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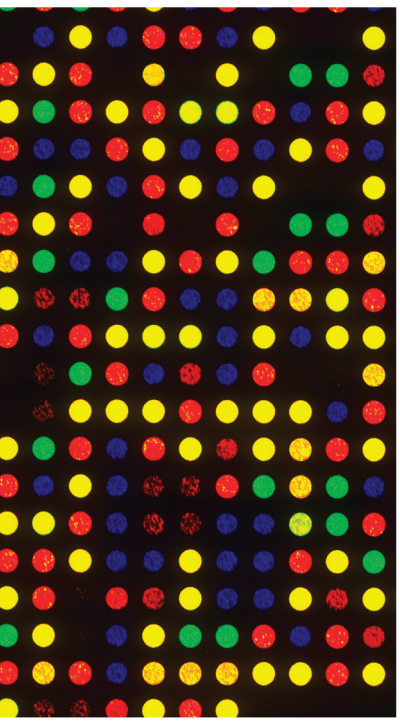

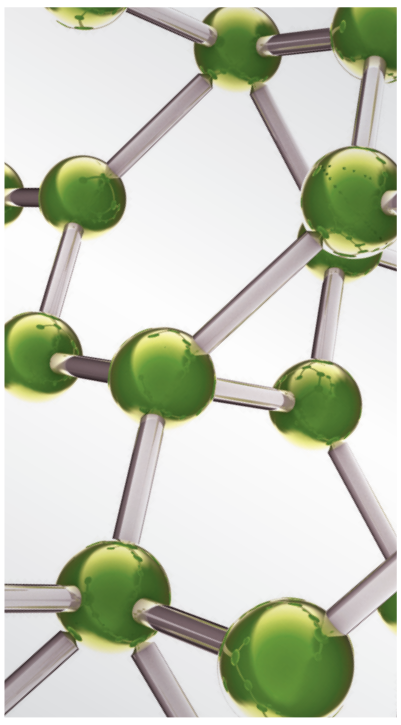


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Editorial Board

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Editorial

Efficacy and Safety of Medicinal Plants Used in the Management of Diabetes Mellitus

Musa T. Yakubu,[1] Taofik O. Sunmonu,[2] Francis B. Lewu,[3] Anafi O. T. Ashafa,[4] Femi J. Olorunniji,[5] and Mohamed Eddouks[6]

[1] *Phytomedicine, Toxicology and Reproductive Biochemistry Research Laboratory, Department of Biochemistry, University of Ilorin, Ilorin 240003, Nigeria*
[2] *Phytomedicine, Environmental Toxicology and Plant Biochemistry Research Laboratory, Department of Biological Sciences, Al-Hikmah University, Ilorin 240001, Nigeria*
[3] *Department of Agriculture, Faculty of Applied Sciences, Cape Peninsula University of Technology, Wellington Campus, Wellington, Western Cape 7655, South Africa*
[4] *Department of Plant Sciences, University of the Free State, Qwaqwa Campus, Phuthaditjhaba 9866, South Africa*
[5] *Institute of Molecular, Cell, and System Biology, College of Medical, Veterinary, and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK*
[6] *Faculty of Sciences and Techniques Errachidia, Moulay Ismail University, 52000 Errachidia, Morocco*

Correspondence should be addressed to Musa T. Yakubu; tomuyak@yahoo.com

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This special issue edited by a team of five renowned scholars led by an Associate Professor of Biochemistry, M. T. Yakubu, Ph.D. (Lead Guest Editor), and other Guest Editors that included Professor T. O. Sunmonu, Dr. F. B. Lewu, Dr. A. O. T. Ashafa, Dr. Femi J. Olorunniji, and Professor Mohammed Eddouks features 15 original research papers covering various aspects of diabetes mellitus.

R. M. P. Gutierrez et al. in the paper entitled "*Ameliorative effect of hexane extract of Phalaris canariensis on high fat diet-induced obese and streptozotocin-induced diabetic mice*" reported the antiobesity effect of a hexane extract of *Phalaris canariensis* seed in high-fat diet and streptozotocin-induced diabetic mice. M. T. Sultan et al. in the paper entitled "*Nigella sativa fixed and essential oil supplementation modulates hyperglycemia and allied complications in streptozotocin-induced diabetes mellitus*" described the modulatory effects of *Nigella sativa* fixed and essential oil supplementation on hyperglycemia and allied complications in streptozotocin-induced diabetes mellitus. In a related study, M. I. Kazeem et al. in the paper entitled "*Protective effect of free and bound polyphenol extracts from ginger (Zingiber officinale Roscoe) on the hepatic antioxidant and some carbohydrate metabolizing enzymes of streptozotocin-induced diabetic rats*" evaluated the protective effect of free and bound polyphenol extracts from ginger (*Zingiber officinale* Roscoe) on the hepatic antioxidant and some carbohydrate metabolizing enzymes of streptozotocin-induced diabetic rats. P.-G. Cheng et al. in the paper entitled "*Polysaccharides-rich extract of Ganoderma lucidum (M.A. Curtis:Fr.) P. Karst accelerates wound healing in streptozotocin-induced diabetic rats*" evaluated the wound healing activity of the hot aqueous extract of *Ganoderma lucidum* in streptozotocin-induced diabetic rats. The work reported by da A. A. Rocha et al. in the paper entitled "*Lectin from Crataeva tapia bark improves tissue damages and plasma hyperglycemia in alloxan-induced diabetic mice*" revealed that lectin extracted from *Crataeva tapia* bark resulted in improvement of tissue damage and plasma hyperglycemia in alloxan-induced diabetic mice.

J. Gu et al. in the paper entitled "*A drug-target network-based approach to evaluate the efficacy of medicinal plants for type II diabetes mellitus*" used drug-target network-based approach to evaluate the efficacy of medicinal plants for type II diabetes mellitus. The study reported by M. M. Zainudin et al. in the paper entitled "*Does oral ingestion of*

Piper sarmentosum cause toxicity in experimental animals?" investigated the potential toxicity of oral administration of an antidiabetic plant, *Piper sarmentosum*, in experimental animals. P. V. Rao et al. in the paper entitled "*Rhinacanthus nasutus improves the levels of liver carbohydrate, protein, glycogen, and liver markers in streptozotocin-induced diabetic rats*" showed that treatment of streptozotocin-induced diabetic rats with *Rhinacanthus nasutus* resulted in improvement in the levels of liver carbohydrate, protein, glycogen, and other hepatic markers. Y. Xiao et al. in the paper entitled "*The effect of Chinese herbal medicine on albuminuria levels in patients with diabetic nephropathy: a systematic review and meta-analysis*" presented a systematic review and meta-analysis of the effect of Chinese herbal medicine on albuminuria levels in patients with diabetic nephropathy. J. Wu et al. in the paper entitled "*Renal protective role of Xiexin decoction with multiple active ingredients involves inhibition of inflammation through downregulation of the nuclear factor-kB pathway in diabetic rats*" presented evidence to suggest that the inhibition of inflammation through downregulation of the nuclear factor-kβ pathway in diabetic rats is the mechanism by which Xiexin decoction with multiple active ingredients exhibits its renal protective role. The paper by L. Zhou et al. in the paper entitled "*Hu-Lu-Ba-Wan attenuates diabetic nephropathy in type 2 diabetic rats through PKC-α/NADPH oxidase signaling pathway*" reported the attenuation of diabetic nephropathy in type 2 diabetic rats through PKC-α/NADPH oxidase signaling pathway by Hu-Lu-Ba-Wan. The evaluation of antidiabetic activity and associated toxicity of *Artemisia afra* aqueous extract in Wistar rats was reported by T. O. Sunmonu and A. J. Afolayan in the paper entitled "*Evaluation of antidiabetic activity and associated toxicity of Artemisia afra aqueous extract in Wistar rats.*" *N. A. Ishak et al. in the paper entitled* "*Antidiabetic and hypolipidemic activities of Curculigo latifolia fruit: root extract in high fat fed diet and low dose STZ induced diabetic rats*" evaluated the antidiabetic and hypolipidemic activities of *Curculigo latifolia* fruit: root extract in high-fat fed diet and low dose STZ-induced diabetic rats. The evaluation of the hypoglycemic properties of *Anacardium humile* aqueous extract was reported by M. A. Urzêda et al. in the paper entitled "*Evaluation of the hypoglycemic properties of Anacardium humile aqueous extract.*" Finally, the decrease of plasma glucose by *Hibiscus taiwanensis* in type-1-like diabetic rats was the focus of the study reported by L.-Y. Wang et al. in the paper entitled "*Decrease of plasma glucose by Hibiscus taiwanensis in type-1-like diabetic rats.*"

The variety of papers on diabetes featured in this special issue highlights the keen awareness of the biomedical community of the potential for exploiting medicinal plants in tackling this debilitating condition. Of particular interest is the large number of contributions from scientists working in developing countries. This level of interest should be encouraged. The gradual improvement in the level of prosperity in developing economies is being accompanied by a significant increase in reported cases of obesity, diabetes, and other metabolic disorders. It is appropriate that scientists and policy makers in these lands devote more resources to exploring newer ways of dealing with these conditions. It is hoped that the encouraging findings reported in this special issue will stimulate further interest in expanding and coordinating efforts on medicinal plants that have clinical potential in the management of diabetes.

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The editorial team would like to thank all the authors who have participated in this issue and the expertise contributions of the reviewers.

Musa T. Yakubu
Taofik O. Sunmonu
Francis B. Lewu
Anafi O. T. Ashafa
Femi J. Olorunniji
Mohamed Eddouks

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Research Article

Hexane Fractions of *Bupleurum falcatum L.* Stimulates Glucagon-Like Peptide-1 Secretion through $G_{\beta\gamma}$-Mediated Pathway

Min-Hee Shin,[1] Eun-Kyeong Choi,[1] Ki-Suk Kim,[1] Kang-Hoon Kim,[1] Young Pyo Jang,[2] Kwang Seok Ahn,[1] Won-Seok Chung,[1] Nam Hyun Cha,[3] and Hyeung-Jin Jang[1]

[1] *College of Korean Medicine, Institute of Korean Medicine, Kyung Hee University, 1 Heogi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea*
[2] *College of Pharmacy, Kyung Hee University, Heogi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea*
[3] *Nursing Department, College of Health Science, Kangwon University, Hyoja 2-dong, Chuncheon-si 200-701, Republic of Korea*

Correspondence should be addressed to Hyeung-Jin Jang; hjjang@khu.ac.kr

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Bupleurum falcatum L. has been used traditionally as a medicinal herb in Korean medicine. The hexane fraction of BF (HFBF), which was profiled with Direct Analysis in Real Time-Mass Spectrometry (DART-MS), activates the secretion of glucagon-like peptide-1 (GLP-1) in NCI-H716 cells significantly. We performed a microarray analysis and GLP-1 ELISA assay, as well as calcium imaging experiments with inhibitors, to investigate the mechanism of action of the HFBF. Through the microarray analysis, it was found that the *ITPR2* gene that encodes the inositol 1,4,5-trisphosphate (IP_3) receptor is up-regulated and the HFBF induces cell depolarization by inhibiting the voltage-gated channel expression in NCI-H716 cells. In addition, we found that the intracellular calcium in NCI-H716 cells, with Gallein, U73122, and 2APB as inhibitors, was decreased. These results suggest that the HFBF activates the GLP-1 secretion through the $G_{\beta\gamma}$ pathways in the enteroendocrine L cells after treatment with the HFBF.

1. Introduction

Bupleurum falcatum L. (BF) has been used traditionally as a medical herb [1]. It is used for the preparation of herbal remedies and also used as an ingredient in herbal tea and traditional fermented beverages. BF is also known for its therapeutic effects in the treatment of diabetes [1]. Despite its outstanding effects during clinical trials on diabetes mellitus, its mode of action has not been examined. To study the effects of a herbal sample, BF was extracted and fractionated as described in [2, 3]. The hexane fractions of *Bupleurum falcatum L.* (HFBF) samples were used to treat enteroendocrine NCI-H716 cells and subsequently a GLP-1 ELISA was performed. The microarray was also examined using isolated RNA from HFBF treated NCI-H716 cells.

The NCI-H716 cell line, the human intestinal cell, is widely used to study glucagon-like peptide-1 (GLP-1) secretion [4, 5]. GLP-1 is an incretin hormone that is released by enteroendocrine L cells in the gastrointestinal tract (GI) and has received considerable interest because of its ability to amplify insulin secretion in pancreatic β-cells [6]. GLP-1 is secreted within minutes of nutrient ingestion and boosts the disposal of ingested nutrients.

Seven-transmembrane receptor guanine nucleotide-binding protein (G protein) coupled receptor (GPCR), also known as a G protein-linked receptor, is composed of G_α, G_β, and G_γ. Both G_α and $G_{\beta\gamma}$ are related to the GPCR signaling pathway. The G_α subunit is related to the cyclic AMP (cAMP), protein kinase A (PKA), and phosphodiesterases (PDEs). In addition, the $G_{\beta\gamma}$ subunit

activates the phospholipase C β (PLC β) that separates the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 then disperses through the cytosol to bind to IP_3 receptors, particularly in the calcium channels in the smooth endoplasmic reticulum (ER). This leads to the increase of Ca^{2+} concentration in the cytosol and causes a cascade of intracellular changes and activities. Also, Ca^{2+} and DAG together activate the protein kinase C (PKC). Hormone secretion such as GLP-1 is highly related to the intracellular Ca^{2+} signaling, which is generally related to the two processes. One is the Ca^{2+} influx due to the depolarization and transmembrane potassium channels. The other is Ca^{2+} release from the intracellular calcium storage area including the endoplasmic reticulum [7].

The G protein-gated ion channels are specific ion channels located in the plasma membrane that are activated by a family of associated proteins. These ion channels allow for the selective movement of certain ions across the plasma membrane in cells. Incretin hormones modulate voltage-gated potassium (Kv) channels [2, 3]. Incretin hormones like GLP-1 also activate the closure of ATP-sensitive potassium channels resulting in membrane depolarization and the activation of voltage-dependent Ca^{2+} channels resulting in the increase in intracellular Ca^{2+}. GLP-1 stimulates insulin secretion through the modulation of the ATP-dependent potassium channel, increasing the Ca^{2+} influx and releasing Ca^{2+} from intracellular stores [8].

In this study, we demonstrate that HFBF induces GLP-1 secretion via the GPCR signaling pathway, especially through the $G_{\beta\gamma}$-mediated pathway. This study provides the important information of the HFBF effects to promote GLP-1 secretion and the possibility that the medical herb can be used as a therapeutic agent of diabetes mellitus.

2. Materials and Methods

2.1. Preparation of Bupleurum falcatum L. Extracts. The herbal extracts and fractions were purchased from Kyung-Hee Oriental Herbal Medicine Research Center. The BF samples were prepared as described in [9, 10]. Briefly, the rind parts of BF were extracted with distilled water (DW) and filtration, evaporation, and freeze drying were performed in order. The extracts were successively partitioned with organic solvents of different polarities to yield the *n*-hexane (HX) fractions.

2.2. Culture of NCI-H716 Cells. Enteroendocrine NCI-H716 cells were purchased from the Korean Cell Line Bank (KCLB, South Korea) and cultured in RPMI 1640 (Welgene, South Korea) with 10% FBS (Welgene, South Korea), penicillin, and streptomycin. To perform the endocrine differentiation, NCI-H716 cells were incubated in the Matrigel- (BD Bioscience, USA) coated 12-well plates, 1×10^6 cells per well with high glucose DMEM (Welgene, South Korea) with 10% FBS, penicillin, and streptomycin. 48 hours later, the media were removed and the plates were washed with PBS, and then the cells were starved with low glucose DMEM (Welgene, South Korea). After 12 hours, the media were removed and the plates were washed again with PBS. 900 μL of 1 mM calcium chloride PBS was added to each well and 100 μL of each sample was (10x) treated for 1 hour in a CO_2 incubator.

2.3. DART-MS Analysis of the Hexane Fraction of Bupleurum falcatum L. A DART ion source (Ion Sense, Saugus, USA) combined with JMS-T100TD was used in the positive ion mode. Analysis conditions were set as needle voltage 3200 V, electrode 1, 2 voltage 100 V, helium gas flow 3 L/min, temperature 250°C, the first orifice lens 15 V, and ring lens voltage 5 V. Analyzed molecular weight peaks were normalized with polyethylene glycol 600 (PEG 600) [10, 11].

2.4. Cell Viability Assay. The 1×10^4 NCI-H716 cells were seeded in 96-well plate after matrigel coating. After two days of differentiation and starvation overnight, BFHF were treated with three concentrations: 10 μg/mL, 100 μg/mL, and 1000 μg/mL. MTT(3-(4,5-dimethythiazol-2-yl)-2,5-Diphenyl tetrazolium bromide) was obtained from Invitrogen (M6494, USA). Before treatment, it was dissolved as a 1 mg/mL stock and treated with cells. After 2 hours, the supernatants were sucked and 100 μL DMSO was added. After shaking for 20 minutes, read plate with wavelength of 570 nm.

2.5. GLP-1 Secretion Study. Three days before the experiments, the cells were seeded at 1×10^6 cells/well into 12-well culture plates precoated with Matrigel (BD Biosciences, USA). One day before the experiments, serum starvation was carried out with a DMEM low glucose medium for 20 hours. On the day of the experiments, the supernatants were replaced by PBS containing 1 mM $CaCl_2$. Cells were incubated for 1 hour at 37°C with the different fractions of BF, and the GLP-1 was measured using a GLP-1 ELISA Kit (Millipore, USA). The data was normalized with total protein concentrations corresponding to each sample well.

2.6. Calcium Imaging. Differentiated NCI-H716 cells grown on cover glass were incubated overnight. Cytosolic free calcium [Ca^{2+}] was measured using fura-2 fluorescence dye. NCI-H716 cells grown on a matrigel-coated coverslide bottom dish were washed three times with PBS and incubated in the dark for 30 min at room temperature with fura-2AM (final concentration 1 μM) in PBS. The cells were again washed with PBS three times and analyzed by being illuminated with alternating light of 340 and 380 nm from a rotating filter wheel.

2.7. RNA Isolation. Experiments were carried out in similar GLP-1 secretion study processes and we used 100 μg/mL of the HFBF. Three days before the experiments, the cells were seeded at 1×10^7 cells/well into 6-well culture plates precoated with Matrigel (BD Biosciences, USA). Other procedures were performed in the same way as described in the GLP-1

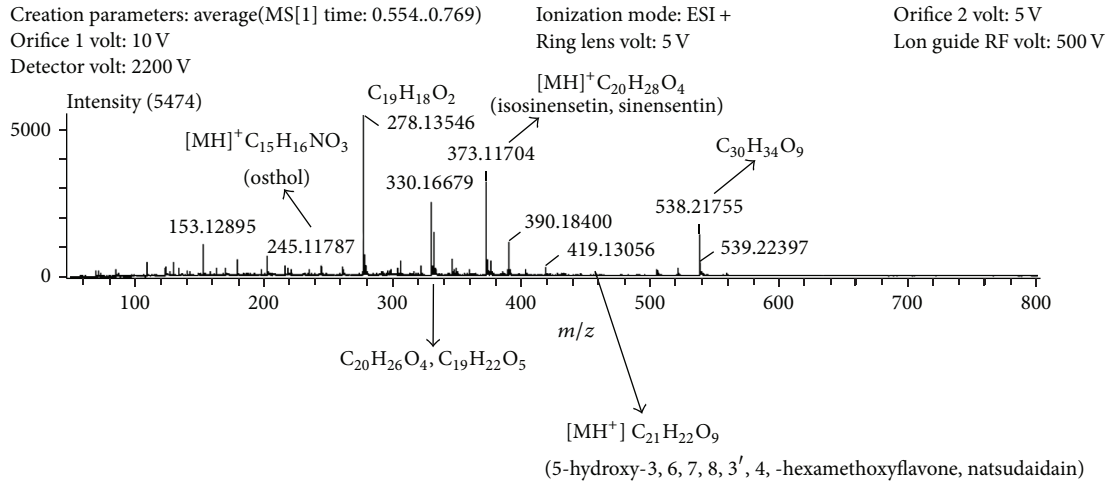

Figure 1: DART-MS profiling of HFBF.

assay. Total RNA was extracted by using Hybrid-^{RTM} (GeneAll Biotechnology, Korea) according to the manufacturer's instructions. The microarray of each total RNA sample (200 ng) was labeled and amplified using a Low Input Quick Amp Labeling Kit (Agilent Technologies, USA) [10], and the Cy3-labeled aRNAs were resuspended in 50 μL of hybridization solution (Agilent Technologies, USA). After the aRNAs were placed on an Agilent SurePrint G3 Human GE 8 × 60 K Array (Agilent Technologies, USA) and covered by a Gasket 8-plex slide (Agilent Technologies, USA), the slides were hybridized for 17 hours in a 65°C oven. The hybridized slides were washed in 2 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 2 min, 1 × SSC for 3 min, and then 0.2 × SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 sec to dry.

2.8. Microarray of NCI-H716 Cells. Each total RNA sample (200 ng) was labeled and amplified using a Low Input Quick Amp Labeling Kit (Agilent Technologies, CA). The Cy3-labeled aRNAs were resuspended in 50 μL of hybridization solution (Agilent Technologies, CA). After the labeled aRNAs were placed on an Agilent SurePrint G3 Human GE 8 × 60 K array (Agilent Technologies, CA) and covered by a Gasket 8-plex slide (Agilent technologies, CA), the slides were hybridized for 17 hours in a 65°C oven. The hybridized slides were washed in 2 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 2 min, 1 × SSC for 3 min, and then 0.2 × SSC for 2 min at room temperature. The slides were centrifuged at 3000 rpm for 20 sec to dry.

2.9. Data Analysis. The arrays were analyzed through the use of an Agilent scanner and the associated software and gene expression levels were estimated with the Feature Extraction v10.7.3.1 (Agilent Technologies, USA). Relative signal intensities for each gene were generated using the Robust Multiarray Average Algorithm. The data were processed according to a quantile normalization method using the GeneSpring GX 11.5.1 (Agilent Technologies, USA). This normalization method used for making the distribution of intensities for each array in the set of arrays was the same. The normalization and log transformed intensity values were analyzed using the GeneSpring GX 11.5.1 (Agilent Technologies, USA). Fold change filters included the requirement that the genes are present in at least 2-fold of the controls for upregulated genes and less than 0.5-fold of the controls for downregulated genes.

2.10. Oral Glucose Tolerance Test. The *db/db* mouse was purchased from DBL (Korea). The *db/db* mouse ($n = 5$) was fasted for 16 hours and then orally administered at the volume of 5 g/kg glucose solution [12]. HFBF was treated at the volume of 100 mg/kg before treatment of the glucose. Blood samples, were obtained through the tail vein for 6 time points: 0 (before the HFBF administration), 10, 20, 40, 90, and 120 min after the glucose injection for the determination of blood glucose levels. Blood samples were obtained before gavage (time 0) and 10, 20, 40, 9, and 120 min after gavage for determination of blood glucose by ACCU-CHEK Performa System (Roche, South San Francisco, CA) [13].

2.11. Statistical Analysis. Each ELISA and calcium imaging set of data represents at least two separate experiments and each experiment was performed in triplicate. The significance of the data was analyzed with Prism 5 software with one-way ANOVA and Bonferroni tests to compare each set of data. Bars show the SEMs of the means of the three assays.

3. Results

3.1. DART-MS Analysis of Hexane Fraction of Bupleurum falcatum L. DART-MS was performed to profile the HFBF (Figure 1). Among the several prominent peaks, isosinensetin (sinensetin) was identified as the second highest peak at m/z 194 ($[MH]^+ = 373.11704$). DART-MS results show isosinensetin is a candidate for the active compound of the HFBF which contributes to the GLP-1 secretion effect. A study using

Table 1: List of genes related to the GPCR signaling pathway which is regulated after HFBF treatment.

Probe name	Description	Gene symbol	*P* value	Fold change
	Inositol 1,4,5-trisphosphate			
A_33_P3298128	Inositol 1,4,5-trisphosphate receptor, type 2 (ITPR2), mRNA [NM_002223]	*ITPR2*	0.004	**1.66**
	Adenylate cyclase			
A_23_P126313	Adenylate cyclase 10 (soluble) (ADCY10), transcript variant 1, mRNA [NM_018417]	*ADCY1*	0.262	**−0.30**
	Phosphodiesterase			
A_24_P197537	Phosphodiesterase 8B (PDE8B), transcript variant 1, mRNA [NM_003719]	*PDE8B*	0.7777	**−0.04**
A_33_P3244951	Phosphodiesterase 8A (PDE8A), transcript variant 1, mRNA [NM_002605]	*PDE8A*	0.397	**−0.14**
A_33_P3301940	Phosphodiesterase 7B (PDE7B), mRNA [NM_018945]	*PDE7B*	0.238	**−0.22**
A_33_P3240552	Phosphodiesterase 4D, cAMP-specific [Source: HGNC Symbol; Acc: 8783] [ENST00000509355]	*PDE4D*	0.474	**−0.08**
A_33_P3389649	Phosphodiesterase 4D, cAMP-specific (PDE4D), transcript variant 4, mRNA [NM_001197218]	*PDE4D*	0.167	**−0.23**
A_33_P3389653	Phosphodiesterase 4D, cAMP-specific (PDE4D), transcript variant 3, mRNA [NM_001165899]	*PDE4D*	0.289	**−0.15**
A_33_P3759611	Phosphodiesterase 4C, cAMP-specific (PDE4C), transcript variant 1, mRNA [NM_000923]	*PDE4C*	0.110	**−0.32**
A_23_P74278	Phosphodiesterase 4B, cAMP-specific (PDE4B), transcript variant d, mRNA [NM_001037341]	*PDE4B*	0.039	**−0.20**
A_24_P322474	Phosphodiesterase 4A, cAMP-specific (PDE4A), transcript variant 4, mRNA [NM_006202]	*PDE4A*	0.336	**−0.23**
A_23_P401106	Phosphodiesterase 2A, cGMP-stimulated (PDE2A), transcript variant 1, mRNA [NM_002599]	*PDE2A*	0.511	**−0.06**

isosinensetin may increase the potential of the HFBF as a therapeutic agent for type 2 diabetes mellitus.

3.2. HFBF Activates Secretion of GLP-1 in NCI-H716 Cells without Cytotoxicity. To confirm the GLP-1 secretion effect due to the HFBF treatment, a GLP-1 assay was conducted. The extracts were diluted with distilled water and were successively partitioned with organic solvents of different polarities as described in the Materials and Methods section. For GLP-1, an ELISA was conducted using a BF fraction including butanol, ethyl acetate, dichloromethane, and hexane. The HFBF activated GLP-1 secretion at higher levels compared to the other extracts (data not shown). As shown in Figure 2(a), GLP-1 secretion was significantly increased in a dose dependent manner compared to the control; DMSO was used as a negative control and 2 mM Quinine was used as a positive control. 1% DMSO was used as a negative control because the entire compound dissolved in it. The HFBF increased GLP-1 secretion by almost 25-fold compared to the negative control. Figure 2(b) shows HFBF has no cytotoxicity to the NCI-H716 cells.

3.3. Hexane Fraction of Bupleurum falcatum L Induces GLP-1 Secretion through GPCR Pathways. Microarray data demonstrated the HFBF activates GLP-1 secretion through GPCR signaling pathways in NCI-H716 cells. GPCR stimulates G protein that is composed of α, β, and γ subunits. G_α and $G_{\beta\gamma}$ act independently through different signaling pathways. As shown in Table 1, the *ADCY1* gene that encodes AC is downregulated and *PDE*-related genes including *PDE8B*, *PDE8A*, *PDE7B*, *PDE4D*, *PDE4D*, *PDE4D*, *PDE4C*, *PDE4B*, *PDE4A*, and *PDE2A* are also downregulated. The *ITPR2* that encodes the inositol 1,4,5-trisphosphate receptor is upregulated (Table 1). To verify the activated pathway of the HFBF, an inhibition study was conducted. Lactisole, which is known as an inhibitor of sweet and umami taste receptors, was also used as an inhibitor of the G_α pathway [14]. There is no significant result in the inhibition study of GLP-1 secretion using lactisole (Figure 3). The HFBF seems to activate GLP-1 secretion through the $G_{\beta\gamma}$-mediated pathway particularly with the activation of PLC. The upregulation of the *ITPR2* gene and downregulation of PDE and AC demonstrate that the HFBF stimulates GLP-1 secretion through the $G_{\beta\gamma}$-mediated pathway [8, 15].

