.8) for 10 min at 37°C. Afterwards, in reaction mixture, 50 μL substrate (5 mM *p*-nitrophenyl-α-D-glucopyranoside prepared in the same buffer) was added and release of *p*-nitrophenol was measured at 405 nm spectrophotometrically after 5 min of incubation. Individual blanks for test samples were prepared to correct background absorbance where substrate was replaced with 50 μL of buffer. Control sample contained 100 μL 10% DMSO instead of test samples. Percentage of enzyme inhibition was

Table 3: Results of HPLC analysis phenolic content in *B. pendula* ethanolic (BP-E) and aqueous (BP-W) leaf extract before and after hydrolysis.

Extract	Before hydrolysis (mg/g)		After hydrolysis		
	Rutin (mg/g)	Chlorogenic acid (mg/g)	Protocatechuic acid (mg/g)	Ellagic acid (mg/g)	Quercetin (mg/g)
BP-E	52.6	1.7	bLOQ	1.15	26.90
BP-W	25.8	1.8	28.55	2.25	23.45

bLOQ: below limit of quantification.

calculated using equation AG = $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$, where A_{control} is absorbance of the mixture without test compound (extract) and A_{sample} represents absorbance of samples containing extracts. As standard reference, acarbose was taken. Applying convenient regression analysis, IC_{50} (concentration of the test sample necessary to inhibit 50% activity of the enzyme) was obtained.

2.10. Alpha-Amylase Inhibition Assay. The assay was performed according to [23]. Extracts (25 μL) at different concentrations and 25 μL of 20 mM phosphate buffer (pH 6.9) containing porcine α-amylase (0.5 mg/mL) were preincubated at 25°C for 10 min. This was followed by addition of 25 μL 0.5% soluble starch solution in the same buffer. The reaction mixture was incubated at 25°C for 10 min and then reaction was stopped with 50 μL of 96 mM 3.5-dinitrosalicylic acid color reagent. Afterwards, the microplate was incubated in a boiling water bath for 5 min and cooled to room temperature. Absorbance was measured at 540 nm, and percent of enzyme inhibition was calculated as mentioned above. Control which represents 100% enzyme activity was prepared by replacing extract with 10% DMSO. Acarbose was used as a standard reference. The concentration of the extract needed to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis.

2.11. Cell Culture and Treatment. Human Caucasian hepatocyte carcinoma (Hep G2) cells from European Collection of Cell Cultures (ECACC) were maintained in an incubator at 37°C with a humidified atmosphere of 5% CO_2 and cultured in MEM medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 20 IU/mL penicillin, and 20 μg/mL streptomycin. The medium was refreshed twice a week. For the experiments, cells were seeded into six-well plates. Plates were changed to FBS-free medium 24 h before the assay. For induction of hyperglycemic conditions, Hep G2 cells were cultured 24 h in MEM, supplemented with additional 20 mM glucose (positive control, D). For determining the influence of *B. pendula* extracts on GSH content in hyperglycemic conditions, Hep G2 cells were treated 24 h with 20 mM glucose plus either 0.5 mg/mL (D-0.5), 0.1 mg/mL (D-0.1), or 0.05 mg/mL (D-0.05) of *B. pendula* extracts. Negative control cells (C) were kept only in MEM medium which contained 5.56 mM glucose.

2.12. Reduced Glutathione Content (GSH). Concentration of GSH was determined in hep G2 cell lysate that were treated with 20 mM glucose solution and different concentrations of extracts. GSH levels were quantified using a spectrophotometric assay, based on 2,2-dithiobisnitrobenzoic acid (DTNB or Ellman's reagent) at 37°C [24]. The production of a yellow colored 5-thio-2-nitrobenzoic acid was measured at 405 nm. Levels of GSH in treated cells were compared to negative and positive control cells.

2.13. Statistical Analysis. The experiments were performed in triplicate. The results were expressed as mean ± SD. Statistical comparisons were made using one-way ANOVA, followed by Dunnett's post hoc test and *t*-test for multiple comparisons with the control and between extracts, respectively. *P* values <0.05 were considered statistically significant. Statistical analyses were performed using the JMP version 6 from SAS software (SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1. Analysis of Total Phenols, Flavonoids, and Phenolic Acids. Chemical solvents interfere with different natural compounds yielding qualitatively and quantitatively different extracts. Those differences are inevitably reflected on antioxidant and other biological properties of the extracts [9]. Therefore, for the purpose of studying its antidiabetic activity, *B. pendula* leaf was extracted using the two most common and relatively nontoxic solvents: water and ethanol. The content of phenolic compounds in the prepared extracts is presented in Table 2. The amount of total phenols and flavonoids in ethanolic extract was approximately twofold higher than the amount of flavonoids in aqueous extracts. The amount of phenolic acids, on the other hand, was higher in the aqueous extracts. This is in line with higher lipophilicity of flavonoids in comparison to phenolic acids which makes them better soluble in relatively nonpolar solvent, ethanol.

The HPLC phytochemical analysis (Figure 1, Table 3) has confirmed that the main phenolic compound in *B. pendula* extracts is flavonoid rutin, while the other components are present in lower amounts. Similar to total flavonoids, the content of rutin was twofold higher in ethanolic than in aqueous extract. Among the phenolic acids, only chlorogenic acid was detected.

Previous studies have found that the flavonoids in *B. pendula* leaf are derivatives of myricetin, quercetin, kaempferol, apigenin, and luteolin. In those studies, quercetin derivatives were the most abundant while chlorogenic acid was present in the highest concentration along with *p*-coumaric acid derivatives [25]. In the extracts prepared in this study, however, analysis of UV spectra has shown that, besides rutin, other prominent peaks in the chromatogram (e.g., peaks

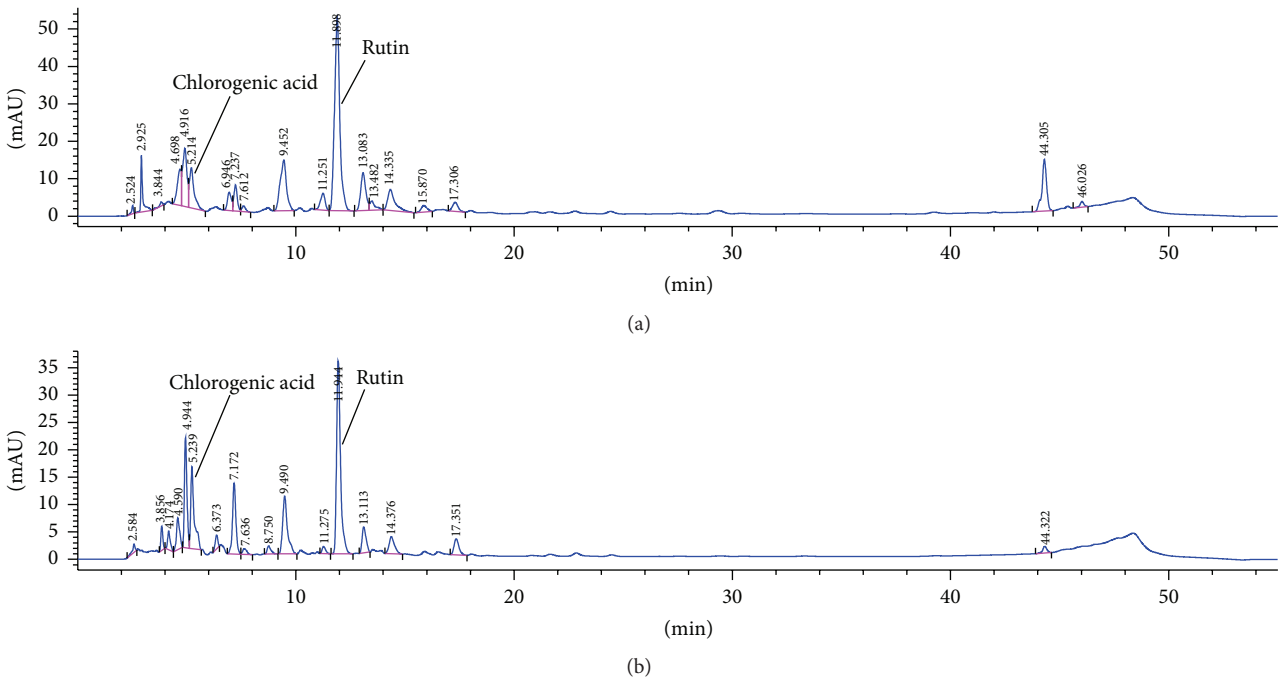

Figure 1: Chromatogram of ethanolic (a) and aqueous (b) *B. pendula* extract recorded at 320 nm.

at 9.49 min, 13.11, and 14.38 min) also belong to quercetin derivatives. This was confirmed by the analysis of the extracts subjected to acid hydrolysis. Both hydrolyzed extracts contained quercetin while a very low amount of myricetin was present only in hydrolyzed ethanolic extracts (the amount of myricetin was too low for quantification). In addition to flavonoids, protocatechuic acid, product of the flavonol degradation [26], was also detected, as well as low amount of ellagic acid. The presence of other used flavonoid aglycone and phenolic acid standards (baicalein, chrysin, hesperetin, luteolin, kaempferol, cinnamic, caffeic, chlorogenic, ferulic, rosmarinic, syringic, vanillic, and sinapic acid) was not detected in the extracts.

The three main proposed mechanisms through which antioxidants may play their protective role are hydrogen atom transfer, single electron transfer, and metal chelation [27]. The proportion of each of those mechanisms in total antioxidant activity of a herbal extract depends on various influences. Therefore, use of more than one method is recommended to give a comprehensive analysis of antioxidant efficiency of complex mixtures such as natural extracts. In the presented study, the following five assays were conducted: total antioxidant activity, DPPH and ABTS radical scavenging assay, and chelating and ferric-reducing antioxidant power assay. BHA, ascorbic acid, EDTA, and Trolox, antioxidants and ion chelators often employed in the food and pharmaceutical industry, were used as positive controls [28].

Comparison of antioxidant activities of the prepared extracts is presented in Table 4. Radical scavenging ability for DPPH free radical did not differ statistically between the two extracts, but ethanolic extract was better ABTS radical scavenger. However, as shown by markedly lower IC_{50} values, aqueous extracts were shown to be better Fe^{3+} ion chelator of metal ions than ethanolic extract. On the other hand, TAA and FRAP, methods that are based on reducing properties of the chemical species, were higher in case of ethanolic extract. Since phenolic compounds are considered to be the major compounds that contribute to the antioxidant activities of herbal extracts [29, 30], better antioxidant activity of ethanolic extract is not surprising.

The prepared *B. pendula* leaf extracts were tested for their α-glucosidase and α-amylase inhibitory properties. While the extracts did not show any inhibitory activity toward α-amylase, their α-glucosidase activity was excellent and comparable to the activity of standard, antidiabetic drug acarbose (Figure 2). Ethanolic extract whose IC_{50} value did not statistically differ from acarbose was especially active (Table 4). In an attempt to determine the phytochemicals responsible for the observed α-glucosidase inhibitory activity, the activity of rutin and chlorogenic acid have also been tested. It was previously reported that chlorogenic acid may suppress postprandial hyperglycemia in rats by inhibiting α-glucosidase [31]. However, in the concentrations present in the active amounts of extracts in this study, rutin and chlorogenic acid did not present observable α-glucosidase inhibitory activity. If we compare the results obtained in this study with the IC_{50} values of rutin and caffeic acid needed for inhibition of α-glucosidase in previously published works [32, 33], we may observe that the concentration of those phenols in the present study may not be sufficient for displaying significant inhibitory potential. However, it has been found that rutin and chlorogenic acid display significantly lower anti-α-glucosidase activity then their nonconjugated counterparts, quercetin and caffeic acid, respectively [32, 33].

Table 4: Radical scavenging activity for DPPH (IC_{50} DPPH RSA) and ABTS (IC_{50} ABTS RSA) free radical, chelating activity (ChA), total antioxidant activity (TAA), ferric-reducing antioxidant power (FRAP), and α-glucosidase activity (IC_{50} AG) of *B. pendula* ethanolic (BP-E) and aqueous (BP-W) leaf extract.

Extract	IC_{50} DPPH RSA (μg/mL)	IC_{50} ABTS RSA (μg/mL)	IC_{50} ChA (μg/mL)	TAA (mg AAE/g DW)	FRAP (mg TE/g DW)	IC_{50} AG (mg/mL)
BP-E	110.3 ± 14.3^{A}	423.5 ± 35.8^{A}	260.2 ± 26.5^{A}	164.0 ± 2.9^{A}	247.5 ± 3.4^{A}	0.60 ± 0.03^{A}
BP-W	87.5 ± 5.4^{A}	563.3 ± 18.8^{B}	118.4 ± 7.4^{B}	142.9 ± 0.9^{B}	150.9 ± 7.2^{B}	2.88 ± 0.6^{B}
Standard	$^{a}6.7 \pm 0.9^{B}$	$^{b}42.9 \pm 3.4^{C}$	$^{c}7.1 \pm 0.4^{C}$	n.a.	n.a.	$^{d}0.50 \pm 0.01^{A}$

$^{A–C}$Differences within column (samples connected by the same capital letter are statistically different at $P < 0.05$).
AAE: ascorbic acid equivalents; TE: Trolox equivalents.
Standard: ^{a}BHA; ^{b}Trolox; ^{c}EDTA; ^{d}Acarbose.
n.a.: not applicable.

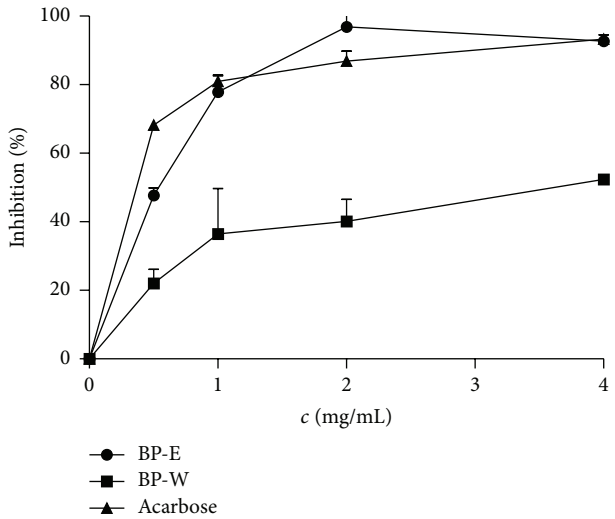

Figure 2: Alpha-glucosidase inhibitory activity of *B. pendula* extracts.

In addition, it seems that the level of glycosylation is inversely related to the inhibitory activity of quercetin derivatives [32]. It has been found that the combination of plant substances may have additive effect on α-glucosidase inhibition [34, 35]. Therefore, we may conclude that quercetin derivatives and other phenolic compounds that have been detected in the investigated extracts may play significant additive or even synergistic role in the observed α-glucosidase inhibitory activity. Besides phenolic substances, *B. pendula* leaf also contains triterpene compounds, betulin, betulinic acid, oleanolic acid, and lupeol [36]. It has been shown that oleanolic acid and related pentacyclic triterpenes may inhibit α-glucosidase *in vitro* in an uncompetitive and dose-dependent fashion with micromolar IC_{50} values [37]. Therefore, it is possible that these triterpenes significantly contribute to the observed inhibitory activity.

One of the consequences of hyperglycemia is elevated production of ROS which leads to the state of pronounced oxidative stress [1, 2]. Even though *B. pendula* extracts have previously been demonstrated to possess significant antioxidant potential in *in vitro* chemical models [38], to the best of our knowledge, birch leaf extracts have not been investigated in neither *in vivo* nor *in vitro* cellular models of diabetes. On the other hand, antioxidant and protective effects of rutin, which was the most prominent phenolic compound in the investigated extracts in our study, have been well investigated in *in vivo* models. For example, rutin can improve the antioxidant defense systems against iron overload-induced hepatic oxidative stress in rats. Such activity may be related to its antioxidant and metal chelation activities [39]. Furthermore, rutin was shown to possess neuroprotective and cardioprotective [40] effects in streptozotocin-induced diabetic models [41], as well as numerous other effects which may be beneficial in amelioration of diabetic complications [42]. Chlorogenic acid, another phenolic compound present in the investigated extracts, has well known antidiabetic properties which have been extensively reviewed [31] and linked to the observed diabetes protection of regular coffee consumption [43]. Some of its activity may be linked to antioxidant mechanisms since chlorogenic acid may ameliorate oxidative stress for renal injury in streptozotocin-induced diabetic nephropathy rats [44]. Therefore, we have aimed to investigate if *B. pendula* leaf extract can ameliorate consequences of hyperglycemia-induced oxidative stress in cellular model of diabetes.

Oxidative stress in diabetes leads to decreased level of one of the most important antioxidants in the body, GSH [7, 45]. In order to evaluate the effect of *B. pendula* leaf extract on GSH concentration, Hep G2 cells were treated with high concentration of glucose. GSH was quantified using Cayman's GSH assay kit. The ability of aqueous and ethanolic extract to reduce oxidative stress in glucose-treated Hep G2 cells was investigated (Figure 3). Level of glutathione in glucose-treated cells (negative control) was significantly lower than in nontreated cells which served as confirmation that glucose has produced the oxidative stress. At the concentrations used in the experiment, both extracts reduced oxidative stress, as seen by significantly increased levels of glutathione in comparison with negative control. In addition to that, ethanolic extract was capable of increasing GSH concentration in comparison with normal control. This finding, along with the observed excellent activity in TAA, RP, and FRAP assay, the tests based on the reducing ability of the sample, might indicate that the electron-donating properties of ethanolic extract are mostly responsible for glutathione regeneration. Due to antioxidant properties of rutin and chlorogenic acid, which have been recorded in numerous

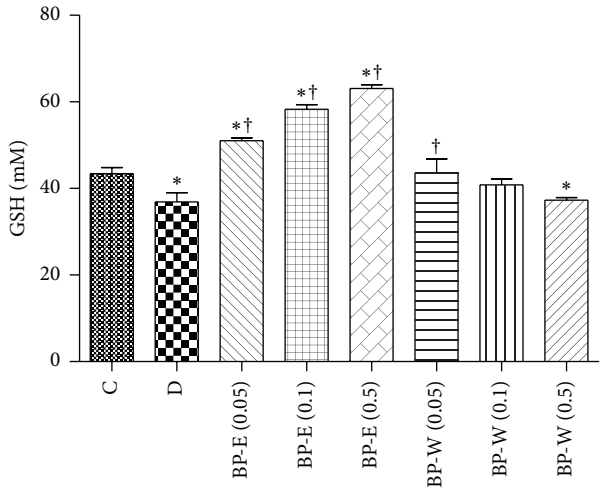

Figure 3: Glutathione (GSH) concentration in Hep G2 cells. C: cells in MEM; D: cells in MEM supplemented with 20 mM glucose; BP-E and BP-W: cells in MEM supplemented with 20 mM glucose and the corresponding *B. pendula* extract (numbers in bracket represent extract concentration in mg/mL); ∗ and †: value statistically different from C and D, respectively ($P < 0.05$, Dunnett's test). Values are average of 3 replications ± SD.

studies [39, 44, 46], it may be concluded that a significant part of the observed activity could be attributed to the presence of those antioxidants.

4. Conclusions

B. pendula extracts posses significant antioxidant and antidiabetic properties as demonstrated by several antioxidant assays, ability to increase intracellular GSH concentration, and inhibition of α-glucosidase. Solvent choice can significantly affect biological properties of herbal extracts. In this study, ethanol was able to efficiently extract more or *B. pendula* leaf bioactive principles yielding the extract with higher content of phenolic antioxidants and better α-glucosidase inhibiting and GSH regenerating properties. Some of the observed biological properties could be attributed to rutin, natural flavonoid which was the main phenolic component of the investigated ethanolic extract. Future *in vitro* and *in vivo* studies are needed to further investigate antidiabetic potential of *B. pendula* ethanolic extract and its mechanism of action.

Competing Interests

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

Acknowledgments

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Research Article

Diosgenin and 5-Methoxypsoralen Ameliorate Insulin Resistance through ER-α/PI3K/Akt-Signaling Pathways in HepG2 Cells

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To determine the effects and the underlying mechanism of diosgenin (DSG) and 5-methoxypsoralen (5-MOP), two main active components in the classical Chinese prescription Hu-Lu-Ba-Wan (HLBW), on insulin resistance, HepG2 cells were incubated in medium containing insulin. Treatments with DSG, 5-MOP, and their combination were performed, respectively. The result showed that the incubation of HepG2 cells with high concentration insulin markedly decreased glucose consumption and glycogen synthesis. However, treatment with DSG, 5-MOP, or their combination significantly reversed the condition and increased the phosphorylated expression of estrogen receptor-α (ERα), sarcoma (Src), Akt/protein kinase B, glycogen synthase kinase-3β (GSK-3β), and the p85 regulatory subunit of phosphatidylinositol 3-kinase p85 (PI3Kp85). At the transcriptional level, expression of the genes mentioned above also increased except for the negative regulation of GSK-3β mRNA. The increased expression of glucose transport-4 (GLUT-4) was meanwhile observed through immunofluorescence. Nevertheless, the synergistic effect of DSG and 5-MOP on improving glycometabolism was not obvious in the present study. These results suggested that DSG and 5-MOP may improve insulin resistance through an ER-mediated PI3K/Akt activation pathway which may be a new strategy for type 2 diabetes mellitus, especially for women in an estrogen-deficient condition.

1. Introduction

The prevalence of diabetes mellitus is increasing year by year around the world. Not only does it reduce people's quality of life, but also it leads to death for its series of complications. According to an estimate by the International Diabetes Federation, the global prevalence of diabetes mellitus among adults with 20–79 years of age was 8.3% in 2013 [1]. In the Southeast Asian region, 19.1% of all-cause mortality in 50–59-year-old men and 25.7% of all-cause mortality in 50–59-year-old women were attributable to diabetes. Menopausal women seemed to have a striking increase in the incidence of type 2 diabetes (T2DM) [2]. A large portion of healthy postmenopausal women showed decreased insulin sensitivity [3]. Therefore, we speculated that estrogen may play a vital role in maintaining glucose homeostasis. Despite abundant evidence of the validity of estrogen-containing therapies on alleviating menopausal symptoms [4], many women resorted to herbal medicines to regulate blood glucose because of side effects.

In China, herbal medicines have been used in the treatment of diabetes for thousands of years. Hu-Lu-Ba-Wan (HLBW) is a significant formula in traditional Chinese medicine described in the book *Yang Shi Jia Cang Fang*. This prescription consisting of *Trigonella foenum-graecum* and *Psoralea corylifolia* was mainly applied to ameliorate sexual dysfunction in the past. In clinic, we have found HLBW to have a positive effect on blood glucose in patients suffering from T2DM. This hypoglycemic effect has also been proved by our previous study on type 2 diabetic rats [5].

DSG (Figure 1(a)) is an important precursor of steroidal hormones and can be found in *Trigonella foenum-graecum*. Many studies have reported the beneficial effect of DSG on

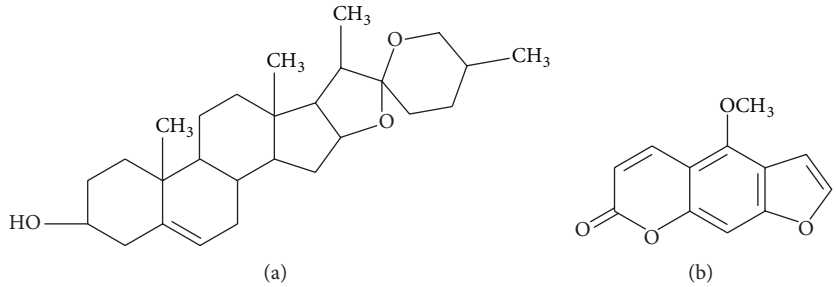

Figure 1: Chemical structure of (a) DSG and (b) 5-MOP.

the treatment of diabetes [6, 7]. 5-MOP (Figure 1(b)) is a kind of phytoestrogen that can be extracted from *Psoralea corylifolia*. Its hypoglycemic effect has also been discovered in vivo and in vitro [8, 9]. Though their estrogen-like effects have been reported [10, 11], relatively little research has examined their direct effect on glycometabolism through the estrogen receptor, which is distributed in multiple organs and mediates estrogen action.

In the human body, glucose is metabolized in specific target organs such as the liver where synthesis and breakdown of hepatic glycogen take place. Under the circumstance of insulin resistance, there is a deficiency in liver glucose uptake and glycogen synthesis, which in turn causes elevated plasma glucose. As a result, decreased insulin sensitivity eventually leads to the occurrence of T2DM. The PI3K/Akt-signaling pathway is the main downstream molecular pathway of insulin and plays an essential role in mobilizing glucose through promoting the expression and translocation of GLUT-4 [12, 13]. Therefore, the objective of this study is to determine whether there is crosstalk between the ER-mediated hypoglycemic effect of the two phytoestrogens and the classical PI3K/Akt-signaling pathway in HepG2 cells.

2. Materials and Methods

2.1. Chemicals and Reagents. Bovine serum albumin was purchased from Biological Industries Israel Beit Haemek Ltd. (Israel). Roswell Park Memorial Institute-1640 (RPMI-1640) was purchased from Hyclone Laboratories Inc. (Logan, UT, USA). DSG was obtained from Aoke Biology Research Co., Ltd. (Beijing, China). β-Estradiol was purchased from Aladdin Industrial Corporation (Shanghai, China). 5-MOP, human insulin, dimethyl sulfoxide (DMSO), and wheat germ agglutinin (WGA) dye were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Trypsin, penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kits, Western blot kit, 4′,6-diamidino-2-phenylindole (DAPI), Triton X-100, and antifade mounting medium were purchased from Guge Biological Technology Co. (Wuhan, China). Bicinchoninic acid (BCA) protein assay kit was obtained from Biosci Biotechnology Co., Ltd. (Wuhan, Hubei, China). Glucose assay kit was purchased from Beijing Applygen Technologies Inc. (Beijing, China). Hepatic glycogen assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Monoclonal antibody against PI3Kp85, ERα, and GLUT-4 were purchased from Millipore Corporation (Billerica, MA, USA). Monoclonal antibody against Akt, p-Akt (Ser473), GSK-3β, p-GSK-3β (Ser9), Src, and p-Src (Tyr-416) were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibody against p-ERα (Tyr-537) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol reagent, PrimeScript RT reagent kit, and SYBR Premix Ex Taq were purchased from TaKaRa Bio Inc. (Dalian, Liaoning, China). Fluorescent-marked second antibody was provided by LI-COR Biosciences (Lincoln, NE, USA). Stripping buffer was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Dylight 488 (Goat Anti-Rabbit IgG) and Dylight 549 (Goat Anti-Mouse IgG) were purchased from Abbkine, Inc. (Redlands, CA, USA).

2.2. Cell Culture and Treatment. HepG2 cells, provided by the Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To ensure cell viability during prolonged incubation of DSG, 5-MOP, and β-Estradiol in modified RMPI 1640 medium, cell viability was evaluated by MTT assay according to the manufacturer's protocol.

Approximately 3×10^5 cells/well were transferred into 6-well plates and allowed to grow overnight to 70% confluence. After 10–12 h starvation in RPMI-1640 medium without FBS, media in model and intervention groups were replaced by RPMI-1640 medium containing insulin (10^{-6} mol/L) for 36 h. For different intervention groups, the medium containing DSG, 5-MOP, DSG + 5-MOP, or β-Estradiol was then added, respectively.

2.3. Measurement of Glucose Content in Cell Supernatant and Intracellular Glycogen Content. After 24 h drug stimulation, the supernatant was collected to determine the glucose consumption by a glucose assay kit using the glucose oxidase method. Cells were then rinsed twice with phosphate-buffered saline (PBS). Cell suspension was obtained by trypsin digestion and then centrifuged and resuspended with normal saline three times. Cells were broken by supersonic technique (VCX150, Sonics & Materials, Newton, CT, USA). Intracellular glycogen content was measured by a hepatic glycogen assay kit using the sulfuric acid anthrone colorimetric method.

2.4. Western Blot Analysis. HepG2 cells were washed with PBS twice and lysed at 4°C with radioimmunoprecipitation assay (RIPA) buffer containing phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail. Cell debris was removed by centrifugation at 12,000 ×g for 15 min at 4°C, and the supernatant was assayed for protein concentration using the BCA method. Fifty μg protein was solubilized in SDS loading buffer and heated in boiling water for 10 min; then it was separated on 10% SDS-PAGE (120 v, 90 min) and transferred onto nitrocellulose (NC) membranes (280 mA, 90 min). The membranes then were blocked with 5% BSA powder in ultrapure water for 1 h, followed by incubation with primary antibodies (p-ERα, ERα, p-Akt, Akt, p-Src, Src, p-GSK-3β, GSK-3β, PI3Kp85, and β-actin) overnight at 4°C. The membranes were washed TBST three times for 5 min each and incubated with fluorescence-labeled secondary antibodies for 1 h at room temperature. Then the membranes were washed four times in TBST for 5 min each. For detection, the bands were visualized using a near-infrared fluorescence imaging system (Odyssey, Lincoln, NB, USA). Band densities were quantified by Image-Pro Plus (version 6.0). The result was presented as the ratio of the optical density of the phosphorylated target band to the total target or the β-actin band.

2.5. Quantitative Real-Time Polymerase Chain Reaction Analysis. Total RNA derived from each group was extracted with Trizol reagent. The purity and concentration of total RNA were measured by a nucleic acid/protein analyzer (Thermo, Rockford, IL, USA). Then 2 μg of total RNA was reverse-transcribed using a PrimeScript RT reagent kit on a Mastercycler gradient polymerase chain reaction (PCR) apparatus (Eppendorf Company, Hamburg, Germany). The total reaction volume was 20 μL. Then 2.0 μL of cDNA was amplified in a 20 μL PCR amplification reaction containing 0.4 μL forward primer, 0.4 μL reverse primer, 6.8 μL ddH_2O, 0.4 μL ROX reference dye (50x), and 10.0 μL SYBR Premix Ex Taq with an Applied Biosystems StepOne Real-Time PCR System (StepOne, Foster City, CA, USA). The whole process has three stages: Stage 1, 95°C for 30 s; Stage 2, 95°C for 5 s; and Stage 3, 60°C for 30 s. The method of $2^{-\Delta\Delta CT}$ was used for data analysis. The primer sequences are given in Table 1.

2.6. Immunofluorescence Analysis. Cells were grown on glass microscope cover slides. After being modeled by insulin for 36 h, an intervention medium was added. Then the cells were fixed in 4% buffered formalin and permeabilized with 0.5% Triton X-100. Sections were incubated with either the GLUT-4 or the ERα antibody at dilution of 1 : 500 overnight at 4°C. After washing with PBS (pH = 7.4) three times, sections were incubated with the secondary antibody at dilution of 1 : 1000 for 1 h at room temperature. Then the sections were lightly counterstained with DAPI or WGA after washing. For immunofluorescence, slides were directly mounted in antifade mounting medium and visualized in a fluorescent microscopy (NIKON ECLIPSE CI or an Olympus Confocal Microscope model FV1000 at 800 × 600 pixel resolution). Image-Pro Plus 6.0 software was used for semiquantitative analysis of immunofluorescence.

2.7. Statistical Analysis. All results were presented as mean ± standard deviation (SD) and analyzed through SPSS 19.0 software. One-way analysis of variance (ANOVA) was used to determine the statistical significance. Based on whether data assumed equal variances or not, LSD or Dunnett's T3 test was used, respectively. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of DSG and 5-MOP on Expression of ERα in HepG2 Cells by Immunofluorescence. Cells in the logarithmic growth phase were used for detecting the expression of ERα. In our study, double staining with ERα and plasma membrane revealed the presence of ERα in plasmalemma, cytoplasm, and nuclei by the use of confocal imaging. The merged images also showed that DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol led to an increase of the ER expression compared with the model group (Figure 2).

3.2. DSG, 5-MOP, and β-Estradiol for Cell Viability. Under the conditions of this study, the maximum nontoxic concentration was as follows: DSG (10^{-5} mol/L), 5-MOP (10^{-6} mol/L), and β-Estradiol (10^{-6} mol/L) (Figure 3).

3.3. Effects of DSG and 5-MOP on Supernatant Glucose Content in HepG2 Cells. Compared with the control group, culturing HepG2 cells in the presence of insulin (10^{-6} mol/L) for 36 h caused a significant decrease in supernatant glucose consumption ($P < 0.01$) (Figure 4). However, treatment with DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol led to an increased glucose consumption ($P < 0.05$, $P < 0.01$). The group treated with DSG + 5-MOP seemed to show a weaker effect on glucose consumption compared with DSG alone ($P < 0.05$). Data were verified by viable cell counts.