3.4. HFBF Induced Downregulation of Potassium Voltage-Gated Channels. The cell creates electrical signal through several types of ion channels such as potassium voltage-gated channels that are important to create membrane depolarization [16, 17]. *KCNC3, KCNA6, KCNQ2, KCNE1, KCNN1*, and *KCTD19* genes encode potassium voltage-gated channels and are downregulated by the HFBF (Table 2). The HFBF seems to induce GLP-1 secretion by changing membrane potential via the potassium voltage-gated channels. HFBF has effects

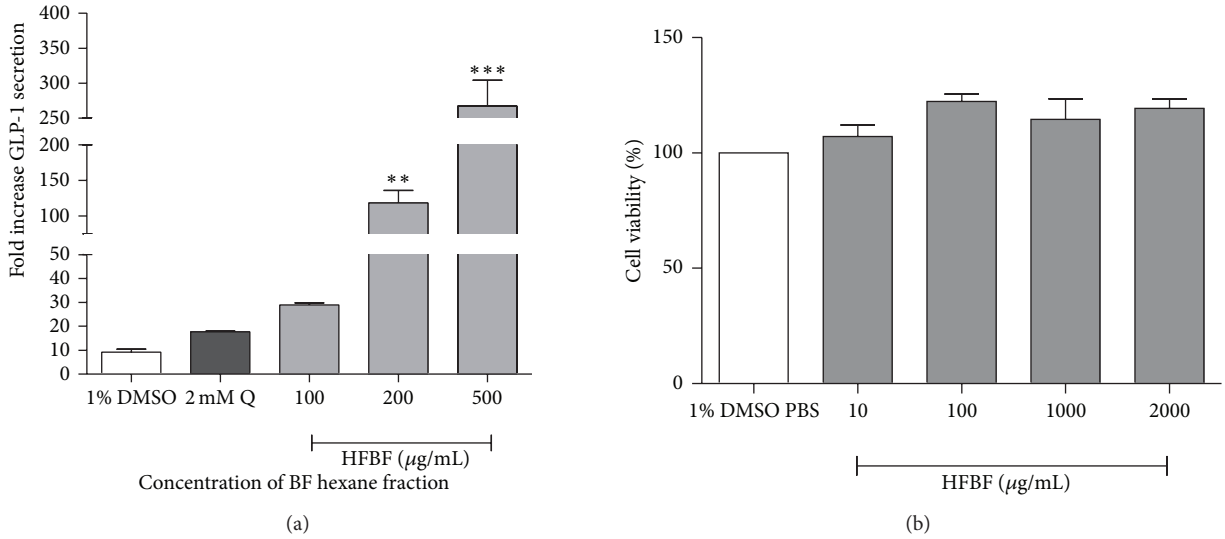

Figure 2: HFBF induced the secretion of GLP-1 in enteroendocrine cells without cell damage. BF extracted was fractionated with *n*-hexane (HX). (a) HFBF was treated with 100, 200, and 500 μg/mL each to the differentiated NCI-H716 cells. GLP-1 secretion was compared with the negative control and positive control. 1% DMSO used as negative control and 2 mM Quinine (Q) used as positive control. Experiments were conducted in triplicate and normalized with protein contents. (b) The effect of HFBF on the cell viability was measured by MTT test. All concentrations of HFBF had no toxicity to the NCI-H716 cell. Statistical significance was determined by a one-way ANOVA and the values are means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0005$.

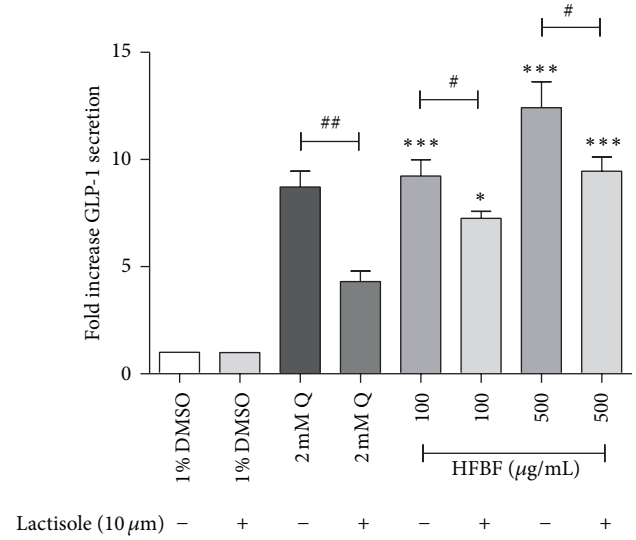

Figure 3: Inhibition study of GLP-1 secretion using lactisole. To test the inhibitory effect of the lactisole, lactisole was treated to the NCI-H716 cells. The BF hexane fraction (HFBF) was treated with 100, 200, and 500 μg/mL each to the differentiated NCI-H716 cells. GLP-1 secretion was compared with the negative control and positive control. 1% DMSO used as negative control and 2 mM Quinine (Q) used as positive control. Statistical significance was determined by a one-way ANOVA and the values are means ± SEM; $^{*}P < 0.05$ and $^{***}P < 0.0001$ versus 1% DMSO. $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ lactisole and HFBF treated group versus only HFBF treated group.

on making cell depolarization and inhibiting the expression of potassium voltage-gated channels by the downregulation of *KCNC3, KCNA6, KCNQ2, KCNE1, KCNN1*,and *KCTD19* genes. This change of membrane potential may cause the secretion of hormones in enteroendocrine cells [18].

3.5. HFBF Induced GLP-1 Secretion through $G_{\beta\gamma}$ Pathway. Lactisole is used as an inhibitor of sweet taste receptors that are known as a G_{α} pathway [19]. We treated lactisole to the NCI-H716 cell to verify the signaling pathway of the HFBF. As shown in Figure 3, lactisole showed no effect on blocking GLP-1 secretion during the treatment of the HFBF. This means the HFBF activates GLP-1 secretion but not through the G_{α} pathway. Thus, we performed calcium imaging experiments using Gallein, 2APB, and U73122 as other $G_{\beta\gamma}$ signaling pathway inhibitors [19, 20].

Intracellular Ca^{2+} is closely related to the GPCR signaling pathway [21]. Intracellular Ca^{2+} ion is produced naturally in the cell after the activation of GPCR. To confirm the signaling pathway of GLP-1 secretion, a $G_{\beta\gamma}$ pathway inhibitor was treated. As shown in Figure 4(a), Gallein which is an inhibitor of the $G_{\beta\gamma}$ subunit was treated to the NCI-H716 cell. Compared to the cells treated with only the HFBF, the Gallein-treated cells showed decreased intracellular calcium concentration. U73122, which is an inhibitor of PLCβ2 activity, and 2APB, which is an inhibitor of both IP_3 receptors and TRP channel treated cells, showed decreased intracellular calcium release in a dose dependent manner (Figures 4(b) and 4(c)). All of these results indicate the effect the HFBF has on GLP-1 secretion via the G protein $\beta\gamma$ pathway.

3.6. Hexane Fraction of Bupleurum falcatum L. Decreases Blood Glucose Level in db/db Mouse. As shown in Figure 5, HFBF regulated the blood glucose level of *db/db* mouse. *db/db* mouse is a model of diabetes because it has no leptin receptor

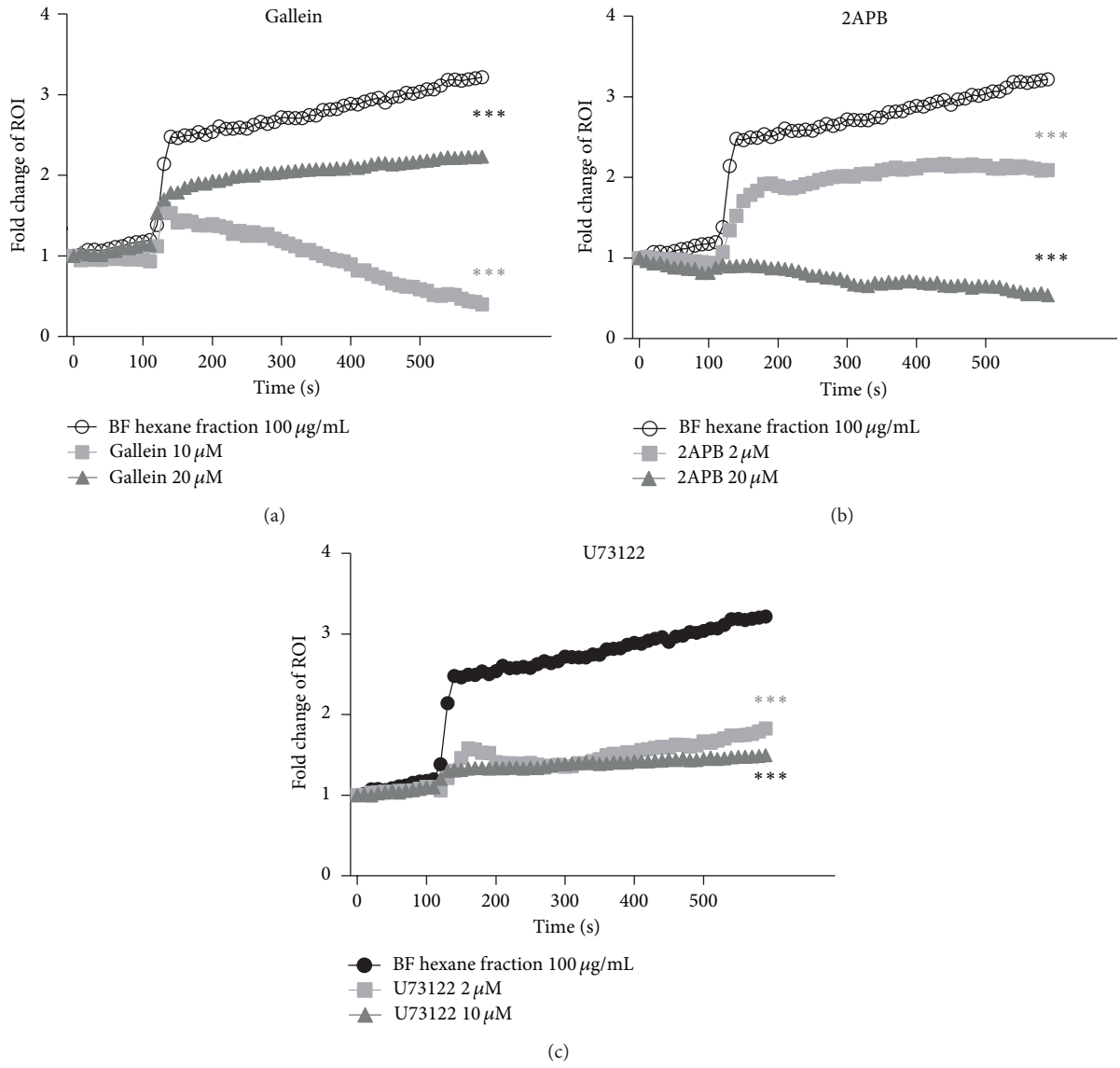

Figure 4: HFBF stimulates GLP-1 secretion through $G_{\beta\gamma}$ pathway. Treatment of BF hexane fraction at 100 seconds into the medium increased the concentration of Ca^{2+} in cytosol. With BF hexane fraction, inhibitors were treated. (a) Gallein which is an inhibitor of $G_{\beta\gamma}$ subunit-dependent signaling pathway reduced GLP-1 secretion dose dependently. (b) 2APB inhibits the IP_3-induced Ca^{2+} release. 2APB also reduced GLP-1 secretion dose dependently. (c) U73122 is an inhibitor of PLC-dependent processes. With this inhibitor, concentration of Ca^{2+} showed less change despite treatment of BF hexane fraction. Data are means of intensity of the NCI-H716 cells ($n = 10$). $^{***}P < 0.0001$ versus BF hexane fraction 100 μg/mL.

activity. It is point mutation in the gene for the leptin receptor. After glucose administration, blood glucose level increased time dependently before 20 min. However, HFBF administration group significantly shows the decreased glucose level compared to PBS-control group.

4. Discussion

This investigation suggests that HFBF stimulates GLP-1 secretion through the $G_{\beta\gamma}$-mediated pathway. Glucagon-like peptide-1 (GLP-1) is an incretin hormone which regulates insulin secretion, appetite, and gut motility [4]. Lately, new drugs that help to release GLP-1 secretion or block the degradation of GLP-1 are issued because GLP-1 regulates the secretion of insulin in pancreatic β-cells. The major source of GLP-1 is the intestinal L cell that secretes GLP-1 as a gut hormone. In this investigation, HFBF shows the antidiabetic effect in cellular level by secreting GLP-1 secretion. HFBF was treated to NCI-H716 cells with three concentrations: 100, 200, and 500 μg/mL. GLP-1 secretion was increased dose dependently. All concentrations of HFBF increased GLP-1 secretion and had significance. We supposed that the HFBF increases the GLP-1 secretion through sweet taste or bitter taste receptor signal pathway in

Table 2: Alteration of genes related to the potassium channel.

Probe name	Description	Gene symbol	*P*-value	Fold change
A_33_P3338793	Potassium voltage-gated channel, S+C38haw-related subfamily, member 3 (KCNC3), mRNA [NM_004977]	*KCNC3*	0.004	**−0.93**
A_33_P3415012	Potassium voltage-gated channel, shaker-related subfamily, member 6 (KCNA6), mRNA [NM_002235]	*KCNA6*	0.004	**−0.59**
A_33_P3395823	Potassium voltage-gated channel, KQT-like subfamily, member 2 (KCNQ2), transcript variant 5, mRNA [NM_172109]	*KCNQ2*	0.001	**−0.74**
A_23_P154855	Potassium voltage-gated channel, Isk-related family, member 1 (KCNE1), transcript variant 2, mRNA [NM_000219]	*KCNE1*	0.014	**−0.72**
A_23_P119573	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1 (KCNN1), mRNA [NM_002248]	*KCNN1*	0.034	**−0.85**
A_33_P3255131	Potassium channel tetramerisation domain containing 19 (KCTD19), mRNA [NM_001100915]	*KCTD19*	0.011	**−0.91**

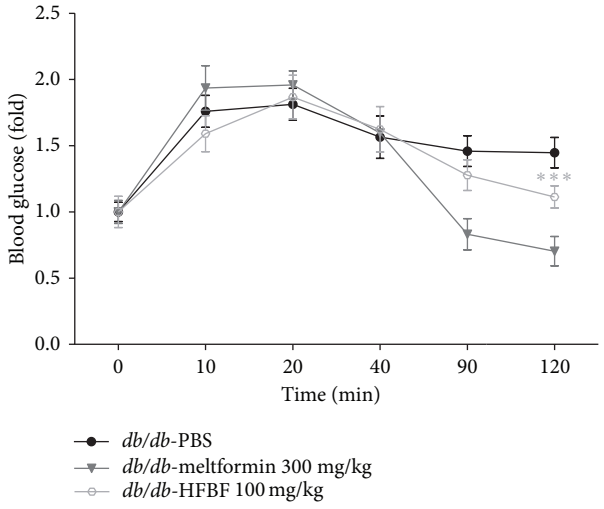

Figure 5: Oral glucose tolerance test of HFBF. To test regulatory effect of HFBF, HFBF treated orally to the *db/db* mouse. Metformin was used as a positive control. To compare with PBS-treated control group, 300 mg/kg of Metformin and 100 mg/kg of HFBF were orally treated just before the glucose administration. Mouse blood sample was collected from tail vein at 0 (before the HFBF treatment) and after 10, 20, 40, 90, and 120 minutes. Statistical significance was determined by student's *t*-test and the values are means ± SEM; $^{***}P < 0.0001$.

enteroendocrine NCI-H716 cells. To verify this hypothesis. Lactisole was used as an inhibitor of sweet taste receptor, exactly, human T1R3. Sweet taste receptor is the heterodimer consisting of T1R2 and T1R3. After treatment of HFBF with lactisole, there is no significant effect comparing to the only HFBF treatment (Figure 3). G protein-coupled receptor is also composed of $G_{\beta\gamma}$ subunit that is related to phospholipase C $\beta 2$ (PLC $\beta 2$), diacyl glycerol (DAG), and inositol 1, 4, 5-triphosphate (IP_3) and increases intracellular calcium. Gallein, 2APB, and U73122 are inhibitors of $G_{\beta\gamma}$ pathways [19, 20]. They inhibit $G_{\beta\gamma}$ subunit, PLC $\beta 2$, and IP_3 receptor. The activated $G_{\beta\gamma}$ activates PLC $\beta 2$ and then divides PIP_2 into DAG and IP_3. DAG regulates protein kinase C (PKC) and triggers phosphorylation of PKC. IP_3 is recruited to IP_3 receptor in ER membrane and, as a result, releases calcium flow into cytoplasm. In Figure 4, Gallein decreased intracellular calcium concentration. U73122 and 2APB also showed decreased intracellular calcium release in a dose dependent manner. To verify the signaling pathway of GLP-1 secretion through the HFBF treatment, we performed microarray analyses. Microarray data show the HFBF activated GLP-1 secretion through the GPCR signaling pathway. HFBF and then following membrane depolarization. The data from the inhibition studies showed the HFBF activates the GPCR signaling pathway, especially the $G_{\beta\gamma}$-mediated pathway, for the release of GLP-1. GLP-1 is one of target molecules for the treatment of Type 2 diabetes mellitus because it activates the GLP-1 receptor on the cell and then mediates insulin secretion. HFBF also showed regulatory effect of blood glucose level in *db/db* mouse OGTT experiments. This investigation suggests that HFBF stimulates GLP-1 secretion through $G_{\beta\gamma}$-mediated signaling pathway. The overall conclusion from the results is that the HFBF could be used as a possible medication for Type 2 diabetes mellitus patients via activation of $G_{\beta\gamma}$-mediated pathway.

Conflict of Interests

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

Authors' Contribution

Min-Hee Shin and Eun-Kyeong Choi contributed equally to this work.

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Research Article

Free Radicals Scavenging Capacity, Antidiabetic and Antihypertensive Activities of Flavonoid-Rich Fractions from Leaves of *Trichilia emetica* and *Opilia amentacea* in an Animal Model of Type 2 Diabetes Mellitus

Kiessoun Konaté,[1,2] Kassi Yomalan,[3] Oksana Sytar,[4] Patrice Zerbo,[5] Marian Brestic,[6] Van Damme Patrick,[7] Paul Gagniuc,[8] and Nicolas Barro[9]

[1] *Unit of Formation in Sciences Applied and Technological (UFR/SAT) and Institute of Sciences of the Environment and the Rural Development (ISEDR), Polytechnic University of Dédougou, Burkina Faso*
[2] *Laboratory of Biochemistry and Applied Chemistry, University of Ouagadougou, 09 P.O. Box 848, Ouagadougou 09, Burkina Faso*
[3] *Laboratory of Animal Physiology, UFR Bioscience, University of Felix Houphouet Boigny of Abidjan, 22 P.O. Box 582, Abidjan 22, Cote D'Ivoire*
[4] *Department of Plant Physiology and Ecology, Taras Shevchenko National University of Kyiv, Volodymyrs'ka Street 64, Kyiv 01601, Ukraine*
[5] *Laboratory of Plant Ecology and Biology, University of Ouagadougou, 09 P.O. Box 848, Ouagadougou 09, Burkina Faso*
[6] *Department of Plant Physiology, Slovak Agriculture University in Nitra, A. Hlinku 2, 94976 Nitra, Slovakia*
[7] *Laboratory of Tropical and Subtropical Agronomy and Ethnobotany, Department of Plants Production, Faculty of Biosciences Ingineering, Gent University, Belgium*
[8] *Institute of Genetics, University of Bucharest, 060101 Bucharest, Romania*
[9] *Laboratory of Biochemistry and Molecular Genetics Microbial, University of Ouagadougou, 03 P.O. Box 7131, Ouagadougou 03, Burkina Faso*

Correspondence should be addressed to Kiessoun Konaté; mehekiessoum@yahoo.fr

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Trichilia emetica and *Opilia amentacea* traditional Burkinabe medicinal plants were investigated to determine their therapeutic potential to inhibit key enzymes in carbohydrate metabolism, which has relevance to the management of type 2 diabetes. *In vitro* and *in vivo* antioxidant and antihypertensive potential and antilipidemia and antihyperglycemia activities in an animal model of type 2 diabetes mellitus have been studied. The antioxidant activity of the flavonoids from leaves of *Trichilia emetica* and *Opilia amentacea* has been evaluated using β-carotene-linoleic acid system, 1,1-diphenyl-2-picrylhydrazyl inhibitory activity, chelation of iron (II) ions, and lipid peroxidation which showed more pronounced antioxidant capacities of *Trichilia emetica*. Total cholesterol concentrations decreased in an animal model of type 2 diabetes mellitus under effects of flavonoid-rich fractions from leaves of *Trichilia emetica* and *Opilia amentacea* has been observed. Extract of flavonoid-rich fractions from *Trichilia emetica* shown maximum radical scavenging activity and possessed marked antiamylase activity which may be due to the presence of certain secondary metabolites. Suggested better antihyperglycemia, antilipidemia, and antihypertensive properties of flavonoid-rich fractions from *Trichilia emetica* compared to the extract of *Opilia amentacea* are demonstrating antidiabetic potential of *Trichilia emetica* as therapeutic targets for the management of type 2 diabetes.

1. Introduction

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia, glycosuria, polyurea, and polydipsia induced by insulin deficiency [1] and insulin resistance [2]. Diabetes mellitus is an important metabolic syndrome. The increasing worldwide incidence of diabetes mellitus in adults constitutes a global public health burden. At least, diabetes

mellitus is possibly the world's fastest growing metabolic disorder, and as the knowledge of the heterogeneity of this disorder increases, so does the need for more appropriate therapy [3]. The World Health Organization (WHO) estimates that currently more than 180 million people worldwide have diabetes and it is likely to double by 2030, with India, China, and United States predicted to have the largest number of affected individuals [4, 5]. Diabetes mellitus is treated by using oral hypoglycemic agents such as sulphonylureas, biguanides, meglitinides, and α-glucosidase inhibitors. Hyperglycemia, a condition characterized by an abnormal excess of sugar in the blood, has been linked to the onset of type 2 diabetes mellitus and associated cardiovascular complications including hypertension [6, 7]. In effect, stress-related disease such as hypertension or high blood pressure has been considered as a high risk factor cardiovascular disease. Twenty percent of the world's population suffers from hypertension [8]. Experimental evidence suggests that antihypertensive or angiotensin I-converting enzyme (ACE) inhibitor treatment can offer a clinical advantage in hypertension [9]. ACE plays a key physiological role in the control of blood pressure by virtue of the rennin-angiotensin system [10]. Captopril (d-3-mercapto-2-methylpranory-l-proline), enalapril, and lisinopril have been developed and used as clinical antihypertensive drugs. Although synthetic ACE inhibitors, including captopril, are remarkably effective as antihypertensive drugs, they cause adverse side effects, such as coughing, allergic reactions, taste disturbances, and skin rashes. Therefore, research and development to find safer, innovative, and economical ACE inhibitors is necessary for the control of blood pressure.

Although a few synthetic antidiabetic drugs are available to combat the impaired insulin secretion, insulin resistance, and hyperglycemia that characterize type 2 diabetes mellitus, some of these drugs can have negative side effects at high doses [11, 12]. A major focus of current antidiabetic research is the development of antihyperglycemic agents that are safe and free of negative side effects. Many plants and their active chemical compounds have demonstrated activity in the treatment of various disorders [13]. According to ethnobotanical information, more than 800 plants are used as traditional remedies in one or other form for the treatment of diabetes [14]. The management of diabetes without any side effects is still a challenge; therefore, plants continue to play an important role in the discovery of new compounds for the treatment of this disease. The management of diabetes can be achieved by reducing postprandial hyperglycemia by delaying the activities of the enzymes α-amylase and α-glucosidase which are responsible for the digestion of carbohydrates and absorption of glucose in the digestive tract, respectively [15, 16]. Drugs derived from natural products have played a major role in the development of pharmaceutical treatments for diabetes. Metformin, the single most prescribed agent for the treatment of diabetes, originated from herbal medicine [17, 18]. A plant-derived antidiabetic agent galegine was isolated from *Galega officinalis*. Experimental and clinical evaluations provided the pharmacological and chemical basis for the subsequent discovery of metformin [17, 19]. 1-Deoxynojirimycin (DNJ), a potent α-glucosidase inhibitor, was isolated from the water extract of leaves of the mulberry tree (*Morus alba* L.) [20]. There are many cellular biochemical pathways and environmental toxins which produce reactive oxygen species (ROS) [21] and contribute to the development of diseases such as cancer, cardiovascular disorders, diabetes, cataracts, and many neurodegenerative diseases [22]. Many studies have confirmed that plants and foods rich in polyphenolic content are effective scavengers of free radicals, thus helping in the prevention of these diseases through their antioxidant activity [23]. Antioxidants which are present in plants, herbs, and dietary sources help in preventing vascular diseases in diabetic patients [24]. Tannins and flavonoids are the secondary metabolites in plants considered to be the natural source of antioxidants which prevent destruction of β cells and diabetes-induced ROS formation [25]. Thus, it is a good strategy to manage diabetes as a whole with plants which show good enzyme inhibitory and antioxidant activities [26].

Trichilia emetica and *Opilia amentacea* are native to sub-Saharan Africa and are essentially tropical in origin. In the western part of Burkina Faso, the leaves of these plants are used to treat cardiovascular diseases. They possess hypotensive, hypolipidemic-delite, antioxidant, antibacterial and anti-inflammatory properties [27]. But none have reported on their antioxidant, antidiabetic, and antihypertensive properties of flavonoid-rich fractions. Lack of scientific data to support these claims prompted this study which was therefore aimed at assessing the possible antioxidant, antidiabetic, and antihypertensive properties of flavonoid-rich fractions from the leaves of these plants in models using rats in order to provide a scientific basis for the traditional use of this plant for better management of type 2 diabetes.

2. Material and Methods

2.1. Plant Materials. Fresh leaves of *Trichilia emetica* and *Opilia amentacea* were collected in October 2011 in Ouagadougou, capital of Burkina Faso, with gardeners. The plants were identified in the Laboratory of Biology and Ecology, University of Ouagadougou, where a voucher specimen was deposited.

2.2. Animals Handling. Swiss NMRI mice (25–30 g) and adult albinos Wistar rats (160–200 g) of both sexes were used for this study. All animals were housed in cages under controlled conditions of 12 h light/and 12 h without light and 25°C. They received pellets of food enriched with 20% protein and water ad libitum. They were deprived of food for 15 h (but with access to drinking water) and weighed before the experiments. Experiments on the animals were performed according to the protocols already approved by the Institute of Health Sciences Research/University of Ouagadougou (Burkina Faso) and met the international standards for animal study [28].

2.3. Chemicals. Streptozotocin and alloxan monohydrate were purchased from Sigma (Germany) and all other chemicals and reagents used in this study were of analytical grade

and were purchased from Sigma Chemical Co. (St. Louis, MO). Glibenclamide, rabbit lung dehydrated by acetone, and captopril were purchased from Sigma-Aldrich, USA.

2.4. Preparation of Extracts for Acute Toxicity Study. 100 grams of leaves (powdered plant materials) dried in laboratory condition were extracted with 500 mL of acetone 80% (400 mL acetone + 100 mL water) for 24 h under mechanic agitation (SM 25 shaker, Edmund BÜHLER, Germany) at room temperature. After filtration, acetone was removed under reduced pressure in a rotary evaporator (BÜCHI, Rotavopor R-200, Switzerland) at approximately 40°C and freeze-dried (Telstar Cryodos 50 freeze-dryer). The extract was weighed before packing in waterproof plastic flasks and stored at 4°C until use.

2.5. Flavonoids Extraction. The fresh harvested plant materials (100 grams of leaves) were dried in the laboratory at room temperature (20–25°C); afterwards samples were ground to pass a sieve of 0.3 mm. Flavonoids were extracted with aqueous acetone (80%, v/v). The extracts were then washed with hexane to remove chlorophyll and other low molecular weight compounds. Acetone was evaporated and then the aqueous extracts with ethyl acetate is used to separate by sequential liquid-liquid extraction. The flavonoid-rich fractions were lyophilized and stored at 22°C prior to biological tests. For the tests, lyophilized sample was dissolved with 10% DMSO in water at the desired concentration.

2.6. Antioxidant Capacity of Flavonoids

2.6.1. β-Carotene-Linoleic Acid Assay. The antioxidant activity of the flavonoids was evaluated using β-carotene-linoleic acid system according to [29]. In short, 1 mL of β-carotene solution in chloroform (0.2 mg/mL) was pipetted into a round-bottom flask. To the solution, 20 mg of linoleic acid and 200 mg of Tween 40 were added. After removing chloroform in a rotary evaporator, 50 mL of aerated distilled water was added to the oily residue. Aliquots (5 mL) of thus obtained emulsion were transferred to a series of tubes containing 2 mg of extract or 0.5 mg of butylated hydroxyanisole (BHA) (positive control). Emulsion without antioxidant served as control. After addition of the emulsion to the tubes, they were placed in a water bath at 50°C for 2 h. During that period, the absorbance of each sample was measured at 470 nm at 15 min intervals, starting immediately after sample preparation ($t = 0$ min) until the end of the experiment ($t = 120$ min). The rate of β-carotene bleaching (R) for the extracts, BHA and water, was calculated according to first-order kinetics. The percent of antioxidant activity (ANT) was calculated as described in [30], using the equation

$$\text{ANT} = \frac{\left(R_{\text{Control}} - R_{\text{Sample}}\right)}{R_{\text{Control}}} \times 100, \quad (1)$$

where R_{Control} and R_{Sample} are average bleaching rates of water control and antioxidant (flavonoids or BHA), respectively.

2.6.2. DPPH Radical-Scavenging Activity. The scavenging effect for DPPH free radical was monitored as described in [31] with minor modification. Briefly, 1.0 mL of 0.16 mM DPPH methanolic solution was added to 1.0 mL of either methanolic solution of extract (sample) or methanol (control). The mixtures were vortexed and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm. Radical-scavenging activity (RSA) for DPPH free radical was calculated using the following equation:

$$\text{RSA} = \frac{\left(A_{\text{Control}} - A_{\text{Sample}}\right)}{A_{\text{Control}}} \times 100, \quad (2)$$

where A_{Control} is the absorbance of the methanol control and A_{Sample} is the absorbance of the flavonoids. Synthetic antioxidant, BHA, was used as positive control. DPPH radical-scavenging activity was calculated as the concentration that scavenges 50% of DPPH free radical and thus has RSA = 50% (EC_{50}).

2.6.3. Chelating Activity (ChA). The chelation of iron (II) ions was studied as described by [32]. An aliquot of the extract in methanol (1.3 mL) was added to 100 μL of 2 mM ferrous chloride. After 5 min, the reaction was initiated by adding 200 μL of 5 mM ferrozine. Following 10 min incubation at room temperature, the absorbance at 562 nm was recorded. For preparation of control, 1.3 mL of methanol was used instead of polyphenols solution. EDTA was used as a chelating standard. The $Fe^{(2+)}$-chelating activity chelating activity (ChA) was calculated using the equation below:

$$\text{ANT} = \frac{\left(A_{\text{Control}} - A_{\text{Sample}}\right)}{A_{\text{Control}}} \times 100, \quad (3)$$

where A_{Control} is the absorbance of the negative control (solution to which no flavonoid was added) and A_{Sample} is the absorbance of the extract solution. Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50% of Fe^{2+} ions and thus has ChA = 50%.

2.6.4. Inhibition of Lipid Peroxidation. Liver of male Wistar rats (160–180) was excised and homogenized (1% w/v) in 0.154 mol/L KCl solution. The homogenate was centrifuged at 3000 rpm at 4°C for 10 min and supernatant was used for the assay. Peroxidation of the liver homogenate was induced by $FeCl_2$-H_2O_2 [33]. Briefly, 1% liver homogenate was incubated with 0.5 mmol/L of each of $FeCl_2$ and H_2O_2 with or without flavonoid-rich fractions (50 μg/mL). After incubation at 37°C for 60 min, the formation of malondialdehyde (MDA) was measured at 535 nm [33]. BHT served as positive control. The equation is

$$\text{Inhibition of peroxidation } (\%) = \left[\frac{(\text{AC} - \text{AA})}{\text{AC}}\right] \times 100, \quad (4)$$

where AC is the absorbance of the control (without any treatment) and AA is the absorbance of the antioxidants.

2.7. In Vitro Antihyperglycemia

2.7.1. Amylase Inhibition Screening Assay. The α-amylase inhibitory assay was modified from [34]. Twenty μL of porcine pancreatic α-amylase solution (EC 3.2.1.1; equivalent to 3000 U in 50 mM phosphate buffer, pH 6.9) was mixed with 15 μL of plant extract and incubated at 37°C for 45 minutes. After incubation, the mixture was applied to a sterile paper disc and placed onto the center of Petri plates containing medium consisting of 1% (w/v) agar and 1% (w/v) starch in distilled water. Plates were allowed to stand for 3 days at 25°C then stained with iodine and allowed to stand for 15 min. The diameter of the clear zone was measured and used to calculate the amylase inhibitory activity. As a control, the enzyme was mixed with the solvent in which the plants were extracted (ethanol) and applied onto the sterile disc. Results were expressed as

$$\text{Percentage (\%) amylase inhibition} = \left\{\frac{(\text{diameter of control} - \text{diameter of sample})}{\text{diameter of control}}\right\} \times 100. \quad (5)$$

2.7.2. Amylase Inhibition Assay by Quantitative Starch Hydrolysis. The α-amylase inhibitory activity was determined [35] using porcine pancreatic α-amylase solution (EC 3.2.1.1) type VI B. To 125 μL of different plant extract concentrations (range 1.56 μg/mL to 500 μg/mL), α-amylase solution (0.5 mg/mL in 0.02 M sodium phosphate buffer) was mixed and the reaction mixture was preincubated for 10 minutes at room temperature. The reaction mixture was diluted by adding 5000 μL of distilled water. The generation of maltose was quantified by measuring the absorbance at 540 nm of 3-amino-5-nitrosalicylic acid (from reduction of 3,5-dinitrosalicylic acid) [36] using a UV-visible spectrophotometer. The control was buffer-treated in the same way as plant samples. The standard used was acarbose (concentration range 1.56 μg/mL to 500 μg/mL). Results were expressed as

$$\text{Percentage (\%) inhibition} = \left\{\frac{(\text{absorbance of control}_{540\,\text{nm}} - \text{absorbance of samples}_{540\,\text{nm}})}{\text{absorbance of control}_{540\,\text{nm}}}\right\} \times 100. \quad (6)$$

2.7.3. Glucosidase Inhibition Assay. The α-glucosidase inhibition assay has been modified from [37] using yeast α-glucosidase (EC 2328898). A volume of 25 μL of plant extract (range 0.35 μg/mL to 100 μg/mL) was mixed with 50 μL of α-glucosidase enzyme 0.1 U/mL in 0.1 M potassium in 96 well plates and incubated at 37°C for 30 minutes. After preincubation, 25 μL of 5 mM pNPG in 0.1 M phosphate buffer was added to each well and the reaction mixture was incubated again at 37°C for 30 minutes. Thirty μL of 0.1 M sodium carbonate solution was added to the previous reaction mixture and incubated again for 20 minutes at 37°C. Before and after incubation, the absorbance was measured at 405 nm and compared to the control that contained 25 μL of buffer solution instead of polyphenols solution. The standard used was acarbose (concentration range 0.35 μg/mL to 100 μg/mL). The α-glucosidase activity was determined by measuring release of p-nitrophenol from p-nitrophenyl α-D-glucopyranoside [38]. The α-glucosidase inhibitory activity was expressed as

$$\text{Percentage (\%) inhibition} = \left\{\frac{(\text{absorbance of control}_{405\,\text{nm}} - \text{absorbance of samples}_{405\,\text{nm}})}{\text{absorbance of control}_{405\,\text{nm}}}\right\} \times 100. \quad (7)$$

2.8. In Vitro Antihypertensive Profile by Measurement of ACE Inhibitory Activity

2.8.1. Assay Buffer. HEPES (297.5 mg, 50 mmol/L), NaCl (438.75 mg, 300 mmol/L), and Na_2SO_4 (1420 mg, 400 mmol/L) were added to a 25 mL volumetric flask (amount and final concentration given in parentheses). After dissolving in 20 mL of distilled water containing 50 mL of saturated NaOH solution, the pH was made up with distilled water. Phosphate buffer (100 mmol/L) was prepared by dissolving 340.2 mg of anhydrous potassium phosphate in 20 mL of distilled water, adjusted to pH 8.5 with 10% NaOH solution, and made up to 25 mL.

2.8.2. Stock and Working Solution of Rabbit Lung Dehydrated by Acetone. The stock solution was prepared as described previously [39] by dissolving 2 g of rabbit lung dehydrated powder in 10 mL of 50 mmol/L phosphate buffer (pH 8.3). The stock solution was highly active and stable for at least 3 months under refrigeration (2–6°C). Working solution (1 g/10 mL) was freshly prepared by diluting the stock solution in the phosphate buffer before performing the assays.

2.8.3. Substrate Solution. 200 mg of hippuryl-glycyl-glycine was dissolved in 4 mL of 1 mol/L ammonium hydroxide solution. After complete dissolution, the volume was increased to 6.8 mL with distilled water.

2.8.4. TNBS Solution. TNBS (2030 μL) was added to a 5 mL volumetric flask and the volume was made up with distilled water to obtain a final concentration of 60 mmol/L. The solution was stored at −20°C and used within 3 months.

2.8.5. Preparation of Flavonoid-Rich Fractions. For antihypertensive screening, the flavonoid-rich fractions were dissolved in 20% methanol and 80% HEPES to a concentration of 5 mg/mL.

2.8.6. Colorimetric Methanol for ACE Inhibition Assay [40]. Ten microlitres of rabbit lung solution (1 g/10 mL) were added to an Eppendorf tube containing 10 μL of flavonoid-rich fractions solution (5 mg/mL) to be tested, or 10 μL of

50 mmol/L phosphate buffer (pH 8.3) (negative control), or 10 μL of captopril solution (5 mg/mL) (positive control). The mixture was homogenized and preincubated for 5 min at 37°C. The enzyme reaction was initiated by adding 60 μL of the assay buffer and 30 μL of the substrate solution. After homogenization, the mixture was incubated for 35 min at 37°C. The reaction was stopped by the addition of 100 μL of sulfuric acid (0.33 mmol/L); the Eppendorf tube was shaken 10 s after the addition of 1000 μL of distilled water. In the sequence, the mixture was centrifuged at 2000 rpm for 10 min. An aliquot of the supernatant (75 μL) was placed on a microtitre plate and mixed with 100 μL of phosphate buffer (100 mmol/L, pH 8.5) and 5 μL of TNBS solution. The plate was kept in the dark at room temperature for 20 min. Its absorbance was read in a microtitre plate reader (Spectramax 340 PC tunable microplate reader) at 415 nm against a blank solution prepared similarly but without adding sodium tungstate and sulfuric acid solutions to the mixture. Assays were performed in triplicate. Calculation of ACE inhibition on a percentage basis was done using the following equation:

$$\text{Inhibition } (\%) = \frac{[100 - (\text{AI} \times 100)]}{\text{AC}}, \quad (8)$$

where AI is the measured absorbance at 415 nm in the presence of an inhibitor and AC is the absorbance of the blank solution.

2.9. Acute Toxicity Study of Aqueous Acetone Extracts. Swiss mice (male and female) were randomly divided into 7 groups (1 control group and 6 treated groups) of 6 animals (3 males and 3 females). The control group received water containing 10% dimethylsulfoxide (DMSO) administered intraperitoneally. The aqueous acetone extracts of *Trichilia emetica* and *Opilia amentacea* suspended in 10% DMSO were administered intraperitoneally at doses of 1, 2, 2.5, 3, 4, 5, and 6 g/kg [35]. The general behaviour of the mice was observed for 120 min after the treatment. The animals were observed for morbidity and mortality once a day for 14 days. The number of survivors after the 14 days period was noted. The toxicological effect was assessed on the basis of mortality for 14 days, which was expressed by the median lethal dose value (Lethal Dose 50 or LD_{50}) estimated from the regression of log-probit mortality rate [41].

2.10. In Vivo Antihyperglycemia and Hypolipidaemia Potential of Flavonoids

2.10.1. In Vivo Antihyperglycemia

(1) Induction of Diabetes. Alloxan monohydrate was first weighed individually for each animal according to its weight and then solubilized with 0.2 mL saline just prior to injection. Diabetes was induced by injecting it at a dose of 100 mg/kg body weight intraperitoneally. After 1 hr of alloxan administration, the animals were got food and 5% dextrose solution was also given in feeding bottle for a day to overcome the early hypoglycemic phase. The animals were kept under observation and after 48 hr, blood glucose was measured. One group served as a control which received vehicle alone. The diabetic rats (glucose level >150 mg/dL) were separated and divided into four different groups for experimental study [42].

(2) Experimental Design

(i) Acute Treatment and Subacute Treatment. Normal rats are kept into group 1; diabetic induced rats are grouped into groups 2, 3, 4, and 5. Each group contains six rats: group I: normal control (saline); group II: diabetic control (saline); group III: standard (glibenclamide 10 mg/kg); group IV: test-dose (100 mg/kg of flavonoid-rich fractions from *Trichilia emetica* and *Opilia amentacea*); group V: test-dose (300 mg/kg of flavonoid-rich fractions from *Trichilia emetica* and *Opilia amentacea*). Drugs are administered via oral route. Treatment continued for seven days [42].

(ii) Acute Study (Single Day Study). Blood samples were collected from rat caudal vein and serum glucose levels were estimated at 0, 1, 3, and 5 h after the extract administration.

(iii) Subacute Stud (Seven Day Study). Blood samples were collected from rat caudal vein and serum glucose levels were estimated at 1, 3, 5, and 7 days. Blood glucose levels were determined by god-pod method.

2.10.2. In Vivo Hypolipidaemic Potential

(1) Induction of Experimental Diabetes. Diabetes was induced by a single intraperitoneal injection of a freshly prepared streptozotocin (STZ) solution (50 mg/kg in citrate buffer 0.01 M, pH 4.5) to overnight-fasted rats [43]. Control rats received normal water alone. Diabetes was identified by polydipsia, polyurea, and measuring nonfasting blood glucose levels 48 h after injection of STZ. Animals which show blood glucose levels more than 250 mg/dL were considered as diabetic rats and used as the experimental animals.

(2) Experimental Design. Adult, male rats of Wistar strain weighing 160–180 g were chosen as animal for present study. They were housed individually in clean, sterile, polypropylene cages under standard conditions and water ad libitum. The animals were acclimatized to the laboratory for one week prior to the start of experiments. The rats were divided into 7 groups comprising of 6 animals in each group as follows: group I: normal rats (controls); group II: diabetic untreated rats; group III: diabetic + glibenclamide treated rats; group IV: normal + *Trichilia emetica* and *Opilia amentacea* treated rats (100 mg/kg of flavonoid-rich fractions from leaves of *Trichilia emetica* and *Opilia amentacea*/day); group V: normal + *Trichilia emetica* and *Opilia amentacea* treated rats (500 mg/kg of flavonoid-rich fractions from leaves of *Trichilia emetica* and *Opilia amentacea*/day); group VI: diabetic + *Trichilia emetica* and *Opilia amentacea* treated rats (100 mg/kg of flavonoid-rich fractions from leaves of *Trichilia emetica* and *Opilia amentacea*/day); and group VII: diabetic + *Trichilia emetica* and *Opilia amentacea* treated rats (500 mg/kg of flavonoid-rich fractions from leaves of *Trichilia*

emetica and *Opilia amentacea*/day). Drugs are administered via oral route; treatment continued for 28 days.

(3) Measurement of Serum Biochemical Parameters. Blood samples were collected by cardiac puncture. The blood samples without anticoagulant were centrifuged at 3000 rpm for 5 min to obtain plasma or serum. Estimation of serum cholesterol was carried out by the method of [44]. Serum triglycerides were estimated by the method of [45] and HDL cholesterol was estimated by the method of [46]. The VLDL cholesterol was calculated using the formula, TG/5 mg/dL. The serum LDL cholesterol was estimated by the method of [47].

(4) Estimation of Triglycerides (TG). Triglycerides in the liver tissue were estimated by modified version method of [48] with slight modifications as given below. Triglycerides were assayed by hydrolyzing them to glycerol and the liberated glycerol was determined. Tissue homogenates were taken and 0.5 mL of 1 N H_2SO_4 and 4 mL of chloroform were added. The contents were centrifuged at 1000 rpm for 15 min. The 0.5 of chloroform layer was taken and to 0.4 mL of methanol and 0.1 mL of alkaline barium solutions was added and the contents were heated for 30 min at 80°C; the total volume was made up to 1 mL with 2 N H_2SO_4 and centrifuged for 10 min at 1000 rpm. The 0.5 mL of this supernatant was taken and 0.1 mL of sodium periodate was added and shaken well for 1 min; 0.1 mL of sodium arsenate and 5 mL of chromotropic acid reagent were added and heated for 30 min and cooled. The samples were evaluated under wavelength 575 nm spectrophotometrically. The results were finally expressed in mg of triglycerides/gram wet weight of the tissue.

(5) Estimation of Total Cholesterol. The total cholesterol content of liver tissue was estimated using Liebermann Burchard reaction as described by [48].

(6) Estimation of Phospholipids. Phospholipids (PL) in the liver tissue were estimated by the method of [49].

2.10.3. Statistical Analyses. Data were expressed as mean ± standard deviation (SD) of six experiments ($n = 6$). Results were analyzed by one-way ANOVA followed by Dunnett's t-test using Prism 4 software. The level of significance was considered at $P \leq 0.05$.

3. Results

3.1. Antioxidant Potential

3.1.1. Antioxidant Capacity of β-Carotene-Linoleic Acid Assay. The basis of β-carotene-linoleic acid assay is degradation of β-carotene in reaction with linoleic acid free radical. Antioxidants present in the solution can hinder this reaction and consequently prevent discoloration of β-carotene solution. The reduction of absorbance of β-carotene-linoleic acid emulsion was shown in presence of the flavonoid-rich fractions. Comparison of the ANT values of the samples (Figure 1) indicates that the flavonoid-rich fractions were less successful at inhibition of bleaching of β-carotene emulsion comparatively to BHA ($P < 0.05$ and $P < 0.001$).

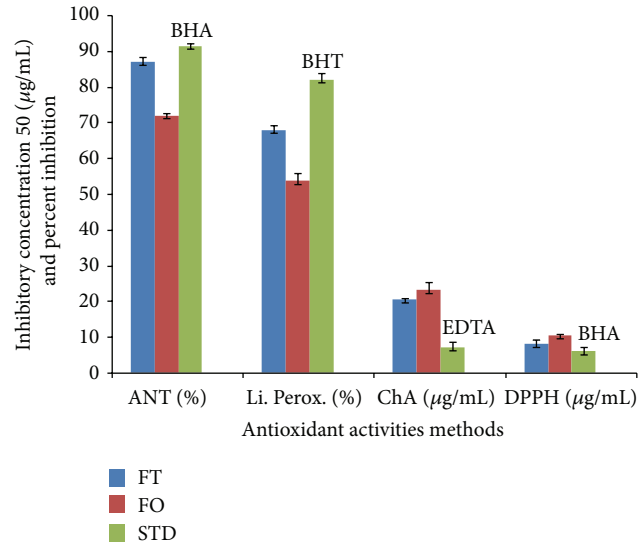

Figure 1: Antioxidant activity in β-carotene-linoleate test (ANT), DPPH radical scavenging activity (EC_{50}), and metal chelating activity ($ChEC_{50}$) of flavonoid-rich fractions from *Trichilia emetica* (FT) and *Opilia amentacea* (FO) and standards (STD).

3.1.2. Antioxidant of DPPH Radical-Scavenging Activity. The basis of DPPH assay is the discoloration of $DPPH^{\bullet}$ solution in presence of an antioxidant. In its radical form, DPPH absorbs with maximum at 517 nm, but upon reduction with an antioxidant. In this study, flavonoid-rich fractions demonstrated notable antiradical activities albeit lower than the activity of BHA ($P < 0.001$). Results are consigned in Figure 1.

3.1.3. Chelating Activity (ChA). The chelating ability of the extracts toward ferrous ions was investigated (Figure 1) in presence of ferrozine, Fe^{2+} ion chelator, which upon binding of the metal ion absorbs with maximum at 562 nm. The investigated flavonoid-rich fractions demonstrated significant chelating ability in the present research, although has been found lower than the ability of EDTA ($P < 0.0001$).

3.1.4. Lipid Peroxidation. The endogenous basal of malondialdehyde in the rat liver homogenate was 40.02 mmol/g tissue. After 30 min of incubation $FeCl_2$-H_2O_2, the incubation of malondialdehyde increase was measured at 535 nm. The inhibition of lipid peroxidation activity by the flavonoid-rich fractions is presented in Figure 1. We noticed that flavonoid-rich fractions showed different statistically significant percentages of inhibition as compared to BHT ($P < 0.001$ and $P < 0.0001$).

3.2. In Vitro Antihyperglycemia. In the amylase assay, the positive control acarbose showed an IC_{50} of 5.70 μg/mL and flavonoid-rich fractions exerted an IC_{50} of 6.12 μg/mL for amylase inhibitory activity comparatively to the amylase inhibition assay by quantitative starch hydrolysis where flavonoid-rich fractions exerted an IC_{50} of 6.23 for glucose inhibition activity ($P < 0.001$). In the investigated flavonoid-rich fractions no statistical significance has been

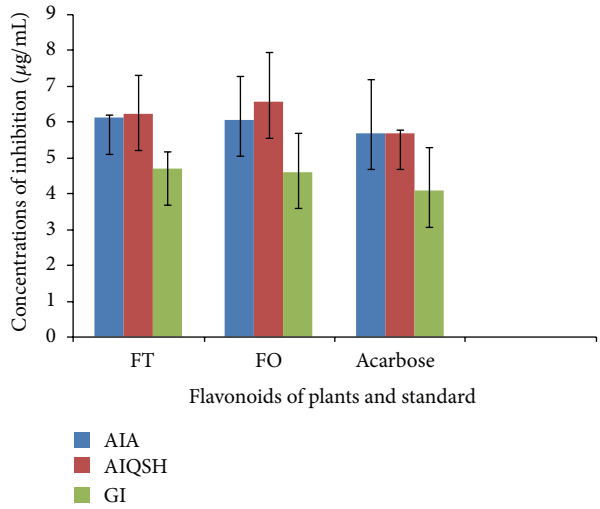

Figure 2: Flavonoid-rich fractions from *Trichilia emetica* (FT) and *Opilia amentacea* (OP) and acarbose (standard) on inhibition of key enzymes (AIA = amylase inhibition screening assay, AIQSH = amylase inhibition assay by quantitative starch hydrolysis, and GI = glucosidase inhibition assay) in carbohydrate metabolism.

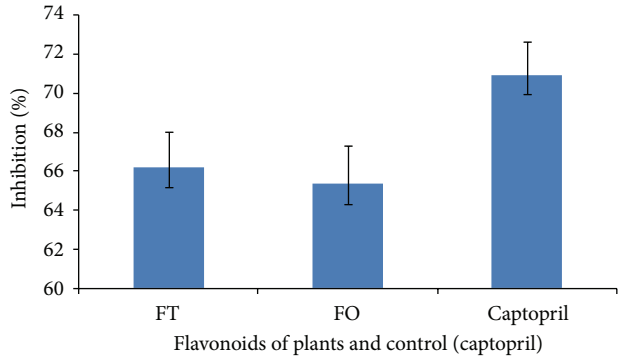

Figure 3: Percent antihypertensive activity upon treatment with flavonoid-rich fractions from *Trichilia emetica* (FT) and *Opilia amentacea* (FO). Captopril was the positive control.

observed (IC_{50} of 4.70 μg/mL and IC_{50} of 4.61 μg/mL) compared to the control, although was lower than IC_{50} of acarbose (4.08 μg/mL) for glucosidase inhibition ($P > 0.05$) (Figure 2).

3.3. In Vitro Antihypertension. Flavonoid-rich fractions which were tested for antihypertension assay showed >65% inhibition of ACE compared to the control Figure 3. Captopril, which was used as a positive control, gave >70%. Comparison of the flavonoid-rich fractions from *Trichilia emetica* and *Opilia amentacea* values to the control, (Figure 3) showed statistically significant percentages of inhibition in both variants ($P < 0.05$).

3.4. Acute Toxicity Study in Mice. It was confirmed the efect of intraperitoneal treatment of aqueous acetone extracts from *Trichilia emetica* and *Opilia amentacea* on mortality, LD_{50} values. The value of LD_{50} is 568.5 mg/kg body weight for intraperitoneal administration for *Trichilia emetica* and 636.2 mg/kg body weight. During 14-day period of acute toxicity evaluation, some signs of toxicity have been observed but were quickly reversible.

3.5. In Vivo Antihyperglycemia Profile of Flavonoid-Rich Fractions. The results of *in vivo* antihyperglycemia of flavonoid-rich fraction are presented in Tables 1 and 2. We noticed that *Trichilia emetica* (300 mg/kg) possess higher antihyperglycimia parameters results than *Opilia amentacea* and comparatively to the control ($P < 0.05$, $P < 0.001$, and $P < 0.0001$).

3.6. In Vivo Antihyperlipidaemia Potential of Flavonoid-Rich Fractions. In this section, we noticed a significant decrease in serum HDL cholesterol levels and a significant elevation in the total cholesterol, triglycerides, and LDL-cholesterol levels in diabetic rats compared to normal rats. Oral administrations of flavonoid-rich fractions for 28 days brought back the levels of serum lipids to near normal levels in diabetic rats and they were restored after administration of flavonoids for a period of 28 days. The total cholesterol, triglycerides, and phospholipids of normal rat hepatic tissue were 48.12 ± 0.53, 1.12 ± 0.10, and 1.21 ± 0.21, respectively, whereas in the diabetic rats these levels are raised to 60.17 ± 2.31, 2.17 ± 0.32, and 10.01 ± 0.33, respectively. The changes of these investigated parameters in the plants after treatment with bioactive fractions were shown in the hepatic tissue: 52.35 ± 1.14, 1.43 ± 0.42, and 8.55 ± 1.1 for *Trichilia emetica* and 53.01 ± 1.10, 1.71 ± 1.10, and 9.02 ± 0.1 for *Opilia amentacea*, respectively ($P < 0.05$). The total cholesterol, triglycerides, LDL-C, and VLDL-C levels of normal rat serum were 87.61 ± 2.01, 111.1 ± 2.51, 66.52 ± 0.51, and 23.2 ± 1.1, whereas in diabetic rats these levels have been raised with the plant bioactive fraction treatment with *Trichilia emetic* extract to 90.27 ± 1.60, 115.28 ± 1.64, 48.21 ± 2.23, and 24.5 ± 1.10 and 118.22 ± 2.2, 93.41 ± 1.81, 70.4 ± 1.12, and 29.43 ± 1.8 for *Opilia amentacea* extract, respectively. The HDL-C levels of normal rats were 45.18 ± 2.26. The levels were regained with the flavonoid-rich fraction treatment to 50.61 ± 1.36. We noticed that extract of flavonoid-rich fractions from *Trichilia emetica* presented better effects on the stabilization of concentrations of total cholesterol, triglycerides, and phospholipids in the liver of experimental rats compared to extract of *Opilia amentacea* (Figures 4 and 5).

4. Discussion

Type 2 diabetes is a global health challenge and the WHO has recommended research and use of complementary medicines for the management of this disease. Type 2 diabetes was previously considered as maturity-onset diabetes but, due to increasing rates of obesity, there is an increasing risk of developing this disease in childhood [46, 47].

In this study, flavonoid-rich fractions antioxidant activity was investigated using four assays which cover different aspects of antioxidant activity. Being a relatively stable free

Table 1: Effect of flavonoid-rich fractions of *Trichilia emetica* (FT) and *Opilia amentacea* (FO) on serum glucose level for acute study (single day study).

	0 h	1 h	3 h	5 h
Normal control	84.12 ± 1.31	85.2 ± 2.18	85.30 ± 1.10	85.10 ± 1.99
Diabetic control	177.1 ± 2.23	179.3 ± 1.30	183.5 ± 2.40	186.1 ± 2.10
Standard	174.1 ± 1.20	165.1 ± 2.21*	152.1 ± 1.40***	144.3 ± 3.20***
FT (100 mg/kg)	176.4 ± 3.08	174.2 ± 2.12	166.4 ± 2.63**	164.3 ± 2.39**
FT (300 mg/kg)	172.5 ± 1.50	177.2 ± 1.30	153.5 ± 1.54***	144.2 ± 2.40***
FO (100 mg/kg)	176.9 ± 1.1	178.1 ± 2.10	166.7 ± 1.33**	167.1 ± 2.37**
FO (300 mg/kg)	173.1 ± 0.10	180.2 ± 3.20	155.2 ± 1.24***	146.6 ± 2.71***

Values are mean ± SEM; $N = 6$. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ versus diabetic control.

Table 2: Effect of flavonoid-rich fractions of *Trichilia emetica* (FT) and *Opilia amentacea* (FO) on blood glucose level for subacute study (multiday study).

	0 day	1 day	3 day	5 day	7 day
Normal control	84.60 ± 1.10	85.68 ± 1.00	84.33 ± 2.10	84.57 ± 1.00	84.53 ± 1.52
Diabetic control	176.2 ± 3.30	183.2 ± 2.31	192.4 ± 2.10	198.2 ± 2.10	203.2 ± 2.04
Standard	174.1 ± 2.20	154.2 ± 1.37**	145.6 ± 2.03***	128.2 ± 2.53***	107.1 ± 1.10***
FT (100 mg/kg)	176.1 ± 1.20	170.1 ± 2.02*	164.2 ± 1.22**	160.4 ± 1.00**	156.6 ± 2.67**
FT (300 mg/kg)	172.5 ± 2.57	155.2 ± 1.42**	145.3 ± 1.10***	128.4 ± 2.17***	113.1 ± 3.53***
FO (100 mg/kg)	176.8 ± 3.10	174.3 ± 1.12*	167.5 ± 1.20**	163.0 ± 1.01**	158.2 ± 2.60**
FO (300 mg/kg)	173.1 ± 1.53	157.1 ± 1.10**	146.9 ± 2.23***	128.6 ± 1.10***	118.7 ± 1.51***

Values are mean ± SEM; $N = 6$. $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ versus diabetic control.

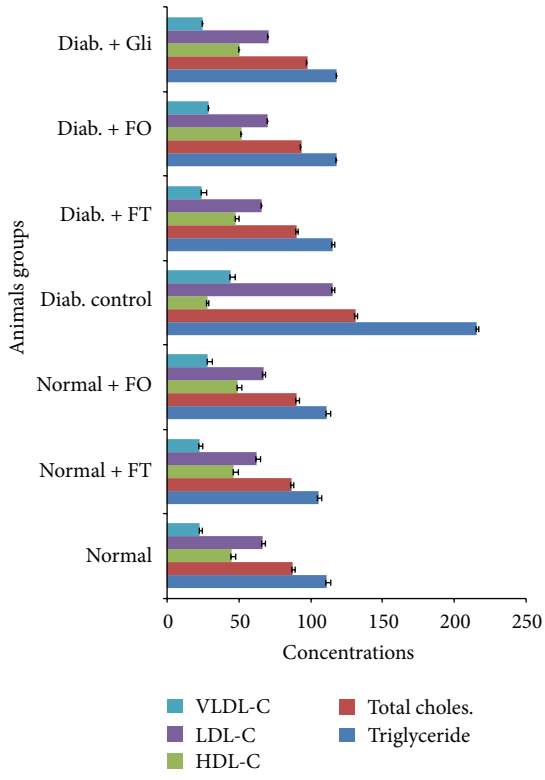

Figure 4: Effect of flavonoids from *Trichilia emetica* (FT) and *Opilia amentacea* (FO) on the concentrations of triglycerides, total cholesterol, HDL-C, LDL-C, and VLDL-C in serum of normal and experimental rats.

radical, DPPH$^{\bullet}$ is frequently used to determine radical-scavenging activity of natural compounds. DPPH assay estimates the ability of sample to scavenge free radicals, species capable of causing damage to natural macromolecules, such as nucleic acids, polysaccharides, and lipids [50]. We noticed that the antiradical activity of flavonoid-rich fractions from *Trichilia emetica* was more pronounced than the activity of the same fractions from *Opilia amentacea*.