3.4. Effects of DSG and 5-MOP on Intracellular Glycogen Synthesis in HepG2 Cells. As shown in Figure 5, the situation of intracellular glycogen synthesis in the model group came to a low degree compared with control group ($P < 0.05$). Meanwhile, intracellular glycogen contents were remarkably increased in the DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol groups ($P < 0.05$, $P < 0.01$). In this regard, the DSG + 5-MOP group did not present a more outstanding performance compared with the two herbal monomer groups.

3.5. Effects of DSG and 5-MOP on the Expression of Proteins in ERα/PI3K/Akt-Signaling Pathway. In order to explore the mechanism of DSG and 5-MOP on improving insulin resistance, we examined the protein expression on the PI3K/Akt pathway including p-Src (Tyr-416)/Src, PI3Kp85/β-actin, p-Akt (Ser473)/Akt, and p-GSK-3β (Ser9)/GSK-3β. We also detected ERα, which might be one upstream protein of this pathway and its phosphorylation site at Tyr-537. As shown in Figure 6, in our study, treatment with either DSG, 5-MOP, or their combination all exhibited a significant increase in the protein expression ratio mentioned above compared with the model group ($P < 0.05$, $P < 0.01$). No preferable effect

Table 1: Real-time PCR primer sequences.

Gene	Forward ($5' \rightarrow 3'$)	Reverse ($3' \rightarrow 5'$)
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
ER-α	CATGAAGTGCAAGAACGTGGTG	AGGAAATGCGATGAAGTAGAGCC
Src	GAGCGGCTCCAGATTGTCAA	CTGGGGATGTAGCCTGTCTGT
PI3K p85	ACCACTACCGGAATGAATCTCT	GGGATGTGCGGGTATATTCTTC
Akt	CCTCAACAACTTCTCTGTGGCG	CACAGTCTGGATGGCGGTTG

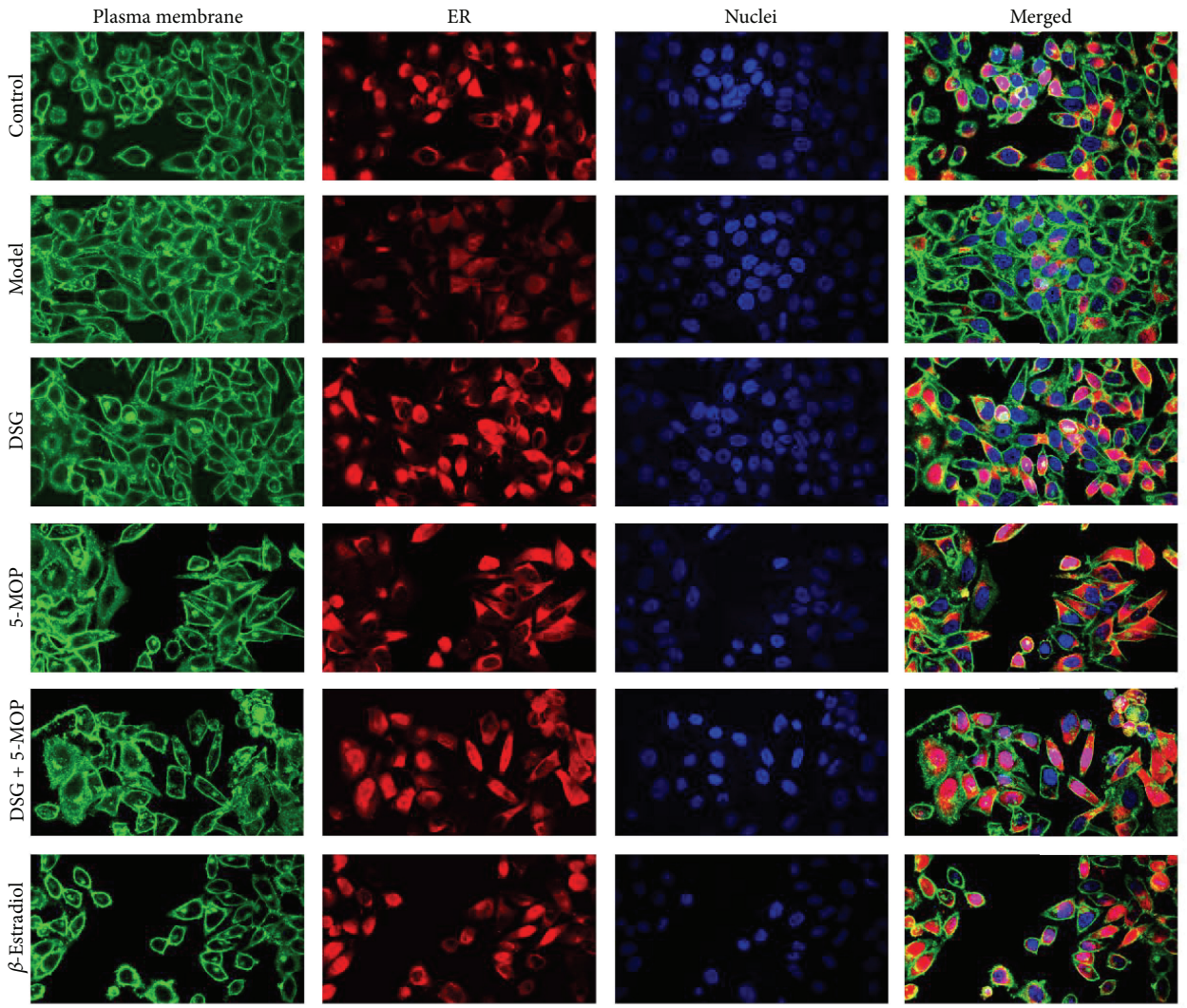

Figure 2: Effects of DSG and 5-MOP on expression of ER in HepG2 cells. HepG2 cells were stained for ER with Dylight 549 (red) and plasma membrane with WGA (green). Additionally, nuclei were stained with DAPI (blue). The merged images showed that DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol led to an increase of the ER expression compared with the model group. Images were collected using an Olympus Confocal Microscope model FV1000 at 800 × 600-pixel resolution with a 60x objective lens.

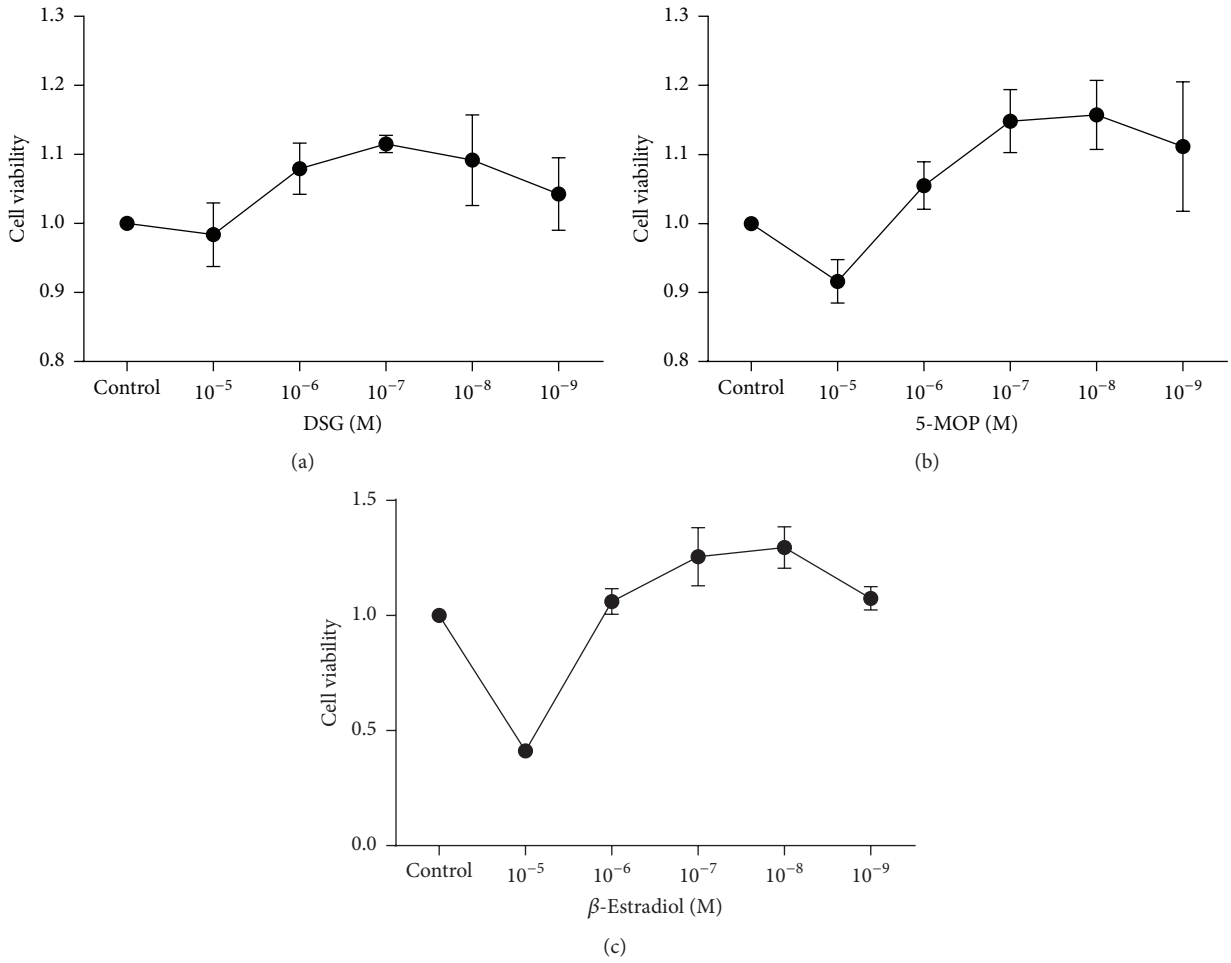

Figure 3: DSG, 5-MOP, and β-Estradiol for the cell viability. (a) DSG (10^{-5} mol/L), (b) 5-MOP (10^{-6} mol/L), and (c) β-Estradiol (10^{-6} mol/L) showed negligible effect on cell viability.

appeared when the combination group was compared with the herbal monomer group except for their influence on p-Src/Src. The combination group (DSG + 5-MOP) led to more increase on p-Src/Src compared with the herbal monomer group ($P < 0.01$). Interestingly, β-Estradiol seemed to have no effects on the expression of p-Akt/Akt compared with the model group. This was different from its influence on other proteins, which showed a significant increase ($P < 0.01$).

3.6. Effects of DSG and 5-MOP on Gene Expression at the Transcriptional Level in ERα/PI3K/Akt-Signaling Pathway. To detect the effects of DSG and 5-MOP on gene expression at the transcriptional level, we conducted RT-PCR. As shown in Figure 7, in our study, treatment with DSG, 5-MOP, their combination, and β-Estradiol all showed an increase in the gene transcription of ERα, Src, PI3Kp85, and Akt compared with the gene levels in the model group ($P < 0.05$, $P < 0.01$). On the contrary, negative regulation gene Gsk-3β displayed a decreasing expression after intervention ($P < 0.05$, $P < 0.01$). Also we found no difference between the combination group (DSG + 5-MOP) and their monomer group with regard to their promoting effects on gene transcription. Interestingly, we also found that the effect of DSG was better than the combination of DSG + 5-MOP on promoting the transcription of Akt ($P < 0.05$).

3.7. Effects of DSG and 5-MOP on Expression of GLUT-4 in HepG2 Cells. To determine the effects of DSG and 5-MOP on the expression of GLUT-4, cells were evaluated by immunofluorescence after incubating with DSG, 5-MOP, DSG + 5-MOP, or β-Estradiol for 24 h. As shown in Figure 8, the expression of GLUT-4 had a significant increase in the treated groups compared with the model group ($P < 0.05$, $P < 0.01$) though no superior effect of the DSG + 5-MOP group appeared. On the contrary, the 5-MOP group showed a more prominent influence on increasing the content of GLUT-4 than did the DSG + 5-MOP group ($P < 0.05$).

4. Discussion

Diabetes mellitus is a chronic metabolic disorder caused by either impaired insulin secretion or a reduction in its biological effectiveness. T2DM is the predominant form of diabetes mellitus characterized by insulin resistance and it accounts

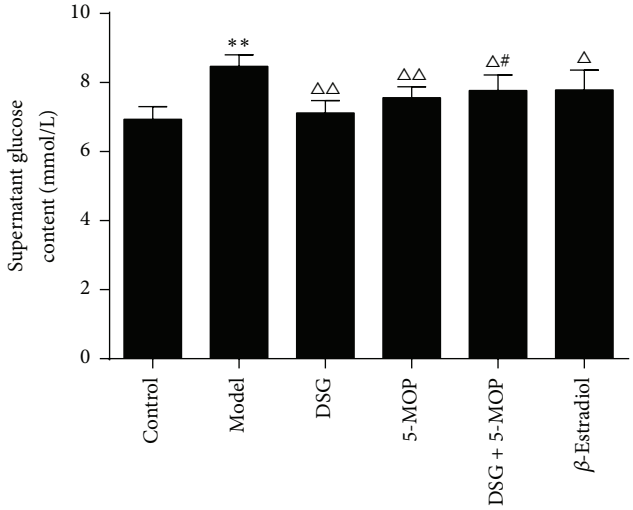

Figure 4: Effects of DSG and 5-MOP on supernatant glucose content in HepG2 cells. DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol led to an increased glucose consumption. $^{**}P < 0.01$: significance from control group. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$: significance from model group. $^{\#}P < 0.05$: significance from DSG-treated group. Each bar represents mean ± SD from three wells. Data were verified by viable cell counts.

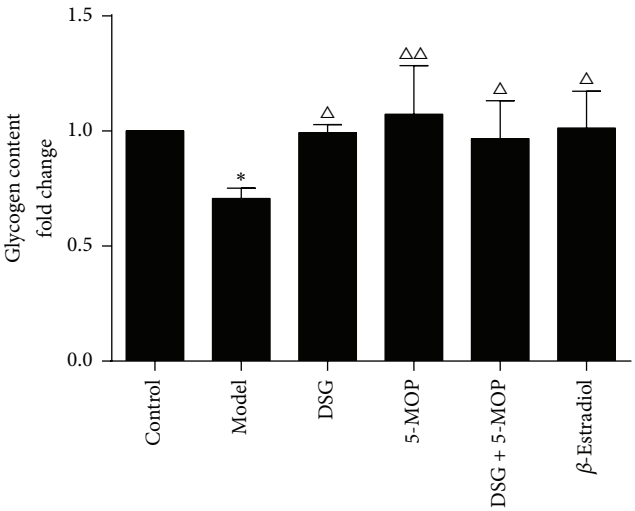

Figure 5: Effects of DSG and 5-MOP on intracellular glycogen synthesis in HepG2 cells. DSG, 5-MOP, DSG + 5-MOP, and β-Estradiol led to an increased intracellular glycogen content. $^{*}P < 0.05$: significance from control group. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$: significance from model group. Each bar represents mean ± SD from three wells.

for 90%–95% of the diabetic population [14]. In physiological conditions, the liver provides a main adjustment for glucostasis through synthesis and decomposition of glycogen, oxidative decomposition of glucose, and gluconeogenesis. When the liver works inappropriately, such as having a low sensitivity to insulin, it may lead to a fluctuation of blood glucose. Thus, improving the insulin resistance of the liver is an important direction for the treatment of T2DM.

In China, herbal medicines have been widely used as an alternative approach for treating T2DM [15]. Plants provide a vast treasure of natural products used as a primary source of medicine. *Trigonella foenum-graecum* and *Psoralea corylifolia* are two common drugs used for T2DM due to their yang-tonic effect. The main ingredients, DSG and 5-MOP, were chosen as the objectives in our study with the method of component compatibility [16]. In previous studies, DSG and 5-MOP showed multiple activities against glycometabolism disorder [17, 18]. Several studies attributed DSG's hypoglycemic effect to its regulation of metabolism-related enzymes [19, 20], its interaction with various target molecules, and the related signaling pathways [21, 22]. A recent study [23] explored the potential of DSG in the management of diabetes by ameliorating oxidative stress. An exploration of the mechanism of 5-MOP's effects on glucose control was also conducted, including its role in the inhibition of protein tyrosine phosphatase 1B [9] and oxidative stress [8]. However, the molecular mechanism concerning the effect of DSG and 5-MOP on glucose metabolism was far from being completely understood. Considering the estrogen-like effects of DSG and 5-MOP, we further explored the probable role of ER signaling in the regulation of glycometabolism in conjunction with the PI3K/Akt-signaling pathway, the direct signaling pathway that regulates glucose metabolism.

In recent years, the PI3K/Akt pathway has gained recognition for its role in metabolism regulation. Based on both in vivo and in vitro studies, PI3K is required for insulin-induced glucose uptake and the inhibition of glucose production [24, 25]. The PI3K regulatory isoform p85α gene has been reported in connection with increased risk of developing T2DM [26]. Many of the metabolic effects, including regulating glycometabolism, require activation of the PI3K downstream target Akt. Complete activation of Akt requires phosphorylation of ser473 at the C terminal. Constitutively active Akt induces translocation of the GLUT-4 to the plasma membrane to promote glucose uptake and also phosphorylate GSK-3β to increase glycogen synthesis [27, 28]. Src is an upstream protein of Akt. Phosphorylated Src at Tyr416 can activate Akt to complete the subsequent signal transduction [29]. A study conducted by Haynes et al. [30] indicated a complex of ER, c-Src, and PI3Kp85, which brought our attention to the ER-initiated membrane-localized steroid hormone receptor-signaling pathway. Therefore, we supposed the probable mechanism of β-Estradiol induced PI3K/Akt activation in HepG2 cell (Figure 9).

Our laboratory results showed that both DSG and 5-MOP could ameliorate insulin resistance as a result of accelerating glucose utility and intracellular glycogen synthesis. This phenomenon was accompanied by phosphorylation of ERα, Src, PI3Kp85, Akt, and Gsk-3β. Measurement of gene transcription was also conducted with a resultant increase in ER, Src, PI3Kp85, and Akt, but not for Gsk-3β after treatment by DSG or 5-MOP. Gsk-3β is a negative regulation molecule downstream of the PI3K/Akt pathway. After treatment, the gene expression of Gsk-3β decreased. Also, by means of immunofluorescence, we detected an increase of ER and

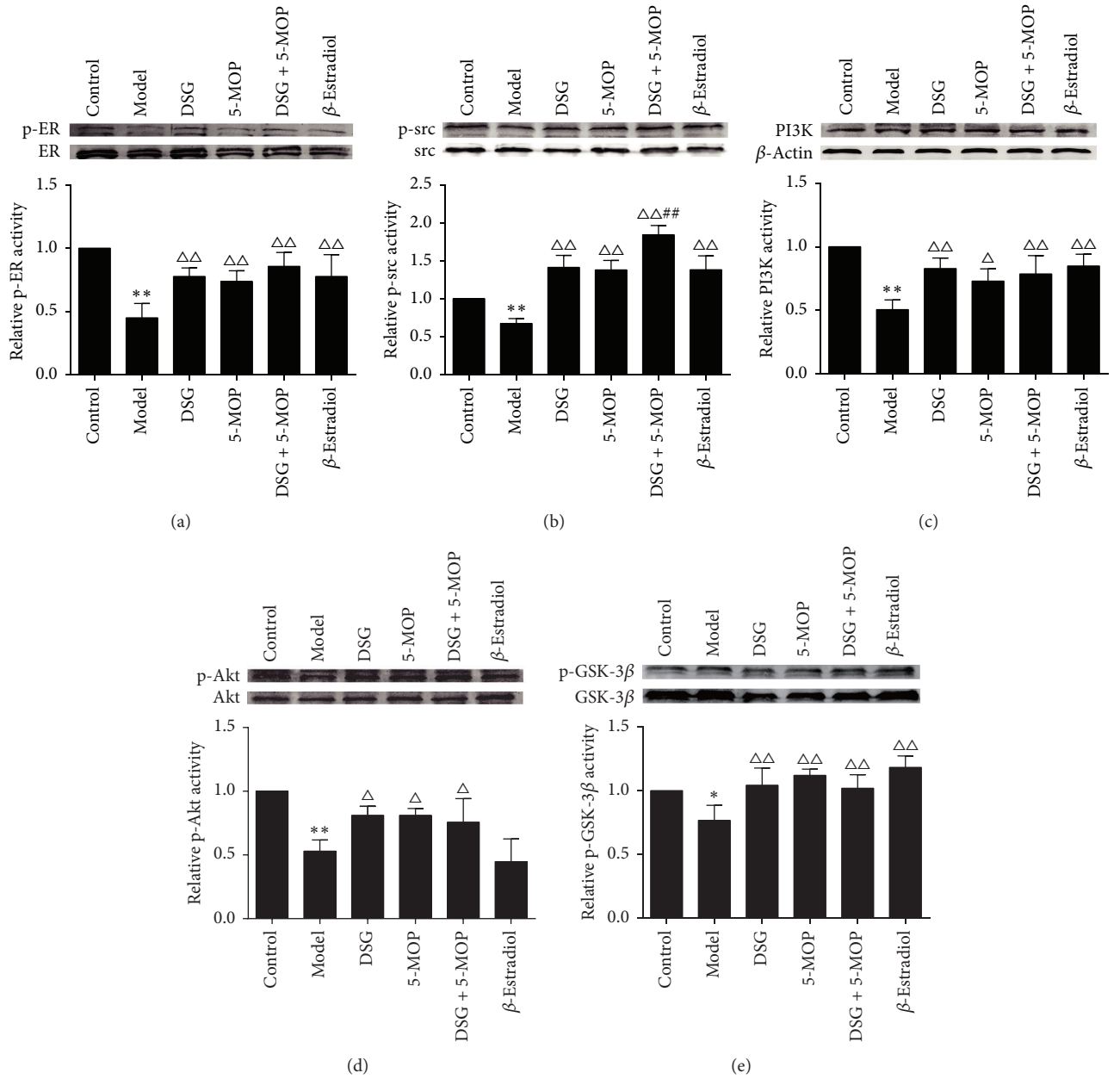

Figure 6: Effects of DSG and 5-MOP on the expression of proteins in PI3K/Akt pathways involving ERα. Representative protein levels for (a) p-ER (Tyr-537)/ERα, (b) p-Src (Tyr-416)/Src, (c) PI3Kp85/β-actin, (d) p-Akt (Ser473)/Akt, and (e) p-GSK-3β (Ser9)/GSK-3β. Each bar represents mean ± SD from three wells. $^{*}P < 0.05$ and $^{**}P < 0.01$: significance from control group. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$: significance from model group. $^{\#\#}P < 0.01$: significance from DSG-treated group.

GLUT-4, a sign of increased glucose utilization. However, the synergistic effect of DSG and 5-MOP on insulin resistance was not obvious in this study. DSG, a plant-derived steroid, serves as a precursor of various natural or synthetic steroidal hormones. It has a similar structure with estrogen, the primary female sex hormone, as discussed in our manuscript. As a mimic, we assumed that DSG might bind to the allosteric site of estrogen receptor immediately and reach a saturation state. In this case, the binding of DSG reduced the affinity of the estrogen receptor for 5-MOP, resulting in convulsions due to lessened inhibition of the estrogen mimic. Without DSG, 5-MOP would potentially exert an effect by activating the estrogen receptor. This may be the reason that no synergistic effect occurred when combining these two compounds. Nevertheless, the exact mechanism requires further investigation.

There are still some limitations of our work. In the present study, no direct evidence was given to certify the linkage

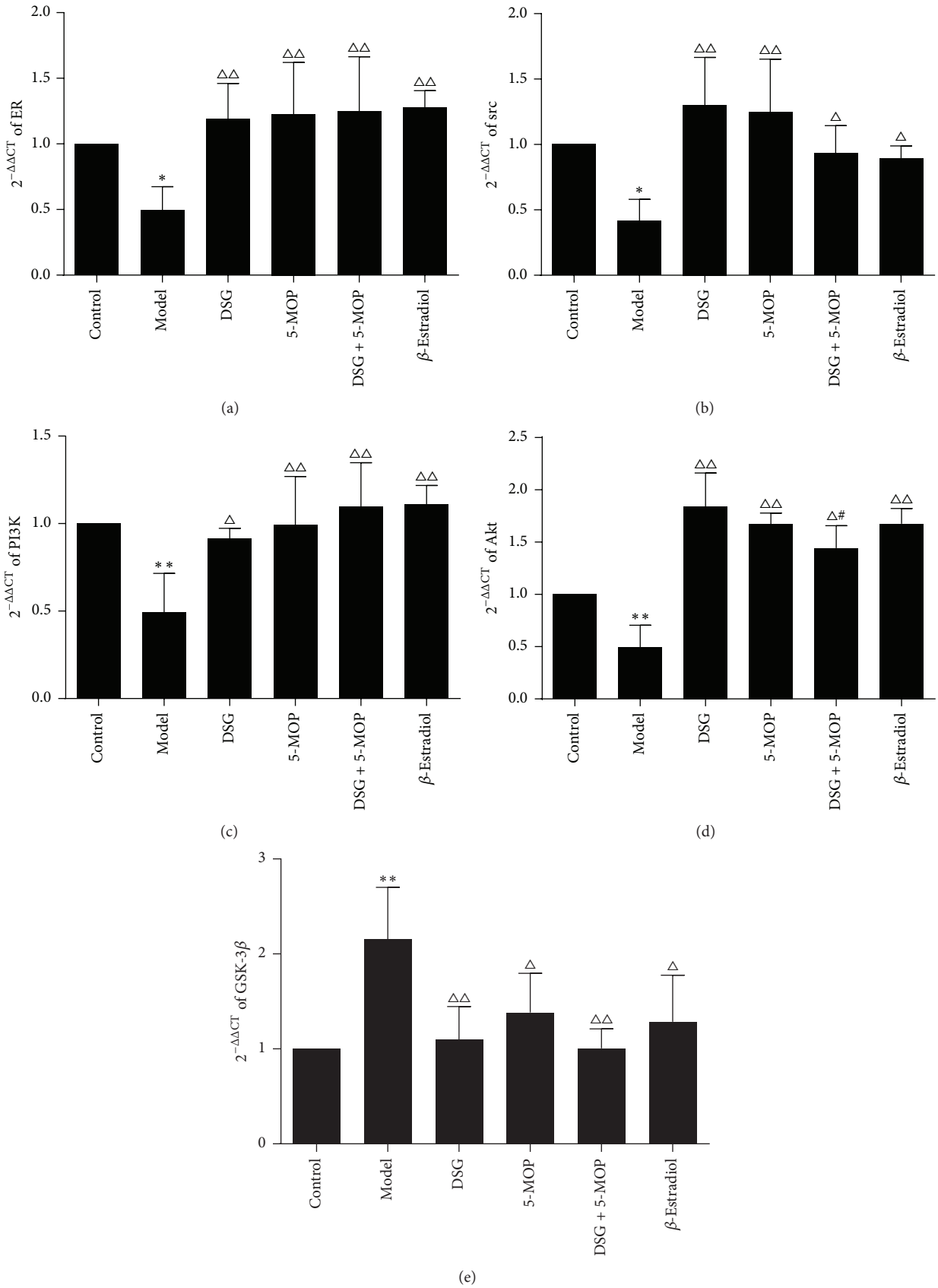

Figure 7: Effects of DSG and 5-MOP on gene expression at the transcriptional level in PI3K/Akt pathways involving ERα. Representative mRNA levels for (a) ERα, (b) Src, (c) PI3K p85, (d) Akt, and (e) GSK-3β. Each bar represents mean ± SD from three wells. $^{*}P < 0.05$ and $^{**}P < 0.01$: significance from control group. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$: significance from model group. $^{\#}P < 0.05$: significance from DSG-treated group.

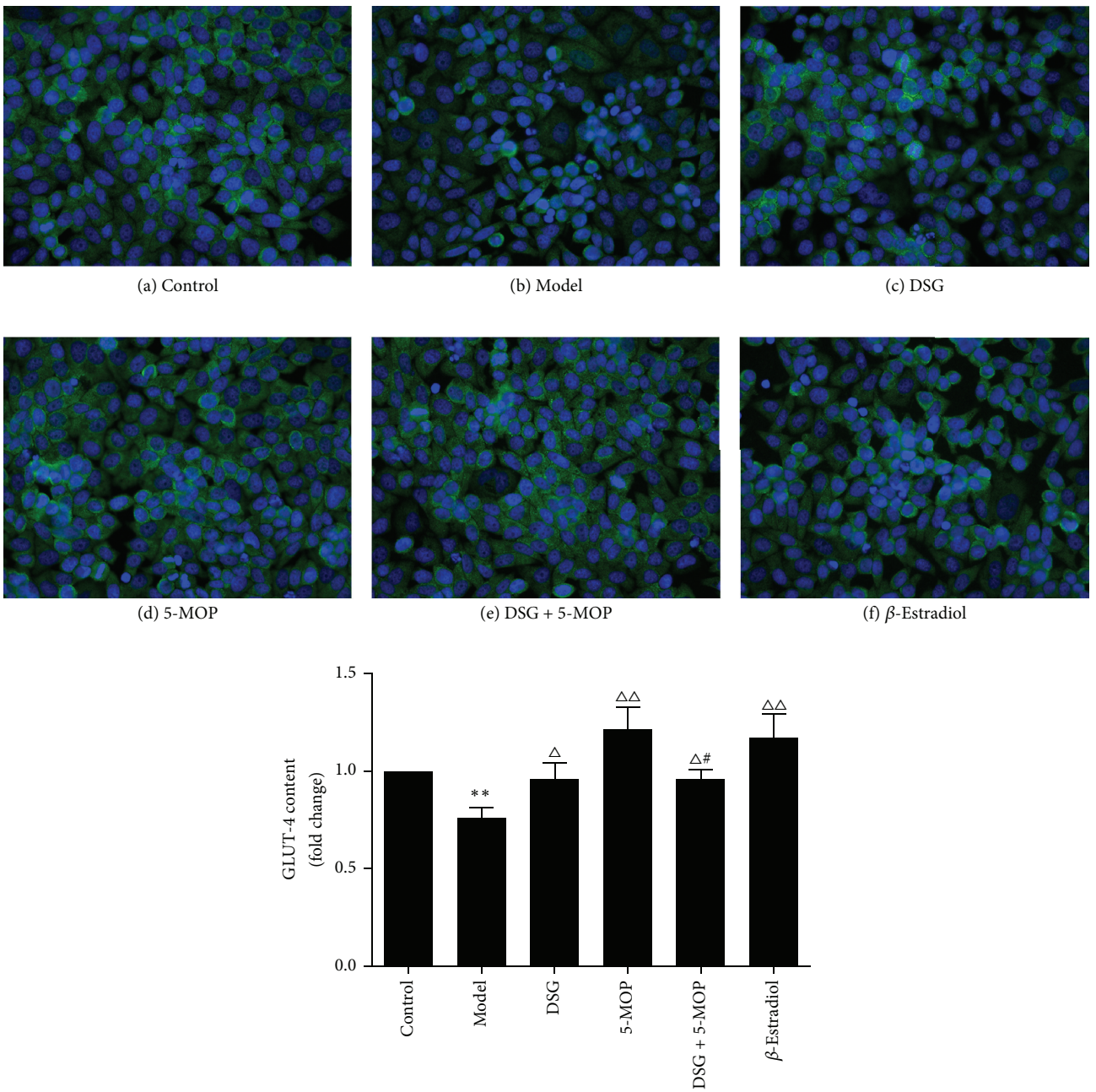

Figure 8: Effects of DSG and 5-MOP on expression of GLUT-4 (green) in HepG2 cells. Nuclei were also stained with DAPI (blue). Immunofluorescence showed that (c) DSG, (d) 5-MOP, (e) DSG + 5-MOP, and (f) β-Estradiol led to an increase of the GLUT-4 expression compared with the (b) model group. Each bar represents mean ± SD from three wells. $^{**}P < 0.01$: significance from control group. $^{\triangle}P < 0.05$ and $^{\triangle\triangle}P < 0.01$: significance from model group. $^{\#}P < 0.05$: significance from 5-MOP-treated group.

between ERα and the PI3K pathway. Therefore further profound research is required.

In conclusion, our in vitro study suggests that DSG and 5-MOP may improve insulin resistance through an ERα-mediated PI3K/Akt activation pathway that implicates a novel therapeutic approach using this natural product in the treatment of metabolic diseases such as T2DM.

Abbreviations

HLBW: Hu-Lu-Ba-Wan
DSG: Diosgenin
5-MOP: 5-Methoxypsoralen
ER: Estrogen receptor
GLUT-4: Glucose transporter-4
T2DM: Type 2 diabetes mellitus.