In β-carotene-linoleic acid assay, the degradation of β-carotene occurs in reaction with linoleic acid free radical formed at elevated temperatures. Subsequent loss of conjugation leads to a decrease in absorbance at 470 nm. Antioxidants present in the solution can prevent the degradation of β-carotene by reacting with the linoleic acid free radical or any other radical formed in the solution [29]. Thus, in this assay, the capacity of antioxidants to prevent degradation of natural lipids, such as linoleic acid, is measured. The reducing power of a compound, on the other hand, is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. It estimates the ability of a substance, or an extract which contains it, to donate a proton and thus cause the transformation of free radicals into a less reactive species. In this study, also *Trichilia emetica* was a more successful inhibitor of β-carotene bleaching than *Opilia amentacea*. Among the biologically relevant ROS (H_2O_2, $O_2^{\bullet -}$, and $^{\bullet}OH$), hydroxyl radicals are the most reactive and dangerous species [51]. Free ferrous iron is sensitive to oxygen and gives rise to ferric iron and superoxide, thereby generating hydrogen peroxide. Thus formed hydrogen peroxide reacts with ferrous iron and

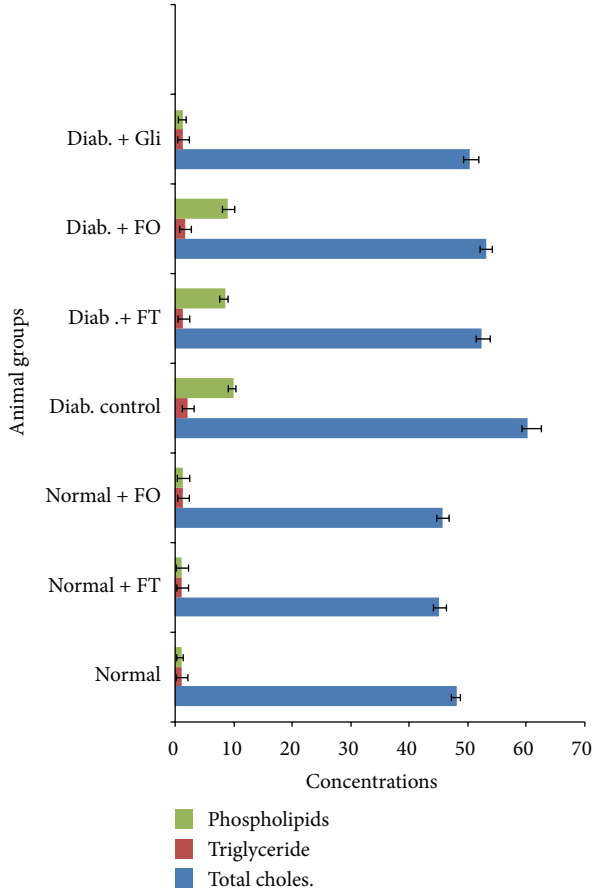

FIGURE 5: Effect of flavonoid-rich fractions from *Trichilia emetica* (FT) and *Opilia amentacea* (FO) on the concentrations of total cholesterol, triglycerides and phospholipids in the liver of normal and experimental rats.

generates the hydroxyl radical, which may subsequently oxidize surrounding biomolecules. The investigated flavonoid-rich fractions from *Opilia amentacea* constituents were incapable of chelating ferrous ions in this assay comparatively to the control. *Trichilia emetica* flavonoid-rich fractions, on the other hand demonstrated significant chelating ability. The differences in chemical compositions have probably caused such difference of ferrous ions. The activity of *Trichilia emetica* extracts could certainly be explained by phenolic compounds [27]. Some studies demonstrated that the extracts having higher phenol content also have higher DPPH radical-scavenging activity and other types of antioxidant activities [52–55]. We noticed that antioxidant activity of *Trichilia emetica* was more pronounced in most of the performed assays which leads to conclusion that besides polyphenols some other compounds may at least partly be responsible for the antioxidant activity of investigated extracts.

The inhibition of key enzyme linked to type 2 diabetes, such as α-amylase and α-glucosidase, has been considered to be an effective strategy to control blood glucose [56]. Agents based on natural products are particularly attractive as side effects are minimal and the therapies are well tolerated compared to the other oral hypoglycemic agents currently available [35, 57]. We noticed that extract with flavonoid-rich fractions from *Trichilia emetica* possessed marked antiamylase activity which may be due to the presence of certain secondary metabolites. In effect, flavonoids, alkaloids, and triterpenoids may be related to the antidiabetic activity of plants. In particular, flavonoids are responsible for variety of pharmacological activities [55]. For example, epicatechin is known to possess insulin-like properties, while epigallocatechin gallate is considered a promising hypoglycemic agent [58]. As shown in previous studies, the enzyme inhibition activity may be related to the polyphenolic content of the plant extract; however, further studies are needed to confirm this.

About antihypertensive property, it is well known that, in recent years, the treatment of hypertension has achieved a breakthrough with the identification of ACE inhibitors as a modern therapeutic tool and considerable interest has focused on the action of ACE inhibitors. ACE inhibitors have potentially improved endothelial function; they are known to increase the plasma concentration of bradykinin, an endothelium-dependent vasodilator, by inhibiting degradation of the peptide [9]. Procyanidins and flavonoids are the major natural products isolated from ethnopharmacologically important plants against *in vitro* ACE inhibitory activity [59, 60]. In addition, other active compounds isolated from medicinal plants include phenylpropanes, xanthones, fatty acids, terpenoids, alkaloids, and peptide amino acids [61]. In the present study, flavonoid-rich fractions from *Trichilia emetica* have shown the best antihypertensive potential compared to the control. This may be connected with capacities of some secondary metabolites like flavonoids.

Nowadays are antihypertensive and antidiabetic effects of these plants known which makes popular a traditional medicine in the developing countries. Medicinal plants are often believed to be harmless because they are natural and commonly used for self-medication without supervision. This increase in popularity and the scarcity of scientific studies on their safety and efficacy have raised concerns regarding toxicity and adverse effects of these remedies [62]. These products of plants contain bioactive principles with the potential to cause adverse effects [63]. The results of the present study indicated that the aqueous acetone extracts of *Trichilia emetica* and *Opilia amentacea* are not toxic. During 14-day period of acute toxicity evaluation some signs of toxicity have been observed, but they were all quickly reversible. According to [64, 65], pharmacological substances where whole LD_{50} less than 5 mg/kg body weight are classified in the range of highly toxic substances, those with LD_{50} between 5 mg/kg body weight and 5000 mg/kg body weight are classified in the range of moderately toxic substances, and those with lethal dose more than 5000 mg/kg body weight not toxic. In this fact, if we refer to this classification we would say that the extracts of *Trichilia emetica* and *Opilia amentacea* are moderately toxic and would be regarded as being safe or have low toxicity.

Traditional plant remedies have been used for centuries in the treatment of diabetes mellitus [66], but only a few have been scientifically evaluated. Alloxan is known for its selective pancreatic islet β cell cytotoxicity and has been

extensively used to induce diabetes mellitus in animals [67]. Generalized increase in the level of blood glucose during diabetes has been consistently reported both in animal models [68] and humans especially those suffering from insulin dependent diabetes mellitus [69]. In this study, increase in blood glucose level was observed on induction of diabetes mellitus in the rats models, which has been reduced in a dose dependent manner with the highest percentage reduction at 300 mg/kg (Tables 1 and 2). At 3rd hours of exposure in variant with dose of 100 mg/kg did not found any significant antidiabetic activity. The flavonoid-rich fractions at the dose of 500 mg/kg show very significant antidiabetic activity from the first day to seventh day; the dose of 100 mg/kg shows significant antidiabetic activity from the 5th day to 7th day. Our study showed that it is possible that extracts may act by undetermined ways apart from stimulating insulin production from the pancreatic islets since these would have been severely damaged by alloxan. The mechanism of the hypoglycaemic effects of flavonoid-rich fractions remains speculative; therefore further studies are required to unravel the pathway of its hypoglycaemic action and to shed more light on the hypoglycaemic constituents of the plants. It is, however, evident that flavonoid-rich fractions contain hypoglycaemic agents capable of lowering blood glucose level in the alloxan diabetic rats (Tables 1 and 2).

It is well known that the prevalence of hyperlipidaemia among diabetics is increasing worldwide. Alteration in serum lipids profile is known in diabetes, which are likely to increase the risk of coronary heart disease [70]. Lipid profile which is altered in serum of diabetic patients [71] appeared to be a significant factor in the development of premature atherosclerosis through increase in serum triglyceride and total cholesterol levels. The significant reduction in serum cholesterol and total lipids in a dose dependent manner as observed in this experimental work has been confirmed with results of previous reports [72]. The marked hyperlipidaemia that characterizes the diabetic state may be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depots [73]. A reduction in lipid profile could be beneficial in preventing diabetic complications as well as improving lipid metabolism in diabetics [74].

Considering flavonoid-rich fractions effects on lipid components [75], it can be assumed a potential hypolipidaemic agent which will be a great advantage both in diabetic conditions as well as in the associated hyperlipidaemic conditions. In effect, the use of synthetic drugs for management of diabetes mellitus has certain adverse effects and therefore there is a need to develop safer and more effective antidiabetic drugs. Consequently, treatment with drugs isolated from plants has an effect on shielding β cells and leveling the oscillation in glucose levels [76]. Elevated serum or tissue lipids and lipoproteins are characteristics of uncontrolled diabetes. Type 2 diabetes mellitus is commonly associated with dyslipidemia which is a significant risk factor for the development of cardiovascular diseases [77]. This is in support with results of our present study. In the present, a marked increase in the lipid content of serum and liver was found in STZ induced diabetic rats which is mainly due to the increased mobilization of free fatty acids (FFAs) from peripheral depots [78]. Interestingly, most of the studies with different plant extracts in diabetic rats were supportive of our results [79]. The rise in serum triacylglycerols, cholesterol, and LDL-cholesterol levels in the present study indicates derangement of lipid metabolism and amplified incidence of cardiac dysfunction in diabetic rats. Rise in serum lipids indicates either the defective overproduction or removal (or both) of one or more lipoproteins [80]. An oral administration of flavonoid-rich fractions for a period of 28 days restored the altered levels of lipids (triglycerides, total cholesterol, and phospholipids) in liver tissue as well as in serum. The decreased levels, that is, restoration levels, of cholesterols and triglycerides are due to the presence of glycosides in the flavonoid-rich fractions. The elevated concentrations of cholesterol can enhance the risk of oxidative disease process due to susceptibility of cholesterol to oxidation while it is in circulation. Insulin deficiency or insulin resistance may be responsible for dyslipidemia, because insulin has an inhibitory action on HMG-coA reductase, a key rate-limiting enzyme which is responsible for the LDL particle metabolism with cholesterol-rich content [81]. A shortage of insulin is associated with rise in cholesterol levels due to the increased mobilization of lipids from the adipose tissue to the plasma. The enlarged concentration of FFAs in liver may be due to lipid catalysis which leads to enhanced generation of NADPH and activation of NADPH dependent microsomal lipid peroxidation [82]. In addition, phospholipids are vital components of biomembranes and play an essential role in the triglycerides transport [83]. In diabetic rats, the gigantic levels of PLs may be due to the high levels of FFAs and total cholesterol [84].

5. General Remarks and Conclusion

The present study assesses the antidiabetic potential of flavonoid-rich fractions from *Trichilia emetica* and *Opilia amentacea*. These plants can be promising agents with good antioxidant activity for the management of hyperglycemia and can be used also in the antihypertensive therapy. However, these results require further investigation to better understand biochemical nature of these effects as they may provide leads for the discovery of new drugs for the management of diabetes type 2 with minimal side effects. Further studies are in progress with isolating these active compounds which are responsible for the antidiabetic and antihypertensive properties.

Conflict of Interests

The authors declare no financial conflict of interests.

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Research Article

Ameliorative Effect of Hexane Extract of *Phalaris canariensis* on High Fat Diet-Induced Obese and Streptozotocin-Induced Diabetic Mice

Rosa Martha Perez Gutierrez,[1] Diana Madrigales Ahuatzi,[2] Maria del Carmen Horcacitas,[3] Efren Garcia Baez,[4] Teresa Cruz Victoria,[2] and Jose Maria Mota-Flores[1]

[1] *Laboratorio de Investigación de Productos Naturales, Escuela Superior de Ingenieria Quimica e Industrias Extractivas, Instituto Politecnico Nacional, Avenida Instituto Politécnico Nacional S/N, Unidad Profesional Adolfo Lopez Mateos, 07708 Mexico D.F., Mexico*
[2] *Departamento de Alimentos, Escuela Nacional de Ciencias Biologicas, IPN Carpio S/N, 11340 Mexico D.F., Mexico*
[3] *Departamento de Biotecnología y Bioingenieria, CINVESTAV, Avenida IPN S/N, 07360 Mexico D.F., Mexico*
[4] *Departamento de Ciencias, Basicas Unidad Profesional de Biotecnologia IPN, Avenida Acueducto S/N, 07340 Mexico D.F., Mexico*

Correspondence should be addressed to Rosa Martha Perez Gutierrez; rmpg@prodigy.net.mx

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Obesity is one of the major factors to increase various disorders like diabetes. The present paper emphasizes study related to the antiobesity effect of *Phalaris canariensis* seeds hexane extract (Al-H) in high-fat diet- (HFD-) induced obese CD1 mice and in streptozotocin-induced mild diabetic (MD) and severely diabetic (SD) mice.AL-H was orally administered to MD and SD mice at a dose of 400 mg/kg once a day for 30 days, and a set of biochemical parameters were studied: glucose, cholesterol, triglycerides, lipid peroxidation, liver and muscle glycogen, ALP, SGOT, SGPT, glucose-6-phosphatase, glucokinase, hexokinase, SOD, CAT, GSH, GPX activities, and the effect on insulin level. HS-H significantly reduced the intake of food and water and body weight loss as well as levels of blood glucose, serum cholesterol, triglyceride, lipoprotein, oxidative stress, showed a protective hepatic effect, and increased HDL-cholesterol, serum insulin in diabetic mice. The mice fed on the high-fat diet and treated with AL-H showed inhibitory activity on the lipid metabolism decreasing body weight and weight of the liver and visceral adipose tissues and cholesterol and triglycerides in the liver. We conclude that AL-H can efficiently reduce serum glucose and inhibit insulin resistance, lipid abnormalities, and oxidative stress in MD and SD mice. Our results demonstrate an antiobesity effect reducing lipid droplet accumulation in the liver, indicating that its therapeutic properties may be due to the interaction plant components soluble in the hexane extract, with any of the multiple targets involved in obesity and diabetes pathogenesis.

1. Introduction

Obesity is a metabolic disease of pandemic proportions largely arising from positive energy balance, a consequence of sedentary life style, conditioned by environmental and genetic factors [1]. Obesity results from an imbalance between energy intake and expenditure. It is often associated with chronic diseases such as hyperlipidemia, hvpertension and noninsulin- dependent diabetes mellitus and with increased risk of coronary heart diseases [2]. It has been reported that variations in total energy intake and diet composition are important in the regulation of metabolic processes [3]. Excessive accumulation of lipids in nonadipose tissues such as liver, heart, skeletal muscle, kidney, and pancreas contributes to the pathogenesis of fatty liver, heart failure, and insulin resistance with the so-called lipotoxicity mechanism, fatty liver is an early hallmark of nonalcoholic fatty liver disease [4], the most common chronic liver disease associated with insulin resistance, obesity and type 2 diabetes [5].

Adipose tissue, besides its primary function of fat storage, also regulates appetite and metabolism by secretion of certain chemicals like an endocrine gland [6]. Thus, persistent insulin

resistance and hyperglycemia lead to the development of diabetes mellitus type 2 (T2DM) [7]. Furthermore, the metabolic syndrome develops atherosclerotic diseases, whose fatality is very high, when the symptom gets worse [8]. It is therefore important to prevent or abate obesity. A growing number of enzymes involved in lipid metabolic pathways are being identified and characterized. They represent a rich pool of potential therapeutic targets for obesity [9]. Triglyceride (TG) metabolism is regulated by several factors such as TG intake from food and synthesis and oxidation in various tissues. Among these factors, the hydrolysis of TG by lipoprotein lipase to free fatty acids is an important determinant in TG metabolism [10]. Canary seed is solely used as food for caged and wild birds. However, the unique composition and characteristics of canary seed make it a promising cereal for food and industrial uses [11]. In the past, canary seed was not seen as a viable cereal for human consumption due to the harmful effects associated with the siliceous hairs that cover the hull of the seed. These hairs are highly irritating when they come in contact with human skin or lungs and have been linked to esophageal cancer [12]. In 1997 CDC Maria was registered in Canada as the first hairless cultivar eliminating the potential health risk associated with hairy varieties [13]. The variety was developed based on mutagenesis and traditional breeding by which a totally hairless variety was developed. Removing the damaging hairs rediscovered canary seed as a potential food crop and industrial crop for fractionation industry.

The canary seed or alpiste, *Phalaris canariensis* L., is a member of a family of grasses (Graminaceae), and it is used in folk medicine in the form of tea as a coadjuvant in the treatment of hypertension, diabetes mellitus, and hypercholesterolemia [14], with or without other forms of traditional therapy [15]; however, such use has no scientific basis. There is only one study related to the hypotensive effect of *P. canariensis* seed infusion in normotensive rats [16]; however, its therapeutic use as an antihypertensive agent and its possible mechanisms of action have not been scientifically demonstrated. The aim of the present study was to establish antiobesity and antidiabetic activities of the seeds of *Phalaris canariensis*.

2. Materials and Methods

2.1. Plant Material. Seeds of *P. canariensis* were collected in Morelos State, Mexico. A voucher specimen (number 8054) was deposited in the Herbarium of the National School of Biological Sciences, for further reference.

2.2. Preparation of Plant Extracts. A total of 1000 g of the seeds of *P. canariensis* were dried and powdered in a mechanical grinder. The grinded material was extracted with 5 L of hexane, chloroform, and methanol consecutively using a soxhlet apparatus. These extracts were filtered and concentrated by a rotary vacuum evaporator and kept in a vacuum desiccator for complete solvent removal.

2.3. Animals. The study was conducted in CD1 mice of both sexes, weighing about 25–30 g. Before and during the experiment, animals were fed a standard laboratory diet (Mouse Chow 5015, Purina) with free access to water. Mice were procured from the bioterium of ENCB and were housed in microloan boxes in a controlled environment (temperature 25 ± 2°C). Animals were acclimatized for a period of three days in their new environment before the initiation of experiment. Litter in cages was renewed three times a week to ensure hygiene and maximum comfort for animals. The experiments reported in this study were carried following the guidelines stated in Principles of Laboratory Animal Care National Institute of Health publication (NIH) publication 85-23, revised 1985 and the Mexican Official Normativity (NOM-062-Z00-1999). All animals procedures were performed in accordance with the recommendations for the care and use of laboratory animals (756/lab/ENCB).

2.4. Mouse Model of Diet-Induced Obesity. Thirty-five male CD1 mice, 2 months old and weighing between 20 and 25 g, were acclimatized under room temperature (28 ± 2°C) with a regular light/dark cycle and free access to food and water for 1 week before use. Following acclimatization, the animals were randomly segregated into five groups of seven rats each. We formulated experimental high-fat diets consisting of 50% normal rat chow pellet, 24% corn oil, 20% full-cream milk powder, and 6% sugar, as suggested by Martinello et al. [17]. Another group, noted as normal control (NC), was given normal rat chow. After the induction period, the mean body weights of the high-fat diet-treated groups were compared with the NC group. Groups with significantly higher mean body weights than the NC group were considered to be obese and were used in the subsequent 10 weeks of experimentation with *P. canariensis* extracts. Each extract at dose 400 mg/kg was prepared by dissolving in 1% of Tween 80. The supplementations were given daily via oral gavage for 10 weeks, at a volume of 0.1 mL. The NC and obese control (OC) groups were given distilled water as a placebo.

2.5. Supplementation of P. canariensis. Treatment with extracts began after 10 weeks of obesity induction and scored until week 10. Two different doses of *P. canariensis* were used in the experiment: 200 and 400 mg/kg. The extracts were prepared by dissolving in 1% of Tween 80. The supplementations were given daily via oral gavage for 10 weeks, at a volume of 0.1 mL. The NC and obese control (OC) groups were given distilled water as a placebo. Contrary from the induction period, all groups were given normal rat chow throughout the treatment period.

2.6. Induction of Severe Diabetes (SD). Severe diabetes was induced in overnight fasting male mice by a single intraperitoneal injection of 50 mg/kg of streptozotocin in a volume 1 ml/kg body weight dissolved in cold citrate buffer (pH 4.5) [18]. Hyperglycemia was confirmed by measuring glucose 72 h after the streptozotocin shot and 7 days after injection, confirming a high glucose level. Mice with permanent high

fasting blood glucose level >300 mg/dl were included in the experiments.

2.7. Induction of Mild Diabetes (MD). Mild diabetes was induced in overnight fasting mice by administering a single intraperitoneal injection of 60 mg/kg *b.w.* STZ in 0.1 mol/L cold citrate buffer (pH 4.5), 15 min after the intraperitoneal administration of 120 mg/kg nicotinamide. The STZ treated animals were allowed to drink 5% glucose solution overnight to overcome drug induced hypoglycemia. After 10 days of development of diabetes, mice with moderate diabetes having persistent glycosuria and hyperglycaemia (blood glucose > 250 mg/dl) were used for further experimentation [19].

2.8. Experimental Design in Diabetic Mice

2.8.1. Effect of Single Oral Administration of Extracts of P. canariensis in Glucose Level in Severe and Mild Diabetic Mice. After the mice had been denied access to food/water overnight, they were randomly divided into eight groups (six mice per group) matched for body weight. Normal mice administered distilled water daily for 30 days. Diabetic control mice administered distilled water daily for 30 days. Diabetic mice administered extract of hexane (100 mg/kg), for 30 days. Diabetic mice administered extract of hexane (200 mg/kg), for 30 days. Diabetic mice administered extract of hexane (400 mg/kg), for 30 days. The other diabetic groups were orally administered 100, 200 and 400 mg/kg body weight (b.w.) of extracts of chloroform (AL-C) and methanol (AL-M) suspended in Tween 80, 1% via gavage). Diabetic treated mice received glibenclamide (GB) a dose of 5 mg/kg b.w as standard drug. Blood samples were collected from the tail vein at 0, 2, 4, 6, 8, and 12 h after the administration. The plasma glucose concentration was determined by an enzymatic colorimetric method using a commercial kit (Sigma Aldrich, USA).

2.8.2. Antidiabetic Test in Chronic Severe and Mild Streptozotocin-Induced Diabetic Mice. In a parallel study eleven groups ($n = 10$) of diabetic mice were used to determine the chronic effect of AL-H extract. Each group was submitted to a specific treatment as follows. Normal control and severe and mild diabetic mice groups were fed with normal diet and drinking water *ad libitum* and were given saline by gastric gavage. Severe and mild diabetic mice that received Al-H extract by gastric gavage (400 mg per kg of body weight) every day [7] were designated as SD + Al-H and MD + AL-H groups. Two groups with severe (SD+GB) and mild diabetes (MD+GB) mice were administered with glibenclamide (GB) 4 mg/kg as positive control.

2.8.3. Determination of Body Weight and Food Intake. The final body weight showed significant increase from the initial body weight in all the groups except in the diabetic group, in which there was significant decrease in body weight compared to the initial body weight (Table 5).

The body weight of each mouse was measured once each week and the total amount of food consumed was recorded 3 times per week.

2.8.4. Collection of Organ Tissues. At the end of obesity and chronic diabetes experiments all mice were anesthetized with 1.0% pentobarbital sodium and blood was obtained from the retroorbital plexus of each animal following the injection of heparin (100 IU kg^{-1} body weight) into a tail vein for 10 min [20]. The liver and kidney were removed according to defined anatomical landmarks [21].

2.8.5. Plasma Biochemical Analysis. Blood samples were collected from tail vein of the mice into microcentrifuge tubes containing heparin (10 μL, 1000 IU mL^{-1}). The blood samples were then centrifuged at 1600 ×g for 15 min at 4°C for the preparation of plasma. Concentrations of plasma glucose, total cholesterol (TC), triglycerides (TG), and HDL-cholesterol were measured with enzymatic assay kit (Genzyme Diagnostics), and LDL-cholesterol was calculated as the remaining difference of total cholesterol and HDL. Blood glucose levels were measured employing the glucose oxidase-peroxidase (GOD-POD) method [22]. Lipid peroxidation, that is, thiobarbituric acid reactive substances (TBARS), was estimated by the method of Fraga et al. [23] and expressed as μM/g of liver and kidney tissue. Serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), and total protein, using a commercial Diagnostic Kit Biocompare**,** BioVision, Biocompare and Thermo scientific, respectively. Malondialdehyde (MDA) as thiobarbituric acid reactive substances was measured at 532 nm spectrophotometrically [24].

2.8.6. Oral Glucose Tolerance Test. Mice of each group were orally administered *P. canariensis* aqueous extracts at doses of 400 mg/kg body weight on a daily basis for 30 days. At the end of the experiment, an oral glucose tolerance test (OGTT) was performed to assess the animals' sensitivity to a high glucose load. Overnight fasting mice were fed orally 2 g glucose/kg b.w. Blood samples were collected from the caudal vein from a small incision at the end of the tail at 0 min (immediately after glucose load), 30, 60, 90, and 120 min after glucose administration.

2.8.7. Assay of Glycogen Content in Liver and Skeletal Muscle. Mice were sacrificed by decapitation; livers and kidneys were extracted. Frozen livers were thawed, weighed, and homogenized in Tris-HCl (5 mmol/L, pH 7.4) buffer containing 2 mmol/L EDTA. Homogenates were centrifuged at 1000 ×g for 15 min at 4°C. The supernatant was mixed with glucose-6-phosphate dehydrogenase and NADPH and the activity of hexokinase was determined at 37°C for 3 min at 30 s intervals at 340 nm [25] The hepatic glycogen content was measured according to the anthrone-H_2SO_4 method [26]. Briefly, liver tissue (64–144 mg) was homogenized in five volumes of an ice-cold 30% KOH solution and the homogenate was placed in a boiling water bath (100°C) for 20 min. The glycogen was redissolved in distilled water

and treated with an anthrone reagent (2 g anthrone/L of 95% (v/v) H_2SO_4) and the absorbance was measured at 620 nm.

2.8.8. Assay of G6Pase Activity in Liver. The hepatic G6Pase (glucose-6-phosphatase) activity was assayed by the method of Baginiski et al. [27]. Shortly, the glucose-6-phosphate in the liver extract was converted into glucose and inorganic phosphate. The inorganic phosphate liberated was determined with ammonium molybdate; ascorbic acid was used as the reducing agent. Excess molybdate was removed by the arsenite-citrate reagent, so that it could no longer react with other phosphate esters or with inorganic phosphate formed by acid hydrolysis of the substrate. The amount of phosphate liberated per time unit, determined as the blue phosphormolybdous complex at 700 nm, was a measure of the glucose-6-phosphatase activity. The protein content of the liver extract was quantified by Bradford reaction (Bio-Rad protein assay kit) [28]. The G6Pase activity (mU) was expressed as mmol of phosphate released/min/mg of protein.

2.8.9. Assay GK Activity in Liver. Glucokinase (GK) activity was measured using a spectrophotometric method as previously described by Panserat et al. [29]. Briefly, liver tissues were homogenized and the supernatant obtained by centrifugation was supplemented with 1 mM NADP, 5 mM ATP, and 100 or 0.5 mM glucose at pH 7.5. The enzymatic reaction was started by the addition of 0.2 unit of glucose-6-phosphate dehydrogenase (E.C.l.l.l.49) and incubated for 5 min at 37°C. NADPH generated by GK was measured using a spectrophotometer at 340 nm. GK activity was estimated by the standard method, that is, subtracting the rate of NADPH formation in the presence of 0.5 mM glucose from that obtained in the presence of 100 mM glucose [30]. Protein concentration was quantified by Bradford and one unit of enzyme activity (mU) was defined as mmol of substrate molecules converted by 1 mg protein per minute. GK activity was estimated as the difference in activity when samples were assayed at 100 mmol/L (GK plus hexokinase activity) and 0.5 mmol/L glucose (hexokinase activity).

2.8.10. Measurement of Antioxidant and Lipid Peroxidation Parameters. After 30 days of treatment, mice fasted overnight and euthanized by anesthesia. Antioxidant enzyme activities in the liver, pancreas, and kidney were assayed using commercial kits: superoxide dismutase (SOD) assay kit Bioxytech SOD-525 for SOD activity (Oxis International), catalase assay kit for catalase activity (CAT) (Cayman Chemical), glutathione reductase (GSH) assay kit Bioxytech GR-340 for GR activity (Oxis International), glutathione peroxidase (GPx) assay kit GPx-340 for GPx (Oxis International), and lipid peroxidation using malondialdehyde level by commercial kit (TBARS assay kit). In the pancreas the protein concentration was determined by the Bradford method as described in the Bio-Rad protein assay kit.