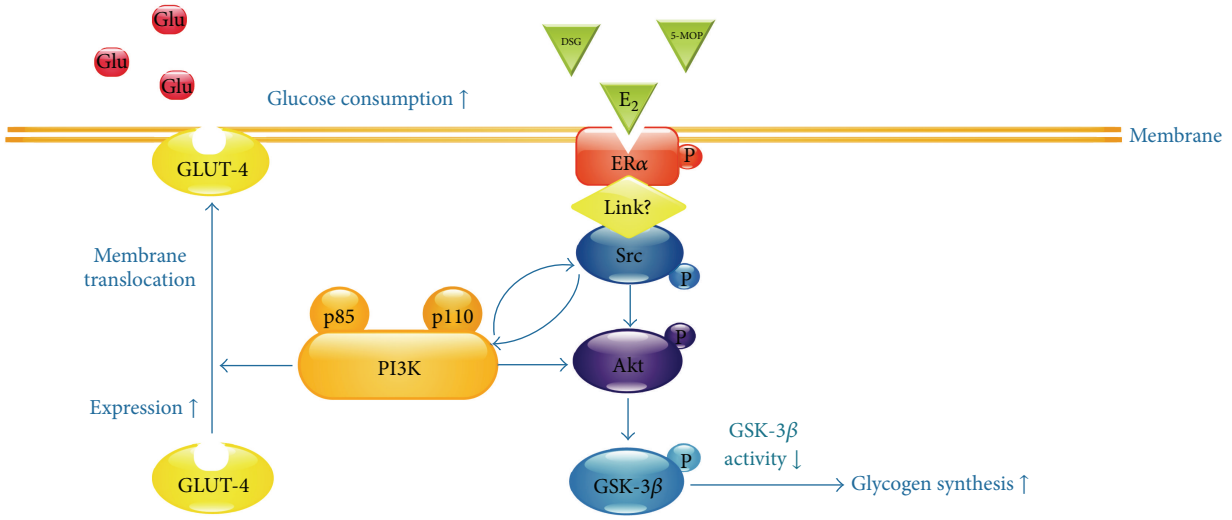

Figure 9: Assumed mechanism for β-Estradiol induced PI3K/AKT activation in HepG2 cells.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Review Article

Euonymus alatus: A Review on Its Phytochemistry and Antidiabetic Activity

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Euonymus alatus (E. alatus) is a medicinal plant used in some Asian countries for treating various conditions including cancer, hyperglycemia, and diabetic complications. This review outlines the phytochemistry and bioactivities of *E. alatus* related to antidiabetic actions. More than 100 chemical constituents have been isolated and identified from *E. alatus*, including flavonoids, terpenoids, steroids, lignans, cardenolides, phenolic acids, and alkaloids. Studies *in vitro* and *in vivo* have demonstrated the hypoglycemic activity of *E. alatus* extracts and its certain constituents. The hypoglycemic activity of *E. alatus* may be related to regulation of insulin signaling and insulin sensitivity, involving PPARγ and aldose reductase pathways. Further studies on *E. alatus* and its bioactive compounds may help to develop new agents for treating diabetes and diabetic complications.

1. Introduction

Euonymus alatus (E. alatus) is a medicinal plant used traditionally in many Asian countries, including China and Korea, for treating various conditions. It has long been used in China as a Chinese Materia Medica for pain and menstrual disorders. The first record of its clinical use in China was documented in *Shen Nong Ben Cao Jing* (The Classic of Herbal Medicine) written between 300 BC and 200 AD. *Ben Cao Gang Mu* (Compendium of Materia Medica, AD1578, written by Li Shizhen) later recorded its applications for vaginal bleeding, abdominal distention, and detoxification, and *Ben Cao Jing Ji Zhu* (Collective Notes to Canon of Materia Medica) recorded its use for abdominal pain, killing worms, and eliminating skin swelling caused by various reasons [1]. The interest in *E. alatus* has been increased recently largely due to the research on its bioactivities against cancer and diabetes. Recent studies have demonstrated a wide range of bioactivities of *E. alatus*, including hypoglycemic, antihypertensive [2], antitumor [3, 4], sedative [2], and regulation of blood lipid [5, 6] and immune functions [7]. There is also clinical evidence for its efficacy against hyperglycemia [8], chronic nephropathy [9], rheumatoid arthritis [10], cor pulmonale [11], bronchial asthma [12], anaphylactic disease [13, 14], urinary tract infection, and prostate diseases [15]. This short review outlines the phytochemistry of *E. alatus* and its pharmacology related to antidiabetic actions.

2. Phytochemistry

More than 128 chemical constituents have been isolated and identified from *E. alatus*. The main chemical classes include flavonoids, terpenoids, steroids, lignans, cardenolides, phenolic acids, and alkaloids.

2.1. Flavonoids. A total of 26 flavonoids have been isolated and identified from *E. alatus*. The main structure types include flavonoid, flavanone, and flavonol. The aglycones of flavonoid glycosides isolated from *E. alatus* include quercetin, kaempferol, naringenin, aromadendrene, and dihydroquercetin. The flavonoids are mainly distributed in the leaves and wings of *E. alatus* [16]. The structures of main flavonoids identified in *E. alatus* are listed in Tables 1–4.

Table 1: Quercetin and glycosides in *E. alatus*.

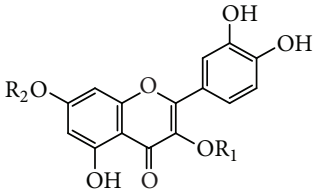
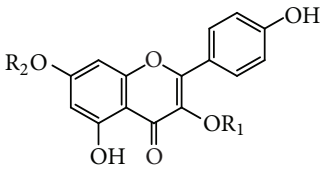
Skeleton	Number	Name	R_1	R_2	Reference
	1	Quercetin	H	H	[24, 39, 48–50]
	2	Quercitrin	α-L-Rhamnose	H	[17, 49]
	3	Quercetin-7-O-α-L-rhamnoside	H	α-L-Rhamnose	[50]
	4	Quercetin-3,7-O-α-L-dirhamnoside	α-L-Rhamnose	α-L-Rhamnose	[17, 50]
	5	Quercetin 3-D-galactoside (hyperin)	3-D-Galactose	H	[17, 20]
	6	Rutin	α-L-Rhamnopyranosyl-(1 → 6)-β-D-glucopyranose	H	[39]

Table 2: Kaempferol and the glycosides in *E. alatus*.

Skeleton	Number	Name	R_1	R_2	Reference
	7	Kaempferol	H	H	[18, 21, 48, 49]
	8	Kaempferol-7-O-α-L-rhamnoside	H	α-L-Rhamnose	[50]
	9	Kaempferol-3,7-O-α-L-rhamnoside (kaempferitrin)	α-L-Rhamnose	α-L-Rhamnose	[17, 50]
	10	Kaempferol-7-O-β-D-glucoside	H	α-L-Glucose	[50]
	11	Apigenin-3-O-L-rhamnopyranoside	α-L-Rhamnose	H	[49]

Other flavonoids include catechin (19) [17–21], symplocoside (20) [17], quercetin-3-galactosyl-xyloside (21) [20], catechin lactone A (22) [17], dehydrodicatechin A (23) [17–21], 3-hydroxycoumarinflavanol (24), 7,4′-dihydroxy-8-C-glucoxylisoflavone (25) [22], and 5-hydroxy-6,7-dimethoxyflavone (26) [23].

2.2. Steroids. Eight steroids including sterols and sterones have been isolated and identified from *E. alatus*. The main structures of the steroids are shown in Table 5. Other steroids include 24R-methyllophenol (34) and α-spinasterol (35) [22].

2.3. Terpenoids. The main terpenoids isolated from *E. alatus* include triterpenes and sesquiterpenes.

2.3.1. Triterpenes. Multiple types of triterpenes were found in *E. alatus*. Most of the triterpenes in *E. alatus* belong to lupane type and oleanane type. Other types include hopane, ursane, and friedelane. Table 6 shows the lupane type and friedelane type triterpenes isolated from *E. alatus*.

Other triterpenes include oleanic acid (45), wilforlide A (46) [24], hop-(22)-29-en-3β-ol (47) [25], 3β-hydroxy-21αH-hop-22(29)-en-30-ol (48), 2α,3β-dihydroxyurs-12,19-dien-23,28-oic acid (49) [21], arborinone (50), taraxerol (51) and germanicol (52) [22], 11-keto-β-boswellic acid (53), acetyl 11-keto-β-boswellic acid (54), camaldulenic acid (55) [23], 3β,28,30-lup-20(29)-ene triol (56), 28,30-dihydroxy-3-oxolup-20(29)-ene (57), glut-5-en-3β-ol (58), maslinic acid (59), hederagenin (60), 3-oxo-11alpha-methoxyolean-12-ene (61), 3β-hydroxy-1-oxo-olean-12-en-28-oic acid (62), ursolic acid (63), and 2α-hydroxy-ursolic acid (64) [26]. The structures of compounds 45–64 are shown in Figure 1.

2.3.2. Sesquiterpenes. Two new sesquiterpenes (65, 66) and two known ones were isolated from 95% ethanol extract of the stems of *E. alatus*. The known ones were identified as 6α,12-diacetoxy-2b,9α-di(b-furancarbonyloxy)-4α-hydroxyl-1β-(2-methylbutanoyloxy)-β-dihydroagarofuran (67), 1α,2α,6β-triacetoxy-4β-hydroxy-9β-(β-) furancarboxy-15-[(amethyl) butyroyloxy]-β-dihydroagarofuran (68) [27]. The structures of sesquiterpenes isolated from *E. alatus* are shown in Figure 2.

2.4. Alkaloids. Five alkaloids have been isolated from *E. alatus* and identified as alatamine (69), alatusamine (70) and alatusinine (71) [28], 1β,2β,5α,8β,11-pentaacetoxy-4α-hydroxy-3α-(2-methylbutanoyl)-15-nicotinoyl-7-oxo-dihydroagarofuran (72), evonine (73), and neoevonine (74) [27]. The structures of alkaloids isolated from *E. alatus* are shown in Figure 3.

Table 3: Apigenin and glycoside in *E. alatus*.

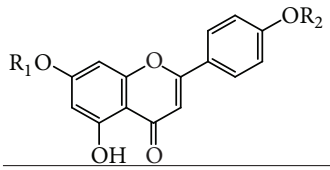
Skeleton	Number	Name	R_1	R_2	Reference
	12	Apigenin	H	H	[48]
	13	Acacetin-7-O-rutinoside (Linarin)	Rutinose	H	[18]

Table 4: Flavanone and the glycoside in *E. alatus*.

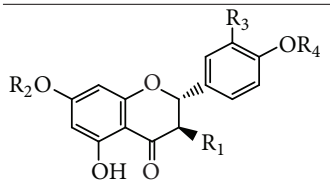
Skeleton	Number	Name	R_1	R_2	R_3	R_4	Reference
	14	Dihydroquercetin	OH	H	OH	H	[49, 50]
	15	Aromadendrene	OH	H	H	H	[19, 39, 49, 50]
	16	Naringenin	H	H	H	H	[48–50]
	17	Naringin	H	Neohesperidose	H	H	[18]
	18	Hesperidin	H	Rutinose	OH	CH_3	[50]

Table 5: Steroids in *E. alatus*.

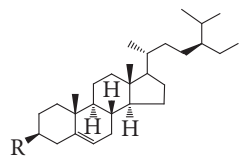
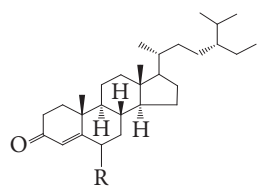
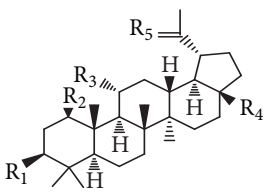
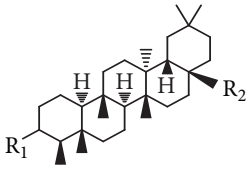
Skeleton	Number	Name	R	References
	28	β-Sitosterol	OH	[18, 20, 24, 39, 51, 52]
	29	β-Sitosterone	=O	[51]
	30	Daucosterol	Glucose	[21, 48]
	31	Stigmast-4-en-3-one (sitostenone)	H	[51, 52]
	32	6β-Hydroxy-stigmast-4-en-3-one	OH	[51, 52]
	33	Stigmast-4-en-3,6-dione	=O	[51]

Table 6: Lupane type and friedelane type triterpenes in *E. alatus*.

Lupane type	Number	Name	R_1	R_2	R_3	R_4	R_5	References
	36	Lupeol	OH	H	H	CH_3	CH_2	[21, 48]
	37	Lupenone	=O	H	H	CH_3	CH_2	[24]
	38	Betulin	OH	H	H	CH_2OH	CH_2	[24]
	39	Betulone	=O	H	H	CH_2OH	CH_2	[26]
	40	Betulinic acid	OH	H	H	COOH	CH_2	[23]
	41	Messagenin	OH	H	H	CH_2OH	O	[26]
	42	(−)-Nepetidone	OH	OH	OH	CH_3	O	[48]

Friedelane type	Number	Name	R_1	R_2	Reference
	43	Epifriedelanol	OH	CH_3	[18, 24, 39, 52]
	44	Friedelin	=O	CH_3	[20, 24]

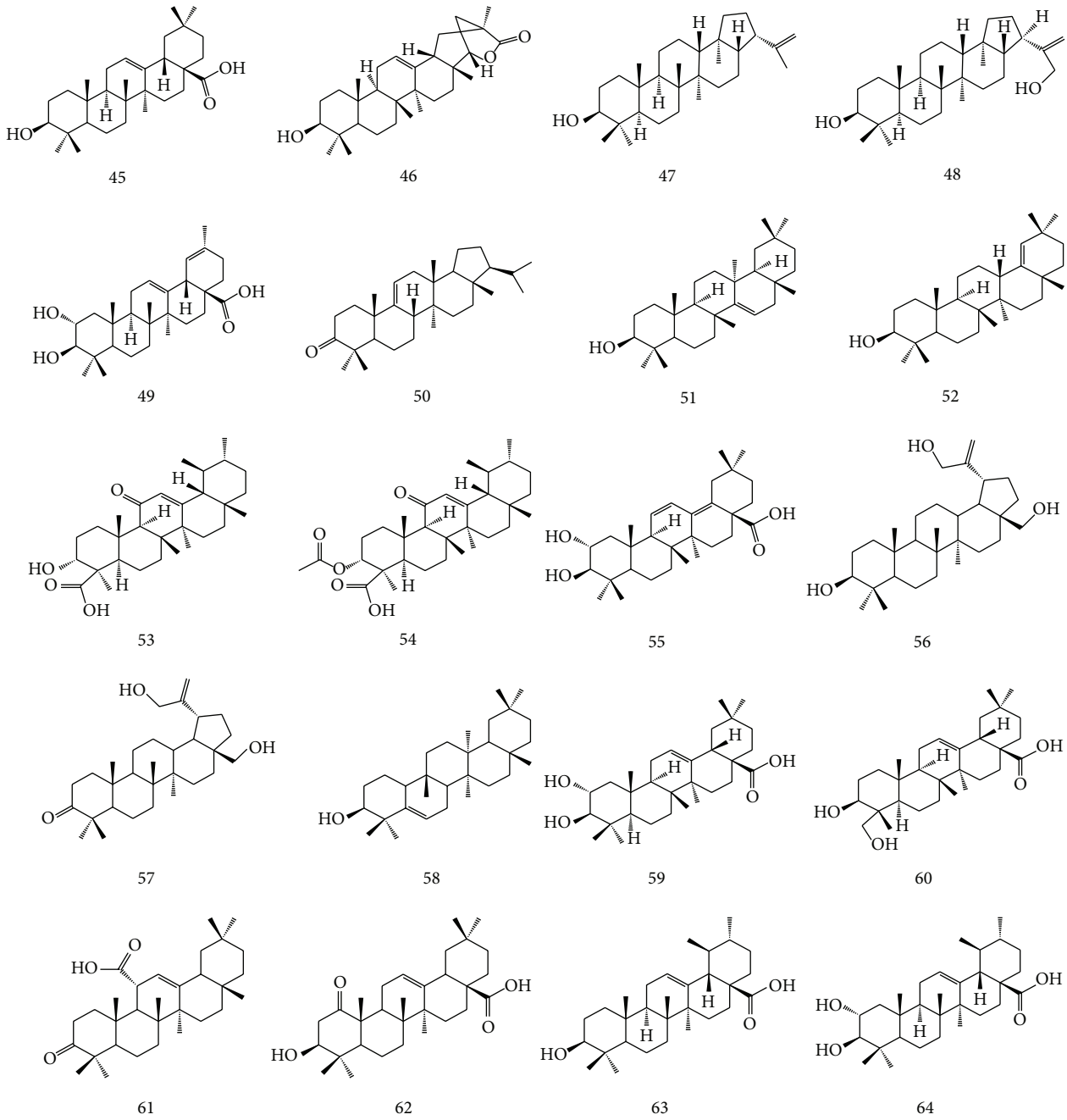


Figure 1: The structures of compounds 45–64 isolated from *E. alatus*.

2.5. Cardenolides. Kitanaka et al. [3] isolated three cytotoxic cardenolides from the woods of *E. alatus* and identified them as acovenosigenin A 3-O-α-L-rhamnopyranoside (75), euonymoside A (76), and euonymusoside A (77).

2.6. Lignans. Jeong et al. [29] identified three new lignans from 80% methanolic extract of *E. alatus* leaves and twigs, including (−)-threo-4,9,4′,9′-tetrahydroxy-3,7,3′,5′-tetramethoxy-8-O-8′-neolignan (78), (−)-threo-4,9,4′,9′-tetrahydroxy-3,5,7,3′-tetramethoxy-8-O-8′-neolignan (79), and (7R,8R,7′R)-(+)-lyoniresinol (80). The other known compounds identified include (+)-simulanol (81), (+)-dehydrodiconiferyl alcohol (82), (−)-simulanol (83), (−)-dehydrodiconiferyl alcohol (84), (+)-dihydrodehyrodiconiferyl alcohol (85), 7R,8S-guaiacylglycerol-8-O-4′-(coniferyl alcohol) ether (86), 7S,8R-guaiacylglycerol-8-O-4′-(coniferyl alcohol) ether (87), 7S,8R-syringylglycerol-8-O-4′-(sinapyl alcohol) ether (88), 7S,8S-guaiacylglycerol-8-O-4′-(sinapyl alcohol) ether (89),7S,8S-4,9,9′-trihydroxy-3,3′-dimethoxy-8-O-4′-neolignan (90), 7R,8R-4,9,9′-trihydroxy-3, 3′-dimethoxy-8-O-4′-neolignan (91), (+)-syringaresinol (92), de-4′-methylyangabin (93), hedyotol C (94), threo-buddlenol B (95), hedyotisol C (96),

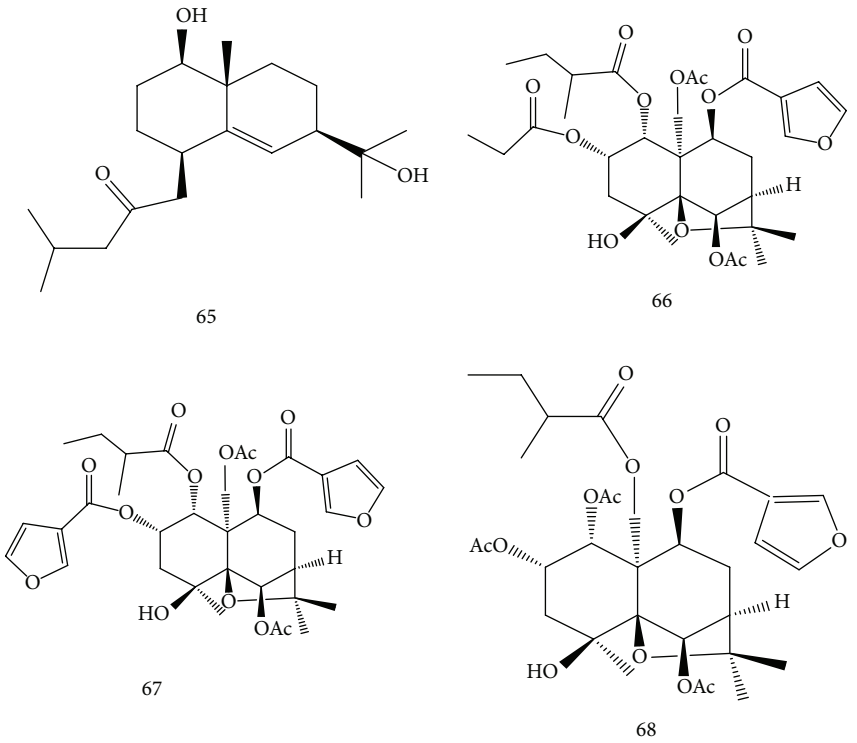


Figure 2: Structures of sesquiterpenes (compounds 65–68) isolated from *E. alatus*.

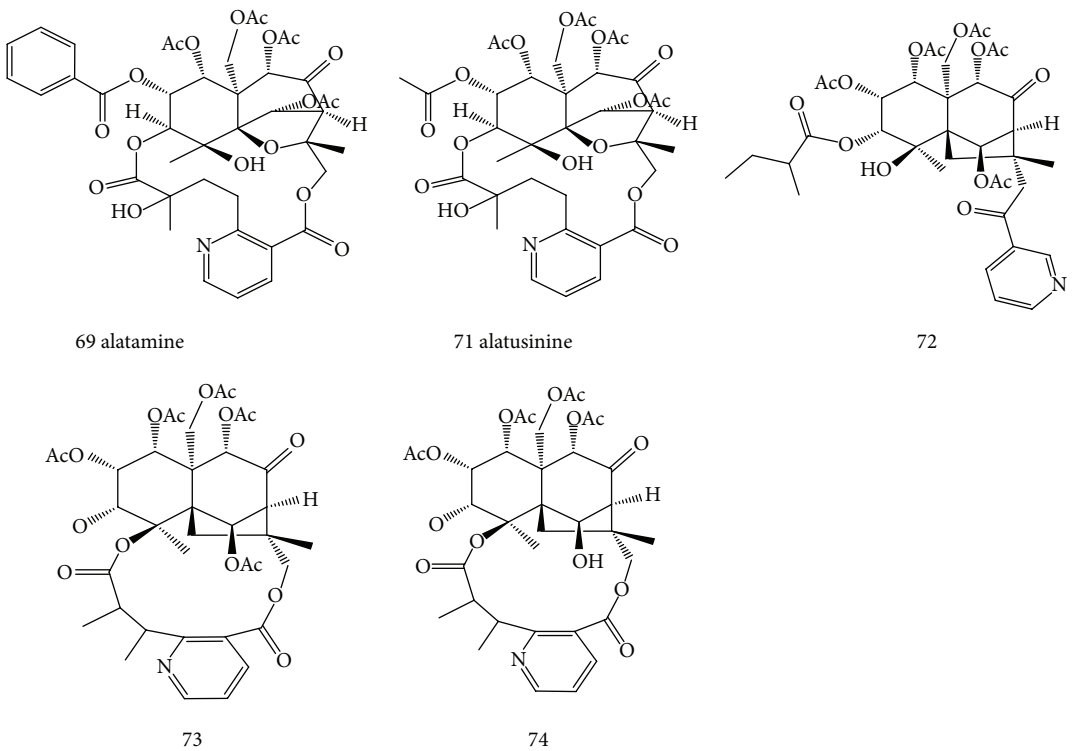


Figure 3: Structures of alkaloids (compounds 69 and 71–74) isolated from *E. alatus*.

and hedyotisol B (97). The structures of compounds 78–97 are shown in Figure 4.

2.7. Other Constituents. E. alatus also contains organic acids, esters, and aldehydes, as illustrated examples in Table 7.

In addition, 3,4-dihydroxybenzoic acid (114), p-propoxybenzoic acid (115), p-coumaric acid (116), ferulic acid (117), 1-feruloyl-β-D-glucoside (118), tetradecyl (E)-ferulate (119) [22], ethyl 2,4-dihydroxy-6-methylbenzoate (120), 4,4′-dimethoxy-1,1′-biphenyl (121) [23], squalene (122) [25], 1-octacosanol (123) [24], n-hexacosanoic acid (124) [18, 24], 1,30-triacontanediol (125), tetracosanoic acid (126), n-octane (127), and n-nonane (128) [21] were also isolated from *E. alatus*. In a study of essential oil from *E. alatus* by using GC-MS, 56 volatile components were identified. The main volatile components include carboxylic acid, aldehyde, ketone, terpenoid, and derivatives of oxygenated terpenoid. Among these the highest content is hexadecanoic acid (39.69%), followed by wintergreen (5.02%) [30].

3. Antidiabetic Activity

The effects of *E. alatus* extracts have been tested *in vivo*. In streptozotocin (STZ) treated diabetic rats, an aqueous extract of *E. alatus* reduced the body weight, the fasting plasma glucose level, and glucose tolerance. The serum levels of insulin, glucagon, cholesterol, and triglyceride were also reduced [31]. Similar results were obtained in high-fat plus low dose STZ diabetic rats, showing that *E. alatus* treated rats had lower levels of fasting blood glucose and insulin and decreased levels of blood lipids and inflammatory mediators (TNF-α, C-reactive protein), indicating that *E. alatus* can improve the glucose-lipid metabolism and insulin resistance in diabetic conditions [32]. Park et al. also demonstrated that an ethanol extract of *E. alatus* reduced the body weight, increased insulin sensitivity, and corrected the associated hyperinsulinemia and hyperlipidemia in high-fat diet-induced hyperglycemic and hyperlipidemic ICR mice [33].

The antihyperglycemic effect of *E. alatus* may involve a protection of functional islet β cells since *E. alatus* treated animals were shown with more positive staining of islet β cells than those in diabetic controls [34]. Other studies in ICR mice indicate that *E. alatus* may affect glucose and lipid homeostasis via a regulation of hepatic lipogenesis related genes (SREBPla, FAS, and GAPT) and PPARγ gene expressions in periepididymal fat. The plausible mechanism of hypoglycemic and hypolipidemic actions of *E. alatus* extract is illustrated in Figure 5 [33].

In addition, a study showed that *E. alatus* protected rats from experimental diabetic nephropathy induced by uninephrectomy plus STZ treatment, with 12-week administration of *E. alatus* extract and irbesartan (positive control) decreased HbA1c and pathological changes (extracellular matrix expansion and glomerulosclerosis) in kidney and improved blood lipids profile and kidney function; the effect was associated with a downregulation of transform growth factor β_1 expression [35]. In addition, *E. alatus* was shown to inhibit polyol pathway, which is known to be associated with chronic diabetic complications such as neuropathy, nephropathy, and retinopathy [36].

Fang et al. studied the antidiabetic effects of different fractions of *E. alatus* extracts (including petroleum ether, diethyl ether, ethyl acetate, n-butanol, and water fraction) in alloxan-induced diabetic mice and high-fat diet diabetic mice and found that ethyl acetate fraction significantly reduced plasma glucose and glucose tolerance in both normal and diabetic mice [37] and also reduced total cholesterol and triglyceride contents and increased SOD activity in diabetic mice [37]. Further analysis revealed that the main components in the ethyl acetate fraction were flavonoids and phenolic acids, including quercetin and kaempferol, which were known for their antioxidant activities [37]. In another study, different extract fractions of *E. alatus*, including aqueous, diethyl ether, and ethyl acetate fractions, were tested in alloxan induced diabetic mice at a dose of 10 g/kg and it was found that the aqueous extract was the most active in decreasing blood glucose and lipid levels and improved glucose tolerance [6]. Thus, *E. alatus* may contain multiple active antidiabetic constituents. Similarly, a study on the hypoglycemic fractions of six fractions of *E. alatus* extracts (including petroleum ether, ethyl acetate, n-butanol, water, residue, and rectified polysaccharide) in diabetic rats found that the fractions of petroleum ether, water, and ethyl acetate had significant antidiabetic effects. Fractions of n-butanol and rectified polysaccharide reduced blood creatinine, and other fractions reduced urea level. The residue fraction decreased the low-density lipoprotein (LDL) and cholesterol contents. The body weight was increased by the treatment with all fractions except rectified polysaccharide. These results indicate that different active compounds in these fractions may be responsible for the observed effects of *E. alatus*, including antidiabetic, antihyperlipidemic, kidney function improvement, blood viscosity decrease, and body weight affecting [38], and the active antidiabetic compounds are likely to be from the petroleum ether, water, and ethyl acetate fractions. In another study, an ethyl acetate extract of *E. alatus* was shown with hypoglycemic effect, and four compounds were isolated from this fraction and identified as p-hydroxybenzoic acid (EA-1), protocatechuic acid (EA-2), 4-hydroxy-3-methoxybenzoic acid (EA-3), and 3, 5-dimethoxy-4-hydroxybenzoic acid (EA-4) [8]. Others reported identification of six compounds with hyperglycemic activity from the 90% ethanol extracts of *E. alatus*, including aromadendrin, epifriedelanol, protocatechuic acid, β-sitosterol, quercetin, and rutin [39]. The active components in protecting experimental diabetic nephropathy as mentioned above have also been suggested to be concentrated in ethyl acetate and n-butanol fractions [36, 40], though the nature of these compounds is still not identified.

Jeong et al. (2015) studied the inhibitory effects of 23 compounds isolated from *E. alatus* on protein tyrosine phosphatases 1B (PTP1B) and α-glucosidase activities and found that lupenone, lupeol, taraxerol, *p*-propoxybenzoic acid, 1-feruloyl-β-D-glucoside, and 3-hydroxycoumarinflavanol exhibited inhibitory activity against PTP1B with IC_{50} values ranging from 5.6 to 18.4 μM. 24R-methyllophenol, arborinone, and *p*-propoxybenzoic acid were shown with a similar

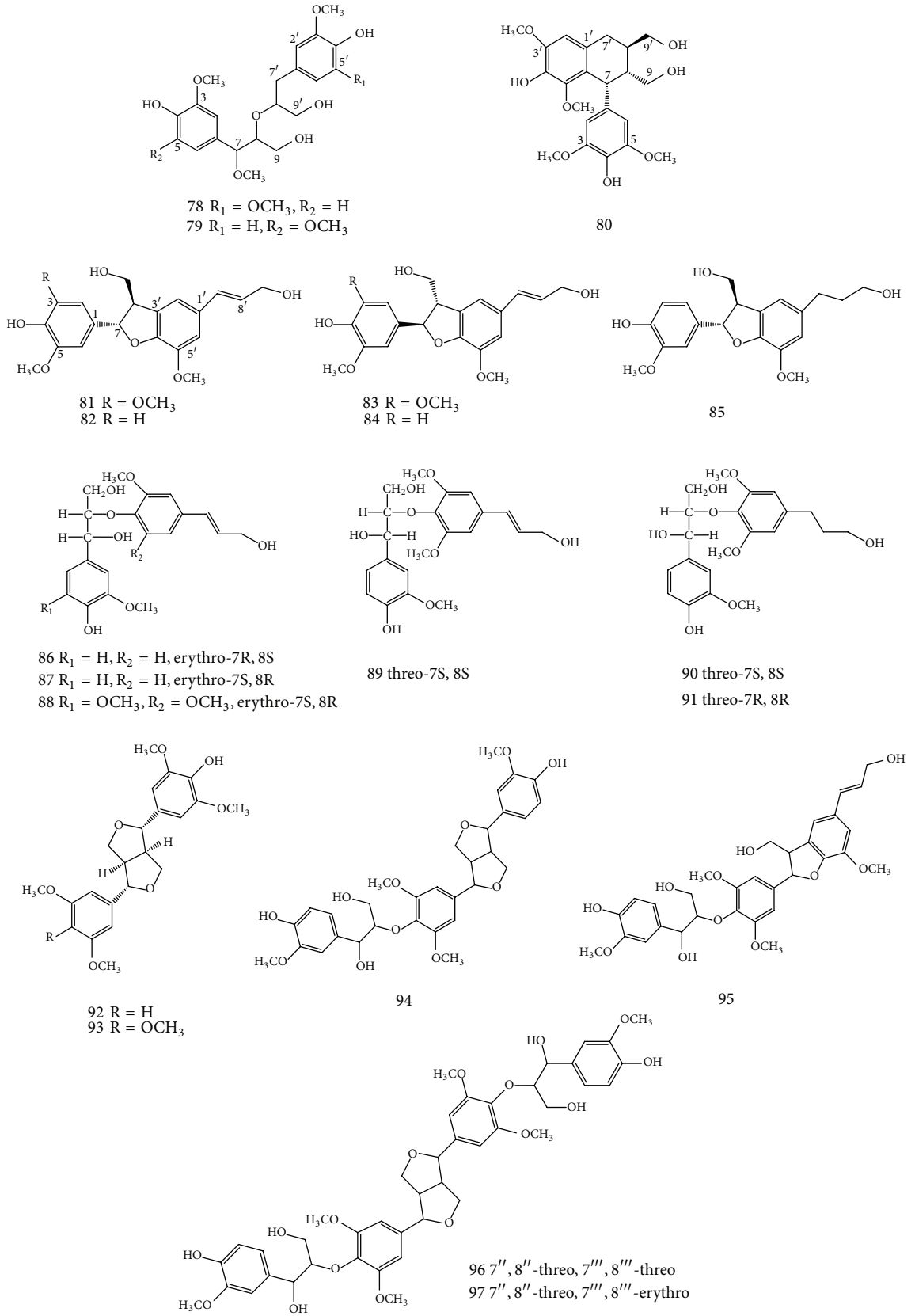


Figure 4: Structures of compounds 78–97 isolated from leaves and twigs of *E. alatus*, modified from [29].

Table 7: Illustrated examples of other constituents in *E. alatus*.

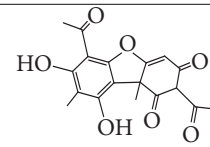
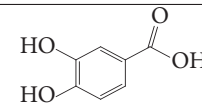
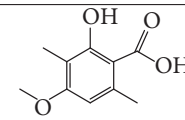
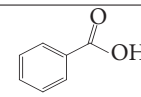
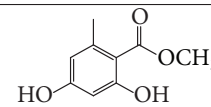
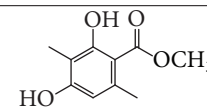
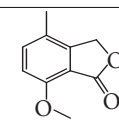
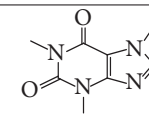
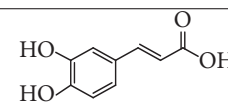
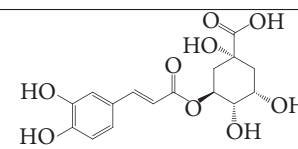
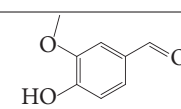
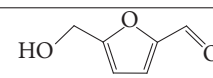
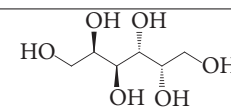
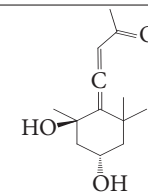
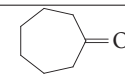
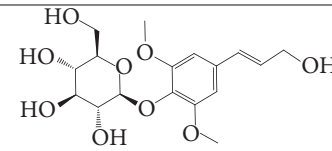
Number	Name	Chemical structure	Reference
98	Usnic acid		[24, 48]
99	Protocatechuic acid		[18, 39]
100	2-Hydroxy-4-methoxy-3,6-dimethylbenzoic acid		[48]
101	Benzoic acid		[24, 48]
102	Methyl 2,4-dihydroxy-6-methyl benzoate		[52]
103	2,4-Dihydroxy-3,6-dimethylbenzoate		[21, 52]
104	7-Methoxy-4-methyl phthalide		[52]
105	Caffeine		[25]
106	Caffeic acid		[49, 53]
107	Chlorogenic acid		[54]
108	Vanillin		[52]
109	5-Hydroxymethyl furfural		[25]
110	Dulcitol		[20]
111	Grasshopper ketone		[49]

Table 7: Continued.

Number	Name	Chemical structure	Reference
112	Suberone		[18]
113	Syringin		[49]

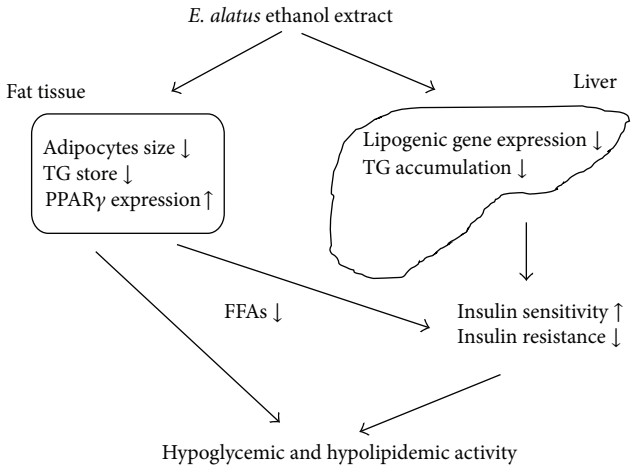


Figure 5: Possible mechanism of hypoglycemic and hypolipidemic actions of *E. alatus* ethanol extract, modified from [33].

activity with IC_{50} values of 15.1, 23.6, and 14.8 μM, respectively. On the other hand, *p*-propoxybenzoic acid, tetradecyl (E)-ferulate, and 3-hydroxycoumarinflavanol exhibited inhibition on α-glucosidase with IC_{50} values of 10.5, 9.5, and 9.1 μM, respectively [22].

Studies on kaempferol and quercetin, the active constituents of *E. alatus,* demonstrated that these compounds improved insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes [41]. kaempferol and quercetin were shown to act as weak partial agonists in the PPARγ reporter gene assay, without inducing differentiation of 3T3-L1 preadipocytes as traditional PPARγ agonists. When kaempferol and quercetin were added together with the PPARγ agonist rosiglitazone, the 3T3-L1 differentiation was inhibited in a dose-dependent manner. Competitive ligand-binding assay confirmed that kaempferol and quercetin competed with rosiglitazone at the same binding pocket site as PPARγ. These compounds were also shown with significant inhibitory effects on NO production in response to lipopolysaccharide treatment in macrophage cells in which the PPARγ was overexpressed. These findings suggest that kaempferol and quercetin may act on multiple targets to ameliorate hyperglycemia [41].

Ivorra et al. studied the effects of daucosterol (β-sitosterol 3-β-glucoside) and its aglycone (β-sitosterol) on plasma insulin and glucose levels in normo- and hyperglycemic rats and found that oral administration of daucosterol or β-sitosterol increased the fasting plasma insulin levels. In addition, both compounds improved the oral glucose tolerance and increased glucose-induced insulin secretion [42]. In addition, rutin, one of the constituents of *E. alatus*, has been shown to decrease the plasma levels of glucose and lipids and increase the expression of PPARγ mRNA and protein in skeletal muscles of db/db mice [43]. Rutin was also demonstrated with an AR inhibition activity (IC_{50} 3.01 μM) [44]. Quercitrin, a flavonoid glycoside in *E. alatus*, was demonstrated as a noncompetitive AR inhibitor. It blocked polyol accumulation in intact rat lenses incubated in medium containing high concentration of sugars [45]. Other compounds, such as linarin (acacetin-7-O-β-D-rutinoside), have also been demonstrated with inhibitory activity against α-glucosidase [46].

3.1. Clinical Evidence. There have been limited clinical studies, mostly case reports, on the antidiabetic actions of *E. alatus* containing formulae (Table 8). In addition, a controlled trial, involving two groups of patients (40 patients in each group) with impaired glucose tolerance, showed that the group treated with diet and exercise intervention plus *E. alatus* formula for 1 month had significantly reduced blood glucose levels, compared to that in the diet and exercise control group. The effective rate was 80% in the *E. alatus* treatment group, compared to that of 55% in the control group [47]. It should be pointed out in most of these studies that *E. alatus* was not used lone, but in combination with other herbs; thus it is not clear if the observed effects are due to *E. alatus* or through interactions with other herbs. Thus, there may be potential bias in these findings. The current evidence for the clinical efficacy for treating diabetes is still weak. Nevertheless, these findings warrant further studies.

4. Conclusion

There is an increasing interest in *E. alatus* as a potential antidiabetic agent. More than 100 chemical constituents have been isolated and identified from *E. alatus*. The main chemical classes include flavonoids, terpenoids, steroids, phenylpropanoids, cardenolides, phenolic acids, and alkaloids. *E. alatus* has been demonstrated with hyperglycemic activity *in vivo*. The hypoglycemic activity *E. alatus* may be related to its effects on insulin signaling and glucose metabolism, including stimulating insulin secretion, improving affinity

Table 8: Clinical studies of *E. alatus for* diabetic conditions.

Number of patients	Preparation/compound	Treatment	Outcome measures/outcome	Reference
57	*E. alatus* formula containing other herbs	Oral, daily per dose, for 3 months	Fasting blood glucose and 24 h urine glucose levels 30 cases with marked improvement 19 cases improved 8 cases no effect Total effective rate: 86%	[55]
58	*E. alatus* formula containing other herbs	Oral, daily per dose, average medication for 37.2 days.	Fasting blood glucose level 21 cases remarkable effect 28 cases effective 9 cases no effect Effective rate: 84.4%	[56]
100	*E. alatus* formula containing other herbs	Oral, daily per dose, for 4 months	Fasting blood glucose and urine glucose levels, clinical symptoms 40 cases showed remarkable effect 51 cases effective 9 cases failed Total effective rate: 91%	[57].
1	*E. alatus* decoction	Oral, daily per dose, for 20 days	Hypoglycemic effects Reduced blood and urine glucose and increased body weight	[58]
80	*E. alatus* formula containing other herbs	Oral, daily per dose, for 30 days	Fasting blood glucose, 2 h postprandial blood glucose values Treatment group: 32 cases effective (80%) and 8 cases no effect (20%) Control group: 22 cases effective (55%) and 18 cases no effect (45%)	[47]

of insulin and receptor, increasing insulin sensitivity and tolerance, and reducing insulin resistance. It may also act as PPARγ agonist and aldose reductase inhibitor. Further study on the bioactive compounds of *E. alatus* and its pharmacology may help to develop new agents for treating diabetes and diabetic complications.

Abbreviations

AR: Aldose reductase
DPP-IV: Dipeptidyl peptidase IV
E. alatus: *Euonymus alatus*
GC-MS: Gas chromatography-mass spectrometry
LDL: Low-density lipoprotein
NO: Nitric oxide
PPARγ: Peroxisome proliferator activated receptor gamma
PTP1B: Protein tyrosine phosphatase 1B
SOD: Superoxide dismutase
STZ: Streptozotocin
TNF-α: Tumor necrosis factor-alpha.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

In Vivo Interrelationship between Insulin Resistance and Interferon Gamma Production: Protective and Therapeutic Effect of Berberine

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This research was conducted to investigate if there is a relation between insulin resistance incidence and inhibition of interferon gamma production or not. Firstly, insulin resistance was induced by high fat diet (HFD) intake for 6 weeks. Secondly, berberine was used as protective/curative compound for insulin resistance. Results revealed that feeding rats HFD for 6 weeks developed features of insulin resistance (IR) syndrome. These features presented in increased body weight, hyperglycemia, hyperinsulinemia, hypercholesterolemia (with increased LDL-cholesterol and decreased HDL-cholesterol), and hypertriglyceridemia. Level of antioxidant enzymes in HFD group was higher than in normal one. Also there was an increasing in level of proinflammatory cytokines as interleukin- (IL-) 6 and IL-12 in HFD group. Feeding rats HFD for 6 weeks also decreased level of interferon gamma (IFN-γ). The decreased level of IFN-γ has been shown to predict infection with infectious diseases especially viral infection. Treatment and protection with berberine 50 mg/kg/day for 2 weeks were found to be effective against the features of insulin resistance syndrome, improved levels of insulin resistance parameters, lipid profile, antioxidant enzymes, proinflammatory cytokines, and IFN-γ.

1. Introduction

The insulin resistance (IR) or metabolic syndrome (MS) is characterized by the clustering of metabolic abnormalities including central obesity, atherogenic dyslipidemia, hypertension, and systemic insulin resistance that leads to an increased risk for type 2 diabetes (DM2) and cardiovascular disease (CVD) [1–3].

IR is a pathological condition in which cells fail to respond to the normal actions of the hormone insulin. IR results from defects at the level of ligand-receptor-response pathway which occur either at the receptor level or in insulin receptor substrate (IRS) molecules by decreasing signaling proteins levels or modulating their activity by phosphorylation.

Due to the change in insulin signaling pathway which occur during IR, several pathophysiological changes took place such as glucose intolerance, obesity, dyslipidemia, and hypertension. Furthermore, several inflammatory cytokines and lipid metabolites like free fatty acids which increased with IR and induced type 2 diabetes mellitus [4].

Interferons (IFNs) are glycoproteins made and released by host cells in response to the presence of pathogens, such as viruses, bacteria, parasites, or tumor cells [5]. In contrast to antibodies, interferons are not virus specific but host specific. Thus, viral infections of human cells are inhibited only by human interferon [6].

Obesity is a major risk factor for cardiovascular disease, certain types of cancer, and type 2 diabetes. The development of type 2 diabetes begins with the onset of insulin resistance, which is correlated with the expansion of visceral fat mass [7]. Obesity is associated with increased release of the proinflammatory cytokines tumor necrosis factor- (TNF-) α

and interleukin- (IL-) 6 from adipose tissue, which leads to a state of chronic inflammation [8, 9].

IL-6 plays an important role in the differentiation of several cell types. IL-6 upregulates suppressor of cytokine signaling 1 (SOCS1) expression in activated cluster of differentiation (CD) 41 T cells, thereby interfering with signal transducer and activator of transcription 1 (STAT1) phosphorylation induced by interferon γ (IFN-γ). Inhibition of IFN-γ receptor-mediated signals by IL-6 prevents autoregulation of IFN-γ gene expression during CD41 T cell activation, thereby preventing T-helper- (Th-) 1 differentiation. Thus, IL-6 promotes CD41 Th2 differentiation and inhibits Th1 differentiation by two independent molecular mechanisms [10]. Therefore, IR alters the immune system response because it shifts T-helper differentiation toward Th-2 and prevented the Th1 differentiation which is responsible for the cytotoxic T-lymphocytes response. The action of IR in immune system is similar to hepatitis C virus (HCV) effect [10].

Nature has been a source of medicinal agents since the beginning of time. The World Health Organization (WHO) estimates that herbal medicine is still the most common source for primary health care of about 75–80% of the world's population, mainly in the developing countries, because of better cultural acceptability, better compatibility with the human body, and fewer side effects [11].

Berberis vulgaris L. is considered as one of the well-known medicinal plants with traditional herbal medical history and used by many civilizations as a curative herbal remedy in homeopathic systems of medicine [12]. The identification of certain alkaloids and phenolic compounds in barberry somehow provides an alternative method for medicine and remedies. Those therapeutic compounds could lead to the development of new drugs derived from that plant, which is believed to be safer and more effective [13].

On one hand, berberine (BRB, the most abundant alkaloid found in *Berberis vulgaris*) was shown to decrease blood glucose, enhance insulin sensitivity, and reduce weight gain in both dietary and genetic rodent models of type 2 diabetes. In high fat diet induced obese rats, berberine decreased fasting blood glucose (FBG), postprandial blood glucose (PBG), fasting insulin, homeostasis model of assessment-insulin resistance (HOMA-IR), and body weight [14–16]. Berberine activates the adenosine monophosphate activated protein kinase (AMPK) which reduces IR through enhancement of adipocytes' glucose uptake. Berberine also increases the expression of hepatocytes insulin receptor and improves cellular glucose consumption [17]. Furthermore, BRB increases glucose transporter-4 (GLUT4) translocation in adipocytes [14]. Moreover, berberine activates the extracellular signal regulated kinase pathway which in turn increases the LDL receptor (LDLR) expression at the posttranscriptional level [17]. Also, berberine reduces hepatocytes lipid synthesis through AMPK activation and decreases peroxisome proliferator-activated receptors- (PPAR-) α mRNA and protein expression, which emphasizes the berberine-hypotriglyceridemic effect [17].

On the other hand, berberine has immune-modulation effect where it has been reported that treatment of macrophages and dendritic cells (DCs) with berberine significantly induced the expression and production of interleukin-12 (IL-12), which consequently increased the interferon gamma (IFN-γ) production and decreased the IL-4 level in antigen-primed CD4C T cells [18]. Therefore, berberine has a stimulatory effect on T-helper lymphocytes subset 1 (Th1) cytokine synthesis in CD4C T cells and an inhibitory effect on Th2 [18]. In case of viral infections as HCV, immune system cannot eradicate the infection as HCV proteins subverts cellular response of the host; it also causes imbalance in TH1/TH2 ratio and targets the body in TH2 way and antibody production which cannot clear the viral particles due to its heterogenecity.

Therefore, the goal of our work was to prove the relation between insulin resistance incidence and the inhibition of interferon gamma production and then assess the effect of berberine as protective and curative compound against IR on interferon gamma production in order to develop a new therapeutic regimen for infection diseases as HCV.

Table 1: Composition of HFD.

Ingredients	Diet (g/kg)
Powdered NPD	365
Butter	310
Casein	253
Cholesterol	10
Vitamin and mineral mix	60
Yeast powder	1
Sodium chloride	1

2. Materials and Methods

Trichloroacetic acid (TCA), 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), reduced glutathione (GSH), and sulphanilic acid were purchased from Sigma-Aldrich (USA). Kits of cholesterol, HDL-cholesterol, and triglyceride were purchased from Biosystems (Spain), glucose was purchased from Spinreact (Spain), ELISA kit of insulin and IFN-γ were purchased from DRG (USA) and Komabiotech (Korea), respectively, and IL-6 and IL-12 were purchased from Sunredbio (Shanghai). All other chemicals and reagents were of analytical grade.

2.1. Animals. Female rats were purchased from experimental animal house, Faculty of Medicine, Alexandria University. The rats aged about 6–8 weeks old and weighted (130–140 g) were used in this study. The animals were housed (six rats/cage) and they were allowed free access to pelleted food and tap water for one week before treatment. All animals were maintained at approximately 23°C–25°C with a 12 h light/dark cycle. Experiments were performed following international ethical standards and according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health [19].

2.2. Diet. The composition and preparation of HFD were described in Table 1. HFD contained 58% fat, 25% protein,

Table 2: Effect of HFD alone or combined with berberine on insulin resistance.

Groups	Body weight (gm)	Glucose (mg/dL)	Insulin (μU/mL)	HOMA-IR	HOMA-β
Control	149.16 ± 10.88[a]	95.70 ± 9.70[a]	11.0 ± 1.43[a]	2.209 ± 0.52[a]	153 ± 11.6[a]
Untreated HFD	178.5 ± 14.33[b]	130.45 ± 15.29[b]	21.8 ± 2.77[b]	6.09 ± 1.33[b]	102.86 ± 23.2[b]
Treated HFD with BRB	157.66 ± 11.80[a]	107.08 ± 3.80[a]	11.7 ± 1.16[a]	2.73 ± 0.61[a]	118 ± 8.6[a]
Protected HFD with BRB	173.5 ± 15.87[b]	110.95 ± 4.27[a]	11.9 ± 1.78[a]	2.72 ± 0.91[a]	93.4 ± 5.7[a]

Mean value in each column having different superscript (a, b) is significant difference at $p < 0.05$.

Table 3: Effect of HFD alone or combined with berberine on lipid profile.

Groups	Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL-chol (mg/dL)	LDL-chol (mg/dL)
Control	100.06 ± 12.60[a]	68.72 ± 14.61[a]	66.20 ± 8.93[a]	28.22 ± 4.56[a]
Untreated HFD	202.05 ± 21.96[b]	165.72 ± 7.11[b]	49.30 ± 6.7[b]	119.61 ± 14.61[b]
Treated HFD with BRB	164.66 ± 15.56[c]	123.47 ± 11.04[c]	55.5 ± 6.88[c]	84.46 ± 8.17[c]
Protected HFD with BRB	180.68 ± 15.84[c]	145.09 ± 14.70[c]	55.0 ± 5.27[c]	96.66 ± 10.32[c]

Mean value in each column having different superscript (a, b, and c) is significant difference at $p < 0.05$.

and 17% carbohydrate, as a percentage of total kcal. HFD was freshly prepared on a weekly basis and stored at 4°C, while normal basal diet (NPD) contained 11% fat, 16% protein, and 15% carbohydrate (Table 1) [20].

2.3. Animal Treatment. The experimental animals were divided into 4 groups each consisting of 6 rats, in Group 1 (control group), healthy rats received normal diet and tap water for evaluation of the basal level of all the experimental parameters, in Group 2 (untreated HFD), rats received HFD for 6 weeks, in Group 3 (treated HFD with BBR), rats firstly received high fat diet for 6 weeks and then received oral administration of berberine daily at a dose of 50 mg/kg/day for 2 weeks, and in Group 4 (protected HFD with BBR), rats firstly received basal diet with oral administration of berberine daily at a dose of 50 mg/kg/day for 2 weeks and then received HFD for 6 weeks.

All animals were fasted for 12 hours before scarification but after 6-hour starvation period blood sample was collected for blood glucose level determination. After complete anesthesia, the abdominal cavity was rapidly opened following the median line of the abdomen. Blood was collected in plain tubes to get sera (for biochemical analysis) and ethylenediaminetetraacetic acid (EDTA) tubes for whole blood (for complete blood count (CBC) analysis) from all groups. The sera tubes were kept at room temperature for 15 min for blood clotting and centrifuged at 3000 r.p.m. for 10 minutes; then the obtained sera were kept at −20°C until analyzed.

The liver from each rat was removed, washed in cold saline, and cleaned. The tissues were weighed, cut into small anatomical pieces, and homogenized to obtain 10% homogenate (1 gm of liver pieces was homogenized in 9 mL cold phosphate buffer, pH 7.4, 0.1 M, and centrifuged for 15 min at 4000 rpm, 4°C, to remove the cell debris).

The standardized methods for determination of serum glucose level [21], blood insulin level [22], HOMA-IR index [23], serum cholesterol level [24], serum triglyceride level [25], serum HDL-cholesterol level [26], serum LDL-cholesterol level [27], liver thiobarbituric acid reactive substances (TBARS) [28], liver glutathione-S-transferase (GST) activity [29], and liver reduced glutathione (GSH) content [30] were used. Moreover, serum Nitric Oxide (NO) level, serum IL-6 level, serum IL-2 level, and serum INF-γ level were carried out using commercial kit according to manufactures' instructions.

3. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Primer of Biostatistics (Version 5) software program. Significance of means ± SD was detected groups by the multiple comparisons Student-Newman-Keuls test at $p < 0.05$.

4. Results

Table 2 shows that feeding rats HFD for 6 weeks significantly ($p < 0.05$) increased body weight, blood glucose, insulin, and HOMA-IR levels by 19.67, 36.31, 98.18, and 175.69% while decreased HOMA-β level by 32.77%. Treatment with berberine normalized body weight, glucose, insulin, HOMA-IR, and HOMA-β levels, at $p < 0.05$, while protection with BRB normalized all parameters except body weight which is still high like untreated HFD group weight at $p < 0.05$.

There was a marked significant increase in lipid profile of HFD group than that of control one where the cholesterol, TG, and LDL levels were significantly increased by 101.9, 141.15, and 323.84% over control level, while HDL level was decreased by 25.53% than that of control group, at $p < 0.05$. Both treatment and protection with BRB significantly reduced level of serum cholesterol, triglycerides, and LDL-cholesterol and elevated HDL-cholesterol when compared with HFD group by the same level but failed to normalize them, at $p < 0.05$ as shown in Table 3.

Table 4: Effect of HFD alone or combined with berberine on cytokines profile, WBCs, and lymphocytes count.

Groups	IL-6 (pg/mL)	IL-12 (ng/mL)	IFN-γ (pg/mL)	WBC $* 10^3$	LYMF
Control	80.21 ± 4.13^a	159.96 ± 13.16^a	91.72 ± 11.85^a	6.7 ± 1.51^a	3.64 ± 0.47^a
Untreated HFD	143.75 ± 13.62^b	105.58 ± 13.92^b	21.16 ± 1.11^b	5.6 ± 1.71^a	1.75 ± 0.71^b
Treated HFD with BRB	91.08 ± 3.61^c	153.46 ± 16.96^a	49.32 ± 5.55^c	5.16 ± 1.11^a	2.15 ± 0.9^c
Protected HFD with BRB	117.01 ± 7.92^d	148.19 ± 18.79^a	46.90 ± 6.17^c	5.96 ± 1.85^a	2.0 ± 0.66^c

Mean value in each column having different superscript (a, b, c, and d) is significant difference at $p < 0.05$.

Table 5: Effect of HFD alone or combined with berberine on liver oxidative stress parameters level.

Groups	TBARS (nmol/mL)	GSH (mg/mL)	GST (μmol/min)	NO (μmol)
Control	4.25 ± 0.159^a	0.187 ± 0.01^a	0.121 ± 0.01^a	72.39 ± 36.26^a
Untreated HFD	5.25 ± 0.113^b	0.208 ± 0.01^b	0.148 ± 0.01^b	163.66 ± 23.42^b
Treated HFD with BRB	4.28 ± 0.166^a	0.188 ± 0.01^a	0.126 ± 0.01^a	92.39 ± 70.77^a
Protected HFD with BRB	4.42 ± 0.161^a	0.191 ± 0.01^a	0.136 ± 0.01^a	95.78 ± 54.84^a

Mean value in each column having different superscript (a, b) is significant difference at $p < 0.05$.

Table 4 reveals that there was a significant ($p < 0.05$) elevation in the level of IL-6 in untreated HFD group when compared to control group. Both treatment and protection with BRB improved the level of IL-6 when compared to HFD but did not normalize it. The level of IL-6 in protected group was higher than that of treated group, at $p < 0.05$.

HFD intake decreased the level of IL-12 when compared to control group, at $p < 0.05$. Both treatment and protection with BRB normalized the level of IL-12, at the same level.

Also, there was a significant ($p < 0.05$) decrease in the level of IFN-γ in untreated HFD group when compared to control group. Both treatment and protection with BRB improved the level of IFN-γ when compared with HFD but did not normalize it.

While there was no significant difference in the count of WBCs among all tested groups, HFD intake decreased lymphocytes number comparing with control one, at $p < 0.05$. Both treatment and protection increased lymphocytes number than those of HFD group by the same level but failed to normalize it.

Table 5 reveals that feeding rats HFD elevated TBARS, GSH, GST, and NO levels when compared to control group. Both treatment and protection with BRB normalized all these parameters, at $p < 0.05$.

5. Discussion

It is reported that IR is associated with increased release of the proinflammatory cytokines TNF-α and IL-6 from adipose tissue, which leads to a state of chronic inflammation, and also stimulated production of a family of proteins known as suppressors of cytokine signaling (SOCS) via IL-6 that participate in a negative feedback loop in cytokine signaling [31–33]. This study was aimed to find out the relation between IR and INF-gamma production and study the modulation effect of berberine.

Our study demonstrated that feeding rats a HFD for 6 weeks resulted in development of features of insulin resistance syndrome. These features presented in increased body weight, hyperglycemia, hyperinsulinemia, hypercholesterolemia (increased LDL-cholesterol and decreased HDL-cholesterol), and hypertriglyceridemia. Also, feeding rats HFD for 6 weeks and induction of IR decreased level of IFN-γ. These findings emphasized the direct correlation between IFN-γ and IR incidence.

With the induction of IR, there is an elevation in the level of proinflammatory cytokines as IL-6 and IL-12 when compared to healthy rats. These results are supported by other studies that showed that adipose tissue has been shown to produce 10–35% of IL-6 in a resting individual, and this production increases with increased adiposity [34]. This suggests that adipose tissue is a source of the increased circulating IL-6 observed in obesity. Also, Senn et al. [35] showed that high level of IL-6 in obese individual induces insulin resistance.

Also, the study of Diehl et al. [10] reported that there is slight reduction in level of IL-12 in cells affected by IL-6 but this reduction was not reproducible.

Interferon γ is a strong activator of inflammatory responses and cellular immunity and is a major activator of macrophages [36]. IFN-γ is produced predominantly by NK, Th1, and $CD8^+$ cells and binds to a heterodimeric cell surface receptor that is ubiquitously expressed. Ligation of the IFN-γ R results in activation of the receptor-associated tyrosine kinases Janus kinase 1 (Jak1) and Jak2, leading to the tyrosine phosphorylation and activation of the transcription factor STAT1 [36, 37].

In the present study the induction of IR increased the level of IL-6 and reduced the level of IFN-γ and the number of lymphocytes compared to normal one but there is no effect on total WBCs count when compared to healthy rats. These findings seem to be in concordance with the study of Diehl et al. [10] that showed the effect of IL-6 high level on IFN-γ production.

IR, a hallmark of type 2 diabetes, is associated with oxidative stress. Although there is substantial evidence that hyperglycemia results in the generation of reactive oxygen species (ROS) and increased oxidant stress in the late complications of diabetes [38], the role of ROS in the development of

insulin resistance [39, 40], especially at the whole-body level, remains virtually unknown. Levels of antioxidant enzymes are greater in obese insulin resistant group than in normal one; this is in agreement with the study of McClung et al. [41].

In the present study, hepatic TBARS level was increased in the HFD group. It is reported that hyperglycemia and hypertriglyceridemia evoke the hepatic lipid peroxidation due to β-oxidation overload that is characterized by high TBARS level [42]. Also level of GSH, GST, and NO are elevated in HFD group.

Berberis vulgaris L. is considered as one of the well-known medicinal plants with traditional herbal medical history and is used in many civilizations as a curative herbal remedy in the homeopathic system of medicine [12]. The most important constituents are isoquinoline alkaloids, such as berberine, berbamine, and palmatine [43]. Berberine represents one of the most studied naturally occurring protoberberine alkaloids, since it possesses a wide range of biochemical and pharmacological activities [44, 45].

Berberis crude extract showed a protective and/or curative capacity with markedly hypoglycemic, hypolipidemic, and antioxidant properties against IR incidence and progression.

Our data showed that oral administration of berberine reduced the key features of insulin resistance syndrome. This is in agreement with several studies that have demonstrated the role of berberine in the protection against HFD induced insulin resistance [14, 46, 47]. This is illustrated when using berberine as a treatment for 2 weeks normalized insulin, glucose, and HOMA-IR levels in both treated and protected groups when compared to HFD group. The observed hypoglycemic effects of berberine are supported by the previous in vitro [48, 49] and in vivo studies [50, 51]. Also, Gomes et al., Kalalian-Moghaddam et al., and El-Sayed et al. [52–54] revealed that berberine supplementation has a positive impact on glucose and insulin homeostasis upon HFD feeding. Also, levels of cholesterol, triglyceride, and LDL-cholesterol are reduced in addition to increased HDL-cholesterol level. These results are supported by other studies that showed the same effect of berberine on lipid profile [53, 54].

The phytochemical constituents of berberine as alkaloids, flavonoids, and phenolic contents would act in synergy in order to increase barberry's bioactivity such as antioxidant and antidiabetic. So, the protection or the treatment with berberine maintained the antioxidant enzymes in the normal level. This is in agreement with the study of El-Sayed et al. [52].

Also, BRB has an effect on proinflammatory cytokines, as after treatment with berberine there is a stimulatory effect on IL-12 and an inhibitory effect on IL-6 production compared to untreated HFD group. From our data we found that berberine has an effect on IFN-γ production, as after treatment for 2 weeks the level of IFN-γ was improved compared to HFD group. These findings come in agreement with the study of Aziz et al. [55] which said that BRB has immune modulatory effect on immune system and after treatment of DCs, there is an increase in level of INF-γ and IL-12.

The present study showed that there is a relation between IR and production of IFN-γ, also showing the powerful effect of berberine as an inhibitor for insulin resistance syndrome development and its usage as a protective compound indicating its importance for people who are infected with viral infection like virus C.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Antidiabetic Properties, Bioactive Constituents, and Other Therapeutic Effects of *Scoparia dulcis*

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This review discusses the antidiabetic activities of *Scoparia dulcis* as well as its antioxidant and anti-inflammatory properties in relation to the diabetes and its complications. Ethnomedical applications of the herb have been identified as treatment for jaundice, stomach problems, skin disease, fever, and kidney stones, reproductory issues, and piles. Evidence has been demonstrated through scientific studies as to the antidiabetic effects of crude extracts of *S. dulcis* as well as its bioactive constituents. The primary mechanisms of action of antidiabetic activity of the plant and its bioactive constituents are through α-glucosidase inhibition, curbing of PPAR-γ and increased secretion of insulin. Scoparic acid A, scoparic acid D, scutellarein, apigenin, luteolin, coixol, and glutinol are some of the compounds which have been identified as responsible for these mechanisms of action. *S. dulcis* has also been shown to exhibit analgesic, antimalarial, hepatoprotective, sedative, hypnotic, antiulcer, antisickling, and antimicrobial activities. Given this evidence, it may be concluded that *S. dulcis* could be promoted among the masses as an alternative and complementary therapy for diabetes, provided further scientific studies on the toxicological and pharmacological aspects are carried out through either *in vivo* or clinical means.