2.8.11. Extraction of Hepatic Lipids. After removal from the animals, parts of the samples of fresh liver were collected for analyzing the lipid content. The liver (1.25 g) was homogenized with chloroform/methanol (1 : 2, 3.75 mL), and then chloroform (1.25 mL) and distilled water (1.25 mL) were added to the homogenate and mixed well. After centrifugation (1500 ×g for 10 min), the lower clear organic phase solution was transferred into a new glass tube and then lyophilized. The lyophilized powder was dissolved in chloroform/methanol (1 : 2) as the hepatic lipid extracts and stored at −20°C for less than 3 days [31]. The hepatic cholesterol and triglycerides in the lipid extracts were analyzed with the diagnostic kits used for the plasma analysis.

2.8.12. Determination of Insulin. Diabetic mice serum and pancreatic insulin were measured by a Glazyme Insulin-EIA Test according to the manufacturer's instructions [32]. Blood samples and pancreas were taken for insulin determination. The level of insulin in serum was expressed in μIU/mL.

2.8.13. Statistical Analysis. Statistical analysis was carried out by using commercially available software SigmaStat 3.5. Values are expressed as mean ± SEM. For multiple comparisons, one-way ANOVA was used followed by Tuckey and Dunnett's test. P value < 0.05 was considered to be significant.

3. Results

Treatment with chloroform and methanol extracts did not significantly inhibit the rise in blood glucose levels in normal and diabetic mice and in oral glucose tolerance. However, mice fed the high-fat diet treated with hexane, chloroform, and methanol extracts showed a decrease similarly; for this reason it was decided to study only hexane extract due to the fact that it presents hypoglycemic and antiobesity activities. However, chloroform and methanol extracts did not.

3.1. Effects of the High-Fat Diet (HFD) on Body Weight and Food Intake. The body weights and food intake are shown in Table 1. No significant difference in food intake was observed throughout the obesity induction period in all groups. Body weight of all groups was significantly increased compared with the control group after 10 weeks of feeding.

3.2. Effect of P. canariensis Extracts on the Weight of Organs and Adipose Tissue. The mice fed on the high-fat diet for 10 weeks had a significantly higher body weight and significantly heavier visceral adipose tissues than the mice fed on the normal diet. In the mice fed on the high-fat diet, the body weight elevation that took place over the initial 6 weeks on the high-fat diet was significantly reduced (Table 2) and the final weights of the visceral adipose tissues were significantly lower than those in the mice fed on the high-fat diet alone. The body weight reduction was proportionally equal to the reduction in the visceral adipose tissue weight after 10 weeks. The weights of organs and adipose tissue of normal and obese mice are shown in Table 3. The high-fat diet led to significant increases in the weights of the visceral adipose tissues, kidney, and liver compared to the weights measured in the normal diet group. Extracts with high-fat diet significantly reduced the final

Table 1: Body weight and food intake of mice during the obesity induction period.

Group (g)	NC	OC	Group 1	Group 2	Group 3
Body weight					
w0	23 ± 0.7	23.2 ± 1.2	22.5 ± 0.5	23.1 ± 0.7	23.5 ± 0.3[a]
w10	30.7 ± 1.5	40.5 ± 1.3[a]	42.3 ± 4.8[a]	41.5 ± 2.8[a]	43.6 ± 3.3
Body weight gain					
w0–10	7.72 ± 2.5	17.52 ± 3.5[a]	19.38 ± 5.4[a]	18.5 ± 2.8[a]	20.6 ± 2.2[a]
Food intake (g/rat/day)					
w0–10	8.9 ± 1.2	10.1 ± 2.1[a]	11.3 ± 1.5[a]	11.6 ± 0.8[a]	12.2 ± 2.3[a]

Each value represents mean ± S.E.M. ($n = 10$ animals in each group). Values for a given parameter in a row that do not share a common superscript are significantly different at [a]$P < 0.05$. W (week); NC: normal control with low-fat diet; OC: obese control with high-fat diet.

Table 2: Effect of *P. canariensis* extracts on changes in body weight in mice.

Group	Treatment	Body weight changes			
		0 weeks	4 weeks	6 weeks	10 weeks
I	NC	30.7 ± 1.5	30.8 ± 0.5	31.0 ± 1.2	31.2 ± 2.4
II	OC	40.5 ± 1.3	41.0 ± 3.5	41.5 ± 5.0	42.0 ± 5.4
III	OC + AL-H	42.3 ± 4.8	40.7 ± 2.1	38.5 ± 3.4[a]	37.7 ± 4.1[a]
IV	OC + AL-C	41.5 ± 2.8	37.9 ± 3.1	36.8 ± 2.5[a]	35.2 ± 3.0[a]
V	OC + AL-M	43.6 ± 3.3[a]	39.1 ± 2.9	37.7 ± 4.5[a]	36.4 ± 1.5[a]

Each value represents mean ± S.E.M. ($n = 10$ animals in each group). [a]$P < 0.05$. NC: normal control with low-fat diet and OC: obese control with high-fat diet. Hexane extract (AL-H), chloroform extract (AL-C), and methanol extract (AL-M) from *P. canariensis*.

Table 3: Effect of different extracts of *P. canariensis* on the weights of organs and adipose tissue of normal and obese mice.

Group	LFD	HFD	AL-H	HFD + AL-C	AL-M
Liver (g/100 g body weight)	5.58 ± 0.95	5.69 ± 0.45	5.56 ± 1.06	5.57 ± 1.23	5.54 ± 1.30
Kidney (g/100 g body weight)	1.61 ± 0.02	1.47 ± 0.08	1.51 ± 0.05	1.53 ± 0.04	1.50 ± 0.02
Plasma total (U mL^{-1})	1.13 ± 0.07	1.58 ± 0.05	1.16 ± 0.03[a]	1.14 ± 0.06[a]	1.19 ± 0.04[a]
Muscle (U mg^{-1})	1.11 ± 0.01	0.76 ± 0.07	0.96 ± 0.04[a]	0.99 ± 0.05[a]	0.98 ± 0.08[a]
VAT (U mg^{-1})	0.76 ± 0.07	1.21 ± 0.06	0.78 ± 0.09[a]	0.75 ± 0.08[a]	0.77 ± 0.03[a]
SAT (U mg^{-1})	1.12 ± 0.05	1.32 ± 0.02	1.17 ± 0.09[a]	1.14 ± 0.06[a]	1.15 ± 0.01[a]

Each value represents mean ± S.E.M. ($n = 10$ animals in each group). LFD: low-fat diet; HFD: high-fat diet; hexane extract (AL-H), chloroform extract (AL-C), and methanol extract (AL-M) from *P. canariensis;* Postheparin plasma (plasma); hind limb muscle (muscle); visceral adipose tissue; the visible mesenteric fat and fat around the liver, kidney, and spleen (VAT); subcutaneous adipose tissue subcutaneous adipose tissue in the abdomen (SAT). [a]$P < 0.05$ versus control group.

weights of the liver and adipose tissues compared to the levels measured in the animals fed on the high-fat diet alone. The kidney weight was decreased in the HFD group compared with the LFD group. The HDF caused the elevation of plasma total. The moderate doses (200 mg/kg per day) of hexane, chloroform, and methanol extracts significantly decreased the level of plasma total (26%, 28%, and 25% resp.). The VAT and liver weight were significantly lower with extracts groups compared with the control mice at 10 weeks, while in SAT weight between the groups of mice at the end point of the experimental period showed decrease of 11%, 14%, and 13%, respectively (Table 3).

3.3. Effect of the P. canariensis Extracts on Lipid Profile and Hepatic Lipid. Table 4 shows the plasma cholesterol, level of the experimental animals. Significant increase in the levels of plasma cholesterol was observed in the HFD fed groups for the initial ten weeks. After treatment the plasma cholesterol was significantly decreased in the treatment to 39% (AL-H) as compared to cholesterol level in HFD-fed control groups. The HFD-fed rats receiving 10 weeks of treatment with *P. canariensis* extracts (200 mg/kg per day) also showed significantly lower values of plasma TG 35%, 44%, and 32% for hexane, chloroform, and methanol extract, respectively, compared with the vehicle-treated counterparts (Table 4). The moderate doses (200 mg/kg per day) of *P. canariensis* extracts of hexane, chloroform, and methanol increased the level of plasma HDL (24%, 27%, and 21% increase resp.). The HFD caused elevation of hepatic TC and TG. All *P. canariensis* extracts decreased the level of hepatic TC (25%, 29%, and 24% reduction, resp.). The moderate doses of *P. canariensis* extracts significantly reduced the TG hepatic (35%, 37%, and 33% reduction, resp.). On the one hand,

there were no significant differences in lipid profile and hepatic lipid in all groups. The hepatic tryglicerides levels in the normal group, high-fat diet group, and high-fat + *P. canariensis* extracts (AL-H, AL-C, AL-M) diet group were 86.2 ± 6.7, 84.5 ± 5.8, and 87.3 ± 2.0 mg/g liver, respectively. In addition, plasma tryglicerides levels tended to be reduced in the high-fat diet group because of a decrease in triacylglycerol secretion from the liver.

3.4. Lipid Peroxidation and Protein Estimation. MDA, which is the final product of lipid peroxidation, was determined spectrophotometrically. HFD increased the MDA level in plasma when compared with that of the normal group. HFD group treatment with AL extracts showed a significant reduction in MDA levels (Table 4).

3.5. Effect of P. canariensis Extracts on the Activities of Hepatic Enzymes. As shown in Table 4, AL-H exhibits a hepatoprotective effect *in vivo*, indicated with reduced ALP, SGOT, and SGPT levels. It was found that mice fed with high-fat diet alone developed a high degree of steatosis. The effect of increased liver enzymes levels and the formation of steatosis in the high-fat diet-fed group correlates with an increase of liver weight (Table 3). The administration of AL-H resulted in the prevention of hepatic fatty deposition in hepatocytes at dose of 400 mg/kg.

3.6. Effect on Diabetic Mice of the Hexane Extract from P. canariensis on Body Weight and Food and Water Intake. During the study period of 4 weeks, body weight and food and water intake of each mouse were recorded daily but data is presented only at day 0 and the end of the experimentation period (Table 5). The body weight, liver and kidney weights of mice from STZ control group (after 15 days) were significantly ($P < 0.001$) decreased when compared with normal control group. The extract at dose of 400 mg/kg b.w. significantly ($P < 0.001$) maintained the body weight and liver and kidney weights toward normal as compared with STZ control.

3.7. Serum Biochemical Parameters. Biochemical parameters like SGOT, SGPT, SALP, and proteins in the STZ control group were significantly ($P < 0.001$) elevated as compared with the normal control group. Treatment with AL at the dose of 400 mg/kg b.w. significantly ($P < 0.001$) brought the SGOT, SGPT, SALP, and serum protein toward the normal values. The total protein was found to be significantly ($P < 0.001$) decreased in the STZ controls groups as compared with the normal control group indicating a lower capacity for protein synthesis in the livers of the diabetic animals; the administration of hexane extract in types SD and MD diabetic animals significantly ($P < 0.001$) prevented the loss of total protein content as compared with the STZ control group (Table 6).

3.8. Effect of P. canariensis Extracts on the Serum Lipid Profile and Hepatic and Renal TBARS. There was a significant elevation in serum triglycerides and total cholesterol and TBARS levels in the liver and kidney of diabetic mice while LDL-cholesterol decreased (Table 7). Daily administration of *P. canariensis* extracts at a dose of 400 mg/kg to diabetic mice for 30 days significantly reduced in severe and mild diabetes total cholesterol and triglycerides by 33%, 45% and 45%, 37%, respectively. TBARS level in diabetic mice also decreased after treatment with the extract, 22.0% and 37% in liver and 28% and 29% in kidney. LDL-cholesterol on the other hand upon treatment also got a decrease. The results also demonstrate a significant control of serum lipid profiles in the *P. canariensis* hexane extract treated diabetic mice, with responses comparable with those of the standard drug.

3.9. Effect of P. canariensis Extracts on the Fasting Blood Glucose Levels in Normal and Diabetic Mice. The oral administration of AL at doses of 100, 200 mg/kg, and 400 mg/kg produced a significant hypoglycaemic effect in normal fasting mice after 4 h. The most pronounced effect of AL was observed after 6 h (Table 8). The dose at 200 mg/kg reduced the blood glucose level of the normal fasting mice from an initial mean value of 102.48 at the initial time (0 h) to a mean value of 50.76 (50%) at the end of the 6 hrs. Whereas, in the group that received 400 mg/kg body weight of the extract, there was a significant reduction in blood glucose level in fasting normal mice (60%) after 6 h ($P < 0.05$). Oral treatment with glibenclamide (4 mg/kg) caused a light reduction in blood glucose levels. Reduction in the blood glucose level caused by AL-H at all doses is higher than that of standard drug, glibenclamide.

3.10. Effect of on Fasting Blood Glucose Levels in STZ-Induced Severe Diabetic Mice and STZ-Nicotinamide-Induced Mildly Diabetic Mice. The antihyperglycemic effect of AL-H on the fasting blood glucose levels in STZ-induced SD and STZ-nicotinamide-induced MD diabetic mice is shown in Table 9. In addition, AL-H given at same concentrations produced significant antihyperglycemic effects ($P < 0.005$) on streptozotocin-induced severe and mildly diabetic mice after 2 weeks of treatment. Treatment of diabetic mice with glibenclamide (4 mg/kg) produced a significant fall in blood glucose after 4 weeks (68%). Hexane extract (AL-H) at 400 mg/kg dose gradually decreased blood glucose level 4 weeks after administration (54% and 52%, resp.). In diabetes mildly, continued glucose lowering effect at the end of the study when compared to diabetic control.

3.11. Oral Glucose Tolerance Test. The effect of AL-H on glucose tolerance was determined after the 30 days of treatment. Postprandial blood glucose levels in mice show a significant change after glucose loading, increasing rapidly in all groups of diabetic mice within the first 30 min and remaining high over the next 120 min in diabetic control mice. Table 11 shows the changes in the levels of blood glucose in normal, diabetic control, and experimental groups after oral administration of glucose (2 g/kg). Oral treatment (400 mg/kg) in diabetic control rats with the hexane extract of seeds of *P. canariensis* produced a significant ($P < 0.05$) reduction of glucose in blood at 120 min, showing a 45% and 47% decrease of glucose in blood in severe and mild diabetes, respectively,

Table 4: Effect of different extracts of *P. canariensis* on the lipid profiles and hepatic enzymes of normal and obese mice.

Group	NC	OC	OC + AL-H	OC + AL-C	OC + AL-M
Serum plasma					
Total cholesterol (mg/dL)	70.3 ± 1.3	120.5 ± 5.4	74.0 ± 3.2[a]	71.4 ± 4.2[a]	72.6 ± 2.5[a]
HDL (mg/dL)	43 ± 2.0	32.0 ± 1.6	39.7 ± 2.2[a]	40.7 ± 3.2[a]	38.9 ± 0.6[a]
Triglycerides (mg/dL)	82.4 ± 3.1	115.8 ± 5.3	86.2 ± 6.7[a]	84.5 ± 5.8[a]	87.3 ± 2.0[a]
MDA (nmol MDA/mg protein)	0.05 ± 0.00	0.08 ± 0.00	0.06 ± 0.00[a]	0.05 ± 0.00[a]	0.06 ± 0.00[a]
Hepatic lipids					
Cholesterol (μmol/g liver)	18.2 ± 4.3	35.6 ± 5.3	26.7 ± 6.1[a]	25.2 ± 7.5[a]	27.0 ± 7.7[a]
Triglyceride (μmol/g liver)	13.8 ± 3.4	26.9 ± 4.8	17.4 ± 3.3[a]	16.8 ± 5.2[a]	18.0 ± 5.0[a]
Hepatic enzymes					
ALP (U/mL)	0.52 ± 0.07	0.63 ± 0.04	0.57 ± 0.04	0.54 ± 0.08	0.59 ± 0.01
SGOT (U/mL)	1.79 ± 0.08	2.01 ± 0.05	1.86 ± 0.03	1.84 ± 0.05	10.88 ± 0.02
SGPT (U/mL)	0.58 ± 0.06	0.72 ± 0.02	0.65 ± 0.02	0.64 ± 0.08	0.68 ± 0.06

Each value represents mean ± S.E.M. ($n = 10$ animals in each group) [a]$P < 0.05$ versus obese control. Hexane extract (AL-H), chloroform extract (AL-C), and methanol extract (AL-M) from *P. canariensis*.

Table 5: Effect of hexane extract of *P. canariensis* on the body weight, organ weight and food and water intake of normal and diabetic mice.

Group (mg/kg)	Body weight (g)			Intake (g/d)		Final (g)	
	Initial	Final	Gain	Food	Water	Liver weight	Kidney weight
Nondiabetic	25.8 ± 4.8	32.4 ± 5.1[a]	6.6 ± 2.2[a] (25)	29.3 ± 3.2[a]	34.1 ± 6.3[a]	6.76 ± 2.7[a]	1.31 ± 0.5[a]
SD	28.0 ± 6.2	30.0 ± 6.3[c]	2.0 ± 0.6[c] (7)	39.2 ± 5.8[c]	189.5 ± 9.6[c]	3.48 ± 1.3[c]	0.81 ± 0.05[c]
MD	25.2 ± 5.4	27.4 ± 6.0[c]	2.2 ± 0.7[c] (8)	37.6 ± 6.1[c]	180.7 ± 10.4[c]	3.40 ± 1.5[c]	0.82 ± 0.04[c]
SD + AL-H	26.6 ± 7.4	30.7 ± 8.2[b]	4.1 ± 1.6[b] (15)	32.2 ± 2.9[b]	139.5 ± 5.2[b]	5.10 ± 2.8[b]	0.98 ± 0.02[b]
MD + AL-H	24.9 ± 7.4	28.3 ± 7.4[c]	3.4 ± 0.4[b] (18)	31.4 ± 7.5[b]	123.0 ± 5.1[b]	5.89 ± 1.9[b]	1.22 ± 0.05[b]
SD + GB	24.5 ± 6.2	28.2 ± 5.8[b]	48.7 ± 4.7[b] (20)	28.2 ± 2.5[b]	113.3 ± 5.0[b]	6.10 ± 3.7[b]	1.29 ± 0.7[b]

Each value represents mean ± S.E.M. ($n = 10$), ANOVA followed by multiple two-tail "t" test. In each vertical column, mean with different superscripts (a, b, and c) differed from "t" each other significantly, $P < 0.05$. () indicates %.

compared with diabetic mice (Table 10). These results reflect the efficiency of the extract to control elevated blood glucose levels.

3.12. Effect on Hepatic Glucose Regulation Enzyme Activities and Skeletal Glycogen Content. Table 11 shows the effect of the hexane extract on G6Pase, GK, and HK activity and glycogen content of liver and skeletal muscle. Administration of AL-Hat 400 mg/kg body weight increased the content of hepatic glycogen, GK, and HK in diabetic mice while G6Pase decreased. Our results showed that the hexokinase activity tended to be reversed to normal values, while normal mice did not exhibit any significant alteration.

3.13. Effect of P. canariensis Extracts on the Antioxidant Enzyme Activities of the Liver, Kidney, and Pancreas. The antioxidant effect of the *P. canariensis* extract over tissue oxidative markers was studied here (Table 12). Diabetic mice showed a significant reduction in SOD, CAT, GSH, and GPx in hepatic and renal tissues. Low levels of SOD, CAT, GSH, and GPx in diabetic mice were reverted to near normal values after treatment with hexane extract. The readings obtained from the treated groups were comparable to that of the standard drug glibenclamide. Administration of *P. canariensis* to diabetic mice showed restoring of liver and kidney activities as reflected by these parameters. Hyperglycemia induced oxidative stress may also cause liver cell damage. Lower activity of antioxidant enzymes such as SOD, GSH, GPx, and CAT and increased rate of glycation oxidation lead to diabetes complications. Levels of these enzymes were back to near normal values after treatment with *P. canariensis* extract.

3.14. Effect of AL on Serum Insulin Level and Pancreatic Insulin Content. In streptozotocin-induced diabetic mice insulin in serum and in pancreas decrease markedly, as low as 1.5 μIU/mL in the STZ untreated mice compared with the nondiabetic control group (3.6 μIU/mL). Treatment with glibenclamide for 30 days restored insulin levels. After three weeks of administration of the *P. canariensis*

Table 6: Effect of hexane extract of *P. canariensis* on some serum biochemical parameters of normal and diabetic mice.

Group	IU/I			g/dL
	SGOT	SGPT	ALP	Total protein
Nondiabetic control	20.9 ± 3.2	24.3 ± 5.8	170.3 ± 10.4	7.9 ± 2.1
SD control	39.2 ± 8.9[a]	42.3 ± 7.1[a]	245.1 ± 11.6[a]	4.3 ± 1.5[a]
MD control	38.7 ± 7.7[a]	42.9 ± 8.6[a]	239.8 ± 9.7[a]	4.0 ± 1.2[a]
SD + AL-H	27.3 ± 6.2[b]	34.6 ± 5.5[b]	209.2 ± 10.7[b]	6.5 ± 1.4[b]
MD +AL-H	24.0 ± 4.8[b]	28.6 ± 6.3[b]	195.8 ± 9.1[b]	6.7 ± 2.4[b]
SD + GB	23.1 ± 8.0[b]	25.1 ± 5.7[b]	192.1 ± 11.8[b]	7.2 ± 3.8[b]

Values are expressed as mean ± SEM ($n = 6$) [a]$P < 0.001$ compared with normal control and [b]$P < 0.001$ compared with STZ control group. ALP: alkaline phosphatase; SGOT: serum glutamate oxaloacetate transaminase; SGPT: serum glutamate; GB: glibenclamide.

Table 7: Effect of hexane extract of *P. canariensis* on lipid profile and malondialdehyde concentration in normal and diabetic mice.

Group (mg/kg)	Mean concentration (mg/g) ± SEM					
	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	TBARS (μM/g)	
					Liver	Kidney
Nondiabetic control	93.44 ± 6.12	130.71 ± 4.26	70.42 ± 4.32	41.34 ± 6.12	0.99 ± 0.003	1.6 ± 0.08
SD control	189.86 ± 8.63[a]	247.23 ± 1.67[a]	35.36 ± 1.57[a]	72.85 ± 5.72[a]	1.61 ± 0.07[a]	2.5 ± 0.07[a]
MD control	173.41 ± 5.34[a]	203.56 ± 7.53[a]	39.72 ± 4.82[a]	68.21 ± 8.33[a]	1.60 ± 0.06[a]	2.4 ± 0.04[a]
SD + AL	127.25 ± 4.12[b]	135.36 ± 2.98[b]	52.40 ± 2.80[ab]	61.51 ± 3.70[a]	1.26 ± 0.03	1.8 ± 0.03[b]
MD + AL	93.78 ± 6.03[b]	127.26 ± 3.17[b]	60.34 ± 3.56[ab]	53.73 ± 4.21[b]	1.01 ± 0.06[b]	1.7 ± 0.05[b]
SD + GB	89 ± 5.28[b]	126.32 ± 2.65[b]	54.39 ± 2.75[ab]	46.94 ± 4.32[b]	0.93 ± 0.09[b]	1.7 ± 0.04[b]

All values are expressed as mean ± SEM, $n = 10$. [a]$P < 0.05$ when compared to normal control group, [b]$P < 0.01$ when compared to diabetic control group, where the significance was performed by one-way ANOVA followed by post hoc Dunnett's test.

hexane extract there was also a notorious elevation in serum insulin and pancreatic insulin levels. This is shown in Table 13.

4. Discussion

Obesity induction via dietary means in animal models has been considered as the most popular reference among researchers due to its high similarity of mimicking the usual route of obesity occurrence in humans. It is generally known that high-fat diet is one of the major factors causing obesity and that the long-term intake of high-fat diet evokes a significant increase in abdominal fat weight in mammals [33]. The present study, which represents the first report on the antiobesity and lipid-lowering effects of seeds of *P. canariensis* in obese animals, has shown that there was a significant difference in the body weights between the high-fat and normal diet groups; no significant difference in the daily food intake between groups was observed. The high-fat diet groups continuously consumed similar quantities of food, regardless of the higher calories content in the diet. As a result, the caloric intake was raised in the high-fat diet group compared to the normal group counterpart. The supplementation of obese rats with AL-H at 400 mg/kg conversely causes a remarkable reduction of body weights compared to the untreated obese group. The findings demonstrated that AL-H is capable of preventing body weight gain and showed a significant reduction in their body weight after 4 weeks of treatment. AL-H has shown that oral administration of *P. canariensis* modulates lipid homeostasis in the development of HFD-induced obesity. In this study, feeding a hyperlipidaemic diet containing 45% energy from fat for 10 weeks caused obesity, hyperlipidaemia, and hyperglycaemia in mice [34], However, *P. canariensis* supplementation not only suppressed excessive gains of weight in body, liver, kidney, visceral adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAT), by affecting adipose tissue growth through modulation of HFD-induced hypertrophy of adipocytes, but also reduced serum lipids, which is defined as the main risk for dyslipidaemia [35]. As shown in our data AL-H decreases in the levels of serum and hepatic lipids such as triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol in mice compared to those for mice fed with HFD only, which may possibly be due to suppressed lipid accumulation [36].

The increased MDA level in the untreated obese group clearly demonstrated that high fat consumption was attributed to increased oxidative stress. AL-H supplementation was found to improve the endogenous antioxidant defense system by enhancing the antioxidant enzymes activities *in vivo*. The groups treated with AL-H showed a significant elevation in their SOD, GPx, GSH, and CAT activities compared to the untreated obese rats. These results are aligned with the reduction of MDA levels, which may be related to the ability of AL-H to suppress lipid peroxidation due to having an increase in the activity of antioxidant enzymes, regardless of the availability of lipid substrates.

Table 8: Acute effect of hexane extract of *P. canariensis* on fasting blood glucose level of normal and diabetic mice.

Group	Dose (mg/kg)	At the time of grouping	Blood glucose levels (mg/dL) at different time intervals (hours)				
			2 h	4 h	6 h	8 h	12 h
Nondiabetic control	—	102.45 ± 4.56	101.7 ± 5.95	100.56 ± 7.89	102.76 ± 7.42	99.55 ± 8.90	103.15 ± 5.76
Nondiabetic + AL-H	100	103.61 ± 7.92	94.23 ± 6.98[a]	84.80 ± 11.04[a]	80.75 ± 9.48[a]	81.59 ± 8.53[a]	94.87 ± 5.69[a]
	200	102.48 ± 8.39	89.18 ± 7.42[a]	57.29 ± 8.59[a]	50.76 ± 6.54[a]	54.37 ± 7.90[a]	73.43 ± 10.01[a]
	400	104.22 ± 6.47	80.38 ± 8.46[a]	48.10 ± 4.79[a]	41.39 ± 5.25[a]	50.49 ± 9.12[a]	70.35 ± 6.28[a]
SD control	—	375.35 ± 4.87	370.42 ± 5.90	371.28 ± 7.41	378.05 ± 5.16	371.50 ± 8.47	378.14 ± 8.46
MD control	—	246.82 ± 7.53	248.67 ± 8.29	245.78 ± 9.53	248.71 ± 6.37	249.73 ± 8.68	250.04 ± 7.90
SD + AL-H	100	245.62 ± 4.81	225.70 ± 5.80[a]	214.52 ± 11.04[a]	190.31 ± 7.58[a]	200.31 ± 7.58[a]	213.31 ± 7.58[a]
	200	281.43 ± 3.69	232.61 ± 5.80[a]	221.68 ± 8.59[a]	182.81 ± 6.85[a]	190.02 ± 8.43[a]	229.57 ± 9.32[a]
	400	274.13 ± 4.23	212.59 ± 5.74[a]	198.39 ± 4.81[a]	149.67 ± 8.44[a]	159.13 ± 7.64[a]	180.42 ± 6.27[a]
MD + AL-H	100	263.26 ± 6.76	251.38 ± 7.56[a]	233.42 ± 10.23[a]	221.49 ± 5.46[a]	230.16 ± 4.68[a]	241.59 ± 6.89[a]
	200	249.51 ± 5.64	235.24 ± 4.80[a]	219.65 ± 6.49[a]	196.08 ± 7.35[a]	199.38 ± 6.72[a]	240.67 ± 9.47[a]
	400	274.38 ± 6.98	258.16 ± 5.39[a]	230.47 ± 9.03[a]	186.14 ± 7.10[a]	191.53 ± 5.37[a]	262.54 ± 8.11[a]
SD + GB	0.5	348.89 ± 5.79	272.68 ± 6.87[b]	201.35 ± 2.59[b]	209.43 ± 2.94[b]	219.60 ± 1.98[b]	266.29 ± 2.83[b]

Each of the values represents mean ± SD ($n = 6$). [a]$P < 0.05$ compared to normal group (ANOVA) followed by Dunnett's test. [b]$P < 0.05$ compared to diabetic group (ANOVA) followed by Dunnett's test. Glibenclamide (GB).

Table 9: Effect of hexane extract of *P. canariensis* on blood glucose level of normal and diabetic mice after 30-day treatment.