1. Introduction

A recent study published by the World Health Organization (WHO) in the Lancet as part of the NCD Risk Factor Collaboration (NCD-RisC) shows that the number of adults with diabetes has quadrupled worldwide in under four decades to 422 million, and the condition is fast becoming a major problem in poorer countries [1]. In one of the largest studies to date of diabetes trends, the researchers of NCD-RisC said aging populations and rising levels of obesity across the world mean diabetes is becoming a defining issue for global public health. This study used data from 4.4 million adults in different world regions to estimate age-adjusted diabetes prevalence for 200 countries. It found that between 1980 and 2014 diabetes has become more common among men than women, and rates of diabetes rose significantly in many low- and middle-income countries, including China, India, Indonesia, Pakistan, Egypt, and Mexico. While the alarming nature of the disease is as such, the inefficacy and inadequacy of current antidiabetic treatments in mitigating the disease condition have also been highlighted from studies such as the Linköping Diabetes Complications Study [2], the Diabetes Control and Complications Trial (DCCT) Research Group [3–5], and the UK Prospective Diabetes Study (UKPDS) Group [6–8]. Current treatments for diabetes include insulin therapy (for type 1 and some type 2 diabetes patients) and administration of oral hypoglycemic agents (i.e., insulin secretagogues, biguanides, α-glucosidase inhibitors, and insulin sensitizers), which nevertheless have been demonstrated to show deteriorations in hyperglycemic control and increased risks of contracting diabetic complications during long-term usage [9]. To further complicate the situation, the hefty

expense of purchasing these drugs is a significant economic burden borne by the diabetic patients, especially given the increased prevalence of the disease in developing countries.

When taking historical elements into account from the perspective of antidiabetic treatments, diabetes is an ancient disease for which remedies have been detailed in many traditional medicinal pharmacopoeias. In Traditional Chinese Medicine (TCM), for instance, the disease has been referred to as "Xiaokezheng" or "Xiaodanzheng," both of which mean diabetes [10]. In the Sri Lankan traditional medicinal system, the disease has been stated as "Madhumeha" [11]. In both of these traditional medicinal systems and many others, the usage of herbs for combating the disease condition and alleviating its symptoms has been practiced for many eons. Plants have always been an exemplary source of drugs and have directly or indirectly yielded many important medicines in the past. For instance, the discovery of the widely used hypoglycemic drug, Metformin, came from the traditional approach of using *Galega officinalis* [12]. Overall, herbal remedies are gaining popularity because of several advantages such as a comparatively lower incidence of side-effects at recommended dosages, better patient tolerance, relatively low cost, and acceptance due to a long history of use. From the perspective of diabetes, the more important cause is that herbal medicines provide rational means for remedying the disease condition as well as many other ailments which are obstinate and incurable in more Western systems of medicine. Therefore, a revival of interest in the use of plants in pharmacy has emerged worldwide recently from both the pharmaceutical industry as a source of new lead molecules and the general public who tend to use plant extracts in many ways as conventional and complementary therapies. Thus, bringing effective herbal remedies into the limelight especially those which have proven antidiabetic effects through at least *in vivo* and *in vitro* studies is of importance given the disturbing incidence of the disease, the voids of effective therapeutic remedies, and the tendency towards searching and promoting complementary and alternative therapies to combat and contain the progression of the disease.

Scoparia dulcis, also known as sweet broomweed (family: Scrophulariaceae), is a perennial herb which is commonly found in tropical and subtropical regions. Figure 1 shows the plant and its flowers in its natural habitat. The leaves of the plant are serrated and the flowers are white in colour. *S. dulcis* has been used in many traditional medicinal systems as an antidiabetic herb and for a variety of ailments. The plant is abundantly found in many countries and can be easily grown and cultivated, should there be a need for mass production. This review highlights the ethnomedicine, antidiabetic properties, antioxidant effects, bioactive chemical constituents, and other therapeutic properties of *S. dulcis*. A schematic outline showing all therapeutic properties of this plant is shown in Figure 2. Through this review, it is hoped that *S. dulcis* could be promoted for downstream scientific investigations where the herb as well as its bioactive constituents would be taken up for further clinical evaluations and thereby discover the true potential as a remedy for combating a global pandemic as well as other noncommunicable disease conditions at large.

2. Ethnomedicine

Before delving into the antidiabetic effects and other related properties of *S. dulcis* from a more scientific perspective, it is of importance to briefly summarize the ethnomedical applications of the herb, so that its therapeutic value is validated by its historical usage. The use of *S. dulcis* plant parts has been recorded from many parts of the world. In a very comprehensive study on the ethnomedical value of plants used in the preparation of traditional rice beer, Bhuyan and Baishya have identified the different plant parts used by several tribal groups in the State of Assam in India [13]. In this study, it was identified that Bodo, Karbi, Ahom, Deori, Rabha, Mising, and Sonowal Kachari tribes of Assam used widely differing herbs and plant parts in their rice beer preparations. Of these, the leaves of *S. dulcis* were used in particular by the Deori and Rabha tribes for alleviation of diabetes, jaundice, stomach problems, skin disease, and piles [13]. Bieski et al. conducted an ethnobotanical survey in the Nossa Senhora Aparecida do Chumbo District (NSACD), located in Poconé, Mato Grosso, Brazil and found that *S. dulcis* was used for several disease categories with diabetes being one of them [14]. In the South Indian State of Tamil Nadu, a survey of the phytotherapeutic agents used by the Nadars revealed that *S. dulcis* juice was taken orally to treat fever and kidney stones [15]. In Trinidad and Tobago, *S. dulcis* juice has been used for cooling babies and for reproductive problems in both men and women [16, 17], while, in Central Laos, in Bolikhamsai Province, the whole plant was used for treating nephritis [18].

Due to the prevalence of malaria in the Amazon Region, Ruiz et al. evaluated the antimalarial potential of traditional remedies used by the indigenous and Mestizo populations around the banks of the Nanay River, a tributary of the Amazon River in Loreto, Peru [19]. These researchers went as far as testing some of these plant extracts for activity on *Plasmodium falciparum*. They found that several of the plants used traditionally, including *S. dulcis*, had antiplasmodium activity. The whole plant of *S. dulcis* is recorded as one of many being used for the treatment of snakebite in the province of Antioquia in Colombia [20]. Over 90% of the bites in this province were caused by the snake *B. asper* (Viperidae) where around 4000 bites are reported annually in Columbia. The rapid effect of the toxins in the venom results in high mortality (5%) and serious long-term effects (6%). The healers and shamans administer plant extracts to neutralize the toxins such as phospholipase A2 present in the venom, where *S. dulcis* was identified as one of the plants being used for this purpose [21]. In Sri Lanka, rice based porridges containing herbal extracts and coconut milk have been traditionally consumed for breakfast. Senadheera et al. [22] reported that three porridges individually made with *Asparagus racemosus*, *Hemidesmus indicus,* or *Scoparia dulcis* were found to have a glycemic index value of less than 55, and peak blood glucose reduction values of around 40%. When diabetic Wistar rats were administered with these porridges, the porridge containing *S. dulcis* resulted in a reduced weight loss while exhibiting hypoglycemic and hypolipidaemic properties with no observed toxic effects.

Figure 1: Leaf and flower structure of *S. dulcis* in its natural habitat.

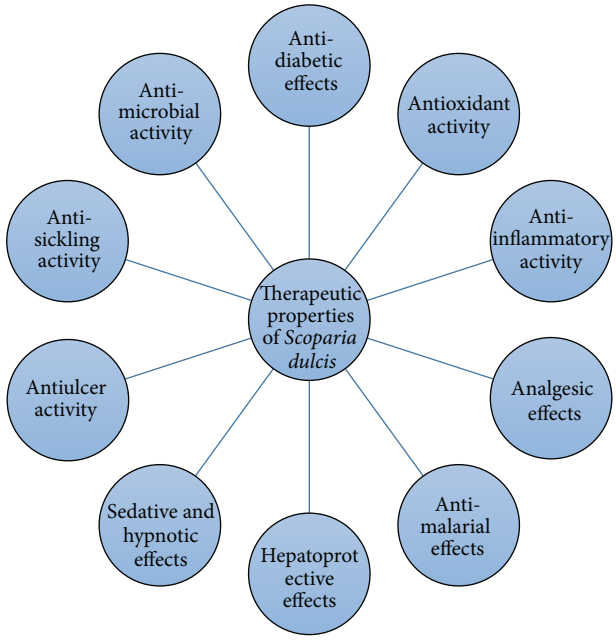

Figure 2: Schematic diagram displaying all the therapeutic properties of *S. dulcis*.

3. Antidiabetic Properties

3.1. Evidence from Studies Based on Evaluating the Antidiabetic Properties of Whole Extracts of S. dulcis. Extracts of *S. dulcis* plant as a whole and numerous compounds isolated from *S. dulcis* all have exhibited antidiabetic or hypoglycemic properties. For this section of the review, the antidiabetic property of *S. dulcis* which was demonstrated by studies based on various types of extracts of the whole plant is highlighted briefly. A diagrammatic representation representing the various actions of different solvent fractions is shown in Figure 3.

Pari and Venkateswaran demonstrated hypoglycemic activity of the aqueous extract of *S. dulcis* leaves using alloxan induced hyperglycemic rats [23]. They, further, indicated that this hypoglycemic effect was highly pronounced when 0.45 g/kg (body weight) of the extract was administrated orally for 45 days. In addition to hypoglycemic effect, the administration of this plant extract prevented weight loss which is characteristic of diabetic patients [24]. Furthermore, it has been shown that the aqueous extract of *S. dulcis*, when administered at a dose of 200 mg/kg (body weight), exhibits a hypoglycemic effect on streptozotocin induced diabetic rats [24]. Similarly, Das and Chakraborty [25] and Attanayake et al. [26] used streptozotocin induced diabetic rats to demonstrate the antihyperglycemic effect of the aqueous extracts of *S. dulcis*. In addition to possessing antidiabetic effects, the aqueous extracts of *S. dulcis* exhibit antihyperlipidemic effects [26]. For example, streptozotocin induced diabetic rats have shown decreased levels of lipids including cholesterol, triglycerides, fatty acids, and phospholipids, decreased levels of very low density lipoprotein and low density lipoprotein cholesterol, and reduced 3-hydroxy-3-methylglutaryl (HMG) CoA reductase activity, as a result of oral administration of the aqueous extract of *S. dulcis* (200 mg/kg of body weight) for 6 weeks [27].

Apart from aqueous extracts, numerous studies have shown that polar organic extracts such as methanol and ethanol extracts of *S. dulcis* possess antidiabetic properties. For example, Sharma and Shah illustrated using streptozotocin induced diabetic rats [28] that the flavonoids from the methanol extract of the aerial parts of *S. dulcis* impart an antihyperglycemic activity comparable to Glibenclamide. Strengthening their claim, Mishra et al. reported that both the aqueous and methanol extracts of *S. dulcis* contain polyphenols that these extracts show antioxidant activity which may most probably be due to the polyphenols and that there is a correlation between the antidiabetic activity and antioxidant activity of these extracts [29]. Another study conducted by Zulfiker et al. using alloxan induced diabetic mice revealed that the ethanol extract of the aerial parts of the plant shows antidiabetic activity [30]. Further, this study also exhibited that this extract shows moderate antioxidant activity compared to ascorbic acid, using DPPH

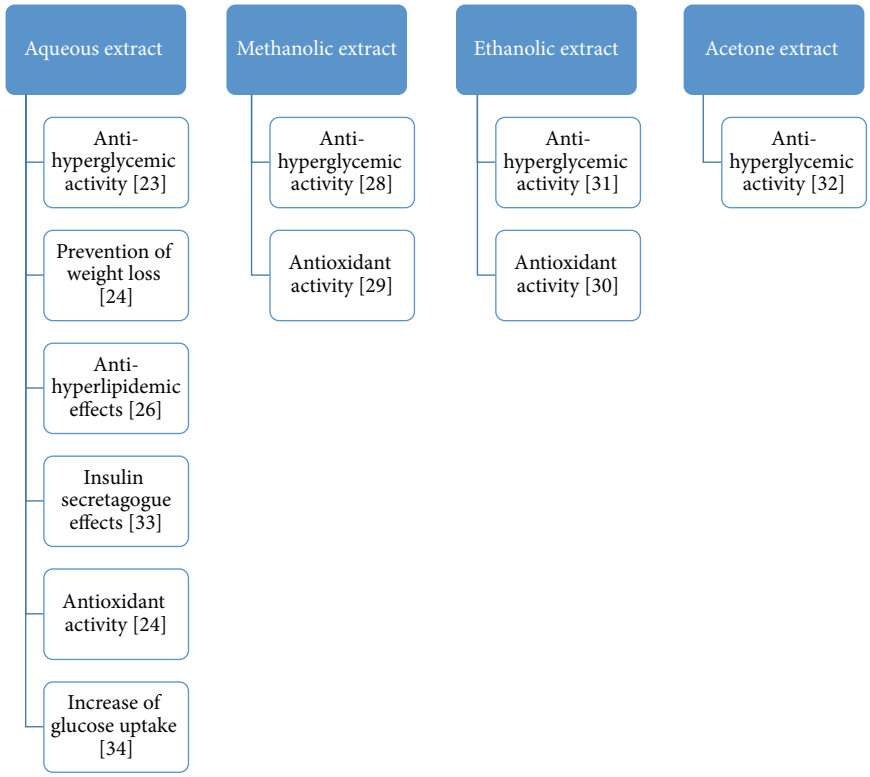

Figure 3: Diagrammatic representation depicting the various actions of different fractions of *S. dulcis*.

(1,1-diphenyl-2-picrylhydrazyl) assay and phosphomolybdenum assay. In addition to the extracts of the whole plant and aerial parts of the plant, the root of *S. dulcis* exhibits significant antidiabetic activity according to Reddy et al. [31]. Specifically, the hydroalcoholic extract of the roots of this plant had imparted a hypoglycemic activity similar to that of Glibenclamide, on alloxan induced rats in this particular study. Interestingly, a comparison of hypoglycemic activity of *S. dulcis* extracts in different organic solvents was carried out, using streptozotocin induced diabetic rats, by Talukder et al. who revealed that the acetone extract was most effective followed by methanol, petroleum ether, and ethanol extracts [32].

The mechanisms of action of *S. dulcis* plant extracts possessing antidiabetic activity have also been elucidated. It has been demonstrated that the antidiabetic activity of the aqueous extracts of *S. dulcis* may be attributable to its insulin secretagogue activity. For example, pancreatic islets isolated from mice have shown a 6-fold increase in secretion of insulin when the islets were exposed to an aqueous extract of *S. dulcis* at a dose of 10 μg/mL [33]. Also, *S. dulcis* imparts its antidiabetic effects via altering the levels of many antioxidant enzymes and enzymes of the polyol pathway. In fact, Latha and Pari showed, using streptozotocin induced diabetic rats, that the aqueous extract of *S. dulcis* significantly decreased the level of sorbitol dehydrogenase while increasing the levels of the antioxidant enzymes glutathione peroxidase and glutathione-S-transferase [24]. Beh et al. demonstrated using L6 rat myoblasts (CRL-1458) that the TLC fraction 7 of the aqueous extract of *S. dulcis* possesses glucose uptake activity comparable to that of insulin [34]. Moreover, they revealed that the TLC fraction 7 of the aqueous extract of *S. dulcis* is more capable than insulin of glucose transport that may be partly responsible for its antidiabetic activity.

3.2. Evidence from Studies Based on Evaluating the Antidiabetic Properties of Bioactive Constituents of S. dulcis. The antidiabetic properties of *S. dulcis* have been primarily owed to the existence of diterpenes [35–38], triterpenes [39, 40], and flavonoids [41] which have been mostly isolated from the aerial parts of the plant. A variety of chemical constituents have led to a plethora of antidiabetic pathways of action initiated by the plant, such as α-glucosidase and peroxisome proliferator-activated receptor gamma (PPAR-γ) agonistic and insulin secretagogue activities. Some of the compounds which have displayed these antidiabetic mechanisms of action by way of *in vitro* and *in vivo* studies are shown in Figures 4 and 5. These pathways of action and the respective compounds responsible for these properties with supporting evidence as provided through *in vitro* and *in vivo* studies are briefly discussed herein.

3.2.1. α-Glucosidase Inhibitory Activities. The use of enzyme inhibitors produces carbohydrate malabsorption and, hence, moderates blood glucose and insulin elevation [42]. This characteristic in particular is shown to be beneficial in the treatment of type 2 diabetes patients [43]. The digestive

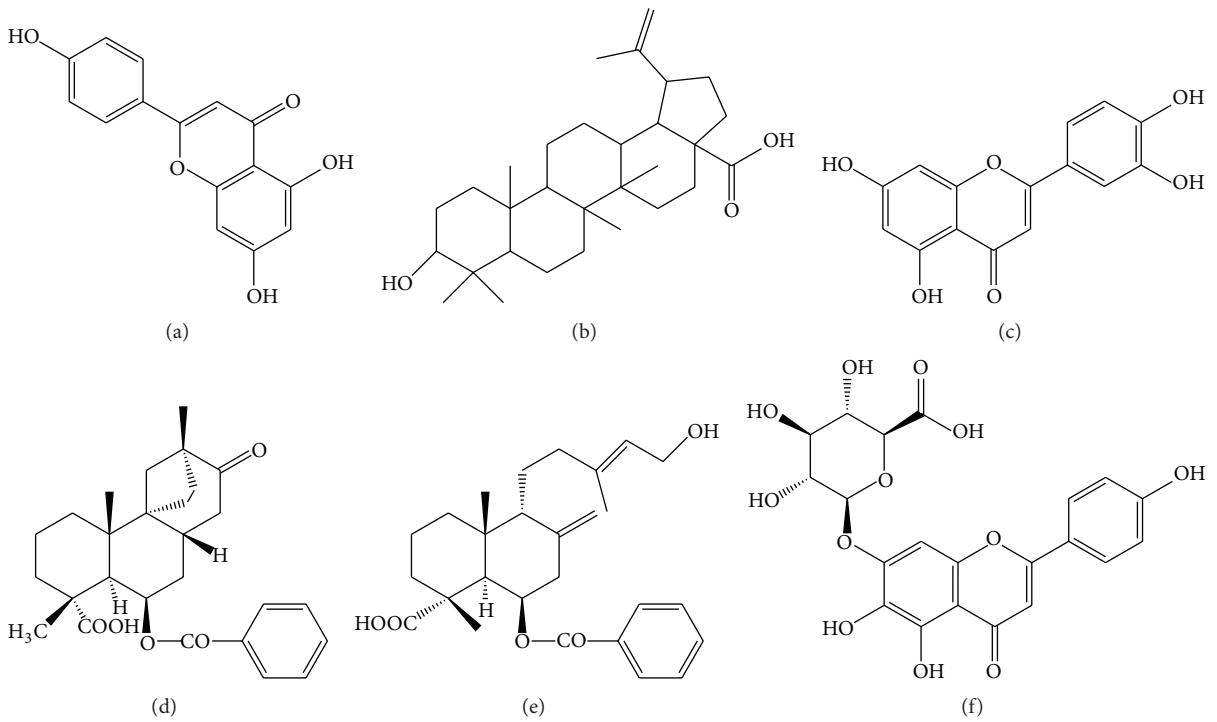

Figure 4: Chemical constituents in *S. dulcis* which have demonstrated PPAR-γ and α-glucosidase inhibitory activities: (a) apigenin, (b) betulinic acid, (c) luteolin, (d) scopadulcic acid B, (e) scoparic acid A, and (f) scutellarin.

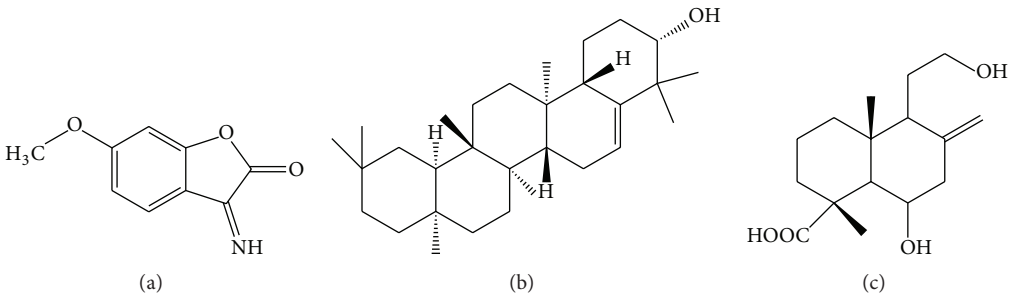

Figure 5: Chemical constituents in *S. dulcis* which have demonstrated insulin secretagogue activities: (a) coxicol, (b) glutinol, and (c) scoparic acid D.

process of starch involves salivary α-amylase, pancreatic α-amylase, and the small intestinal brush border α-glucosidase, that is, maltase-amyloglucosidase and sucrase-isomaltase [44–46]. While inhibition of all 3 of these enzymes is deemed important in terms of control and release of blood glucose levels, inhibition of α-glucosidase in particular is considered as a priority, given that it is the final step upon which the rate of breakdown of carbohydrates is decided. Acarbose is one of the commonly used synthetic starch hydrolase inhibitors which is prescribed as an antidiabetic drug. However, it has been known to cause adverse health effects such as promotion of hypoglycemia at higher doses, as well as diarrhea and flatulence due to the gut microflora fermentation on the undigested carbohydrates [47]. Thus, the occurrence of natural starch hydrolase inhibitors is currently being explored, an aspect in which compounds of *S. dulcis* have been investigated as well. In the study by Liu et al. [48], the *in vitro* α-glucosidase inhibitory activities of the flavonoids scutellarein, apigenin, and luteolin and the terpenoids scopadulcic acid B and betulinic acid which were present in *S. dulcis* were observed to be more potent than the positive control, acarbose (with IC_{50} values in the range 13.7–132.5 μM). A confirmation of these properties of the bioactive compounds was stated by Mishra et al. in their review [49].

3.2.2. PPAR-γ Agonistic Activities. PPAR-γ is a member of the nuclear hormone receptor superfamily which can be activated by many kinds of ligands, such as flavonoids, terpenoids, and unsaturated fatty acids [50–52]. Its activation

results in increased insulin sensitivity partly by reversing lipotoxicity-induced insulin resistance [53]. Thiazolidinediones (TZDs), which are well-known insulin resistance ameliorating agents in use for the treatment of type 2 diabetes since the 1980s, have been identified as potent PPAR-γ inhibitors [48]. Nevertheless, as with many of the synthetic drugs in use for diabetes, TZDs are also fraught with many side-effects [48]. Thus, screening of new PPAR-γ agonists from herbal medicine seems a reasonable strategy for the discovery of antidiabetic agents or their lead compounds. In the study by Liu et al. [48], scoparic acid A, scutellarein, apigenin, and luteolin had exhibited PPAR-γ agonistic activities *in vitro*, with EC_{50} values ranging from 0.9 to 24.9 μM. This study could be considered as the first of its kind to report the PPAR-γ agonistic activities of apigenin and luteolin in particular. Through this study, it was also evident that the bioactive constituents impart antidiabetic properties through multiple biochemical pathways, with individual compounds being able to modulate and mitigate the disease condition through numerous mechanisms of action.

3.2.3. Insulin Secretagogues. The importance of insulin secretagogues as a remedy for combating diabetes was highlighted in the Kyoto declaration of 2013, where nonobese type 2 diabetic patients mostly of Asian origin were observed to possess defects in insulin secretion rather than insulin resistance [54]. The insulin secretory activity and cytoprotective properties of aqueous *S. dulcis* extract have been previously reported by Latha et al. [55]. Identification of compounds responsible for these properties was also reported by Sharma et al. [56]. In this study, coixol and glutinol (Figure 4) were found to be potent and mildly active, respectively, in terms of insulin secretagogue activity. Coixol was further evaluated for insulin secretory activity on MIN-6 cells and was further subjected to *in vitro* cytotoxicity assay against MIN-6, 3T3 cell lines, and islet cells and *in vivo* acute toxicity test in mice which was found to be nontoxic. Thus, the study confirmed the insulin secretory activity of coixol and glutinol which supported the ethnobotanic uses of *S. dulcis* as an antidiabetic agent containing potent insulin secretagogues. Additionally, the study by Latha et al. [55] further verified the insulin secretagogue properties of scoparic acid D (SAD) in particular. In brief, streptozotocin (STZ) induced diabetic Wistar rats were administered with SAD at dosages of 10, 20, and 40 mg/kg bodyweight for 15 days. At the end of the experimental period, the SAD-treated STZ diabetic rats showed decreased levels of glucose as compared with diabetic control rats. The improvement in blood glucose levels of SAD-treated rats was associated with a significant increase in plasma insulin levels. SAD at a dose of 20 mg/kg bodyweight exhibited a significant effect when compared with other doses. Further, the effect of SAD was tested on STZ-treated rat insulinoma cell lines (RINm5F cells) and isolated islets *in vitro*. SAD at a dose of 20 mg/mL had evoked twofold stimulation of insulin secretion from isolated islets, indicating its insulin secretagogue activity. This study also demonstrated the cytoprotective effects of SAD.

4. Other Therapeutic Properties of *S. dulcis* Relevant to Diabetes

Oxidative stress and elevated inflammatory levels have been biochemically determined to be the root cause of diabetic complications as shown by epidemiological and biochemical data [57, 58]. Thus, the antioxidant and anti-inflammatory properties of *S. dulcis* are discussed here, in view of the potential of this plant to mitigate the deleterious effects resulting from hyperglycemia-induced oxidative stress and inflammatory reactions.

4.1. Antioxidant Activity of S. dulcis. Several researchers have studied the effect of *S. dulcis* extracts on the ability to scavenge free radicals. Though many report the activity of the water extract, some have reported the activity of methanolic, ethanolic, chloroform, and hexane extracts. Babincová and Sourivong used the DPPH assay to demonstrate strong antioxidant activity of the extract [59]. Coulibaly et al. [60] studied the antioxidant property of the hexane, chloroform, and methanol extracts of the plant by the DPPH and FRAP assays. In addition, inhibition of lipid peroxidation was measured by the TBARS assay and inhibition of lipoxygenase and xanthine oxidase by the extracts was determined [60]. They found that the chloroform extract exhibited the highest activity and concluded that the phytochemical content being the highest in this extract was the responsible factor for the observation [61, 62]. Three extracts, aqueous, ethanolic, and chloroform, were shown to have significant antioxidant capacity [DPPH, FRAP, β-carotene bleaching, and (TBARS) assay] [63, 64]. The water extract showed the highest activity. Zulfiker et al. studied the effect of the ethanolic extract of S. *dulcis* on alloxan induced diabetic mice [30]. The extract, at a dose of 100 and 200 mg/kg, reduced glucose level by 31.87% and 46.97%, respectively, while a 50.74% reduction was given by Metformin after 2 weeks. The antioxidant potential assessed by DPPH free radical scavenging assay was moderate in comparison to ascorbic acid (IC_{50} of 243.82 μg/mL for plant extract and 58.92 μg/mL for ascorbic acid).

4.2. Anti-Inflammatory Activity of S. dulcis. Inflammation is a complex nonspecific immune response triggered by damage to living tissues that protects higher organisms from infection and injury. There are two types of inflammation, and their effects can be either beneficial (defense against agents interfering with homeostasis, that is, acute inflammation) or harmful (causing damage to cells and tissues, that is, chronic inflammation) [65]. de Farias Freire et al. reported that the ethanol extracts were superior to water extracts of *S. dulcis* in eliciting effects probably related to the anti-inflammatory activity of the plant [66]. Ethnobotanical studies in many parts of the world have revealed the use of *S. dulcis* for anti-inflammatory activity [67, 68]. Since anti-inflammatory medications are used to relieve menstrual discomfort, plants having anti-inflammatory activity may explain their use in traditional medicines for relieving these symptoms. Hence, Michel et al. researched into plants used to treat symptoms related to menstruation and menopause by the Q'eqchi, the third largest Maya population in Guatemala [69]. They

found that *S. dulcis* was used to alleviate labour pains. An ethnobotanical survey conducted by Souza et al. in north eastern Brazil indicated that root of *S. dulcis* was used for treating inflammation and uterine inflammation [70]. Sala et al. [71] have investigated the anti-inflammatory pathway of *S. dulcis* aqueous extracts *in silico* and have discovered its ability to inhibit human inhibitor nuclear-factor κB kinase 2 (hIKK-2). hIKK-2 is a serine-threonine protein kinase belonging to the IKK complex and is the primary component responsible for activating nuclear-factor κB transcription factor (NF-κB) in response to inflammatory stimuli. The NF-κB pathway is deemed important in the regulation of gene expression controlling cellular immune and inflammatory responses and has motivated research groups in both academia and the pharmaceutical industry to devote increasing efforts to developing synthetic ATP-competitive inhibitors for hIKK-2 [72]. Thus, the hIKK-2 inhibitory activity of *S. dulcis* could be considered as an important therapeutic characteristic in its promotion as an anti-inflammatory agent.

5. Other Therapeutic Properties of *S. dulcis*

Several systematic studies have been carried out on various other therapeutic properties of *S. dulcis*. While these studies are not numerous as compared with those on antidiabetic, antioxidant, and anti-inflammatory activities, the outcomes could be considered as stepping stones for investigating these therapeutic properties of *S. dulcis* in detail, through further *in vitro*, *in vivo,* or clinical means.

5.1. Analgesic Properties. The analgesic properties of the plant have been substantiated by *in vitro* investigations which have been verified to have extended from the existence of glutinol [30, 73]. Additionally, the analgesic and hyperanalgesic properties of *S. dulcis* aqueous extracts have been verified in rat models by Ratnasooriya et al. [74]. This is an important characteristic in possession by this plant since current analgesics, especially opiates, have been known to cause adverse side-effects such as gastric lesions.

5.2. Antimalarial Properties. The antimalarial effects of *S. dulcis* have been investigated by Bourdy et al. [75]. Aqueous extracts of the aerial part of *S. dulcis* were explored for the inhibitory activity versus *Plasmodium falciparum*. Although the antimalarial effects were not as significant in comparison with the rest of the plants selected for this particular study, positive observations were noted in terms of its activity against the parasite.

5.3. Hepatoprotective Effects. Hepatoprotective effects of *S. dulcis* have been observed by Li et al. [10] in mice containing CCl_4-induced acute liver injuries. In this study, an oral dose of 800 mg/kg had exhibited a significant ($P < 0.01$) protective effect against the CCl_4-induced changes in serum aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), total protein (TP), and liver histopathology, compared with the positive control. Similar observations have been made by Ediriweera and Ratnasooriya [11], where crude ethanolic and aqueous extracts of *S. dulcis* were evaluated against CCl_4-induced liver cirrhosis in Sprague Dawley rats. These values had been comparable with the standard, and silymarin and were associated with the ability of *S. dulcis* to scavenge free radicals. Both of these studies provided evidence as to the traditional practice of administering *S. dulcis* for hepatic ailments.

5.4. Sedative and Hypnotic Effects. The study by Moniruzzaman et al. [76] evaluated the sedative and hypnotic effect of the ethanolic extract of whole plants of *S. dulcis*. The sedative and hypnotic activity were then investigated using hole cross, open field, hole-board, rotarod, and thiopental sodium-induced sleeping time determination tests in mice at the doses of 50, 100, and 200 mg/kg of the ethanolic extracts of the plant. A significant dose-dependent inhibition of locomotor activity of mice in both hole cross and open field tests was observed in this instance, suggesting that *S. dulcis* may possess sedative principles with potent hypnotic properties.

5.5. Antiulcer Activity. Among other miscellaneous therapeutic properties, the study by Babincová et al. [77] explored the antiulcer activity of water extracts of *S. dulcis* in Sprague-Dawley rats. For the first time, *S. dulcis* water extract was verified to possess gastroprotective activity as evidenced by its significant inhibition in the formation of ulcers induced by indomethacin in this study.

5.6. Antisickling Activity. The antisickling activity of *S. dulcis* was recently investigated by Abere et al. [78]. Sickle cell disease (SCD) is one of the most prevalent morbidity and mortality diseases in Africa [79]. Management of SCD is aimed at relieving pain, preventing infections and management of complications. First-line clinical management of sickle cell anaemia includes use of hydroxyurea, folic acid, amino acids, and blood transfusion to stabilize the patient's haemoglobin level [80]. These are quite expensive and have attendant risk factors, thereby gradually paving way for the consideration of condiments from natural sources as antisickling remedies [81]. Ethnomedicinally, *S. dulcis* is used to manage sickle cell disease in Nigeria [78]. Abere et al. [78] confirmed traditional usage of *S. dulcis* in the management of SCD and a candidate for further investigations.