Group (mg/kg)	Fasting blood glucose level (mg/dL)				
	0	1	2	3	4
No-diabetic control	100.1 ± 4.7	101.6 ± 8.5[a]	105.4 ± 7.4[a]	107.5 ± 9.9[a]	103.3 ± 6.8[a]
SD control	323.7 ± 6.7	327.6 ± 9.5[b]	341.2 ± 6.1[b]	401.3 ± 7.6[b]	411.4 ± 15.9[b]
MD control	268.5 ± 17.7	277.3 ± 19.2[b]	297.5 ± 9.4[b]	320.6 ± 17.4[b]	334.6 ± 18.2[b]
SD + AL	356.2 ± 14.2	313.9 ± 12.2[c] (12)	190.5 ± 17.1[c] (46)	170.3 ± 15.7[c] (52)	161.8 ± 14.6[c] (54)
MD + AL	239.4 ± 13.7	196.5 ± 21.3[c] (18)	132.3 ± 20.6[c] (45)	124.7 ± 17.9[c] (48)	114.2 ± 5.6[c] (52)
SD + GB	352.6 ± 6.6	270.5 ± 6.9[c] (14.3)	152.7 ± 9.2[c] (51)	109.4 ± 5.3[c] (65)	100.2 ± 2.1[c] (68)

Each value represents mean ± S.E.M. ($n = 10$), ANOVA followed by multiple two-tail "t" test.
In each vertical column, mean with different superscripts (a, b, and c) differ from "t" each other significantly, <0.05. Glibenclamide (GB) at doses 5 mg/kg. () % inhibition.

P. canariensis is a kind of traditional medicine which has long been used to effectively treat obesity and diabetesl by local people. In the present findings, we evaluated the antidiabetic effect of AL-H on SD and MD induced by STZ. Our date clearly showed hypoglycemic activity and glucose tolerance pattern was significantly altered comparable to that of glibenclamide. This activity improved glucose tolerance suggesting a decrease in insulin resistance and helping to maintain blood glucose levels steady which may indicate certain induction of peripheral utilization of glucose.

Streptozotocin is known for its selective pancreatic islet beta cells cytotoxicity and has been widely used to induce diabetes mellitus. Besides alteration in the carbohydrate and lipid metabolism, rats treated with STZ also exhibited reduced total protein and liver glycogen levels, increased liver glucose transfer, and decline in liver glycogen content in STZ diabetic rats [37]. From glucose tolerance test it has been indicated that this extract did not execute the antihyperglycemic effect by modulating the absorption of glucose in the intestine. In glucose loaded rats AL-H inhibited significantly the rise of glycemia. This improved glucose tolerance and also suggests that a decrease in insulin resistance and maintenance of blood glucose levels may indicate induction of increased peripheral utilization of glucose [37].

Furthermore, the attenuating effect of this extract on experimental physiological symptoms of streptozotocin-induced severe and mild diabetes has been confirmed here by the study of glucose-6-phosphatase activity in liver, as well as the quantification of glycogen in liver and skeletal muscle, which are very important indicators of diabetes mellitus. In our study, the hexane extract of AL-H seeds had a beneficial effect in terms of peripheral glucose utilization.

Control of hepatic glucose (HGO) may occur through regulation of gluconeogenesis or glycogenolysis. However, the common final pathway of glucose uptake and release involved the phosphorylation and dephosphorylation of glucose via GK and G6Pase, respectively [38]. In this study,

Table 10: Effect of hexane extract of *P. canariensis* on postprandial blood glucose level of normal and diabetic mice.

Groups (mg/kg)	Blood glucose levels (mg/dL)				
	0 min	30 min	60 min	90 min	120 min
Nondiabetic control	92.34 ± 1.78	178.78 ± 3.42	160.12 ± 2.47	126.42 ± 2.59	96.58 ± 4.67
SD control	368.78 ± 3.42	344.16 ± 7.36[a]	409.23 ± 6.21[a]	373.41 ± 6.71[a]	348.53 ± 13.71[a]
MD control	274.56 ± 5.94	352.21 ± 8.44[a]	399.60 ± 9.68[a]	362.31 ± 8.84[a]	332.19 ± 10.28[a]
SD + AL	366.19 ± 14.03	335.38 ± 8.63[a]	255.11 ± 10.39[c]	219.23 ± 11.36[c]	199.08 ± 9.46[c]
MD + AL	254.46 ± 10.21	317.49 ± 12.39[a]	268.49 ± 9.56[c]	213.47 ± 8.46[c]	134.89 ± 11.83[c]
SD + GB	265.30 ± 8.00	315.25 ± 12.25[a]	295.05 ± 8.4[c]	263.11 ± 6.78[c]	167.36 ± 10.83[c]

Values are expressed as mean ± SD ($n = 10$), [a]significantly ($P < 0.05$) different from normal rats. [b]Significantly ($P < 0.05$) different from diabetic rats. [c]Significantly ($P < 0.05$) different from normal and diabetic rats, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test. ALH extract 400 mg/kg.

Table 11: Effect of hexane extract of *P. canariensis* on hepatic glucose regulation enzyme activities of normal and diabetic mice.

Group	G6Pase activity (mU)	GK activity (mU)	Glycogen (mg/g)		HK activity (U/mg protein)
			Liver	Skeletal muscle	
Normal control	0.40 ± 0.006	3.38 ± 0.05	19.64 ± 2.45	11.74 ± 4.39	1.69 ± 0.07
SD control	0.71 ± 0.005[a]	1.36 ± 0.06[a]	9.67 ± 3.12[a]	4.89 ± 1.34[a]	1.31 ± 0.03[a]
MD control	0.69 ± 0.002[a]	1.27 ± 0.08[a]	10.23 ± 4.35[a]	5.27 ± 1.56[a]	1.29 ± 0.05[a]
SD + AL-H	0.53 ± 0.008[ab]	2.8 ± 0.04[ab]	[d]17.53 ± 5.18[b]	[d]9.78 ± 1.54[b]	[d]1.53 ± 0.09[ab]
MD + AL-H	0.46 ± 0.007[ab]	3.09 ± 0.03[ab]	[d]18.13 ± 5.80[b]	[d]10.89 ± 2.41[b]	[d]1.59 ± 0.05[ab]
GB 4 (mg/kg)	0.39 ± 0.004[b]	3.11 ± 0.04[b]	17.78 ± 2.30[b]	11.01 ± 1.96[b]	1.60 ± 0.03[ab]

Each value represents mean ± SD, ($n = 10$); ANOVA followed by multiple two-tail "t" test. In each vertical column, mean with different superscripts (a, b) differed from each other. Significant difference of diabetic control from normal control [a]$P < 0.001$. Significant difference of treated groups from diabetic control [b]$P < 0.01$, [c]$P < 0.05$, [d]$P < 0.01$ when compared with glibenclamide 4 mg/kg treated group.

AL-H caused a marked increase in hepatic glycogen content in STZ-induced diabetic mice, which indicates that mice may decrease HGO by increasing glycogen content. In addition, *P. canariensis* decreased G6Pase activity and increased GK activity in liver, which indicates that this can be an increase in hepatic glucose uptake and decrease in hepatic glucose release. Therefore, this study strongly suggests that *P. canariensis* enhances hypoglycemic activity probably by reducing HGO via decreasing G6Pase activity and increasing GK activity. One of the key enzymes in the catabolism of glucose is hexokinase, which phosphorylates glucose and converts it into glucose-6-phosphate. The increased activity of hexokinase can promote glycolysis and increase utilization of glucose for energy production [39]. The administration of *P. canariensis* to the diabetic mice increased the activity of hepatic hexokinase causing an increase in glycolysis. The hepatic glycogen was found to be increased in both liver and skeletal muscle in diabetic rats and suggested also a reduction in glycogenolysis and an increase in glycogenesis.

Insulin deficiency is associated with hypercholesterolaemia and hypertriglyceridaemia. STZ-induced diabetes showed increased plasma levels of cholesterol, triglyceride, free fatty acid, and phospholipids. Insulin deficiency or insulin resistance could be responsible for dyslipidaemia because insulin increases fatty acid as well as triglyceride synthesis in adipose tissue and liver. It inhibits lipolysis, partly via dephosphorylation (and hence inactivation) of lipases [40]. Insulin deficiency leads to fall in lipoprotein lipase activity. In our study, STZ mice showed hypercholesterolaemia and hypertriglyceridaemia and the treatment with AL-H significantly decreased both cholesterol and triglyceride levels. STZ induction of diabetes in mice leads to lipid peroxidation. TBARS are an index of endogenous lipid peroxidation and oxidative stress as an intensified free radical production. TBARS levels in both liver and kidney of diabetic control group were high and were significantly reduced upon administration of the hexane extract. These findings supported the hypothesis that *P. canariensis* improved insulin sensitivity.

We studied the antioxidant effect of the AL-H extract over tissue oxidative markers. Diabetic mice showed a significant reduction in SOD, CAT, GSH, and GPx in hepatic and renal tissues as a result of a persistent oxidative stress. One of the main consequences of chronic hyperglycemia is the enhanced oxidative stress resulting from the imbalance between production and neutralization of reactive oxygen species (ROS), in particular, the diabetes-associated free radical injury, accumulation of lipid peroxidation products, depletion of GSH, decrease in GSH/GSSG ratio, and downregulation of key antioxidant enzymes [40]. Accordingly, we measured a decrease in GSH in the liver of diabetic mice, probably due to a higher demand, following the diabetes-induced oxidative stress. The glutathione system, SOD, GPx, GSH, and CAT comprise the most important endogenous antioxidant defense against ROS-induced damage of the cell membrane. SOD protects tissues from oxygen free radicals by catalyzing the removal of free radical superoxide anion $O_2^{\bullet -}$; GPx and CAT were shown to be responsible for the detoxification of

Table 12: Effect of hexane extract of *P. canariensis* on antioxidant enzyme activities of normal and diabetic mice.

Parameters	Normal C	SD C	MD C	SD + AL-H	MD + AL-H	SD + GB
SOD—liver	7.54 ± 2.19	3.79 ± 0.82[a]	4.05 ± 1.38[b]	5.87 ± 0.89[b]	6.01 ± 1.05[b]	6.80 ± 0.41[c]
SOD—kidney	13.82 ± 3.51	7.35 ± 1.37[a]	8.36 ± 2.53[b]	10.24 ± 2.63[b]	11.47 ± 3.41[b]	13.025 ± 1.57[b]
SOD—pancreas	54.52 ± 5.21	36.47 ± 3.28[a]	35.99 ± 5.42[b]	43.88 ± 5.39[b]	46.90 ± 5.48[b]	50.71 ± 4.36[b]
CAT—liver	82.36 ± 6.17	44.53 ± 3.13[a]	47.32 ± 7.14[b]	64.56 ± 6.38[b]	67.12 ± 6.74[b]	70.26 ± 2.16[b]
CAT—kidney	35.61 ± 2.33	20.76 ± 1.65[a]	22.01 ± 9.53[c]	29.56 ± 2.81[c]	32.07 ± 2.53[c]	34.17 ± 1.79[b]
CAT—pancreas	59.32 ± 5.38	25.56 ± 4.19[a]	23.19 ± 5.46[a]	40.24 ± 3.67[a]	43.79 ± 5.18[a]	47.83 ± 4.36[c]
GSH—liver	47.68 ± 7.12	21.80 ± 1.97[a]	29.15 ± 6.36[b]	36.52 ± 4.86[b]	39.48 ± 4.36[b]	42.38 ± 2.28[b]
GSH—kidney	24.45 ± 3.71	5.79 ± 2.06[a]	7.21 ± 3.53[b]	15.29 ± 3.83[b]	17.20 ± 3.53[b]	19.27 ± 3.58[b]
GSH—pancreas	12.48 ± 3.39	5.19 ± 0.94[a]	7.42 ± 2.56[b]	6.91 ± 1.80[b]	8.47 ± 1.56[b]	10.79 ± 1.92[b]
GPx—liver	7.26 ± 1.59	4.31 ± 1.30[a]	5.39 ± 2.01[b]	5.35 ± 1.83[b]	5.64 ± 1.87[b]	5.92 ± 1.26[b]
GPx—kidney	5.92 ± 1.42	3.53 ± 0.92[a]	3.40 ± 1.43[b]	4.53 ± 1.34[b]	4.60 ± 1.73[b]	4.78 ± 0.94[b]
GPx—pancreas	4.64 ± 1.19	2.32 ± 0.36[a]	3.42 ± 1.26[b]	3.31 ± 0.61[b]	3.70 ± 1.26[b]	3.89 ± 0.27[b]

All values are expressed as mean ± SEM, $n = 6$ values. [a]$P < 0.01$ when compared to normal control group; [b]$P < 0.01$, [c]$P < 0.05$ compared to diabetic control group, where the significance was performed by Oneway ANOVA followed by post hoc Dunnett's test. Glibenclamide (GB). Control (C); the values are given in U/mg of protein.

Table 13: Effect of hexane extract of *P. canariensis* on serum and pancreatic insulin concentration of normal and diabetic mice.

Groups	Before administration (0 h)	Plasma insulin (μIU/mL)	Pancreatic insulin (μIU/mL)
Normal control	3.59 ± 0.52	3.60 ± 0.15	25.80 ± 3.76
SD control	0.77 ± 0.084	1.50 ± 0.29[a]	15.21 ± 5.43[a]
MD control	0.76 ± 0.032	[d]1.61 ± 0.36[b]	15.02 ± 5.13[bc]
SD + AL	0.74 ± 0.019	[d]3.17 ± 0.51[b]	21.24 ± 6.38[bc]
MD + AL	0.77 ± 0.037	[d]3.27 ± 0.54[b]	21.68 ± 6.18[bc]
SD + glibenclamide 4 mg/kg	0.75 ± 0.070	3.49 ± 0.34[b]	19.35 ± 3.23[bc]

All values are expressed as mean ± SD, $n = 6$ values. Plasma insulin values at 0 h before drug administration are significantly different compared to respective 30 days after drug treatment. Significant difference of diabetic control from normal control [a]$P < 0.001$. Significant difference of treated groups from diabetic control [b]$P < 0.01$, [c]$P < 0.05$. [d]$P < 0.01$ when compared with glibenclamide 4 mg/kg treated group.

significant amounts of H_2O_2. In addition, administration of AL-H extract showed increased activities of SOD, GPx, and CAT after 30 days of treatment in STZ rats indicating that the AL-H extract can reduce reactive oxygen free radicals and improve the activities of the antioxidant enzymes [41]. The enhanced activities of the antioxidant enzymes promoted by AL-H protect against STZ-induced damage; therefore, hyperglycemia does not develop. The protection against lipid peroxidation offered by GPx and the effect of AL-H on this enzyme appear to be relevant responses to ROS-induced membrane damage [42].

The effects of the AL-H on transaminase (i.e., ALP, SGPT, and SGOT) activity was also studied in hyperlipemic mice. Transaminases are important enzymes for the study of liver toxicity. ALP is found predominately in the liver, with lesser quantities in the kidneys, heart, and skeletal muscles. As a result, ALP is a more specific indicator of liver inflammation than SGPT and SGOT and may also be elevated in diseases that affect other organs, such as the heart and muscles. Our results indicate that only treatment with the AL-H of hyperlipemic mice reduced the activity of these enzymes, suggesting that this extract effectively reduced the toxic effect on these enzymes. These results suggest that *P. canariensis* prevents oxidative stress, acts as a suppressor of liver cell damages and inhibit, the progression of liver dysfunction induced by chronic hyperglycemia. AL-H extract has a potent effect over antioxidant enzymes activities in pancreatic tissue, enhancing them compared to diabetic control.

P. canariensis improves glucose metabolism by reducing insulin resistance and by protecting pancreatic β-cells from oxidative stress and exhibited excellent hypoglycemic activity, enhancing glucose uptake by adipose and muscle tissues, along with beneficial lipid regulation ability. A reduction in the activities of SGOT, SGPT, and ALP in plasma and an increase in glucokinase and HK and a decrease in G6Pase indicated the hepatoprotective role. We demonstrated that daily consumption of *P. canariensis* tended to suppress body weight in fatty mice with hypertriglyceridemia. Although the effect of *P. canariensis* is important, it is interesting that *P. canariensis* suppressed body weight without affecting food consumption. *P. canariensis* possesses lipid lowering effect in obesity-induced mice, as well as its weight-reducing ability. Due to the promising effects of *P. canariensis* in STZ-induced diabetes mice and diet-induced obesity, further studies are sought in order to determine the active principle from this plant.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Nigella sativa Fixed and Essential Oil Supplementation Modulates Hyperglycemia and Allied Complications in Streptozotocin-Induced Diabetes Mellitus

Muhammad Tauseef Sultan,[1] Masood Sadiq Butt,[2] Roselina Karim,[1] M. Zia-Ul-Haq,[3] Rizwana Batool,[2] Shakeel Ahmad,[4] Luigi Aliberti,[5] and Vincenzo De Feo[5]

[1] *Faculty of Food Science and Technology, University of Putra Malaysia (UPM), Malaysia*
[2] *National Institute of Food Science & Technology, University of Agriculture, Faisalabad, Pakistan*
[3] *The Patent Office, Karachi, Pakistan*
[4] *Department of Agronomy, Bahauddin Zakariya University, Multan 60800, Pakistan*
[5] *Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, Fisciano, 84084 Salerno, Italy*

Correspondence should be addressed to Vincenzo De Feo; defeo@unisa.it

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In the recent era, diabetes mellitus has emerged as one of the significant threats to public health and this situation demands the attention of the researchers and allied stakeholders. Dietary regimens using functional and nutraceutical foods are gaining wide range of acceptance and some traditional medicinal plants are of considerable importance. The main objective of this instant study was to explore the antidiabetic potential of *Nigella sativa* fixed oil (NSFO) and essential oil (NSEO). Three experimental groups of rats received diets during the entire study duration, that is, D_1 (control), D_2 (NSFO: 4.0%), and D_3 (NSEO: 0.30%). Experimental diets (NSFO & NSEO) modulated the lipid profile, while decreasing the antioxidant damage. However, production of free radicals, that is, MDA, and conjugated dienes increased by 59.00 and 33.63%, respectively, in control. On the contrary, NSFO and NSEO reduced the MDA levels by 11.54 and 26.86% and the conjugated dienes levels by 32.53 and 38.39%, respectively. *N. sativa* oils improved the health and showed some promising anti-diabetic results.

1. Introduction

In the domain of diet-based therapies, functional foods are important to combat lifestyle related disorders, that is, hyperglycemia, high cholesterol, and immune dysfunction. Such functional foods, nutraceuticals, and pharma foods are modern trends [1] and utilization of medicinal plants is gaining wide range of recognition [2, 3]. Various plants rich in functional ingredients possess the ability to reduce hyperglycemia and hypercholesterolemia along with quenching free radicals [4]. The bioactive molecules present in them, including antioxidants, phytosterols, and flavonoids, are responsible for health claims associated with the plants. Recent research studies also validated some traditional health claims of certain plants and whole scenario led dietetics/nutritionists to consider them suitable in diet-based medication of various illnesses [5].

Diabetes mellitus is one of the leading causes of mortality all over the globe and targets multiorgan systems [6]. According to World Health Organization estimates, more than 376 million people will be diabetic globally in 2030 and about two billion people would be at risk due to poor dietary habits, obesity, and lack of physical exercise [7]. It is worth mentioning that healthy lifestyle and dietary measures can prevent 30–40% of all kinds of diseases. Diet diversification or slight changes in the daily diet can possibly prevent the onset of diabetes mellitus [8]. Therefore, the utilization of natural foods rich in bioactive compounds/functional ingredients is gaining wide range of acceptance. These natural compounds act as micronutrients and there are growing

efforts in exploring molecular basis for their therapeutic mechanisms [4]. However, diet selection is imperative for the management of diabetes and its allied complications. Scientists over the globe believe that dietary modifications are important along with pharmaceuticals for the treatment of diabetes mellitus and allied complications [9].

In the last few decades, scientists explored many plants possessing antidiabetic perspectives, for example, garlic, bitter melon, green tea, fenugreek, pelargonium, turmeric, rice bran, oat, mulberry, amaltas, and so forth, [10]. Researchers over the globe have recently focused their studies on the possible role of *Nigella sativa* L. (*Ranunculaceae*) or black cumin for management of diabetes. Extracts of *Nigella sativa* (also known as black cumin or black seeds) possessed blood glucose lowering effects, but the exact anti-diabetic mechanism is not yet established. Hypoglycemic effects of black cumin oil might be due to presence of some phytochemicals including thymoquinone and carvacrol [11]. *N. sativa* fixed oil is rich in polyunsaturated fatty acids and some minor dihomolinolenic acids, tocopherols, and phytosterols. In comparison, *N. sativa* essential oil is rich source of antioxidants including thymoquinone, *p*-cymene, carvacrol, anethole, and 4-terpineol [12, 13]. These fractions of *N. sativa* might be helpful in lowering hyperglycemia through β-cell integrity and enhancing the insulin secretions. Moreover, presence of functional ingredients and bioactive molecules could provide protection against diabetes complication [14].

In the present study, we attempted to explore *N. sativa* fixed (NSFO) and essential oils (NSEO) for their antidiabetic properties, through the estimation of values of blood glucose, insulin, serum lipid profile, and indices of oxidative damage.

2. Materials and Methods

2.1. Plant Material. The Barani Agricultural Research Institute, Chakwal, provided *N. sativa* seeds. A voucher specimen (Voucher/Specimen no. Chk. Pk-926) of the plant is preserved in the herbarium of the same Institute.

2.2. Chemicals. Chemical reagents (analytical and HPLC grade) like xylenol orange (o-cresosulfonphthalein-3,3-bis (sodium methyl iminodiacetate)) and standards were purchased from Sigma-Aldrich Tokyo, Japan, and Merck KGaA, Darmstadt, Germany.

2.3. Extraction of Nigella sativa Fixed and Essential Oils. Following the standard procedures, the seeds of *N. sativa* were slurred with hexane (in the ratio of 1 : 6 using a Soxlet apparatus and rotary evaporator was used to remove solvent) to extract the fixed oil. In contrast, the essential oil was extracted using locally assembled hydrodistillation apparatus.

2.4. Animals. The National Institute of Health (NIH), Islamabad, provided infectious free 30 Sprague Dawley rats that were further divided into three groups of ten rats each. The animals were maintained according to standard guidelines of Animal Institute of Nutrition (AIN), USA, that is, temperature $23 \pm 2^{\circ}$C, relative humidity $55 \pm 5\%$, and 12-hr light-dark cycle. In the first week, the feed of the rats was a basal diet in order to acclimatize them to new environment. Later, rats received their respective experimental diets for a period of eight weeks (56 days) as reported in Table 1.

Table 1: Diet plan used in the study.

	Diets
Group-I	D_1: (control/placebo diet)
Group-II	D_2: (4.0% fixed oil)
Group-III	D_3: (0.3% essential oil)

The analytical procedures carried out include parameters measured daily (feed and water intake) and body weight (weekly basis). At 28 and 56 days of feeding trials, five rats from each group were decapitated to collect blood through cardiac and neck puncture [15].

2.5. Induction of Diabetes Mellitus. Diabetic mellitus was induced in rats (weight 150–200 g) by injecting intravenously streptozotocin (STZ) in a dose of 60 mg/Kg body weight, dissolved in 0.01 M citrate buffer (pH 4.5). The blood glucose level of each rat was monitored after injecting STZ to check the glucose response. The rats received experimental diets after the mean values for blood glucose reached >200 mg/dL.

2.6. Blood Glucose and Insulin Levels. In order to check the hypoglycemic effects of NSFO and NSEO, we determined the glucose concentration of individual rat in each study using GOD-PAP method as described by [16], while insulin level was determined following the method of Besch et al. [17].

2.7. Blood Lipid Profile. The blood samples from each group of rats were centrifuged at 3000 rpm and the serum was collected as supernatant layer. The collected serum was used for the estimation of serum lipid profile and cholesterol by CHOD-PAP method [18], high density lipoprotein (HDL) by HDL cholesterol precipitant method [19], total triglycerides by liquid triglycerides (GPO-PAP) method [20], and low density lipoproteins (LDL) following the procedure of McNamara et al. [21]. For estimations of the aforementioned parameters, the standard procedures mentioned on the commercial kits without any further modifications were used. Briefly, serum cholesterol and triglyceride levels were measured by using enzymatic reagents adapted to a MicroLab-300 (Merck, Germany). Similarly, phosphotungstic acid and magnesium chloride based assay was used to estimate high-density lipoprotein (HDL).

2.8. Indices of Oxidative Damage. Indicators of lipid peroxidation were estimated including MDA level [22], total antioxidant capacity using xylenol orange assay [23], and conjugated dienes according to protocol described by Corongiu and Milla, [24]. For total antioxidant capacity, reagent-1 (containing xylenol orange, NaCl, and glycerol) was added to 35 μL of collected serum and then to 11 μL of reagent-2

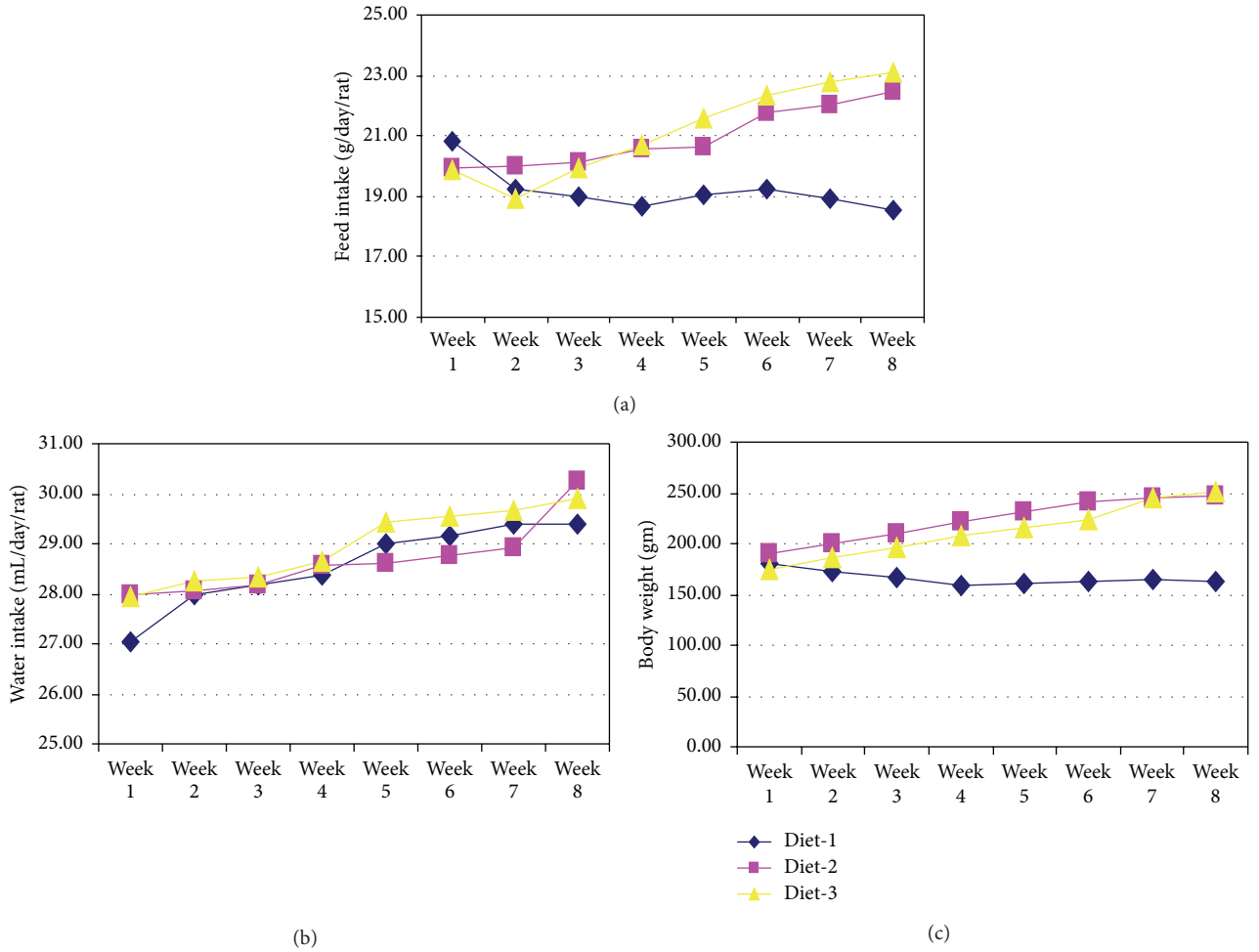

FIGURE 1: Feed and water intake and body weight of diabetic rats. D_1 (control), D_2 (NSFO), and D_3 (NSEO).

(containing ferrous ion and *o*-dianisidine in H_2SO_4 solution). The readings were taken at 560 nm and 800 nm at the start of the reaction and at the end of the reaction (3-4 min). Briefly, we measured MDA with a colorimetric method using tetraethoxypropane as standard in nmol/mL. The detection limit was 0.02 mmol/L and the intra- and interassay coefficients of variation were 6.1% and 7.3%, respectively. For the determination of conjugated dienes, serum was homogenized and centrifuged at 3000 g. Then the supernatant or organic layer was transferred in screw-capped micro test tubes and flushed with nitrogen gas for semidrying. The spectra obtained at wavelength of 232–247 nm using hexane as standard solution were further used to estimate conjugated dienes and values were expressed in nmol/g of lipids.

2.9. Statistical Analysis. Statistical package, that is, Cohort V-6.1 (Co-Stat Statistical Software, 2003), was used for data analysis. Briefly, values presented in tables are means ± standard deviation. In order to check the level of significance, analysis of variance (ANOVA) technique was applied. The diets (factor A), intervals (factor B), and their interaction (A × B) were used as source of variations. Duncan's multiple range test (DMRt) further clarified the effects of diets in a comprehensive manner.

2.10. Ethics. The experiments were carried out following the instructions of "Animal Care Committee, NIFSAT-Faisalabad, Pakistan".