5.7. Antimicrobial Activity. In the study by Coulibaly et al. [82], the antimicrobial activity of the acetone : water (70 : 30) extract of *S. dulcis* was investigated against the bacterial cultures of *Bacillus licheniformis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* and the fungal cultures of *Gloeophyllum trabeum*, *Pycnoporus sanguineus*, *Fomitopsis palustris*, *Schizophyllum commune,* and *Trametes versicolor*. They concluded that the antifungal activity of *S. dulcis* was more pronounced than its antibacterial activity in this study. Niveditha and Prabavathy [83] tested for the ability of the ethanolic extract of the leaves of the plant to inhibit virulence factors of the two pathogens *Escherichia coli* and *Staphylococcus aureus*. They used untreated and ethanolic extract treated bacteria to study the effect of the extract on inhibition of haemagglutination,

haemolysis, proteolysis, lipolysis, and gelatinase production. The ability of the extract to inhibit the tests indicates the potential of *S. dulcis* for use as a commercial drug against urinary tract infections. Uma et al. had tested six extracts (petroleum ether, toluene, chloroform, methanol, ethanol, and water) of *S. dulcis* for activity towards the gram positive strains *Bacillus* sp. and *Corynebacterium* sp. and the gram negative bacteria *Escherichia coli*, *Klebsiella pneumoniae,* and *Acinetobacter* sp. using the agar well diffusion assay [84]. They report activity of the extracts towards both gram positive and gram negative strains. However the activity of the extracts was mild in comparison to the ampicillin standard which was used. In another study on the ethanolic extract of S. *dulcis*, Zulfiker et al. performed disc diffusion assays on several gram positive and negative strains [85]. Their results also indicated very mild activity of the extract compared to the kanamycin standard. When they tested the same extract against three fungal strains (*Aspergillus niger*, *Saccharomyces cerevisiae,* and *Candida albicans*), the results indicated smaller zones of inhibition (6–10 mm) in comparison to that of the nystatin standard (20–25 mm).

6. Conclusions

It is evident from the scientific studies carried out to date on *S. dulcis* that this plant is worthy of being explored and promoted as complementary and alternative means of combating diabetes. Additional biochemical avenues of exploration in this aspect would be to evaluate whether the extracts or bioactive compounds are capable of decreasing insulin resistance which is a typical characteristic of type 2 diabetes. Studies which go in proximity to exploring this characteristic have been carried out but none which specifically focuses on type 2 diabetes and insulin resistance. This could be potentially explored using genetically modified rat models such as the Goto-Kakizaki model. Additionally, it would be of value to investigate whether the plant and its bioactive compounds are capable of regenerating pancreatic β-cells. This aspect could be easily and systematically verified using *in vitro* and *in vivo* studies. Nevertheless, as compared with other types of plants which have been ethnomedically used for diabetes, it is possible that *S. dulcis* has progressed quite notably in terms of scientific studies, from evaluation of crude extracts, to identification of chemical constituents, to identification of bioactive compounds and discovering their specific mechanisms of action. Additionally, although the plant may specifically be promoted for antidiabetic purposes, given the scientific evidence as to its miscellaneous therapeutic properties, it may also be used for overall health and wellness purposes. Nevertheless, despite the plethora of systematic studies on *S. dulcis*, it is imperative to investigate the toxicological and pharmacological aspects of the plant either through *in vivo* or clinical means. As with many other herbal remedies, while the plant is currently being administered or consumed by way of traditional practices, identification of recommended dosages and consumption limits should be determined before formal promotion of the plant, its extracts, or bioactive constituents for antidiabetic or other therapeutic purposes.

Competing Interests

The authors have no conflict of interests to declare, financial or otherwise.

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Research Article

The Hypoglycemic and Antioxidant Activity of Cress Seed and Cinnamon on Streptozotocin Induced Diabetes in Male Rats

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The present study aimed to estimate the stimulation of pancreas of rats with streptozotocin induced diabetes using 20% (w/w) garden cress seed (*Lepidium sativum*) and cinnamon methanol extracts. The positive control diabetic group showed a significant increase in fasting blood sugar, lipid peroxide, interleukin-6, carboxymethyl lysine, serum uric acid, urea, creatinine, immunoglobulins, and urine albumin and a significant decrease in antioxidant enzymes, sodium ions, potassium ions, and urine creatinine. Severe histopathological changes in the kidney and pancreas tissues in hyperglycemic rats were also shown in the positive control diabetic group. Meanwhile, the groups that were treated with 20% garden cress seed and cinnamon methanol extracts showed a significant decrease in fasting blood sugar and all elevated abovementioned biochemical parameters and an increase in the lowered ones restoring them nearly to the normal levels of G1. Kidney and pancreas tissues were also ameliorated and restored nearly to the normal status. Both garden cress seed and cinnamon methanol extracts succeeded in controlling hyperglycemia in rats with streptozotocin induced diabetes and ameliorated the biochemical and histopathological changes because of their antioxidant activity acquired by their possession of phenolic phytochemicals.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia linked with total or partial deficiencies in insulin secretion or function. It is one of the most frequent chronic diseases affecting millions of people globally leading to morbidity and mortality worldwide particularly in developing countries of Africa, Asia, and South America [1–3]. Diabetes mellitus is considered an extended chronic metabolic disease that causes several other complications, such as cardiovascular diseases; fixed cost used for its treatment placed a huge burden on the economy and health systems worldwide [4, 5].

Traditional medicines and plant-based systems continue to play an essential role in healthcare [6]. Cinnamon and garden cress seeds are members of a list containing 150 plants used in treatment of diabetes mellitus [7]. Cinnamon is a spice obtained from the inner bark of several trees from the genus *Cinnamomum* (family: Lauraceae) [8]. The active compounds of cinnamon have been reported, such as water-soluble polyphenol type-A polymers, cinnamaldehyde, and cinnamic acid [1]. It is used for treating abdominal and chest pains, chronic diarrhea, hypertension, kidney disorders, and rheumatism [9]. Cinnamon extracts were shown to have antidiabetic effects as a number of cell studies demonstrated an insulin-like action. Additionally, cinnamaldehyde promoted glucose uptake into skeletal muscle through glucose transporter 4 translocation [10].

Lepidium sativum L. (garden cress) contains mucilage in its dry seed coat that has been isolated using dissimilar solvents and utilized by researchers as an excipient in a variety of pharmaceutical formulations for preferred functionality [11]. Garden cress seed mucilage is extensively used in many traditional medicinal arrangements such as cough syrups. It also has antihyperglycemic properties which help to control glucose level in diabetics [12, 13]. The seeds of *L. sativum* are aperient, diuretic, tonic, demulcent, carminative, galactagogue, and emmenagogue, are used to induce an abortion, and also possess antibacterial and antifungal properties [14].

This study aimed to estimate the stimulation of the pancreas by the antidiabetic effect of 20% (w/w) garden cress seed (*Lepidium sativum*) and cinnamon methanol extracts in male rats with STZ induced diabetes.

2. Materials and Methods

The experimental work of the present study was conducted at king Fahd Medical Research Center and Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

2.1. Materials. Cinnamon and garden cress seed were purchased from a local herbal medicine shop in Jeddah, Saudi Arabia.

2.2. Conventional Animal Basal Diet. The conventional animal basal diet was obtained from a grain mill in Jeddah. Every 100 g consists of the following: 12% protein (17.14 g of 70% casein), 4 g corn oil (4% fat), 0.3 g methionine (0.3%), 0.2 g choline chloride (00.2%), 4 g minerals (4% minerals), 1 g vitamin mixture (1% vitamin), 4 g cellulose (4% fiber), and 69.36 g corn starch (69.36%). The basal diet was stored in a dry place away from direct sunlight.

2.3. Animals. Forty adult male albino rats weighing 180 to 200 g were used in this study. All animal experiments were carried out under protocols approved by the Institutional Animal House of the University of King Abdulaziz at Jeddah, Saudi Arabia. The animals were housed in cages and received normal rat chow and tap water *ad libitum* in a constant environment (room temperature 28 ± 2°C, room humidity 60 ± 5%) with a 12 h light and 12 h dark cycle. The animals were kept under observation for two weeks prior to the start of the experiments.

2.4. Study Design. Ten rats were used as control group (the first group, G1) and received a single tail vein injection of 0.1 mol/L citrate buffer only. The other 30 rats were intravenously injected with freshly prepared streptozotocin (65 mg/kg body weight) in a 0.1 mol/L citrate buffer (pH 4.5), after fasting for 12 hours to induce diabetes [15]. After five days of injection, rats with blood glucose higher than 200 mg/dL were considered diabetic in the fasting state. Rats with blood glucose lower than 200 mg/dL were excluded from the study. Glucose measurement was done by using *OneTouch Select* Analyzer (LifeScan, Inc., UK). The study was started one week after STZ injection. The 30 diabetic rats were randomly divided into 3 groups. The second group (G2) was the diabetic control positive group fed normal basal diet. The third group (G3) was diabetic group treated with 20% (w/w) garden cress seeds methanol extract, orally using stomach tube for 28 days. The fourth was diabetic group treated with 20% (w/w) cinnamon methanol extract, orally using stomach tube for 28 days.

2.5. Phytochemical Analysis. The total flavonoid contents of cinnamon and garden cress seed were determined by a colorimetric method as follows: each sample (0.5 mL) was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a $NaNO_2$ solution (15%). After 6 minutes, 0.15 mL of aluminum chloride ($AlCl_3$) solution (10%) was added and allowed to stand for 6 minutes, and then 2 mL of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 mL and the mixture was thoroughly mixed and allowed to stand for another 15 minutes. Absorbance of the mixture was then determined at 510 nm versus prepared water blank.

Total phenol estimation was carried out using Folin-Ciocalteu reagent according to the method of Malick and Singh [16]. Phenols react with phosphomolybdic acid in Folin-Ciocalteu reagent in alkaline medium and produce blue-colored complex. The absorbance of mixture was measured using spectrophotometer at 650 nm against a reagent blank. A standard curve was prepared using different concentrations of catechol and then total phenols were expressed as g of phenols/100 g material. Total carotenoids were extracted with acetone-hexane mixture and determined with a spectrophotometer at wave length 440 nm as described by Dubois et al. [17].

2.6. Preparation of Methanol Extract. Cinnamon and garden cress seed were milled by mixer, and then methanol extract was prepared according to the method of Adebayo et al. [18] as follows: 200 g of each of cinnamon and garden cress seed was soaked in 1 liter of 90% methyl alcohol under shaking for 5 days and kept in a refrigerator. The methanol was evaporated using a rotatory evaporator apparatus attached with a vacuum pump. Twenty grams of either extract (semisolid) was suspended in 100 mL distilled water with 2 mL of tween 80 (suspending agent) to prepare a 20% alcoholic solution.

2.7. Samples Collection. At the end of the experiment, rats were fasted 14–16 hours after their last feeding and blood samples were collected from the heart of each rat under anesthesia with diethyl ether. Blood sample of rats was centrifuged at ×2,000 g for 10 minutes at 4°C, and serum was removed and stored at −80°C until analysis.

2.8. Urine Sample. Before induction of diabetes and the day before the end of the experiment, urine samples were collected by placing the rats in individual metabolic cages for 24 h.

2.9. Dissection. Animals were sacrificed using ether anesthesia by cervical dislocation, and then the abdomen was dissected and heart, liver, right kidney, left kidney, left testis, and right testis were dissected and weighed. In addition, one kidney and a piece of the pancreas were saved in saline buffer (0.9% NaCl) for histopathological investigations and the other kidney was kept in ice for homogenate preparation.

2.10. Kidney Homogenate Preparation. Kidney tissue was cut into small pieces and washed by phosphate-buffered saline and then ground in a homogenization buffer, and then the homogenate was prepared as described in Al-Malki and El Rabey [19]. 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 levels of IL-6. The other kidney from each group was used for histopathological examination.

2.11. Determination of Glucose. Glucose was assayed using a kit from Human (Germany) according to the method of Barham and Trinder [20].

2.12. Determination of Carboxymethyl Lysine (CML). Carboxymethyl lysine (CML) was estimated in the serum according to the method of Monnier and Cerami [21] using ELISA kits from MyBIOSOURCE (Canada). This kit employs Double Antibody Sandwich Technique. The principle of Double Antibody Sandwich is based on characteristics of the tested antigen with more than two valances which can identify coated antibody and detection antibody at the same time.

2.13. Determination of Interleukin-6 (IL-6). Interleukin-6 (IL-6) was estimated in the serum and the kidney tissue homogenate by Sayed [15] using immunoassay kit from R&D Systems Inc. (USA).

2.14. Antioxidants Enzymes Activity. Superoxide dismutase (SOD) activity was estimated in the serum and in the kidney tissue homogenate according to the method described by Nishikimi et al. [22] using colorimetric kit from Biodiagnostic Chemical Company (Egypt). Catalase (CAT) activity was estimated in the serum and in the kidney tissue homogenate according to the method described by Aebi [23] using colorimetric kit from Biodiagnostic Chemical Company (Egypt). Glutathione-S-transferase (GST) was estimated in the serum and in the kidney tissue homogenate according to the method described by Habig et al. [24] using a special kit from Biodiagnostic Chemical Company (Egypt).

2.15. Determination of Lipid Peroxidation (MDA). Lipid peroxidation was assayed in the serum and in the kidney tissue homogenate using a kit from Biodiagnostic Chemical Company (Egypt) according to the method of Ohkawa et al. [25] according to the instructions of the supplier.

2.16. Kidney Functions. Uric acid was estimated according to Barham and Trinder [20] using enzymatic colorimetric kit, PAP-method from Human (Germany). Creatinine was assayed in the serum and in the urine by Bartels et al. [26] using a photometric colorimetric kit, Jaffe reaction from Human (Germany). Urea was estimated in the serum according to the method described by Berthelot [27] and Fawcett and Scott [28] using enzymatic colorimetric kit from Human (Germany).

2.17. Determination of Electrolytes. Sodium (Na^+) was assayed using a kit from Human (Germany) according to the colorimetric method of Trinder [29]. Potassium (K^+) was estimated according to the method of Terri and Sesin [30] using Human (Germany).

2.18. Urine Analysis. Creatinine and albumin were estimated in urine. Urine albumin was estimated using a Nephrat II Albumin Kit from Exocell Inc., Philadelphia, PA, USA, according to the method of Sayed [15].

Table 1: The phytochemical analysis of *L. sativum* and cinnamon as revealed by spectrophotometric analysis.

Material	Total phenols	Flavonoids	Carotenoids
L. sativum	58.8 mg/100 g	42.35 mg/100 g	1.43 mg/100 g
Cinnamon	52.7 mg/100 g	1235 mg/100 g	567.3 mg/100 g

2.19. Determination of Immunoglobulins. Immunoglobulins (IgA, IgM, and IgG) were estimated in the serum according to Berne [31] using commercially available kits from Genway Biotech (USA) according the instruction of the suppliers.

2.20. Physiological Parameters. The following physiological parameters were estimated according to the method of Davies and Morris [32] as follows:

(i) Food intake and water consumption were calculated every week.

(ii) Total body weight: rats were weighed every week.

(iii) Food intake (FI) body weight gain (BWG) and food efficiency ratio (FER) were calculated.

(iv) Heart, liver, right kidney, left kidney, left testis, and right testis were weighed after dissection and the relative organ weight was calculated by dividing the organ weight on the total body weight of each rat and then multiplied by 100.

2.21. Histopathological Examination. Five μm thick sections of kidney and pancreatic tissues were prepared and stained with hematoxylin and eosin (H&E) dye for microscopic investigation according to Drury et al. [33]. The stained sections were examined and photographed under an Olympus light microscope with a digital camera.

2.22. Statistical Analysis. Values were analyzed using SPSS program to calculate the t-test and the mean ± SD and then analyzed using one-way analysis of variance (ANOVA, $P < 0.05$) using a protected least significant difference (LSD) test of SAS package.

3. Results

3.1. Phytochemical Analysis of Cress Seeds and Cinnamon. Table 1 shows the phytochemical analysis of *L. sativum* and cinnamon. *L. sativum* seeds that contain 58.8 mg/100 g DW total phenols, 42.35 mg/100 g DW flavonoids, and 1.43 mg/100 g DW carotenoids, whereas cinnamon contains 52.7 mg/100 g DW total phenols, 1235 mg/100 g DW flavonoids, and 567.3 mg/100 g DW carotenoids.

3.2. Fasting Blood Sugar, CML, and IL-6. Table 2 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum fasting blood sugar, CML, and IL-6 in diabetic rats. Induction of diabetes in

Table 2: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on blood sugar, CML, and IL-6 in diabetic rats.

	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
FBS mg/dL	Mean ± SE	92.666 ± 1.145[d]	283.333 ± 2.472[a]	206.333 ± 2.444[b]	147.000 ± 1.211[c]
	LSD 0.05 = 6.256				
	t-test	—	−63.63***	16.67***	53.15***
Carboxymethyl lysine (CML)%	Mean ± SE	188.16 ± 2.38[d]	276.00 ± 2.58[a]	243.66 ± 1.72[b]	227.16 ± 1.30[c]
	LSD 0.05 = 6.415				
	t-test	—	−24.84***	13.53***	16.07***
Serum interleukin-6 (SIL-6) pg/mL	Mean ± SE	5.600 ± 0.260[d]	24.483 ± 0.892[a]	15.883 ± 0.612[b]	8.733 ± 0.230[c]
	LSD 0.05 = 1.864				
	t-test	—	−17.24***	6.55***	21.77***
TIL6 pg/mL	Mean ± SE	48.800 ± 2.010[d]	90.433 ± 1.551[a]	74.216 ± 0.772[b]	63.966 ± 1.275[c]
	LSD 0.05 = 4.276				
	t-test	—	−14.14***	16.14***	12.93***

Data are represented as mean ± SE. For *t*-test values, *** is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

the positive control group (G2) significantly ($P < 0.001$) increased the mean values of serum fasting blood sugar in the positive control compared with that of the negative control. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts in G3 and G4, respectively, significantly ($P < 0.001$) decreased the mean values of serum fasting blood sugar compared with the positive control. In addition, treating the diabetic rats in G4 with cinnamon methanol extract was much more efficient than *L. sativum* methanol extract in G3.

Table 2 also shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on carboxymethyl lysine (CML) in diabetic rats. The mean values of carboxymethyl lysine in the positive control were significantly ($P < 0.001$) higher than that of the negative control. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts in G3 and G4, respectively, significantly ($P < 0.001$) decreased the mean values of CML compared to that of the positive control. Treating diabetic rats with cinnamon methanol extract in G4 was more efficient than treating with *L. sativum* in G3.

Table 2 also shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum interleukin-6 (SIL-6) and kidney tissue homogenate (TIL-6) of diabetic rats. The mean values of serum IL-6 and kidney tissue homogenate in the positive control were higher than that of the negative control. Treating the diabetic rats in G3 and G4 with *L. sativum* and cinnamon methanol extract significantly ($P < 0.001$) decreased the mean values of SIL-6 and TIL-6 more than that of the positive control. Cinnamon methanol extract (in G4) was much more efficient than *L. sativum* methanol (in G3) extract in lowering SIL-6 and TIL-6.

3.3. Antioxidants Enzymes. Table 3 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on antioxidants enzymes in the serum and kidney tissue homogenate of diabetic rats. The mean values of catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) in serum and kidney tissue homogenate of the positive control were very high and significantly ($P < 0.001$) decreased compared with that of the negative control. Treating the diabetic rats in G3 and G4 with *L. sativum* and cinnamon methanol extract significantly ($P < 0.001$) increased the mean values of CAT, SOD, and GST compared with that of the positive control. Generally, treating the diabetic rats with cinnamon methanol extract in G4 increased all the studied antioxidant enzymes in serum more than *L. sativum* methanol extract in G3.

3.4. Lipid Peroxide. Table 4 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on lipid peroxide in diabetic rats. The mean values of MDA in the serum and kidney tissue homogenate of the positive control were significantly ($P < 0.001$) higher than that of the negative control. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts for 4 weeks in G3 and G4, respectively, very highly significantly ($P < 0.001$) lowered the mean values of lipid peroxide compared to that of the positive control. It is worth mentioning that treating diabetic rats with cinnamon methanol extract in G4 was more efficient than *L. sativum* extract in G3.

3.5. Renal Function. Table 5 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum urea, creatinine, uric acid, and sodium and potassium ions in diabetic rats. Induction of diabetes significantly ($P < 0.001$) increased the mean values of serum urea, creatinine, and uric acid in the positive control compared with that of the negative control due to the diabetic nephropathy. Treating the diabetic rats in G3 and G4 with *L. sativum* and cinnamon methanol extract, respectively,

Table 3: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on antioxidants enzymes in the serum of diabetic rats.

Parameters serum	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
Catalase (CAT) U/I	Mean ± SE	2.405 ± 0.193^{a}	0.151 ± 0.011^{d}	0.950 ± 0.036^{c}	1.808 ± 0.016^{b}
	LSD 0.05 = 0.295				
	t-test	—	11.49***	−24.48***	−73.76***
Superoxide dismutase (SOD) U/mL	Mean ± SE	638.683 ± 1.561^{a}	120.833 ± 2.411^{d}	239.683 ± 1.597^{c}	487.016 ± 1.469^{b}
	LSD 0.05 = 5.922				
	t-test	—	178.65***	−37.04***	−168.71***
Glutathione reductase (GSST) U/mL	Mean ± SE	813.200 ± 2.320^{a}	120.933 ± 2.381^{d}	239.683 ± 1.597^{c}	714.250 ± 2.478^{b}
	LSD 0.05 = 7.090				
	t-test	—	228.70***	−37.37***	−169.43***
Catalase (CAT) U/g kidney tissue	Mean ± SE	5.028 ± 0.085^{a}	0.385 ± 0.023^{d}	1.495 ± 0.065^{c}	2.720 ± 0.056^{b}
	LSD 0.05 = 0.205				
	t-test	—	51.41***	−14.99***	−37.78***
Superoxide dismutase (SOD) U/g. kidney tissue	Mean ± SE	917.183 ± 2.597^{a}	175.583 ± 4.539^{d}	585.566 ± 2.396^{c}	735.700 ± 2.848^{b}
	LSD 0.05 = 10.754				
	t-test	—	117.11***	−70.70***	−90.93***
Glutathione reductase (GSST) U/g kidney tissue	Mean ± SE	826.200 ± 2.755^{a}	315.683 ± 3.560^{d}	623.600 ± 2.735^{c}	719.600 ± 2.848^{b}
	LSD 0.05 = 7.978				
	t-test	—	109.14***	−118.29***	−119.70***

Data are represented as mean ± SE. ∗∗∗: significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

Table 4: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on lipid peroxide in the serum and kidney tissue homogenate in diabetic male rats.

Parameters	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
MDA nmol/mL	Mean ± SE	0.936 ± 0.035^{d}	4.500 ± 0.057^{a}	3.601 ± 0.061^{b}	2.240 ± 0.038^{c}
	LSD 0.05 = 0.164				
	t-test	—	−52.66***	8.71***	34.09***
MDA nmol/g kidney tissue	Mean ± SE	2.586 ± 0.069^{d}	16.081 ± 0.183^{a}	7.581 ± 0.071^{b}	4.731 ± 0.090^{c}
	LSD 0.05 = 0.347				
	t-test	—	−70.12***	36.50***	51.68***

Data are represented as mean ± SE. For t-test values, ∗∗∗ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

significantly ($P < 0.001$) decreased the mean values of all renal function parameters compared with that of the positive control.

Table 5 also shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on sodium and potassium ions in the serum of diabetic rats. The mean values of sodium and potassium ions in serum of the positive control were significantly ($P < 0.001$) lower than that of the negative control. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts in G3 and G4, respectively, significantly ($P < 0.001$) increased the mean values of sodium and potassium ions in the serum compared with that of the positive control.

3.6. Urine Analysis. Table 6 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on urine albumin and creatinine in diabetic rats. The mean values of urine albumin in the positive control were significantly ($P < 0.001$) higher than that of the negative control. Treating the diabetic rats in G3 and G4 significantly ($P < 0.001$) decreased the mean values of urine albumin compared to that of the positive control.

Table 5: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum urea, creatinine, uric acid, Na^+, and P^+ in diabetic rats.

Parameters mg/dL	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
Urea	Mean ± SE	24.50 ± 1.11[d]	74.83 ± 0.87[a]	46.66 ± 0.88[b]	36.83 ± 0.60[c]
	LSD 0.05 = 2.720				
	t-test	—	−29.16***	24.75***	28.32***
Creatinine	Mean ± SE	0.68 ± 0.03[d]	3.63 ± 0.18[a]	2.78 ± 0.07[b]	1.62 ± 0.30[c]
	LSD 0.05 = 0.547				
	t-test	—	−14.90***	3.47***	7.12***
Uric acid	Mean ± SE	3.33 ± 0.08[d]	6.68 ± 0.04[a]	5.60 ± 0.05[b]	4.70 ± 0.07[c]
	LSD 0.05 = 0.147				
	t-test	—	−59.53***	13.67***	23.80***
Sodium	Mean ± SE	143.833 ± 0.945[a]	118.333 ± 0.881[d]	125.833 ± 0.477[c]	133.000 ± 1.154[b]
	LSD 0.05 = 3.523				
	t-test	—	19.85***	−7.83***	−8.06***
Potassium	Mean ± SE	4.866 ± 0.033[a]	3.033 ± 0.088[d]	3.516 ± 0.054[c]	4.183 ± 0.060[b]
	LSD 0.05 = 0.392				
	t-test	—	17.39***	−5.54***	−10.88***

Data are represented as mean ± SE. For *t*-test values, *** is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

Table 6: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on urine albumin and creatinine in diabetic rats.

Parameters mg/dL	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
Urine albumin	Mean ± SE	22.16 ± 1.70[d]	411.50 ± 7.74[a]	291.50 ± 2.36[b]	163.50 ± 2.98[c]
	LSD 0.05 = 13.638				
	t-test	—	−47.24***	16.17***	24.64***
Urine creatinine	Mean ± SE	85.00 ± 0.85[a]	27.00 ± 0.36[d]	45.83 ± 1.57[c]	62.16 ± 1.19[b]
	LSD 0.05 = 3.148				
	t-test	—	84.90***	−11.61***	−37.18***

Data are represented as mean ± SE. For *t*-test values, *** is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

The mean values of urine creatinine in the positive control were significantly ($P < 0.001$) lower than that of the negative control. Treating the diabetic rats in G3 and G4 *L. sativum* and cinnamon methanol extracts for 4 weeks significantly ($P < 0.001$) increased the mean values of urine creatinine compared to that of the positive control.

3.7. Immunoglobulins (IgG, IgA, and IgM). Table 7 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum immunoglobulins (IgG, IgA, and IgM) in diabetic rats. Induction of diabetes in G2 significantly ($P < 0.001$) increased the mean values of the serum IgG, IgA, and IgM in the positive control compared with that of the negative control. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts in G3 and G4, respectively, significantly ($P < 0.001$) decreased the mean values of IgG, IgA, and IgM compared with that of the positive control. Moreover, treating diabetic rats with cinnamon methanol extract in G4 was more efficient in ameliorating immunoglobulins more than treating them with *L. sativum* in G3.

3.8. Total Body Weight. Table 8 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on the total body weight in diabetic rats under study. The mean values of total body weight of the positive control in the 1st, 2nd, 3rd and 4th weeks were significantly ($P < 0.01$) lower than that of the negative control. Treating the diabetic rats in G3 and G4 significantly ($P < 0.01$) increased the mean values of total body weight compared with that of the positive control. Moreover, treating diabetic rats with cinnamon methanol extract in G4 was more efficient in ameliorating immunoglobulins more than treating them with *L. sativum* in G3.

Table 7: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on serum immunoglobulins in diabetic rats.

Immunoglobulins mg/dL	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
IgG	Mean ± SE LSD 0.05 = 6.952	530.66 ± 1.05^{d}	754.33 ± 3.46^{a}	702.00 ± 3.04^{b}	581.16 ± 1.66^{c}
	t-test	—	−63.91***	14.09***	52.62***
IgA	Mean ± SE LSD 0.05 = 6.261	99.16 ± 1.88^{d}	359.83 ± 1.74^{a}	267.66 ± 2.74^{b}	135.50 ± 1.28^{c}
	t-test	—	−85.42***	31.37***	159.51***
IgM	Mean ± SE LSD 0.05 = 4.776	129.83 ± 1.07^{d}	357.16 ± 2.24^{b}	250.33 ± 1.60^{c}	581.16 ± 1.66^{a}
	t-test	—	−138.06***	51.15***	−81.25***

Data are represented as mean ± SE. For *t*-test values, $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

Table 8: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on the total body weight in diabetic rats.

Total body weight (g)	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
1st week	Mean ± SE LSD 0.05 = 1.922	188.83 ± 0.40^{c}	182.83 ± 0.79^{d}	194.83 ± 0.83^{b}	201.66 ± 0.66^{a}
	t-test	—	8.78***	−17.56***	−19.21***
2nd week	Mean ± SE LSD 0.05 = 2.276	194.00 ± 0.81^{c}	187.16 ± 0.79^{d}	202.50 ± 0.76^{b}	210.66 ± 0.42^{a}
	t-test	—	4.72***	−20.17***	−41.76***
3rd week	Mean ± SE LSD 0.05 = 2.349	207.83 ± 0.70^{b}	192.33 ± 1.08^{c}	210.16 ± 0.54^{b}	215.00 ± 0.77^{a}
	t-test	—	10.82***	−15.28***	−28.23***
4th week	Mean ± SE LSD 0.05 = 3.091	210.33 ± 0.91^{c}	194.00 ± 1.69^{d}	213.66 ± 0.61^{b}	219.00 ± 0.44^{a}
	t-test	—	7.32***	−10.30***	−17.11***

Data are represented as mean ± SE. For *t*-test values, $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference.

3.9. Food Intake. Table 9 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on food intake in diabetic rats. In the 1st, 2nd, 3rd and 4th weeks, the mean values of food intake in all groups were approximately equal. The differences were nonsignificant.

3.10. Water Consumption. Table 10 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on water consumption in diabetic rats. Water consumption was nonsignificantly affected as a result of diabetes for all groups in all weeks except for the 2nd and 3rd week of the positive control (G2) and G3 and G4 of the 1st week.

3.11. Organs Weight. Table 11 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on organ weight in diabetic rats. The mean values of heart weight in the positive control were nonsignificantly higher than that of the negative control in all weeks, whereas treating the diabetic rats in G3 and G4 with cress seed and cinnamon methanol extract significantly increased all organ weights in all weeks.

3.12. Physiological Evaluation. Table 12 shows the effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on food intake, body weight gain (BWG), and food efficiency ratio (FER) in diabetic rats. The mean values of body weight gain (BWG)/day, BWG g/4 weeks, and BWG% in the positive control were lower than that of the negative control, whereas treating the diabetic rats with cress seed and cinnamon methanol extract significantly increased these BWG parameters. On the other hand, FER and FER% in the positive control group were significantly lower than that of the negative control. In G3 and G4, the mean values of FER and FER% were higher than those of the positive control.

Table 9: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on food intake in diabetic rats.

Food intake g/day	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
1st week	Mean ± SE	14.50 ± 0.22^{a}	14.50 ± 0.22^{a}	14.66 ± 0.21^{a}	14.50 ± 0.22^{a}
	LSD 0.05 = 0.698				
	t-test	—	0.00^{NS}	-0.41^{NS}	0.00^{NS}
2nd week	Mean ± SE	16.66 ± 0.21^{a}	16.66 ± 0.21^{a}	16.50 ± 0.22^{a}	16.50 ± 0.22^{a}
	LSD 0.05 = 0.622				
	t-test	—	0.00^{NS}	0.54^{NS}	0.54^{NS}
3rd week	Mean ± SE	16.66 ± 0.21^{a}	16.50 ± 0.22^{a}	16.83 ± 0.30^{a}	16.66 ± 0.21^{a}
	LSD 0.05 = 0.686				
	t-test	—	0.54^{NS}	-1.00^{NS}	-0.54^{NS}
4th week	Mean ± SE	17.66 ± 0.21^{a}	18.66 ± 0.42^{a}	19.00 ± 0.44^{a}	18.66 ± 0.42^{a}
	LSD 0.05 = 1.329				
	t-test	—	-1.93^{NS}	-0.41^{NS}	0.00^{NS}
Food intake g/day	Mean ± SE	16.37 ± 0.26^{a}	16.58 ± 0.33^{a}	16.75 ± 0.35^{a}	16.58 ± 0.33^{a}
	LSD 0.05 = 0.925				
	t-test	—	-1.04^{NS}	-0.70^{NS}	0.00^{NS}

Data are represented as mean ± SE. For t-test values, $*$ is significant at $P < 0.05$, $**$ is significant at $P < 0.01$, and $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference, NS: nonsignificant.