3. Results

The injection of 60 mg/Kg body weight streptozotocin resulted in onset of diabetic mellitus, due to damage to pancreas thus reducing the concentrations of insulin. During the course of study, feed intake (Figure 1) increased significantly ($P < 0.05$) in the experimental diets, that is, D_2 (NSFO) and D_3 (NSEO). In contrast, feed intake decreased significantly in D_1 (control) group. However, water intake increased non-significantly with the passage of study from 27.65 ± 0.53 to 29.86 ± 0.43 mL/rat/day. Body weight varied significantly ($P < 0.01$) as a function of diets; maximum body weight 223.59 ± 21.49 g was observed in D_2 (NSFO) followed by

Table 2: Effects of diets containing NSFO and NSEO on blood glucose and insulin levels in diabetic rats.

Parameters	Diets	Study intervals (days)		
		0	28	56
Glucose (mg/dL)	D_1 (control)	211.78 ± 12.22^{c}	241.67 ± 7.83^{b}	282.10 ± 8.20^{a}
	D_2 (NSFO)	217.41 ± 9.09^{c}	214.28 ± 7.20^{c}	208.50 ± 9.18^{c}
	D_3 (NSEO)	210.47 ± 8.01^{c}	195.36 ± 9.51^{d}	188.94 ± 8.39^{e}
Insulin (μU/mL)	D_1 (control)	42.11 ± 1.35^{b}	32.07 ± 1.04^{d}	20.33 ± 0.59^{e}
	D_2 (NSFO)	40.35 ± 1.03^{bc}	38.68 ± 0.70^{c}	42.62 ± 0.89^{b}
	D_3 (NSEO)	42.38 ± 0.52^{b}	45.44 ± 1.34^{a}	46.80 ± 2.08^{a}

Means sharing same letters in a column/row do not differ significantly at $P < 0.05$.

Table 3: Effects of diets containing NSFO and NSEO on cholesterol, HDL, LDL, and triglycerides levels in diabetic rats.

Parameters	Diets	Study intervals (days)		
		0	28	56
Cholesterol (mg/dL)	D_1 (control)	106.56 ± 3.42^{de}	130.37 ± 4.22^{b}	147.76 ± 4.30^{a}
	D_2 (NSFO)	110.36 ± 2.81^{cd}	108.90 ± 1.98^{cd}	98.97 ± 2.08^{e}
	D_3 (NSEO)	116.31 ± 1.42^{c}	113.92 ± 3.36^{c}	105.97 ± 4.70^{de}
HDL (mg/dL)	D_1 (control)	38.36 ± 2.21	36.79 ± 1.19	37.40 ± 1.90
	D_2 (NSFO)	40.45 ± 1.69	42.46 ± 1.43	42.68 ± 1.88
	D_3 (NSEO)	39.08 ± 1.49	41.79 ± 2.03	41.82 ± 1.86
LDL (mg/dL)	D_1 (control)	47.71 ± 2.75^{d}	72.79 ± 2.36^{b}	83.41 ± 4.23^{a}
	D_2 (NSFO)	51.13 ± 2.14^{c}	48.54 ± 1.63^{d}	38.99 ± 1.72^{e}
	D_3 (NSEO)	57.44 ± 2.19^{c}	53.20 ± 2.59^{c}	46.02 ± 2.04^{d}
Triglycerides (mg/dL)	D_1 (control)	102.45 ± 5.91^{b}	103.96 ± 3.37^{b}	130.34 ± 6.61^{a}
	D_2 (NSFO)	93.89 ± 3.93^{d}	89.50 ± 3.01^{e}	86.48 ± 3.81^{e}
	D_3 (NSEO)	98.94 ± 3.77^{c}	94.64 ± 4.61^{d}	93.67 ± 4.16^{d}

Means sharing same letters in a column/row do not differ significantly at $P < 0.05$.

212.49 ± 26.83 g in D_3 (NSEO), while minimum 165.99 ± 7.03 g was recorded for D_1 (control).

3.1. Hypoglycemic Potential. Blood glucose and insulin levels varied significantly ($P < 0.05$) due to diets, study intervals, and interaction. The maximum glucose level was observed in D_1 (control) followed by D_2 (NSFO), while minimum level was recorded in D_3 (NSEO) treatment. During the course of the eight-week study, D_3 group exhibited pronounced decrease in glucose from 210.47 ± 8.01 mg/dL to 188.94 ± 8.39 mg/dL (Table 2). Likewise, glucose decreased from 217.41 ± 9.09 to 208.50 ± 9.18 mg/dL in D_2 group, whereas D_1 group showed significant increase in glucose from baseline value of 211.78 ± 12.22 to 282.10 ± 8.20 mg/dL at the end of study. Insulin levels differed due to the diets; the diet D_3 showed the maximum insulin concentrations followed by D_2, while D_1 group recorded the least insulin concentrations. Insulin level improved in fixed and essential oils group from from 40.35 ± 1.03 to 42.62 ± 0.89 and 42.38 ± 0.52 to 46.80 ± 2.08 μU/mL, respectively, as compared to the substantial decrease in D_1 group from 42.11 ± 1.35 to 20.33 ± 0.59 μU/mL during the 56-day study.

3.2. Serum Lipid Profiles. It is obvious from statistical analysis that cholesterol and LDL differed significantly ($P < 0.05$) with diets, study intervals, and interaction. Likewise, diets affected triglycerides and HDL momentously. Means for cholesterol contents (Table 3) indicated significant variations due to diets and maximum cholesterol was recorded in D_1 (control), while D_2 (NSFO) and D_3 (NSEO) groups had lower values. During the eight-week trial, in D_1 (control) cholesterol contents increased from 106.56 ± 3.42 to 147.76 ± 4.30 mg/dL. Experimental diets containing oils of *N. sativa* fixed and essential oils decreased the same trait significantly ($P < 0.05$), with more pronounced effects in D_2 from 110.36 ± 2.81 to 98.97 ± 2.08 mg/dL, whereas D_3 decreased that said trait from 116.31 ± 1.42 to 105.97 ± 4.70 mg/dL. The D_1 diet showed the maximum LDL contents (67.97 ± 10.58 mg/dL), while minimum contents 46.22 ± 3.69 mg/dL recorded in D_2 group. The results regarding HDL indicated that the maximum HDL, 41.86 ± 0.71 and 40.90 ± 0.91 mg/dL was recorded in D_2 and D_3 groups, respectively, as compared to minimum 37.52 ± 0.46 mg/dL in D_1. Similarly, maximum triglycerides 112.25 ± 9.06 mg/dL were recorded in groups of rats fed on D_1 followed by D_2 and D_3 with mean triglycerides of 89.96 ± 2.15 and 95.75 ± 1.62 mg/dL, respectively.

Table 4: Indices of antioxidants damage in diabetic rats treated with NSFO and NSEO.

Parameters	Diets	Study intervals (days)		
		0	28	56
Total antioxidants capacity (TAC) (IU/mL)	D_1 (control)	0.52 ± 0.03^{de}	0.44 ± 0.01^{e}	0.29 ± 0.02^{f}
	D_2 (NSFO)	0.56 ± 0.02^{d}	0.65 ± 0.02^{c}	0.78 ± 0.03^{b}
	D_3 (NSEO)	0.47 ± 0.02^{de}	0.67 ± 0.03^{c}	0.91 ± 0.04^{a}
MDA (nmol/g)	D_1 (control)	6.61 ± 0.38^{c}	9.02 ± 0.29^{b}	10.51 ± 0.53^{a}
	D_2 (NSFO)	7.00 ± 0.29^{c}	5.86 ± 0.20^{d}	4.89 ± 0.21^{e}
	D_3 (NSEO)	7.06 ± 0.27^{c}	3.66 ± 0.18^{f}	3.28 ± 0.15^{f}
Conjugated dienes (CD) (nmol/g)	D_1 (control)	2.25 ± 0.13^{c}	2.63 ± 0.08^{b}	3.01 ± 0.15^{a}
	D_2 (NSFO)	2.08 ± 0.09^{c}	1.90 ± 0.06^{cd}	1.84 ± 0.08^{d}
	D_3 (NSEO)	2.08 ± 0.08^{c}	1.58 ± 0.08^{e}	1.41 ± 0.061^{e}

Means sharing same letters in a column/row do not differ significantly at $P < 0.05$.

Table 5: Correlation matrix of some important parameters in diabetic rats.

	Chl	HDL	TG	LDL	Gl.	Insulin	MDA	TAC	CD
Chl	1.00								
HDL	-0.82^{**}	1.00							
TG	0.94^{**}	-0.80^{*}	1.00						
LDL	0.99^{**}	-0.87^{**}	0.90^{**}	1.00					
Gl	0.93^{**}	-0.75^{*}	0.89^{**}	0.92^{**}	1.00				
Insulin	-0.57^{ns}	0.27^{ns}	-0.65^{ns}	-0.50^{ns}	-0.74^{*}	1.00			
MDA	0.74^{*}	-0.69^{ns}	0.78^{*}	0.73^{*}	0.86^{**}	-0.72^{*}	1.00		
TAC	-0.68^{ns}	0.66^{ns}	-0.70^{*}	-0.68^{ns}	-0.77^{*}	0.69^{ns}	-0.96^{**}	1.00	
CD	0.86^{**}	-0.72^{*}	0.87^{**}	0.83^{**}	0.96^{**}	-0.79^{**}	0.94^{**}	-0.86^{**}	1.00

*Significant ($P < 0.05$); **highly significant ($P < 0.01$); and ns: non-significant.
Chl: cholesterol; HDL: high density lipoprotein; TG: triglycerides; LDL: low density lipoprotein; Gl: glucose; MDA: malonaldehyde; TAC: total antioxidant capacity; CD: conjugated dienes.

3.3. Indices of Oxidative Damage. Indicators of oxidative damage like total antioxidant capacity (TAC), serum MDA, and conjugated dienes levels exhibited significant differences due to diets ($P < 0.01$) and interaction ($P < 0.05$). However, study interval remained nonsignificant except for TAC (Table 4). It is evident that during the 56-day trial, D_2 (NSFO) and D_3 (NSEO) improved the TAC of the serum from 0.56 ± 0.02 to 0.78 ± 0.03 IU/mL and from 0.47 ± 0.02 to 0.91 ± 0.04 IU/mL, respectively. However, total antioxidant capacity decreased momentously in D_1 (control) from 0.52 ± 0.030 to 0.29 ± 0.02 IU/mL. Maximum MDA level (8.71 ± 1.136 nmol/g) was recorded in D_1, whereas the minimum MDA level was noted in D_3 group (4.67 ± 1.202 nmol/g). Conjugated dienes varied significantly ($P < 0.05$); however, maximum values were observed in D_1 (control) groups followed by D_2 (NSFO), and least conjugated dienes were observed in D_3 (NSEO).

3.4. Correlation Matrix. The association of various parameters has been evaluated using correction matrix. This was evaluated by using the technique of multiple regression or order to check the interdependence of various variables on each other (Table 5). It is obvious from the correlation coefficients that serum glucose levels are positively associated with lipid profile, that is, cholesterol, LDL, and triglycerides ($P < 0.01$) and indices of oxidative damage, that is, MDA and conjugated dienes ($P < 0.01$); However, glucose is in negative association with insulin ($P < 0.01$) and total antioxidant capacity ($P < 0.05$). Insulin level was also found to be inversely associated with lipid profile, however, the same parameter was in linear association with that of total antioxidant capacity ($P < 0.01$). Cholesterol, LDL, and triglycerides were positively correlated with each other ($P < 0.01$) but were found to be inversely associated with HDL ($P < 0.01$) in diabetic rats modeling.

4. Discussion

Diabetes mellitus is one of the most common noncommunicable diseases that targets multiorgan systems. In the recent years, scientists over the globe directed their efforts for the exploration of some novel food sources as hypoglycemic agents [6]. In the early stages, drugs and diet can mediate the adverse consequences. If there arose complications like cardiovascular disorders or renal malfunction, the strategies need to focus through some alternative arrangements. However, prevention of root causes and managing diabetes at early stages is the better remedy. Under such circumstances,

natural therapies including the use of medicinal plants, functional foods, and nutraceuticals are important [5, 25].

Many researches have studied the possible role of *Nigella sativa* fixed and essential oils for the management of diabetes. In the present investigation, *N. sativa* fixed and essential oils decreased the blood glucose significantly. The group of rats fed on control diet witnessed the abrupt increase by twofold in blood glucose during the 56 days of study duration. Diets containing NSFO and NSEO resulted in enhanced insulin secretions as compared to substantial decrease in control group. The increased insulin secretion certainly is a possible reason for the drop in glucose level in experimental diets groups. The present statistics proved that *N. sativa* fixed and essential oils hold insulinotropic potential and mediated by extra pancreatic action. Supportive evidences were presented by Kanter [26], who observed that *N. sativa* and its constituent thymoquinone, at a dose of 400 and 50 mg/kg body weight/day, caused a marked decrease in glucose and increased the serum insulin concentrations in streptozotocin-induced diabetic rats. Earlier, Meral et al. [27] observed lower glucose levels in diabetic rats treated with *N. sativa* in comparison to control. Moreover, hepatic glucose production contributes significantly to hyperglycemia in diabetic patients. It tends to decrease, due to *N. sativa* treatments as reported by Fararh et al. [28]. The aforementioned reports suggested that the mechanisms behind hypoglycemic potential could include increased insulin concentrations, protection of the β-cells of islets of Langerhans, prevention of oxidative damage, and extra pancreatic action [11].

Diabetes mellitus results in frequent changes in the plasma lipid concentration that certainly contribute to the development of vascular diseases [1]. In the present investigation, cholesterol, triglycerides, and LDL increased in the control group during the entire study. In contrary, NSFO treatment reduced the same traits by 10.32, 7.89 and 23.74%, respectively, whilst NSEO treatment decreased the 8.89%, 5.33, and 19.89% cholesterol, triglycerides, and LDL, respectively. Lipid lowering potential is substantially beneficial for the subjects with diabetes and multiple cardiovascular risk factors. Kanter et al. [11] and Meral et al. [27] hypothesized that the cholesterol deposition is due to increased activities of cholesterol synthesizing enzyme and reduced activities of antioxidants. Considering the fact that the serum cholesterol was in negative association with tocopherols, the supplementation of diets with NSFO (rich source of tocopherols) and NSEO (rich source of antioxidant) can reduce the elevated cholesterol level [29].

N. sativa inhibited the lipid peroxidation of biological membranes and prevented the lipid-peroxidation-induced liver damage in diabetic rabbits [27]. The results of El-Missiry and El-Gindy [30] showed marked increase in lipid peroxides and decreased antioxidant enzymes in diabetes mellitus. Later, Türkdoĝan et al. [31] observed occurrence of oxidative decomposition of liver because of increased lipid peroxidation [27]. This effect might be due to increased level of antioxidants production that protects tissues from the hazards of free radicals. The bioactive molecules present in such functional foods, for example, α-lipoic acid, tocopherols, and selenium, are helpful to control diabetes and its complications. In similar type of rats modeling, Fararh et al. [28] enumerated the differences in feed intake and body weight of diabetic rats. *N. sativa* essential oil might mediate hypoglycemic impact through extra pancreatic actions, stimulated insulin release, and partial regeneration/proliferation of pancreatic β cells [11].

There are several evidences that complications related to diabetes are associated with oxidative stress, induced by reactive oxygen species. Accordingly, interest has grown in using natural antioxidants for prevention or protection against oxidative damage [14]. The results of the present exploration indicated that production of free radicals, that is, MDA and conjugated dienes, increased by 59.00 and 33.63%, respectively, in control, whilst groups of rats fed on *N. sativa* fixed and essential oils based diets improved the antioxidant defense mechanism by reducing the MDA and conjugated dienes levels. In this study, hyperglycemia was linearly associated with antioxidant damage, that is, MDA and conjugated dienes levels, whilst it was inversely correlated with total antioxidant capacity and glutathione contents. STZ results in depletion of antioxidant system in both blood and tissues and promotes the generation of free radicals [32]. *N. sativa* essential oil owing to its antioxidant potential is useful in controlling the diabetic complications in experimental diabetic rats. Results of present study supported the traditional use of *N. sativa* and its derived products as a treatment for hyperglycemia and related abnormalities. Moreover, *Nigella sativa* fixed and essential oils significantly ameliorate free radicals and improve antioxidant capacity, thus reducing the risk of diabetic complications.

5. Conclusion

The experimental diets containing *Nigella sativa* fixed and essential oil possess hypoglycemic properties; NSEO was more effective in reducing the extent of oxidative damage. Moreover, both extracts improved significantly the lipid profile including total cholesterol, triglycerides, and LDL and reduced the serum malonaldehyde level, improving antioxidant capacity of the body. Data obtained in the present study are helpful in designing further studies in human subjects in order to validate the use of *N. sativa* oils in prevention and in treatment of diabetes and related conditions.

Conflict of Interests

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

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Research Article

Protective Effect of Free and Bound Polyphenol Extracts from Ginger (*Zingiber officinale* Roscoe) on the Hepatic Antioxidant and Some Carbohydrate Metabolizing Enzymes of Streptozotocin-Induced Diabetic Rats

Mutiu Idowu Kazeem,[1] Musbau Adewunmi Akanji,[1] Musa Toyin Yakubu,[1] and Anofi Omotayo Tom Ashafa[2]

[1] *Department of Biochemistry, University of Ilorin, PMB 1515, Ilorin, Nigeria*
[2] *Phytomedicine and Phytopharmacology Research Group, Department of Plant Sciences, University of the Free State, Qwaqwa Campus, Phuthaditjhaba, South Africa*

Correspondence should be addressed to Anofi Omotayo Tom Ashafa; tom.ashafa@gmail.com

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This study investigated the hepatoprotective effects of polyphenols from *Zingiber officinale* on streptozotocin-induced diabetic rats by assessing liver antioxidant enzymes, carbohydrate-metabolizing enzymes and liver function indices. Initial oral glucose tolerance test was conducted using 125 mg/kg, 250 mg/kg, and 500 mg/kg body weight of both free and bound polyphenols from *Z. officinale*. 28 day daily oral administration of 500 mg/kg body weight of free and bound polyphenols from *Z. officinale* to streptozotocin-induced (50 mg/kg) diabetic rats significantly reduced ($P < 0.05$) the fasting blood glucose compared to control groups. There was significant increase ($P < 0.05$) in the antioxidant enzymes activities in the animals treated with both polyphenols. Similarly, the polyphenols normalised the activities of some carbohydrate metabolic enzymes (hexokinase and phosphofructokinase) in the liver of the rats treated with it and significantly reduced ($P < 0.05$) the activities of liver function enzymes. The results from the present study have shown that both free and bound polyphenols from *Z. officinale* especially the free polyphenol could ameliorate liver disorders caused by diabetes mellitus in rats. This further validates the use of this species as medicinal herb and spice by the larger population of Nigerians.

1. Introduction

Diabetes is a chronic metabolic disorder which has been in existence since time immemorial and affects about 4-5% of the population worldwide [1]. Its complications cause disability in its sufferers leading to frequent hospitalization and huge financial burden [2]. It is a "modern day epidemic" and is given attention as a worldwide public health problem. The number of people suffering from this disease globally is rising on a daily basis with an estimated 366 million people likely to be affected by the year 2030 as against 191 million estimated in 2000 [3]. The management of diabetes mellitus is considered a global problem and successful treatment is yet not available.

Studies have shown that diabetes mellitus is related to oxidative stress, leading to an increased generation of free radicals such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) or reduced antioxidant defense mechanism [4, 5]. Effect of oxidative stress in the progression of diabetes mellitus is not only by free radical generation but also due to non-enzymatic protein glycation, impaired antioxidant enzyme system and formation of peroxides [6] which may lead to liver disorder.

Pharmaceutical agents from plants such as polyphenols have been utilised in the treatment of many diseases including diabetes and its complications [7, 8]. Polyphenols are integral part of human diet and are present in plant extracts that have been used in alternative medicine. The antioxidant potential

of polyphenols is believed to account in large part for their pharmacological activities [9]. Polyphenols show several pharmacological activities including apoptotic, antidiabetic, antitumor, cardiovascular protection, hepatoprotective, and cell proliferation activities [10].

This study was aimed at evaluating the hepatoprotective effects of polyphenols extracted from *Zingiber officinale* in streptozotocin-induced diabetic rats. Since the medicinal attribute of this species has not been reported in any scientific literature, yet *Z. officinale* is a medicinal herb/spice in Nigeria and may also help in the amelioration of liver damages caused by diabetes.

2. Materials and Methods

2.1. Plant Material. *Zingiber officinale* was purchased from the Central Spices Market in Mile 12 area, Ketu, Lagos, Nigeria. The identification and authentication of the sample were done by Dr. Kadiri at the Department of Botany of the University of Lagos, Akoka, Lagos, and voucher specimen (LUH 4730) was deposited in the university herbarium.

2.2. Experimental Animals. Albino rats were obtained from the Animal House of the Department of Biochemistry, Lagos State University, Ojo, Lagos. All the animals were maintained under laboratory conditions of temperature (22 ± 2°C), humidity (45 ± 5%), and 12 h day: 12 h night cycle and were allowed access to food (standard pellet diet) and water *ad libitum*.

2.3. Chemicals. Streptozotocin (STZ) was a product of Alexis Biochemical, San Diego CA (92101), USA, while glibenclamide was a product of Sigma, St. Louis (93101), MO, USA. The assay kit for glucose was obtained from Randox Laboratories, Antrim (BT41), UK. Other chemicals and reagents were of Analar grade, and water used was glass distilled.

2.4. Extraction of Free Phenolic Compounds. A known mass (3 kg) of *Zingiber officinale* was crushed in 80% acetone (1 : 2 w/v) using a Waring blender (Waring Commercial, Torrington, CT) for 5 minutes [11]. The sample was homogenized in a Polytron homogenizer (Glen Mills Inc., Clifton, NJ) for 3 minutes. The homogenates were filtered under vacuum using Buchner funnel and Whatman no. 2 filter paper (Whatman PLC, Middlesex, UK). The filtrate was concentrated using a rotary evaporator under vacuum and later freeze-dried in a lyophilizer (Ilshin Lab. Co. Ltd, Seoul, Republic of Korea). The extract was stored frozen at −20°C for 24 h before the commencement of the experiment.

2.5. Extraction of Bound Phenolic Compounds. Residue from the free phenolic extraction was drained and hydrolyzed with 2 L of 4 M NaOH for 1 h with constant shaking [11]. The mixture was acidified with concentrated hydrochloric acid to pH 2. The acidified mixture was extracted six times with ethyl acetate by partitioning and the supernatant pooled together and concentrated using rotary evaporator and subsequently freeze-dried using Virtis Bench Top (SP Scientific Series, USA) freeze dryer. The extract was stored frozen at −20°C for 24 h before the commencement of the experiment.

2.6. Induction of Diabetes. Rats were fasted for 18 h after which diabetes mellitus (type 2) was induced by single intraperitoneal injection of freshly prepared STZ (50 mg/kg bw) in 0.1 M citrate buffer (pH 4.5) [12]. Diabetes was confirmed in these rats within a period of 7 days. The control animals were administered citrate buffer (pH 4.5). To overcome the initial hypoglycaemic shock, which may occur as a result of the STZ administration, diabetic rats were given 5% glucose solution *ad libitum* for 24 h. Blood was collected from the tail, and the blood glucose level of each rat was determined. Rats with a fasting blood glucose range of 15–20 mmol/L were considered diabetic and included in the study [12].

2.7. Oral Glucose Tolerance Test (OGTT). Twenty-four rats were randomised into 8 groups of 3 animals each. All the rats were fasted overnight (12–14 h) prior to this test. Group 1 was made up of normal rats while the others were streptozotocin-induced diabetic rats. Groups 3, 4, and 5 were administered 125, 250, and 500 mg/kg body weight of free polyphenol from *Z. officinale* while groups 6, 7, and 8 were administered 125, 250, and 500 mg/kg body weight of bound polyphenol from *Z. officinale,* respectively. Forty-five (45) minutes following the various treatment schedules, each rat was administered an oral glucose load (3 g/kg body weight). All rats were tested for blood glucose levels at 45 minutes before the administration of the extracts, 0 minutes (just before the oral administration of glucose load), and 30, 45, 60, and 120 minutes after the glucose load.

2.8. Experimental Design. A total of 40 male rats weighing 200 ± 10 g (8 normal; 32 STZ-diabetic rats) were used. The rats were randomised into five groups of eight animals each. Group 1 comprises normal rats administered with vehicle alone (distilled water) and serve as normal control; group 2 consisted of STZ-induced diabetic rats only; groups 3 and 4 comprise STZ-induced diabetic rats administered with 500 mg/kg bw free and bound polyphenol extracts of *Z. officinale*, respectively. Group 5 consisted of STZ-induced diabetic rats administered with glibenclamide (0.6 mg/kg bw). The extract was suspended in distilled water and was orally administered daily for 28 days using orogastric tube. Day 1 was regarded as the first day of treatment with polyphenol extracts or glibenclamide. After 28 days of administration, animals were humanely sacrificed under halothane euthanasia, and blood was collected through cardiac puncture, and serum was separated immediately. The rats were dissected; the liver was excised, freed of surrounding tissues, blotted with clean tissue paper, weighed, and homogenized in ice-cold 0.25 M sucrose solution (1 : 5 w/v). The homogenates were centrifuged at 105 ×g for 15 minutes to obtain the supernatants that were kept frozen overnight at −20°C before being used for the assays.

2.9. Biochemical Parameters

2.9.1. Determination of Glucose Concentration. Glucose concentration was estimated by the glucose oxidase method described by Trinder [13].

2.9.2. Determination of Hepatic Antioxidant Enzymes. Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were determined using the procedure described by Aebi [14], S. Marklund and G. Marklund [15], and Paglia and Valentine [16], respectively. Reduced glutathione (GSH) content was determined according to the method described by Ellman [17].

2.9.3. Determination of Hepatic Carbohydrate Enzymes. Hexokinase, phosphofructokinase, glucose-6-phosphatase, and fructose-1,6-bisphosphatase activities were assayed in the liver by the methods of Brandstrup et al. [18], Castano et al. [19], Hikaru and Toshitsugu [20], and J. M. Gancedo and C. Gancedo [21], respectively. Glycogen content was determined according to the procedure described by Ong and Khoo [22].

2.9.4. Liver Function Test. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin and bilirubin were measured by standard techniques using Reflotron Plus Dry chemistry analyzer (Roche Diagnostics, Mannheim, Germany).

2.9.5. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5 statistical package (GraphPad Software, USA). The data were analysed by one way analysis of variance (ANOVA) followed by Bonferroni test. All the results were expressed as mean ± SE for 8 rats in each group.

3. Results

3.1. Effect on Postprandial Blood Glucose. The effects of administration of free and bound polyphenol extracts from *Z. officinale* on postprandial blood glucose of male Wistar rats are presented in Figures 1 and 2. The groups of diabetic rats treated with 500 mg/kg of both free and bound polyphenol extracts of *Z. officinale,* respectively, displayed most significant reduction ($P < 0.05$) at all periods tested in comparison to the diabetic control group. Generally, the reduction in postprandial blood glucose was more pronounced in the free polyphenol treated animals compared to the bound polyphenol treated animals and control groups, respectively.

3.2. Effect on Fasting Blood Glucose. The results of fasting blood glucose (FBG) of the polyphenol treated animals during the 28-day experimentation are presented in Table 1. The diabetic control animals had increasing fasting blood glucose throughout the period of the experiment rising from 18.34 on the 1st day to 24.87 mmol/L on the 28th day. The FBG of all groups was significantly different from the normal control ($P < 0.05$). However, the animals treated with *Zingiber officinale* free polyphenol displayed significant reduction ($P < 0.05$) in fasting glucose reaching 12.22 mmol/L from the initial 20.44 mmol/L at the end of the experimental period. Similarly, there was significant reduction in fasting glucose level in the animals treated with bound polyphenol (from 21.80 to 16.56 mmol/L) but not compared to the free polyphenol and glibenclamide treated animals.

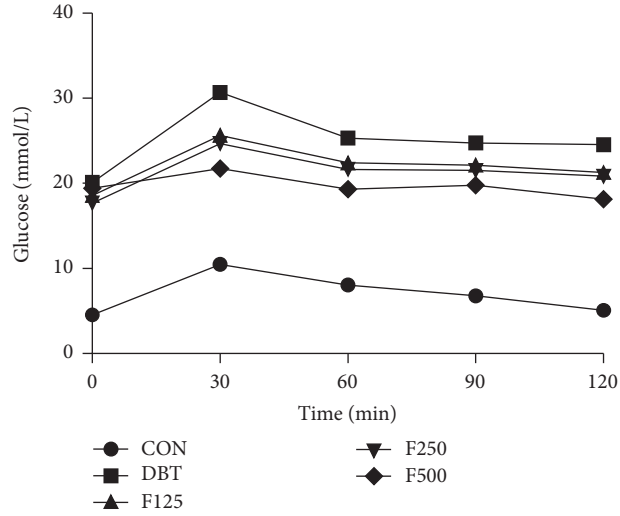

Figure 1: Effect of free polyphenol extract of *Z. officinale* on postprandial blood glucose in streptozotocin-induced diabetic rats. Values are given as mean ± SEM, $n = 3$. CON: normal control, DBT: diabetic control, F125: diabetic rats + 125 mg/kg bw free polyphenol, F250: diabetic rats + 250 mg/kg bw free polyphenol, F500: diabetic rats + 500 mg/kg bw free polyphenol.