Table 10: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on water consumption by diabetic rats.

Water consumed mL/day	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
1st week	Mean ± SE	33.33 ± 1.05^{ab}	32.50 ± 1.11^{b}	36.33 ± 0.88^{a}	36.33 ± 0.88^{a}
	LSD 0.05 = 3.257				
	t-test	—	0.415^{NS}	-4.60^{***}	-2.49^{**}
2nd week	Mean ± SE	33.8 ± 1.24^{b}	37.16 ± 0.79^{a}	36.83 ± 0.87^{a}	36.16 ± 1.40^{ab}
	LSD 0.05 = 2.578				
	t-test	—	-2.65^{**}	0.26^{NS}	0.69^{NS}
3rd week	Mean ± SE	34.66 ± 1.05^{a}	36.83 ± 0.87^{a}	37.50 ± 0.92^{a}	37.16 ± 0.79^{a}
	LSD 0.05 = 3.035				
	t-test	—	-2.13^{*}	-0.38^{NS}	-0.21^{NS}
4th week	Mean ± SE	29.16 ± 1.53^{a}	29.66 ± 1.33^{a}	33.33 ± 1.54^{a}	33.00 ± 1.91^{a}
	LSD 0.05 = 5.155				
	t-test	—	-0.24^{NS}	-1.67^{NS}	-1.28^{NS}

Data are represented as mean ± SE. For t-test values $*$ is significant at $P < 0.05$, $**$ is significant at $P < 0.01$, and $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

3.13. Histopathology of Kidney. Kidney of the negative control group (G1) exhibited the general structure of normal kidney with normal histological structure of renal parenchyma, normal renal tissue, blood vessels, and normal interstitial tissue with no histopathological changes (Figure 1(a)). The positive control (G2) group shows collapsed glomerular tuft with marked tubular atrophy interstitial inflammation and interstitial hemorrhage (Figure 1(b)). The kidney of diabetic rats in the third group (G3) that were treated with *L. sativum* shows normal glomeruli regenerated tubule with persistent interstitial hemorrhage (Figure 1(c)). Similarly, the kidney tissue of a rat from group G4 treated with cinnamon (Figure 1(d)) shows near normal renal cortical tissue.

3.14. Histopathology of Pancreas. The histology of pancreas is shown in Figure 2. Pancreas tissues of rat from negative control group showing normal pancreatic acini Langerhans cells and interductal glands are shown in Figure 2(a). Pancreas tissues of the positive control diabetic group (G2) showed mild degeneration of pancreatic acini cells with periductal inflammation and edema with mild congestion (Figure 2(b)). Figure 2(c) shows pancreas tissues of rat from group G3 that

Table 11: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on organ weight in diabetic rats.

Organs weight, g	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
Heart	Mean ± SE	0.433 ± 0.091^{a}	0.450 ± 0.018^{a}	0.550 ± 0.022^{a}	0.566 ± 0.021^{a}
	LSD 0.05 = 0.127				
	t-test	—	-0.20^{NS}	-3.46^{**}	-11.06^{***}
Liver	Mean ± SE	4.100 ± 0.821^{a}	4.866 ± 0.084^{a}	5.383 ± 0.153^{a}	5.200 ± 0.077^{a}
	LSD 0.05 = 1.290				
	t-test	—	-0.94^{NS}	-2.79^{**}	-2.59^{**}
Right kidney	Mean ± SE	0.483 ± 0.101^{b}	0.633 ± 0.021^{ab}	0.666 ± 0.021^{a}	0.633 ± 0.021^{ab}
	LSD 0.05 = 0.151				
	t-test	—	-1.62^{NS}	-1.00^{NS}	0.00^{NS}
Left kidney	Mean ± SE	0.516 ± 0.104^{a}	0.683 ± 0.016^{a}	0.633 ± 0.021^{a}	0.633 ± 0.021^{a}
	LSD 0.05 = 0.168				
	t-test	—	-1.53^{NS}	2.23^{*}	1.46^{NS}
Right testes	Mean ± SE	0.933 ± 0.194^{a}	0.965 ± 0.020^{a}	1.133 ± 0.021^{a}	1.133 ± 0.021^{a}
	LSD 0.05 = 0.299				
	t-test	—	-0.15^{NS}	-4.99^{***}	-7.76^{***}
Left testes	Mean ± SE	0.966 ± 0.201^{a}	0.956 ± 0.019^{a}	1.1833 ± 0.030^{a}	1.1833 ± 0.016^{a}
	LSD 0.05 = 0.298				
	t-test	—	0.04^{NS}	-5.14^{***}	-7.37^{***}

Data are represented as mean ± SE. For t-test values, $*$ is significant at $P < 0.05$, $**$ is significant at $P < 0.01$, and $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

Table 12: Effect of administration of *L. sativum* and cinnamon methanol extracts for 4 weeks on food intake (FI), body weight gain (BWG), and food efficiency ratio (FER) in diabetic rats.

Biological evaluation	Statistics	G1 Negative control	G2 Positive control	G3 *L. sativum* extract	G4 Cinnamon extract
BWG g/day	Mean ± SE	0.822 ± 0.026^{a}	0.422 ± 0.061^{b}	0.755 ± 0.014^{a}	0.738 ± 0.035^{a}
	LSD 0.05 = 3.379				
	t-test	—	5.13^{***}	-5.77^{***}	-9.58^{***}
BWG g/4 weeks	Mean ± SE	24.666 ± 0.802^{a}	12.666 ± 1.855^{b}	22.666 ± 0.421^{a}	22.166 ± 1.077^{a}
	LSD 0.05 = 0.153				
	t-test	—	5.13^{***}	-5.77^{***}	-9.58^{***}
BWG %	Mean ± SE	13.284 ± 0.425^{a}	6.991 ± 1.029^{c}	11.867 ± 0.223^{ab}	11.274 ± 0.592^{b}
	LSD 0.05 = 1.792				
	t-test	—	4.91^{***}	-5.18^{***}	-7.76^{***}
FER g/day	Mean ± SE	0.050 ± 0.001^{a}	0.025 ± 0.003^{b}	0.045 ± 0.000^{a}	0.044 ± 0.002^{a}
	LSD 0.05 = 0.006				
	t-test	—	5.34^{***}	-5.77^{***}	-9.58^{***}
FER%	Mean ± SE	5.022 ± 0.163^{a}	2.546 ± 0.373^{b}	4.510 ± 0.083^{a}	4.456 ± 0.216^{a}
	LSD 0.05 = 0.127				
	t-test	—	5.24^{***}	-5.63^{***}	-9.57^{***}

Data are represented as mean ± SE. For t-test values, $*$ is significant at $P < 0.05$, $**$ is significant at $P < 0.01$, and $***$ is significant at $P < 0.001$. For ANOVA analysis, within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means superscripts with the same letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

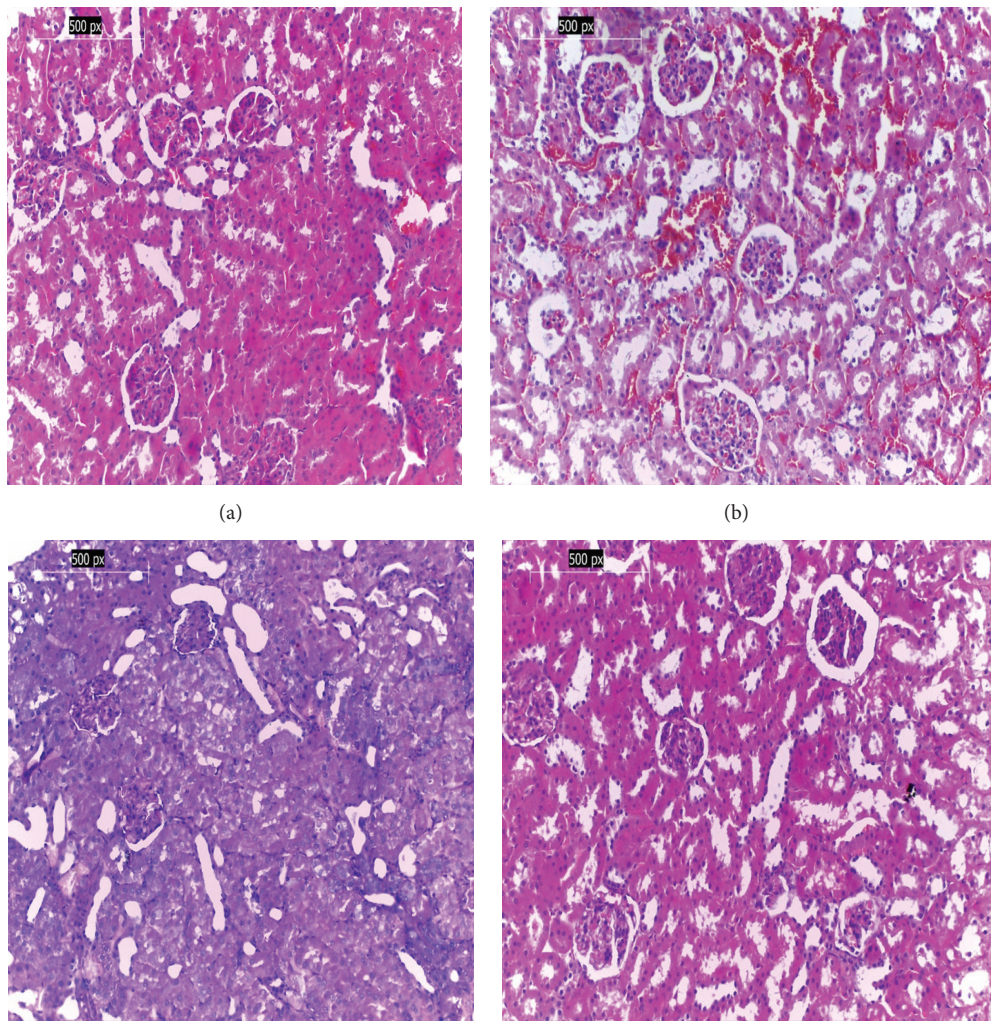


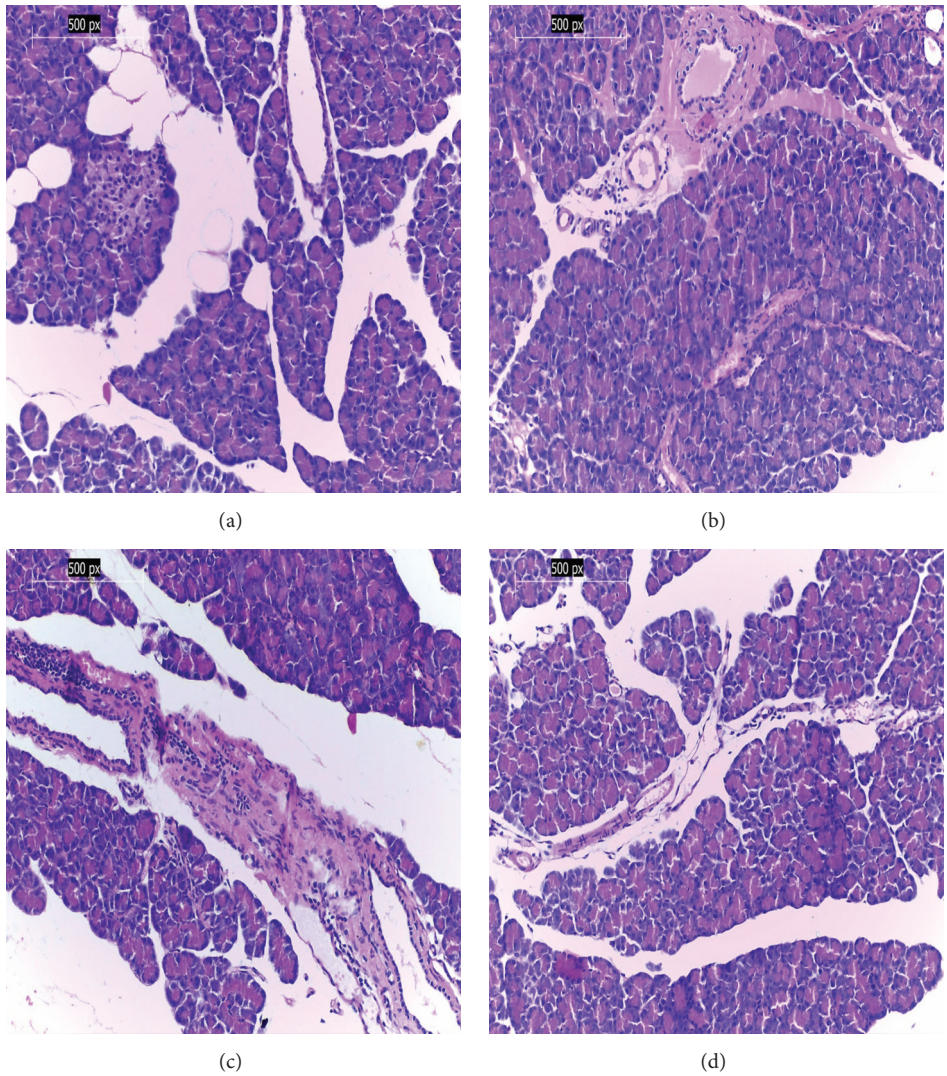
Figure 1: (a) Photomicrography of a kidney of negative control group (G1) reveals normal histological structure; (b) photomicrography of a kidney of the positive control group with pathological changes; (c) photomicrography of a kidney of G3 treated with *L. sativum* shows nearly normal tissues; (d) photomicrography of a kidney of G4 group treated with cinnamon shows nearly normal renal cortical tissue (H&E ×200).

were treated with *L. sativum* for 4 weeks with very mild inflammation and edema. Figure 2(d) shows pancreas of rat from group G4 treated with cinnamon for 4 weeks with normal pancreatic tissue.

4. Discussion

This study was conducted in order to find out the antidiabetic activity of *L. sativum* and cinnamon methanol extract in rats with STZ induced diabetes. Induction of diabetes increased the fasting blood sugar in the diabetic male rats of the positive control group. The phenolic, flavonoid, and carotenoid content of both garden cress and cinnamon encouraged us to test their antioxidant potential in controlling STZ induced diabetes and diabetic nephropathy as one of diabetic complications. The flavonoid group of compounds in *L. sativum* has anti-inflammatory activity [34]. Phenolic phytochemicals have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, and anti-inflammatory activities [35].

The current results showed a decrease in the levels of FBS in G3 and G4 after treatment with *L. sativum* and cinnamon methanol extract, respectively, for 4 weeks compared with the positive control group. This result is consistent with that of Abdelwahab et al. [8] using the aqueous *L. sativum* extract that significantly reduced the blood glucose levels after a single or repeated administration. The strong hypoglycemic action of *L. sativum* extract is due to the presence of benzyl isothiocyanate [1, 11]. Similarly, cinnamon acquired its antidiabetic activity because it contains several phenolic compounds as catechin, epicatechin, procyanidin B2, and phenol polymers that showed significant inhibitory effects on the formation of advanced glycation end products [36]. It also possesses insulin mimetic properties because its biologically



(a) (b)

(c) (d)

Figure 2: (a) Photomicrography of pancreas of rat from the negative control group showing normal pancreatic islets and glands; (b) photomicrography of pancreas of rat from the positive control group shows sever pathological changes; (c) photomicrography of pancreas of rat from G3 treated with *L. sativum* with very mild inflammation and edema; (d) photomicrography of pancreas of rat from group G4 treated with cinnamon shows normal pancreatic tissue (H&E ×200).

active substances enhance glucose uptake by activating insulin receptor kinase activity, autophosphorylation of the insulin receptor, and glycogen synthase activity [37].

In the current study, interleukin-6 levels were increased in the serum and kidney tissue homogenate as a result of diabetes in G2 as a result of STZ induced diabetes. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts showed a significant decrease in levels of interleukin-6 in G3 and G4 compared with the positive control. Several inflammatory cytokines including TNF-a, IL-1b, and IL-6 have been identified as being involved in the development of insulin resistance [19, 38]. Their interference with insulin signaling leads to hyperglycemia and proinflammatory changes. IL-6 influences insulin sensitivity by directly impairing insulin signaling in primary mouse hepatocytes and 3T3-L1 adipocytes with decreased activity. In hamsters with fructose feeding induced insulin-resistant diabetes, the serum levels of TNF-a and IL-6 were found to be significantly higher compared with those of the chow-fed hamsters [39].

In the current study, the levels of carboxymethyl lysine which is an advanced glycation end product (AGE) were increased in the serum as a result of diabetes in G2. Treating the diabetic rats with *L. sativum* and cinnamon methanol extracts showed a significant decrease in CML levels in G3 and G4 compared with the positive control. Adisakwattana et al. [40] indicated that cinnamic acid and its derivatives significantly inhibit the formation of advanced glycation end products (AGEs) by approximately 11.96–63.36% in a concentration-dependent manner.

Our result showed a significant decrease in the levels of catalase, SOD, and GST in serum and kidney tissue homogenate of the positive control group (G2) compared

with that of the negative control group (G1) as a result of induction of diabetes. This result is consistent with Eidi et al. [41] and Al-Malki and El Rabey [19]. In addition, Baynes and Thorpe [42] reported that hyperglycemia increases the generation of free radicals by glucose autooxidation and the increment in free radicals thereby depleting the activity of antioxidant defense system and thus promoting de novo free radical generation that may lead to liver cell damage. However, in G3 and G4 the levels of catalase, SOD, and GST in serum and kidney tissue homogenate was increased compared with the positive control group as a result of treatment of diabetic rats with *L. sativum* or cinnamon methanol extract for 4 weeks. Both *L. sativum* and cinnamon contain polyphenols, which are among the natural dietary antioxidants found in cinnamon and have been shown to reduce oxidative stress via the inhibition of 5-lipoxygenase [43].

The current study showed that lipid peroxide was increased in serum and kidney tissue homogenate as a result of diabetes induction in the positive control group (G2). This result is in agreement with that of Al-Malki and El Rabey [19]. Meanwhile, after treatment with *L. sativum* or cinnamon in G3 and G4, respectively, a significant decrease in the levels of MDA was encountered compared with the positive control. Cinnamon contains high level of phenolic groups that cause scavenging of free radicals which is one of the major antioxidation mechanisms to inhibit the chain reaction of lipid peroxidation [44]. In addition, our results concerning the elevation of MDA and reduction in antioxidants enzyme activity due to induction of diabetes and amelioration after *L. sativum* and cinnamon treatments are consistent with other studies [36].

The renal function showed that an increase in serum urea, creatinine, uric acid, and urine albumin was disrupted by diabetes induction in the positive control group (G2). This result is consistent with the fact that STZ induced diabetes leads to diabetic nephropathy and consistent with the studies of Sayed [15] and Al-Malki and El Rabey [19]. Treating the diabetic rats of G3 and G4 with *L. sativum* and cinnamon methanol extract, respectively, showed a significant decrease in serum urea, creatinine, uric acid, and urine albumin and increase in urine creatinine. This result agrees with that of Mogensen and Christensen [45] and Kumar et al. [46]. This amelioration in renal function is due to the presence of flavonoids and steroidal compounds [46]. The current results also showed significant decrease in serum sodium and potassium ions level as a result of diabetes, whereas the methanol extracts of cinnamon and cress seeds restored them to the normal levels. Cinnamon has the most potent inhibitory effect on the intestinal ATPase as compared to extracts of other spices [47]. They also inhibited the *in vitro* Na^+-K^+-ATPase activity in a crude kidney homogenate and the activity of an isolated dog kidney $Na^{(+)}$-$K^{(+)}$-ATPase [48, 49].

The immunoglobulins (IgG, IgA, and IgM) results showed a significant increase in immunoglobulins (IgG, IgA, and IgM) compared with the negative control group. Treating the diabetic rats in G3 and G4 with cress seed and cinnamon methanol extract, respectively, restored these immunoglobulins to the normal levels. This result is consistent with that of Muthenna et al. [50] who stated that cinnamon is able to prevent cross-linking of IgG on red blood cell surface (RBC-IgG). During diabetic conditions, there is a considerable increase in RBC-IgG cross-linking that provides an index of AGE mediated protein cross-linking. Cinnamon obtained from twigs of *Cinnamomum osmophloeum* contains oils reported by Tung et al. [51] to have powerful anti-inflammatory properties. The essential oils and major constituents are primarily represented by trans-cinnamaldehyde, caryophyllene oxide, L-borneol, L-bornyl acetate, eugenol, beta-caryophyllene, E-nerolidol, and cinnamyl acetate. These oils were able to reduce chronic inflammation in granulomatous responses [52].

The total body weight (g) in G2 showed a significant decrease as a result of induction of diabetes, whereas it increased with treating with cinnamon and cress seed methanol extract. This result is consistent with that of Beejmohun et al. [53]. In our study, weight of heart, testes, left kidney, and liver in all groups showed a significant increase as a result of diabetes, whereas the right kidney showed the no significant change. Restoring the normal organ's weight as a result of treating diabetic rats with cinnamon and cress seed methanol extract is consistent with Elgawish and Abdelrazek [54]. For food intake, our result showed no significant change in all groups and the values were very close. Similar results by Torki et al. [55] stated that food intake and body weight were not affected by dietary zinc and cinnamon essential oil.

Water consumption showed no significant changes in all groups, except the first week that showed significant increase in G3 and G4 rats compared with the control group. For the physiological evaluation our result showed significant increase in BWG g/day, BWG g/4 week, BWG%, FER g/day, and FER% compared with the control group. The result of Al-Yahya et al. [56] agrees with our result.

The histological studies showed altered pathological changes in the tissues of kidney and pancreas as a result of diabetes in the positive control group [1, 19], whereas treating the diabetic rats with cress seed and cinnamon methanol extract restored the altered tissues nearly to the normal conditions. Ullah et al. [57] stated that cinnamon significantly attenuated aminoglycosides-kidney toxicity by improving the urea, creatinine, uric acid, urinary protein levels, and histopathological alterations of the kidneys. In addition, our result is in agreement with that of Al-Malki and El Rabey [19].

It could be concluded that both cress seed (*Lepidium sativum*) and cinnamon extracts methanol extracts succeeded in controlling hyperglycemia in rats with streptozotocin induced diabetes. They also ameliorated all biochemical tests and kidney and pancreas functions and tissues and restored them to the normal state because of their antioxidant activity acquired by their possession of phenolic phytochemicals.

Abbreviations

AGEs: Advanced glycation end products
ATP: Adenosine triphosphate

BWG: Body weight gain
CAT: Catalase
CML: Carboxymethyl lysine
CRE: Creatinine
DM: Diabetes mellitus
FBS: Fasting blood sugar
FER: Food efficiency ratio
FI: Food intake
g/dL: Gram per deciliter
G1: Negative control group
G2: Hyperglycemic positive control group
G3: Hyperglycemic rats treated with 20% cress seeds methanol extract
G4: Hyperglycemic rats treated with 20% cinnamon methanol extract
GST: Glutathione S-transferase
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IGT: Impaired glucose tolerance
IL-6: Interleukin-6
LS: Lepidium sativum
MDA: Malondialdehyde
mg/dL: Milligram per deciliter
mmol/L: Millimole per liter
MODY: Maturity-onset diabetes of the young.
ROS: Reactive oxygen species
SOD: Superoxide dismutase
STZ: Streptozotocin
U/g: Unit per gram
U/L: Unit per liter
U/mL: Unit per milliliter
UA: Uric acid
w/w: Weight to weight ratio
μmol/L: Micromole per liter.

Competing Interests

The authors of this paper have no competing interests.

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Research Article

Protective Effects of *Panax notoginseng* Saponins against High Glucose-Induced Oxidative Injury in Rat Retinal Capillary Endothelial Cells

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Diabetic retinopathy, a leading cause of visual loss and blindness, is characterized by microvascular dysfunction. Hyperglycemia is considered the major pathogenic factor for diabetic retinopathy and is associated with increased oxidative stress in the retina. In this study, we investigated the potential protective effects of *Panax notoginseng* Saponins (PNS) in retinal capillary endothelial cells (RCECs) exposed to high glucose conditions. We found a pronounced increase in cell viability in rat RCECs incubated with both PNS and high glucose (30 mM) for 48 h or 72 h. The increased viability was accompanied by reduced intracellular hydrogen peroxide (H_2O_2) and superoxide (O_2^-), decreased mitochondrial reactive oxygen species (ROS), and lowered malondialdehyde (MDA) levels. PNS also increased the activities of total superoxide dismutase (SOD), MnSOD, catalase (CAT), and glutathione peroxidase (GSH-PX). The glutathione (GSH) content also increased after PNS treatment. Furthermore, PNS reduced NADPH oxidase 4 (Nox4) expression. These results indicate that PNS exerts a protective effect against high glucose-induced injury in RCECs, which may be partially attributed to its antioxidative function.

1. Introduction

Diabetic retinopathy is a common complication of diabetes mellitus and a leading cause of acquired blindness in adults in developed countries. Diabetic retinopathy is characterized by progressive alterations in the retinal microvasculature. Retinal microvascular dysfunction is clinically characterized by the loss of endothelial cells and pericytes, capillary occlusion, and blood-retinal barrier (BRB) breakdown during the early stages [1, 2]. Hyperglycemia is considered the main contributing factor for developing diabetic retinopathy, triggering a cascade of pathological metabolic and biochemical changes. Several reports demonstrate that hyperglycemia increased damage in microvascular cells in the retina [3, 4].

The retina has high polyunsaturated fatty acid content and the highest relative oxygen uptake and glucose oxidation when compared to many tissues; these phenomena render the retina more susceptible to oxidative stress [5]. Indeed, a large body of evidence supports the idea that an oxidative stress increase in the retinal microvasculature is a key factor for developing diabetic retinopathy [3]. Endothelial cells, similar to other nonphagocytic cells, generate reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Although low concentrations of ROS might serve as intracellular signaling molecules to induce repair mechanisms against tissue injury, large amounts of ROS are considered toxic products that can cause cell death [6]. In diabetes, the activities of antioxidant defense enzymes are responsible for scavenging free radicals and maintaining redox homeostasis. Superoxide dismutase (SOD) is considered a first-line defense against ROS. This enzyme is present in nearly all cells and converts O_2^- into H_2O_2. As H_2O_2 can react with ROS, it is further degraded by one of two antioxidant enzymes, glutathione peroxidase (GSH-PX) or catalase (CAT) [7, 8]. Furthermore, the cell is equipped with glutathione (GSH), an intracellular antioxidant that is probably the most important defense in the cell. It can act as an ROS scavenger and modulate the intracellular redox state [9].

Panax notoinseng, known as Sanqi in Chinese, is mainly cultivated in the Yunnan and the Guangxi provinces of China. The medicinal properties of the *Panax notoginseng* root include promoting blood clotting, relieving swelling, and

alleviating pain [10]. *Panax notoginseng* Saponins (PNS) is the main active chemical ingredient of *Panax notoginseng*, which is mainly composed of ginsenoside Rg1, ginsenoside Rb1, notoginsenoside R1, and so forth, and belongs to the Araliaceae family. Numerous studies reported that PNS had a protective effect on oxidative stress-induced damage and apoptosis in cultured rabbit bone marrow stromal cells (BMSCs) [11], primary astrocytes, and a neuroblastoma cell line, SH-SY5Y [12]. We postulated that PNS executes a protective function against high glucose-induced injury in RCECs, which may be partially attributed to its antioxidative properties. Therefore, we evaluated the effect of PNS on ROS; MDA content; the activities of total SOD, MnSOD, CAT, and GSH-PX; GSH content; and expression of Nox4 in rat RCECs treated with high glucose. The present findings provide evidence for a functional role of PNS in RCECs in the prevention of diabetic retinopathy.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley (SD) rats weighing 180–200 g were obtained from the Vital River Laboratory Animal Technology Co., Ltd., in Beijing, China. The certificate number was SCXK (Jing) 2012-0001. The laboratory animal care guidelines, approved by the Animal Ethics Committee at the Beijing University of Chinese Medicine, were strictly followed. All efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

2.2. Cell Culture and Identification. Capillary endothelial cells derived from rat retinas and confirmed to be positive for vWf and CD31 proteins by immunofluorescence microscopy were used in this study. To establish the primary cell culture of RCECs, retinas isolated from 20 rat eyes were minced into small pieces, washed in Phosphate Buffered Saline (PBS) containing 5% penicillin/streptomycin, with the retinal fragments retained on a 100 μm cell strainer. Following harvesting, the fragments from sieve were incubated in 3 mL of 0.1% type II collagenase (Sigma Aldrich St. Louis, MI, USA) at 37°C for 30 min with agitation. The retinal fragment suspension was filtered through a 70 μm cell strainer. After centrifugation at 174 ×g for 5 min, the pellets containing microvessel fragments were suspended in endothelial cell medium (ECM, Sciencell, San Diego, CA, USA). The above suspension was transferred to T-25 cm^2 flask precoated with 1% gelatin (Gibco, Life Technologies Inc., Grand Island, NY, USA) and cultured at 37°C in a 5% CO_2 humidified incubator. After 48 h, half of the medium was renewed and thereafter the medium was changed every 2-3 days. The confluent cultures were passaged by detaching the cells using 0.1% trypsin (Sigma Aldrich, St. Louis, MI, USA) in PBS for 2-3 min and plating at a split ratio of 1 : 2. The cells used in this study were passaged 3–5 times.

Endothelial cells were stained with primary antibodies recognizing vWf and CD31. Briefly, the cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. After fixation, the cells were washed with PBS three times and blocked with normal goat serum (NGS) for 30 min. The cells were then incubated with primary antibodies (rabbit anti-vWf, 1 : 100; rabbit anti-CD31, 1 : 100, Santa Cruz, Dallas, Texas, USA) at 4°C overnight. The cells were then washed with PBS and probed with FTIC-labeled anti-rabbit secondary antibody (1 : 100, Zhong Shan Jin Qiao, Beijing, China). 4′,6-Diamidino-2-phenylindole (DAPI, Cell Signaling Technology, Danvers, MA, USA) was used to stain nuclei in the final step. The images were captured using a fluorescence microscope.

2.3. MTT Assay. The rat RCECs were seeded in gelatin-coated 96-well plates, allowed to attach overnight, and then incubated with either 5.5 mM of glucose or 30 mM of glucose with or without varying concentrations of PNS (20, 50, 100, and 200 μg/mL) for 24 h, 48 h, and 72 h. Four hours before the culture was terminated, MTT (5 mg/mL) was added to each well. After 4 h of incubation with MTT and medium at 37°C, the supernatants were removed and 150 μL of dimethylsulfoxide [(DMSO), Sigma Aldrich, St. Louis, MI, USA] was added to each well to dissolve the blue formazan. The 540 nm absorbance of each well was read on an enzyme-linked immunosorbent assay reader (Thermo Labsystems, Finland). All of the experiments were repeated three times.

2.4. Trypan Blue Staining Assay. Rat RCECs were seeded in gelatin-coated 24-well plates, allowed to attach overnight, and then exposed to experimental conditions. After treatment, the supernatant was removed, 0.1% trypsin was added to each well to suspend the cells, and then 0.4% trypan blue solution (Sigma Aldrich, St. Louis, MI, USA) was added to each well to stain the cells. The cell numbers were observed by an inverted microscope (LEICA, Germany).

2.5. Fluorescent Probe Assay. The fluorescent probes DCFH-DA (Sigma Aldrich, St. Louis, MI, USA), DHE (Sigma Aldrich, St. Louis, MI, USA), and MitoTracker Red CM-H_2XRos (Invitrogen, Life Technologies Inc., Grand Island, NY, USA) were used to measure the production of intracellular H_2O_2 and O_2^- and mitochondrial ROS. Briefly, confluent rat RCECs plated in 35 mm^2 confocal laser special disks were exposed to varying experimental conditions. The reactions were stopped by removing the medium, washing with PBS, staining with DCFH-DA (at a final concentration of 10 μM for 30 minutes), DHE (at a final concentration of 5 μM for 30 minutes), and MitoTracker Red CM-H_2XRos (at a final concentration of 2.5 μM for 20 minutes) at 37°C, washing with PBS, and then observing under an ECLIPSE Ti laser confocal microscope (Nikon, Japan). Confocal images were processed with ImageJ software.

2.6. Biochemical Assay. Rat RCECs were seeded in gelatin-coated T-25 cm^2 flasks and allowed to attach for 24 h before treatment. After 72 h of treatment, the cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS], and the protein concentrations were determined by a BCA protein assay. Aliquots were stored at −80°C until use in assays to detect MDA, SOD, Mn-SOD, CAT, GSH-PX, GSH, and GSSG. MDA SOD, Mn-SOD, CAT, and GSH-PX were

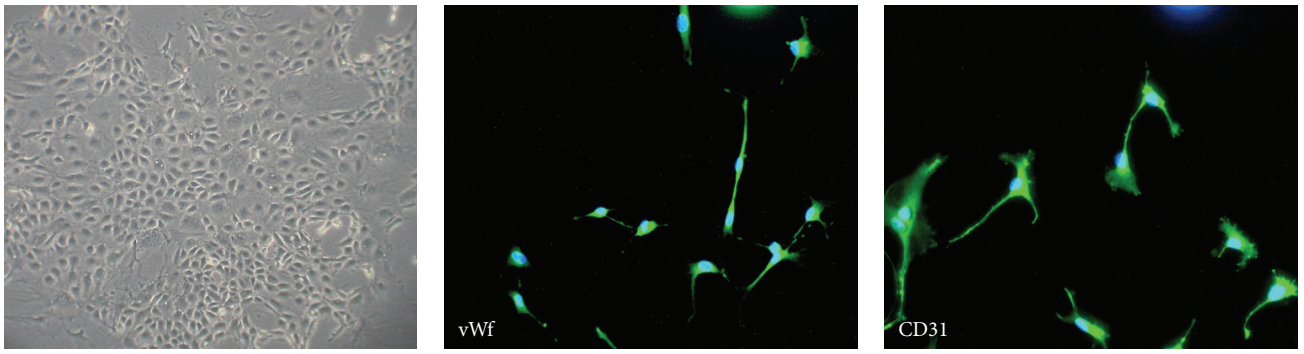

Figure 1: Morphology and immunofluorescence in RCECs. RCECs from rat retinal microvascular fragments were cultured in endothelial cell medium. When the RCECs were cultured for 10 days, the characteristic cobble-stone morphology of RCECs was observed (a). The RCECs were positive for both vWf (b, green fluorescent) and CD31 (c, green fluorescent), as determined by an immunofluorescent assay. All nuclei were stained with DAPI (blue fluorescent).

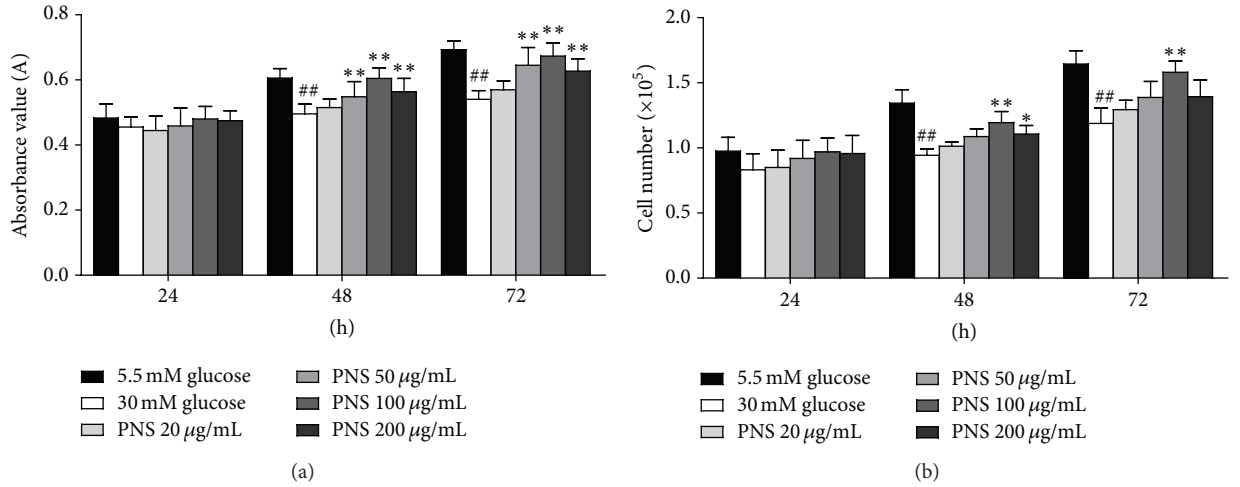

Figure 2: PNS increased cell viability in rat RCECs exposed to high glucose. Seeded in 24-well or 96-well plates, the rat RCECs were incubated with varying concentrations of PNS (20, 50, 100, and 200 μg/mL) in 30 mM glucose. After culturing for 24 h, 48 h, or 72 h, MTT and trypan blue staining assay were used to examine cell viability. The MTT assay showed that 50, 100, and 200 μg/mL of PNS increased cell viability after 48 and 72 h (a). The trypan blue assay showed that 100 and 200 μg/mL of PNS increased cell viability after 48 h and that 100 μg/mL of PNS increased cell viability after 72 h (b). The experiment was repeated three times. Data are expressed as the mean ± SD ($n = 5$). $^{\#\#}P < 0.01$ versus 5.5 mM glucose; $^{**}P < 0.01$ and $^{*}P < 0.05$ versus 30 mM glucose.

detected using the respective kits (Nanjing, Jiancheng Bioengineering Institute, Nanjing, China) and according to the manufacturer's instructions. The MDA content was measured through a reaction with thiobarbituric acid that forms a stable chromophoric product, visible at a wavelength of 532 nm. The MDA levels were expressed as nanomoles per milligram of protein. The SOD activity was measured according to its ability to inhibit the production of a water-soluble formazan dye. The CAT activity was determined by the conversion rate of hydrogen peroxide into H_2O and O_2. The GSH-PX activity was determined by measuring the rate of oxidation of GSH to GSSG, which is monitored by the dismutation of cumene hydroperoxide that is then catalyzed by GSH-PX. The total SOD, MnSOD, CAT, and GSH-PX activities were expressed as units per milligram of protein. GSH and GSSG contents were determined using commercially available kits (Beyotime Biotech, Shanghai, China). All of the procedures complied with the manufacturer's instructions. GSH and GSSG levels were expressed in nanomoles per milligram of protein.

2.7. Immunofluorescence. Rat RCECs were seeded in gelatin-coated 24-well plates, allowed to attach overnight, and then exposed to various experimental conditions. After treatment, the rat RCECs were fixed in 4% PFA for 30 min at room temperature. After fixation, the cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Non-specific binding sites were blocked using a 30 min incubation NGS. The cells were incubated with primary antibody (rabbit polyclonal anti-Nox4, 1 : 50, Santa Cruz, Dallas, Texas, USA) at 4°C overnight. Cells were then washed with PBS and incubated with FITC-labeled secondary antibody (goat anti-rabbit IgG/FITC, 1 : 100, Zhong Shan Jin Qiao, Beijing, China) for 1 h at 37°C. DAPI was used to stain nuclei in the final incubation step, and then the cells were observed under

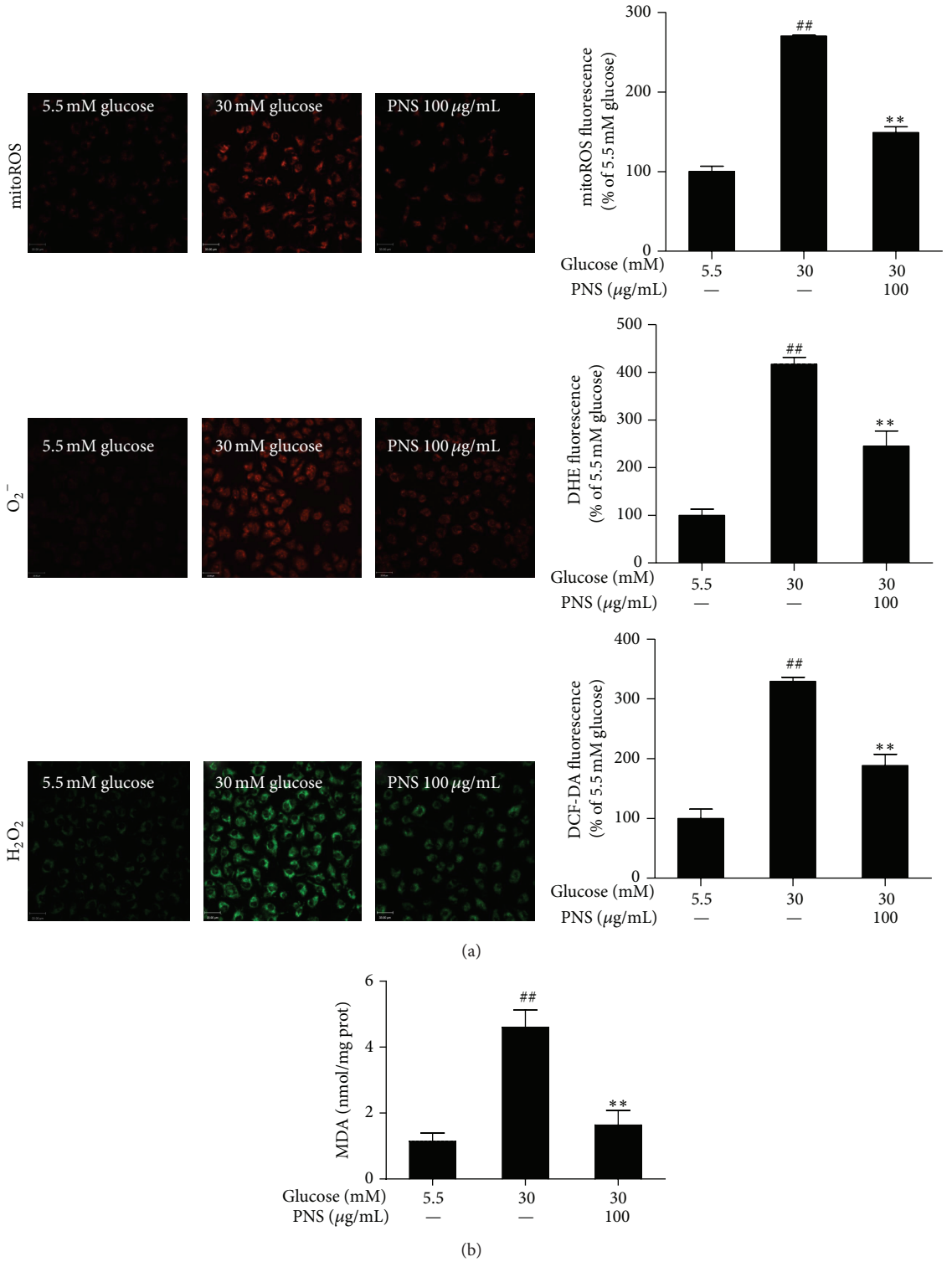

Figure 3: PNS inhibited the ROS and MDA induced by high glucose in rat RCECs. Seeded in plates or flasks, rat RCECs were incubated with PNS (100 μg/mL) in 30 mM glucose for 72 h. Fluorogenic probes (DCFH-DA, DHE, and MitoTracker Red CM-H_2XRos) showed that, after 72 h of PNS treatment, the intracellular H_2O_2 and O_2^- and mitochondrial ROS were decreased (a). A lipid peroxidation assay showed that PNS could decrease the MDA content (b). Data are expressed as the mean ± SD ($n = 4$). $^{\#\#}P < 0.01$ versus 5.5 mM glucose; $^{**}P < 0.01$ versus 30 mM glucose. Scale bar: 33 μm.

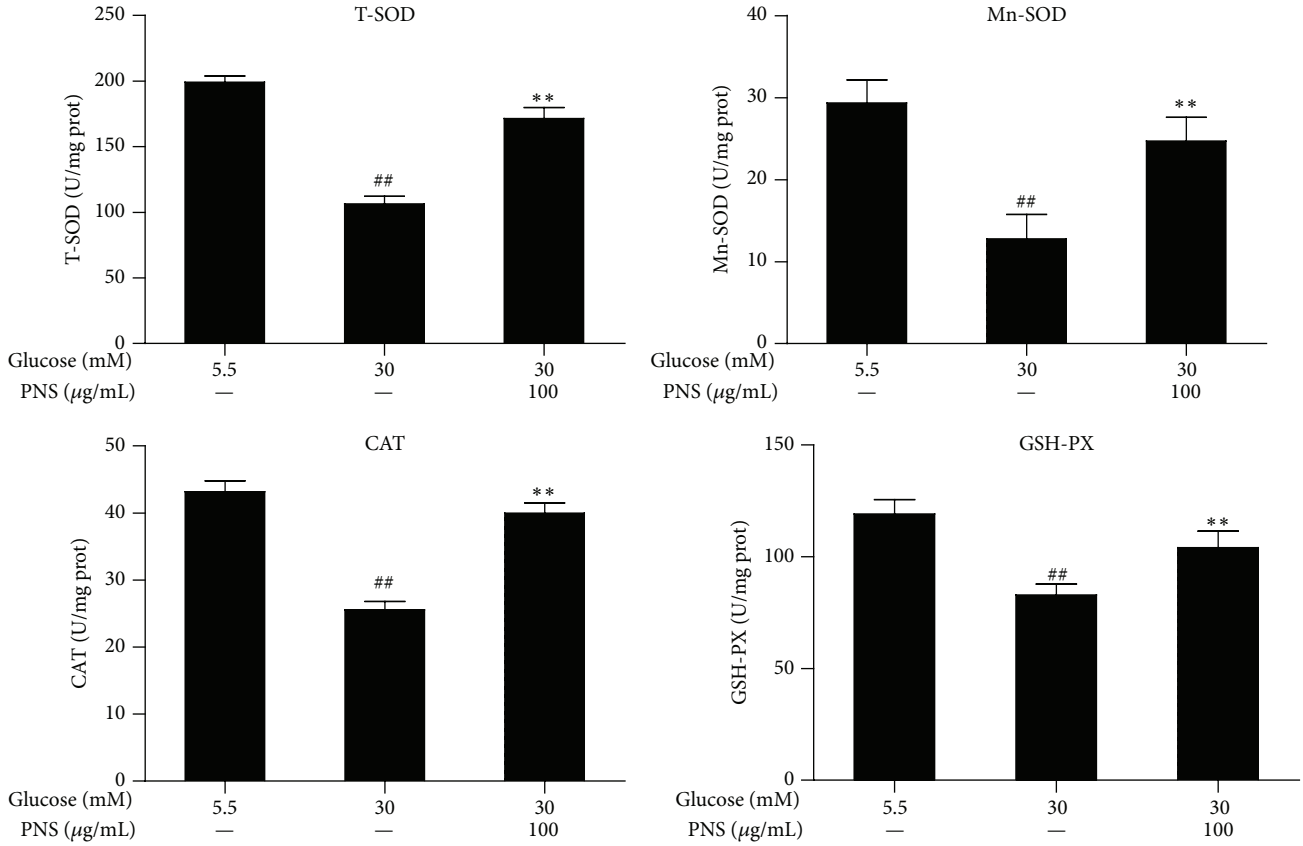

Figure 4: PNS increased the activities of total SOD, Mn-SOD, CAT, and GSH-PX in rat RCECs exposed to high glucose. Rat RCECs were incubated with concentration of PNS (100 μg/mL) in 30 mM glucose for 72 h; cells were collected and subjected for total SOD, MnSOD, CAT, and GSH-PX determination. The results showed that PNS increased the activities of total SOD, MnSOD, CAT, and GSH-PX in rat RCECs incubated with 30 mM glucose. Data are expressed as the mean ± SD ($n = 4$). $^{\#\#}P < 0.01$ versus 5.5 mM glucose; $^{**}P < 0.01$ versus 30 mM glucose.

an ECLIPSE Ti laser confocal microscope (Nikon, Japan). Confocal images were processed with ImageJ software.

2.8. Western Blot Analysis. Rat RCECs were seeded in gelatin-coated T-25 cm^2 flasks, allowed to attach overnight, and then exposed to varying experimental conditions. Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA and 0.1% SDS], and the protein concentrations were determined by a BCA protein assay. The protein (8 μg) was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and incubated with primary antibody (rabbit polyclonal anti-Nox4, 1 : 500, Santa Cruz, Dallas, Texas, USA) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 40 min. The immunoreactive bands were visualized using an enhanced chemiluminescence kit. The relative densities of the bands were determined by image analysis with Image-Pro Plus software.

2.9. Statistical Analysis. All of the data are presented as the mean value ± standard deviation (SD). One-way ANOVA, followed by a Student-Newman-Keuls test was used to analyze all of the data. A value of $P < 0.05$ and $P < 0.01$ was considered significant. The statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, USA).

3. Results

3.1. Validation of Rat RCECs. In this study, the cells showed a cobble-stone morphology and a contact-inhibited monolayer (Figure 1(a)) when observed under an inverted microscope. The cells expressed von Willebrand factor (vWf) (Figure 1(b)) and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Figure 1(c)), which are well-known endothelial cell-specific markers.

3.2. PNS Increases Cell Viability in Rat RCECs Exposed to High Glucose. In this study, we observed that stimulation with 30 mM glucose for 48 h and 72 h significantly decreased cell viability in comparison with 5.5 mM glucose-treated cells ($P < 0.01$). In the MTT assay, 50 μg/mL, 100 μg/mL, and 200 μg/mL of PNS significantly increased cell viability ($P < 0.01$) after 48 h or 72 h of treatment (Figure 2(a)). In the trypan blue assay, we found that 100 μg/mL and 200 μg/mL of PNS increased cell viability after 48 h of treatment ($P < 0.05$), and 100 μg/mL of PNS increased cell viability after 72 h of treatment ($P < 0.01$) (Figure 2(b)). These data suggest

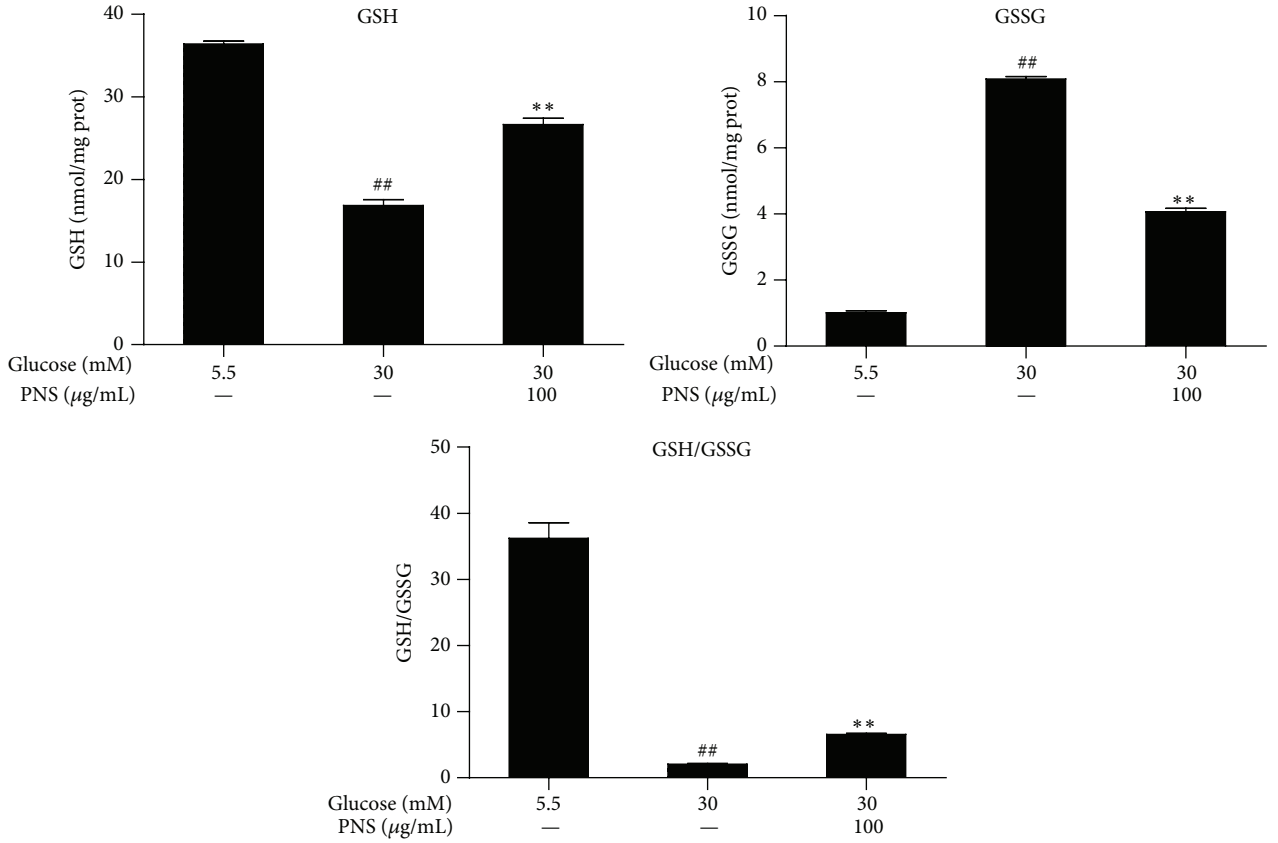

Figure 5: PNS increased GSH and reduced GSSG contents in rat RCECs exposed to high glucose. Rat RCECs were incubated with PNS (100 μg/mL) in 30 mM of glucose for 72 h; then cells were collected and GSH and GSSG were quantified. The results showed that PNS increased GSH content and reduced GSSG content after incubation with 30 mM of glucose. Data are expressed as the mean ± SD ($n = 4$). $^{\#\#}P < 0.01$ versus 5.5 mM glucose; $^{**}P < 0.01$ versus 30 mM glucose.

that PNS could increase cell viability in high glucose-treated RCECs.

3.3. PNS Inhibits the High Glucose-Induced Increase in ROS and MDA in Rat RCECs. As shown in Figure 3, 30 mM glucose significantly enhanced intracellular H_2O_2 and O_2^- and mitochondrial ROS ($P < 0.01$), observed by staining with 3 different fluorogenic probes: dichloro-dihydro-fluorescein diacetate (DCFH-DA), dihydroethidium (DHE) and Mito-Tracker Red CM-H_2XRos. PNS at 100 μg/mL reduced the 30 mM glucose-induced increase in intracellular H_2O_2 an, O_2^- and mitochondrial ROS ($P < 0.01$). The malondialdehyde (MDA) content was significantly increased after 72 h of 30 mM glucose treatment ($P < 0.01$) compared with cells treated with 5.5 mM glucose. Treatment with PNS (100 μg/mL) inhibited the elevated MDA observed in RCECs treated with 30 mM glucose ($P < 0.01$). These data suggest that PNS significantly reduced the levels of ROS and MDA, showing an effective attenuation of high glucose-induced oxidative injury.

3.4. PNS Increases SOD and CAT Activity and Regulates Glutathione Redox in Rat RCECs Exposed to High Glucose. The total SOD, Mn-SOD, CAT, and GSH-PX activity in the RCECs significantly decreased when exposed to 30 mM glucose compared with 5.5 mM glucose ($P < 0.01$). Treatment with PNS (100 μg/mL) increased the total SOD, Mn-SOD, CAT, and GSH-PX activity ($P < 0.01$), as shown in Figure 4. When exposed to 30 mM glucose, the GSH level in the RCECs markedly decreased while the GSSG level increased, subsequently enhancing the GSH/GSSG ratio ($P < 0.01$). Treatment with PNS (100 μg/mL) increased GSH levels, decreased GSSH levels, and enhanced the GSH/GSSG ratio ($P < 0.01$) (Figure 5). These data suggest that PNS had significant antioxidative effects against high glucose-induced oxidative stress in rat RCECs.

3.5. PNS Inhibited the NADPH Oxidase 4 (Nox4) Expressions in Rat RCECs Exposed to High Glucose. As shown in Figure 6, western blot and immunofluorescence showed increased expression of Nox4 protein in the rat RCECs treated with 30 mM glucose ($P < 0.01$). Treatment with PNS (100 μg/mL) markedly inhibited the high glucose-induced increase of Nox4 expression ($P < 0.05$).

4. Discussion

In this study, we found that PNS treatment attenuates high glucose-induced injury in rat RCECs and is accompanied by reduced intracellular ROS and lowered MDA contents. PNS

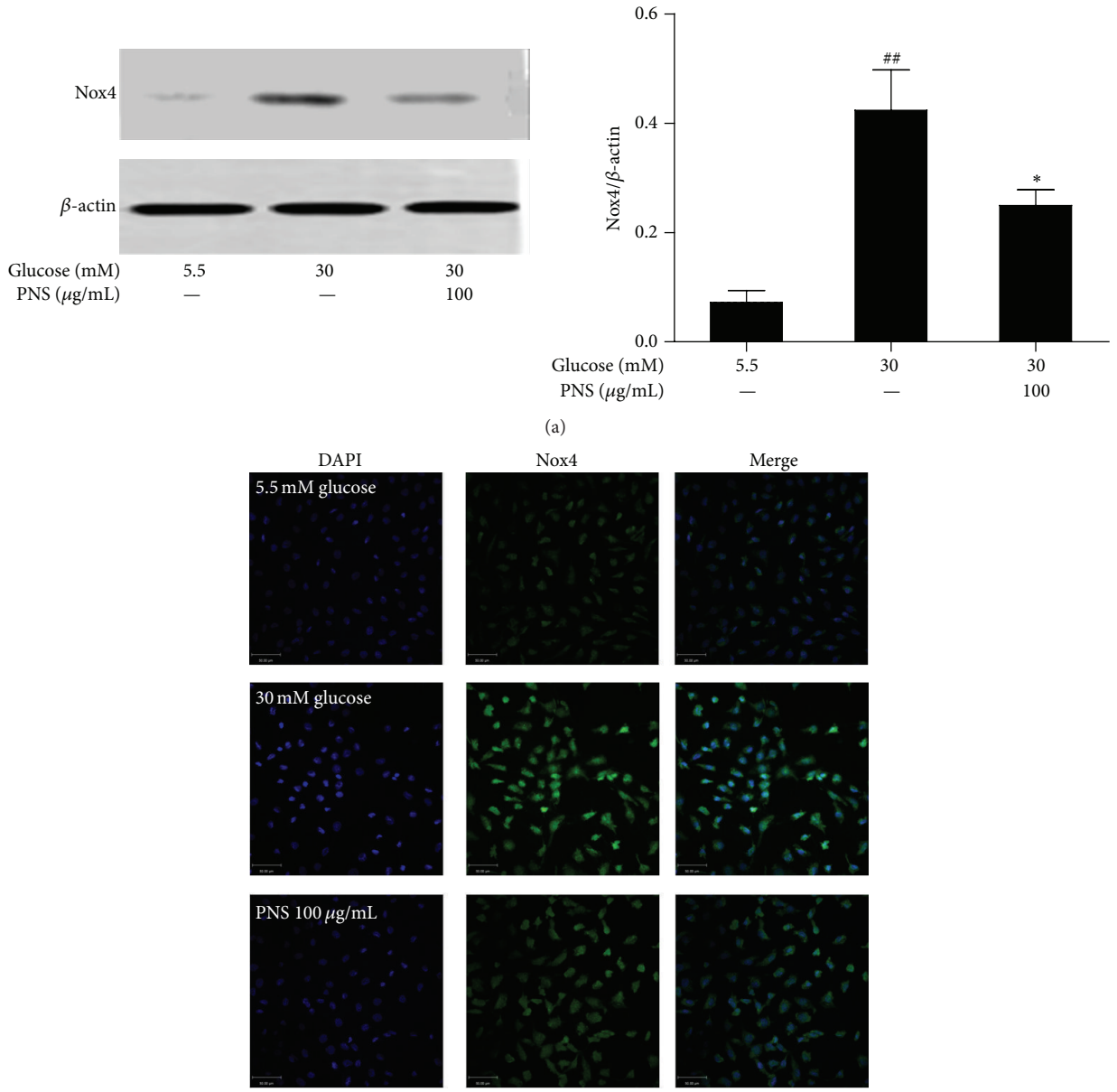

Figure 6: PNS inhibited NADPH oxidase 4 (Nox4) expression in rat RCECs exposed to high glucose. Western blot (a) and immunofluorescence (b) analyses were performed to detect Nox4 by PNS in rat RCECs exposed to 30 mM of glucose for 72 h. We found that PNS could reduce Nox4 expression. Data are expressed as the mean ± SD ($n = 4$). $^{\#\#}P < 0.01$ versus 5.5 mM glucose; $^{*}P < 0.05$ versus 30 mM glucose. Scale bar: 50 μm.

increased the intracellular SOD, mitochondrial SOD, CAT, and GSH-PX activities. The GSH content also increased after PNS treatment. These results indicate that PNS may exert its protective effect through the promotion of antioxidation.

Diabetic retinopathy is characterized by progressive alterations in the retinal microvasculature, and hyperglycemia is considered the prime triggering factor for increased BRB permeability [13]. Studies have found that rat RCECs [14], human RCECs [15], or bovine RCECs [16] display increased apoptosis when cells are exposed to high glucose. Similar results were seen in the TR-iBRB2 rat RCEC cell line [17]. Here, we employed a cell-based experimental model in which cultured RCECs were exposed to 30 mM glucose and confirmed the detrimental effect of hyperglycemia on the rat RCEC functionality. At same time, the coincubation with PNS improved RCEC viability, indicating that PNS protected the RCECs against high glucose-induced injury.

A large body of evidence has demonstrated an increase in ROS in different tissue and cell types during diabetes or after exposure to high glucose [18, 19]. These data are the basis for the claim that high glucose contributes to the vascular alterations observed in diabetic retinopathy. Some studies have shown that mitochondrial ROS levels are increased in diabetic rat retinas and in retinal cells incubated in

high glucose and that downregulation of mitochondrial ROS can inhibit glucose-induced apoptosis in both endothelial cells and pericytes [3, 4]. In this study, we found that, after culturing RCECs 30 mM glucose medium for 72 h, the mitochondria ROS was significantly increased and intracellular H_2O_2 and O_2 were concomitantly increased. High glucose increased MDA, which suggests that hyperglycemia can increase oxidative damage in RCECs, an observation that agrees with previous studies. PNS reduced intracellular H_2O_2 and O_2^- and mitochondrial ROS, as indicated by the decreased fluorescence intensity of DCF-DA, DHE, and MitoTracker Red CM-H_2XRos. Meanwhile, PNS markedly decreased the MDA levels. These data suggest that PNS may exert antioxidant effects in the intracellular compartment. It should be acknowledged that intracellular ROS may come from nonmitochondrial sources, although the mitochondrion is main source. Previous studies have confirmed that Nox4, a homolog of gp91phox/Nox2, was abundantly expressed in endothelial cells. Overexpression of Nox4 can cause an imbalance between the production of free radicals and the antioxidant defense system [18, 20]. Our study showed a significant increase in Nox4 expression after high glucose exposure, indicating elevated ROS production. PNS significantly reduced the Nox4 expression levels. These data suggest that PNS may reduce the glucose-induced ROS via decreasing the high glucose-induced Nox4 expression.

Oxidative stress causes cell damage when the antioxidant enzymes and antioxidative substrates are exhausted [21]. During oxidative stress, the glutathione redox system plays an important role in endothelial cells. This process has been reported in many other cell types and proposed as a mechanism of cellular self-defense [22]. Thus, in order to investigate the possible mechanism by which PNS protects RCECs from high glucose-induced oxidative injury, the glutathione and glutathione related enzymes were examined. The results indicated that PNS treatment caused a significant enhancement of the GSH content and GSH-PX activity. Undoubtedly, these contribute to the restored cell viability and the antioxidative capacity. In addition to the glutathione system, CAT is also an important antioxidant pathway in the removal of H_2O_2 [21]. The data show that PNS could decrease CAT activity. SOD, an endogenous antioxidant, catalyzes the breakdown of O_2^- into H_2O_2 scavenging O_2^- [22]. Because intramitochondrial O_2^- does not readily cross mitochondrial membranes, MnSOD, a superoxide scavenging enzyme in mitochondria, converts intramitochondrial O_2^- into H_2O_2 that can diffuse out of mitochondria [21]. In our study, PNS treated RCECs inhibited the SOD and MnSOD levels induced by 30 mM glucose. Thereby, it was reasonably presumed that reducing the ROS overload, coupled with improving antioxidant enzymes activities and glutathione redox system, may be the main protection mechanism of PNS in RCECs.

In conclusion, our findings indicated that PNS is endowed with a significant protective function against high glucose-induced oxidative injury in RCECs. The protective effect of PNS may be due to its ability to reduce oxidative stress. Therefore, it is plausible to conduct further investigation aimed at the clinical application of PNS in the treatment of diabetic retinopathy.

Disclosure

Yue Fan and Yuan Qiao are co-first authors.

Conflict of Interests

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

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