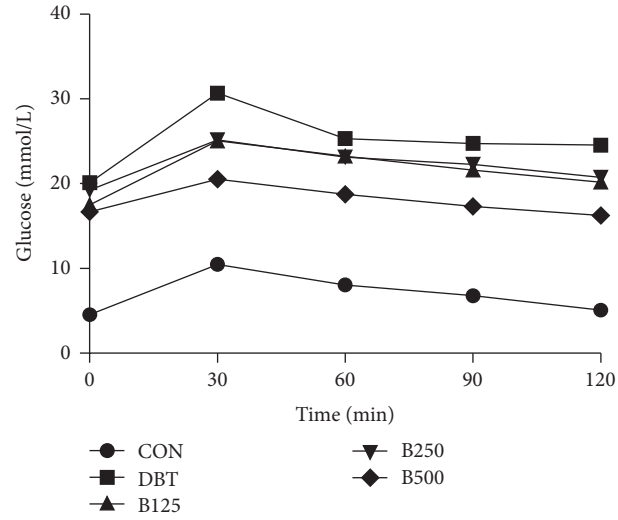

Figure 2: Effect of bound polyphenol extract of *Z. officinale* on postprandial blood glucose in streptozotocin-induced diabetic rats. Values are given as mean ± SEM, $n = 3$. CON: normal control, DBT: diabetic control, B125: diabetic rats + 125 mg/kg bw bound polyphenol, B250: diabetic rats + 250 mg/kg bw bound polyphenol, B500: diabetic rats + 500 mg/kg bw bound polyphenol.

3.3. Effect on Hepatic Antioxidant Enzymes. The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase were significantly decreased ($P < 0.05$)

Table 1: Effect of administration of polyphenols from *Z. officinale* on the fasting blood glucose of normal and streptozotocin-diabetic rats.

Group	Glucose (mmol/L)		
	Day 1	Day 14	Day 28
Normal control	4.51 ± 0.18^{a}	4.44 ± 0.20^{a}	4.78 ± 0.36^{a}
Diabetic control	18.34 ± 0.47^{b}	21.8 ± 0.67^{b}	24.87 ± 1.13^{b}
Diabetic + free polyphenol	20.44 ± 0.72^{b}	21.38 ± 0.95^{b}	12.22 ± 1.63^{d}
Diabetic + bound polyphenol	21.8 ± 0.67^{b}	22.46 ± 0.91^{b}	16.56 ± 0.95^{c}
Diabetic + glibenclamide	20.48 ± 0.83^{b}	17.56 ± 0.39^{c}	9.22 ± 0.78^{d}

Values are mean ± S.E.M. of 8 rats per group. Test values down the vertical columns carrying different superscripts for each day are significantly different ($P < 0.05$).

Table 2: Effect of administration of polyphenols from *Z. officinale* on antioxidant enzymes' activities in the liver of normal and streptozotocin-diabetic rats.

Group	U/mg protein			
	CAT	SOD	GPx	GSH
Normal control	65.20 ± 2.89^{a}	33.60 ± 1.73^{a}	51.63 ± 2.75^{a}	16.21 ± 1.16^{a}
Diabetic control	11.25 ± 1.16^{b}	7.83 ± 1.21^{b}	19.63 ± 1.24^{b}	28.74 ± 2.67^{b}
Diabetic + free	34.56 ± 2.31^{c}	24.35 ± 2.17^{c}	25.47 ± 1.68^{b}	23.37 ± 1.19^{b}
Diabetic + bound	21.27 ± 1.83^{d}	18.53 ± 1.17^{d}	23.75 ± 2.76^{b}	25.46 ± 1.70^{b}
Diabetic + GBN	40.42 ± 1.76^{c}	25.45 ± 1.29^{c}	35.20 ± 1.79^{c}	20.26 ± 1.12^{c}

Values are mean ± S.E.M. of 8 rats. Test values down the vertical columns carrying different superscripts for each parameter are significantly different ($P < 0.05$). CAT: catalase, SOD: superoxide dismutase, GPx: glutathione peroxidase, GSH: reduced glutathione.

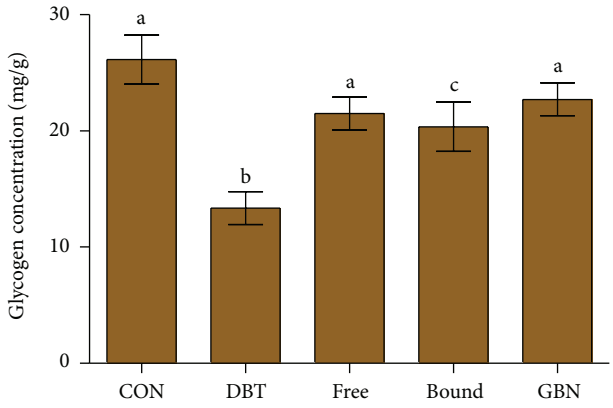

Figure 3: Effect of oral administration of polyphenols from *Z. officinale* on the glycogen concentration in the liver of normal and streptozotocin-induced diabetic rats. Values are mean ± S.E.M. of 8 rats. Bars carrying different superscripts are significantly different ($P < 0.05$). CON: normal control, DBT: diabetic control, Free: diabetic rats + free polyphenol, Bound: diabetic rats + bound polyphenol, GBN: diabetic rats + glibenclamide.

in the diabetic control rats while the reduced glutathione had significant increase ($P < 0.05$) (Table 2). The activities of CAT and SOD in the free polyphenol treated animals were compared favourably with those treated with GBN. Although there were increases in the CAT and SOD activities of the bound polyphenol treated animals but the increase was not comparable to what is obtained in the CAT and SOD of free polyphenol treated animals. Similarly, there was significant increase in the GPx and GSH of GBN treated animals but these enzymes level remained similar in both diabetic control and polyphenol-treated animals.

3.4. Effect on Carbohydrate Metabolizing Enzymes. Table 3 shows the effect of administration of polyphenols from *Z. officinale* on the activities of carbohydrate metabolic enzymes in the liver of normal and streptozotocin-induced diabetic rats. There were fluctuations in the activities of hexokinase and phosphofructokinase in all the groups of animals studied but was not significantly different ($P > 0.05$) from one another. The activities of fructose-1,6-bisphosphatase and glucose-6-phosphatase, significantly reduced ($P < 0.05$) in the diabetic control rats compared to the normal control. The 28-day administration of polyphenols especially free polyphenol from *Z. officinale* significantly increased fructose-1,6-bisphosphatase and glucose-6-phosphatase activities in the diabetic rats.

3.5. Effect on Glycogen Concentration. There was a significant reduction ($P < 0.05$) in the glycogen content of diabetic control rats in comparison to the normal control (Figure 3). Diabetic rats treated with polyphenols of *Zingiber officinale* increased the level of glycogen and it is particularly significantly elevated ($P < 0.05$) in the free polyphenol treated animals. The results were compared favourably with the glycogen, concentration of the glibenclamide-treated diabetic rats.

3.6. Effect on Liver Function Indices. There was significant elevation ($P < 0.05$) in the activities of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the diabetic untreated animals when compared to the normal

Table 3: Effect of administration of polyphenols from *Z. officinale* on carbohydrate metabolic enzymes' activities in the liver of normal and streptozotocin-diabetic rats.

Group	U/mg protein			
	HK	PFK	F-1,6-BP	G-6-P
Normal control	2.95 ± 0.03^{a}	3.81 ± 0.06^{a}	3.56 ± 0.58^{a}	10.50 ± 1.16^{a}
Diabetic control	0.93 ± 0.36^{a}	2.56 ± 0.23^{a}	8.42 ± 1.16^{b}	25.62 ± 2.89^{b}
Diabetic + free	2.55 ± 0.23^{a}	3.34 ± 0.17^{a}	4.23 ± 0.29^{a}	13.25 ± 1.16^{a}
Diabetic + bound	2.17 ± 0.29^{a}	2.86 ± 0.58^{a}	5.96 ± 0.58^{c}	15.63 ± 1.73^{a}
Diabetic + GBN	2.68 ± 0.21^{a}	3.69 ± 0.17^{a}	4.06 ± 0.51^{a}	12.36 ± 2.05^{a}

Values are mean ± S.E.M. of 8 rats. Test values down the vertical columns carrying different superscripts for each parameter are significantly different ($P < 0.05$). HK: hexokinase, PFK: phosphofructokinase, F-1,6-BP: fructose-1,6-biphosphatase, G-6-P: glucose-6-phosphatase.

Table 4: Effect of administration of polyphenols from *Z. officinale* on liver function parameters of normal and streptozotocin-diabetic rats.

Group	Liver function			
	AST (U/L)	ALT (U/L)	ALB (mg/dL)	BIL (mg/dL)
Normal control	67.2 ± 4.67^{a}	131.1 ± 9.44^{a}	5.29 ± 1.10^{a}	3.30 ± 0.54^{a}
Diabetic control	191.4 ± 22.94^{b}	251.4 ± 36.96^{b}	5.84 ± 1.50^{a}	4.84 ± 0.53^{a}
Diabetic + free	95.60 ± 15.91^{c}	156.6 ± 13.20^{c}	5.04 ± 0.93^{a}	3.10 ± 0.49^{a}
Diabetic + bound	121.0 ± 3.54^{d}	181.2 ± 7.87^{d}	6.27 ± 0.71^{a}	4.15 ± 0.72^{a}
Diabetic + GBN	83.3 ± 5.54^{c}	136.5 ± 11.92^{a}	4.89 ± 0.87^{a}	2.31 ± 0.60^{a}

Values are mean ± S.E.M. of 8 rats per group. Test values down the vertical columns carrying different superscripts for each parameter are significantly different ($P < 0.05$). AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALB: albumin, BIL: bilirubin.

control (Table 4). In contrast, there were significant reductions ($P < 0.05$) in these enzyme activities in animals treated with 500 mg/kg polyphenols from *Zingiber officinale* and glibenclamide in comparison to the diabetic control rats. There was no significant difference in the concentration of albumin (ALB) and bilirubin (BIL) of the animals in all groups studied.

4. Discussion

The choice of 500 mg/kg body weight of both free and bound polyphenol extracts of *Zingiber officinale* used in the 28-day treatment of diabetic rats in this study was predicated upon the most effective reduction of postprandial blood glucose of diabetic rats following single dose of oral administration in diabetic rats within two hours (oral glucose tolerance test). Though, 125 mg/kg and 250 mg/kg of the polyphenols also decreased postprandial blood glucose but were not comparable to the reduction elicited by the 500 mg/kg dosage (Figures 1 and 2).

Antioxidant enzymes (SOD, CAT, and GPx) play important role in the maintenance of physiological concentrations of oxygen and hydrogen peroxide by enhancing the dismutation of oxygen radicals and mopping up organic peroxides generated from exposure to STZ [23]. The data generated from the present study indicated that STZ-induced diabetes disrupted the activities of hepatic antioxidant enzymes [24]. SOD mop up superoxide radicals by converting them to H_2O_2 and oxygen while both CAT and GPx are involved in the elimination of H_2O_2 [5]. The observed decrease in the activities of SOD, CAT, and GPx in the liver of diabetic rats may be due to the rise in generation of ROS such as superoxide (O_2^-) and hydroxyl (OH^-) radical [5, 25] by STZ. It may also be that the free radicals generated inactivated the activities of these enzymes [26, 27]. This may be responsible for the insufficiency of antioxidant defences in mitigating ROS mediated damage [6]. However, administration of free polyphenol extract of *Zingiber officinale* reduced the imbalance between the generation of ROS and antioxidant enzymes' activities in diabetic rats. Therefore, treatment with free polyphenol extract of *Zingiber officinale* improved the activities of these antioxidant enzymes and may help to control the production of free radicals in sufferers of diabetes.

Glucose-6-phosphatase (G-6-P) is an important enzyme in the last step of gluconeogenesis and glycogenolysis where it catalyzes the hydrolysis of glucose-6-phosphate to glucose. Glucose is transported out of the liver to increase blood glucose concentration. Physiologically, insulin slows down hepatic glucose production by reducing glucose-6-phosphatase and fructose-1,6-bisphosphatase activities [28, 29]. The hepatic gluconeogenic enzymes, glucose-6-phosphatase, and fructose-1,6-bisphosphatase were elevated significantly in diabetic rats. This may be due to the increased synthesis of the enzymes contributing to the rise in glucose production during diabetes by the liver [30]. The administration of free polyphenols from *Zingiber officinale* normalizes the activities of these enzymes, and this is comparable to both the normal control and glibenclamide-treated diabetic rats. The observed activity may primarily be by modulating the activities of these enzymes, either through the regulation by cyclic adenosine monophosphate (cAMP) or inhibition of glycolysis and gluconeogenesis [30, 31].

Glycogen is the primary intracellular form in which glucose is stored and its levels in various tissues, particularly the liver, are a direct indication of insulin activity as insulin enhances intracellular glycogen deposition by stimulating

glycogen synthase and inhibiting glycogen phosphorylase [32]. Because streptozotocin causes selective destruction of β-cells in the pancreas, resulting in a noticeable reduction in insulin levels, it implies that glycogen levels in the liver reduce because they depend on insulin for the influx of glucose [33]. Oral administration of free polyphenol extracts of *Z. officinale* significantly improved hepatic glycogen levels of diabetic animals. This is possibly due to the reactivation of the glycogen synthase system as a result of improved insulin secretion following 28-day administration of polyphenol extract to diabetic rats [33]. It may also be due to insulinomimetic activity of the polyphenols giving rise to direct peripheral glucose uptake [34].

Aspartate aminotransferase, alanine aminotransferase, albumin, and bilirubin are considered as part of liver toxicity markers [35]. In streptozotocin-induced diabetic animals, change in the serum enzymes is directly related to alteration in the physiological functions of aspartate aminotransferase, alanine aminotransferase, albumin, and bilirubin [36]. It has been reported that the elevated activities of transaminases under insulin deficiency [37] were responsible for the increased gluconeogenesis and ketogenesis during diabetes. The increase in the activities of these serum enzymes indicated that liver dysfunction might be induced due to diabetes. Previous report has shown that the induction of diabetes in rats with STZ usually leads to necrosis of the liver tissues [38]. Therefore, increase in the activities of serum AST and ALT in diabetic untreated control animals may be due to the leakage of these enzymes from the liver cytosol into the blood stream [39] which is a pointer to the hepatotoxic effect of STZ. Conversely, treatment of the diabetic rats with free and bound polyphenol extracts of *Zingiber officinale* caused reduction in the activity of these enzymes when compared to the diabetic control group and consequently alleviated liver damage caused by STZ-induced diabetes [40].

The difference in the activities of both free and bound polyphenol extracts of *Z. officinale* may not be unconnected to the difference in their structures and compositions. Free polyphenols occur as phenolic acids and flavonoids. They are freely available and more readily absorbed, and, thus, exert beneficial bioactivities in early digestion [41]. Bound polyphenolic compounds, on the other hand, are present as a component of plant cell walls. They are present as monomeric, dimeric, or oligomeric compounds, which are esterified to the cell wall. Bound phytochemicals may not be digested by human enzymes and could survive stomach and intestinal digestion to reach the colon and be digested by bacteria flora releasing phytochemicals with health benefits [11, 42]. Therefore, the potent antioxidant and antidiabetic activities observed in the free polyphenol treated animals may be due to its reported beneficial bioactivity coupled with the ease of digestibility that made it to permeate into the blood system of the treated animals.

5. Conclusion

It can be concluded from this study that polyphenols from *Zingiber officinale* offer protection to the liver of diabetic rats. However, free polyphenols of this plant elicited better effect possibly due to the fact that they are freely available and more readily absorbed and exert beneficial bioactivities in early digestion. This study supports the ethnobotanical usage of *Zingiber officinale* rhizome in the treatment of diabetes and its associated complications.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Polysaccharides-Rich Extract of *Ganoderma lucidum* (M.A. Curtis:Fr.) P. Karst Accelerates Wound Healing in Streptozotocin-Induced Diabetic Rats

Poh-Guat Cheng,[1,2] Chia-Wei Phan,[1,2] Vikineswary Sabaratnam,[1,2] Noorlidah Abdullah,[1,2] Mahmood Ameen Abdulla,[1,3] and Umah Rani Kuppusamy[1,3]

[1] *Mushroom Research Centre, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[3] *Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Vikineswary Sabaratnam; viki@um.edu.my

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Ganoderma lucidum (M.A. Curtis:Fr.) P. Karst is a popular medicinal mushroom. Scientific reports had shown that the wound healing effects of *G. lucidum* were partly attributed to its rich polysaccharides. However, little attention has been paid to its potential effects on wounds associated with diabetes mellitus. In this study, we evaluated the wound healing activity of the hot aqueous extract of *G. lucidum* in streptozotocin-induced diabetic rats. The extract of *G. lucidum* was standardised based on chemical contents (w/w) of total polysaccharides (25.1%), ganoderic acid A (0.45%), and adenosine (0.069%). Six groups of six rats were experimentally wounded in the posterior neck region. Intrasite gel was used as a positive control and aqueous cream as the placebo. Topical application with 10% (w/w) of mushroom extract-incorporated aqueous cream was more effective than that with Intrasite gel in terms of wound closure. The antioxidant activity in serum of rats treated with aqueous extract of *G. lucidum* was significantly higher; whereas the oxidative protein products and lipid damage were lower when compared to those of the controls. These findings strongly support the beneficial effects of standardised aqueous extract of *G. lucidum* in accelerating wound healing in streptozotocin-induced diabetic rats.

1. Introduction

Diabetes mellitus is a metabolic disorder characterised by hyperglycemia with impaired carbohydrate, fat, and protein metabolism. The diabetic condition can be due to defects in insulin secretion, action, or both [1]. It affects more than 180 million individuals worldwide and by 2030 these numbers are projected to double [2]. Diabetes mellitus has led to debilitating consequences such as vasculopathy, retinopathy, and neuropathy [1]. More than 80% of diabetes mellitus is Type 2 diabetes (noninsulin dependent) characterised by peripheral resistance to the action of insulin and decreased peripheral glucose uptake or increased hepatic glucose output [1].

According to the statistics provided by the National Diabetes Information Clearinghouse, 15% of diabetic individuals suffered from diabetic foot ulcers that caused lower limbs to be amputated [3]. The hyperglycemic state in diabetic patients, especially those with peripheral vasculopathy interrupts proper wound healing. Wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. Many growth factors and cytokines released by these cell types are needed to coordinate and maintain healing [1, 4]. Furthermore, poor blood circulation and the oxygen supply in the affected area could lead to infection and gangrene formation [5]. This often leads to increased morbidity and mortality. Despite the existence of protocols to standardise wound care, the physiological impairments that can result in a diabetic foot ulcer (DFU) further complicate the healing process [6]. Currently, the wounds in diabetic patients are managed with antiseptic or antibiotic creams or

gels such as Intrasite gel for wound dressing. Wound healing takes prolonged periods and the healed wounds leave scars. In the search and discovery of safe and effective wound healing agents, natural products from plant and mushrooms are currently being explored.

Mushrooms have enormous potential as a source of both dietary protein and health-enhancing dietary supplements [7]. Among the medicinal mushrooms, *Ganoderma* species are much sought after for their wide array of medicinal properties. *Ganoderma lucidum* (M.A. Curtis:Fr.) P. Karst which belongs to the Polyporaceae family has long been known and used extensively in traditional Chinese medicine. In Malaysia, due to the high humidity and temperature throughout the year this mushroom is widely cultivated. Chemical analysis of the fruiting bodies of *G. lucidum* showed the presence of polysaccharides and triterpenoids [8–10] that may have therapeutic values in the treatment or prevention of peripheral or central inflammatory diseases. *Ganoderma* spp. are a natural source of potent bioactive antioxidant metabolites [11]. Total phenols were the major naturally occurring antioxidant components found in both *G. lucidum* and *G. tsugae* [12]. It has been reported that some plants, for example, *Annona squamosa* L. (Annonaceae), commonly known as the custard apple [13], as well as *Catharanthus roseus* L (Apocynaceae) flower [14], promoted wound healing in diabetic rats via free radical scavenging activity of flavonoids. To date, reports of the applications of the medicinal properties of mushrooms in the healing of wounds in diabetic rats are rather rare. Kwon et al. [15] reported that the cauliflower mushroom, *Sparassis crispa* Wulf.:Fr. (Aphyllophoromycetideae), improved the healing of diabetic wounds. The high β-glucan (more than 40%) accounted for the increase in the migration of macrophages and fibroblasts as well as elevated collagen synthesis. Sacchachitin and chitin membrane prepared from the aqueous extracts of *G. tsugae* were also found to have wound healing properties [16, 17].

In our preliminary study using normal rats without diabetes, the period of reepithelialisation and wound closure showed no significant difference ($P > 0.05$) between the Intrasite-gel- and *G. lucidum*-treated groups (unpublished data). The effect of wound healing by the hot aqueous extract of *G. lucidum* was comparable to that by Intrasite gel and this might be due to its high content of polysaccharides (25.1%) and the synergistic reactions combining all the medicinal properties as a whole. As wound healing in hyperglycemic state is difficult and challenging, the aim of this study was to further investigate the effect of the hot aqueous extract of *G. lucidum* on wound healing and the oxidative damage in streptozotocin-induced diabetic rats.

2. Materials and Methods

2.1. Preparation and Standardisation of Hot Aqueous Extract of G. lucidum. The fresh fruiting bodies of *G. lucidum* were obtained from Ganofarm Sendirian Berhad, a mushroom farm in Tanjung Sepat, Selangor, Malaysia. The production of *G. lucidum* was reported previously [18, 19]. The powdered fruiting bodies were subjected to hot water extraction (5 : 200, w/v) at 100°C for 8 hours. The resulting aqueous extract was freeze-dried and kept at −20°C prior to use. Heavy metal composition and microbial load of selected pathogens were analyzed by Nova Laboratory, Sepang, Malaysia, using proprietary methodology. A voucher specimen of *G. lucidum* (KLU-M 1233) was deposited in the herbarium of Mushroom Research Centre, University of Malaya.

2.2. Determination of Total Polysaccharides Content in Hot Aqueous Extracts of G. lucidum. The total polysaccharide content of the hot aqueous extract was determined using the phenol-sulphuric acid method with D-glucose as in [20]. Briefly, one mL of 5% (w/v) phenol was added to one mL of sample solution, followed by five mL of concentrated H_2SO_4. The absorbance was measured using a spectrophotometer (Shimadzu series 1601 UV/Vis) after 10 minutes at 483 nm.

2.3. Quantification of Ganoderic Acid and Adenosine Using HPLC. For determination of ganoderic acid A, Perkin Elmer Series 200 liquid chromatography equipped with a Perkin Elmer Series 200 UV detector was used. The detector signal was recorded by the Turbochrom workstation software. The column was Hypersil BDS C18 (4.6 × 250 mm) with Alltech refillable C18 Guard column (10 × 4.6 mm) (Alltech, USA). The mobile phase consisted of 5% (v/v) acetic acid in methanol and the flow rate was 1.0 mL/min. The calibration curve was prepared by injecting a series of ganoderic acid A (Sigma) reference standard dilutions. Quantification and validation of adenosine were also performed in Perkin Elmer Series 200 liquid chromatography as mentioned. The mobile phase was methanol: 10 mM monobasic potassium phosphate (15 : 85), pH 5.0, and the flow rate was 1.5 mL/min. Both ganoderic acid A and adenosine were quantified by means of calibration curves obtained from commercial standards of these compounds (Sigma).

2.4. Determination of Cupric Reducing Antioxidant Capacity (CUPRAC) of Hot Aqueous Extract of G. lucidum. The cupric reducing antioxidant capacity (CUPRAC) of the hot aqueous extract of *G. lucidum* was determined according to the method of Apak et al. [21]. Briefly, to a mixture of one mL of copper(II) (10^{-2} M), neocuproine (7.5×10^{-3} M), and ammonium acetate buffer solution (1 M), freshly-prepared mushroom extracts of varying concentrations were added to make up a final volume of four mL. After incubation at room temperature (25 ± 2°C) for 30 minutes, the absorbance at 450 nm was recorded against a reagent blank. The results of antioxidant activity were expressed in absorbance at 450 nm and compared with ascorbic acid as a positive control.

2.5. Preparation of Mushroom Extract-Incorporated Treatment Cream. The hot aqueous extract at concentrations of 5%, 10%, 15%, and 20% (w/w) was mixed with aqueous cream homogeneously. Aqueous cream was obtained from the Department of Pharmacy, Faculty of Medicine, University of Malaya (a product of Sunward Pharmaceutical Sendirian Berhad, MAL 19920890 X).

2.6. Experimental Animals. Healthy adult male Sprague Dawley rats were obtained from the animal house, Faculty

of Medicine, University of Malaya. The rats were divided randomly into six groups of six rats each. Each rat weighed between 180 to 250 g and was housed separately (one rat per cage). The animals were maintained on a standard pellet diet and tap water. The study conformed to the Principles of Laboratory Animal Care and was approved by the Ethics Committee of University of Malaya with the Ethic number ISB/14/10/2009/CPG (R). All animals received care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

2.7. Diabetes Induction. Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). After an overnight fast, diabetes mellitus was induced in six groups of rats by a single intraperitoneal injection of streptozotocin (STZ; 45 mg/kg) dissolved in citrate buffer (0.1 M, pH 4.0). Blood was drawn from the tail vein on the 7th day after the STZ injection, and the fasting blood glucose levels were estimated using a glucometer (Ames, Bayer Diagnostic). Rats with fasting blood glucose levels higher than 10 mmol/L (or 200 mg/dL) were considered diabetic and were included for the experiment [22].

2.8. Excision Wound Creation. Excision wounds were created on the 7th day after the induction of diabetes in all rats. The animals were anaesthetised with 2 mL of diethyl ether (Sigma, 98% purity). The skin was shaved using an electric clipper, disinfected with 70% alcohol, and 0.5 mL of lignocaine HCl (2%, 20 mg/mL) was injected as a local anaesthetic agent. The area of wound was outlined with methylene blue using a circular stencil. A full thickness of the excision wound of 2.0 cm in length and 0.2 cm depth was created as described by Nayak and Pinto Pereira [14]. Care was taken to avoid injuring the muscle layer, and the tension of skin was kept constant during the procedure. The wound areas were measured using a graph paper.

2.9. Topical Application of Treatment Creams. The wounds of Group 1 rats were dressed with a thin layer of aqueous cream as a blank placebo twice daily. The wounds of Group 2 rats were dressed topically twice daily with 0.2 mL of Intrasite gel as the positive control. Intrasite gel, which is a trademark of Smith and Nephew Ltd., was purchased from the University Malaya Medical Centre Pharmacy. Rats in Group 3, 4, 5, and 6 rats were treated with a thin layer of aqueous cream containing 5%, 10%, 15%, and 20% (w/w) aqueous extracts of *G. lucidum*, respectively. All applications of cream were performed with appropriate care twice a day.

2.10. Determination of the Period of Reepithelialisation. The wounds of all animals under the different treatments were observed daily. The period of reepithelisation was assessed by counting the number of days required for the complete healing including eschar falloff without any residual raw wound [13].

2.11. Determination of the Wound Closure. The wound area (mm^2) was measured at 0, 1, 4, 8, 12, and 16 days after wounding using transparency paper and a permanent marker. The rate of wound closure which is expressed as the percentage of wound reduction from the original wound was calculated using the following formula [14]:

$$\begin{aligned}&\text{Percentage of wound closure } (\%)\\&= \big[(\text{wound area on day 0} - \text{wound area on postoperation day})\\&\quad \times (\text{wound area on day 0})^{-1}\big] \times 100\%.\end{aligned} \tag{1}$$

2.12. Histological Evaluation of Healed Wounds. The specimens of skin from healed wounds and surrounding tissues were excised and stained for histological studies. Three sections (5 μm thickness) from each rat were prepared for hematoxylin and eosin (H&E) and Masson Trichrome Staining, and stained sections of each wound were examined by light microscopy. Scar width (mm) which is the junction gap between the normal dermis and dermis in the wound tissues was measured [23]. The morphological changes (fibroblast, inflammatory cell, neovascularisation, and collagen) were recorded.

2.13. Determination of In Vivo Antioxidant Activity of Experimental Rats. Blood sample was collected from the experimental rats on postoperation days 7 and 16. Antioxidant activity in the blood serum was determined by the CUPRAC method [21], which utilises copper(II)-neocuproine reagent as the chromogenic oxidising agent. Briefly, the mixture of 1 mL of $CuCl_2$ (10^{-2} M), neocuproine (7.5×10^{-3} M), and ammonium acetate buffer solution (1 M) were added into a cuvette. Then, 1090 μL of distilled water with 10 μL of blood serum was added into the reagent mixture and incubated at room temperature ($25 \pm 2^{\circ}$C) for 30 minutes. The absorbance at 450 nm was recorded against a reagent blank. The result of antioxidant activity was expressed as absorbance at 450 nm against blank. Ascorbic acid was used as positive control. Each sample of serum was triplicated for absorbance reading, $n = 6$ for each group of experimental animals.

2.14. Assessment of Oxidative Damage

2.14.1. Advanced Oxidation Protein Product (AOPP) Assay. Advanced oxidation protein product (AOPP) was determined by the method of Witko-Sarsat et al. [24]. Briefly, AOPP was determined spectrophotometrically using a microplate reader and was calibrated with chloramine-T solutions. Reagent mixture was prepared by adding 81 mL of PBS solution, 15 mL of acetic acid (50%), and 4 mL of potassium iodide. Then, 18 μL of plasma sample was added to 200 μL of reagent mixture in a 96-well microplate reader. The absorbance at 340 nm was recorded against a reagent blank. AOPP assay for each plasma sample was carried out in triplicates, $n = 6$ for each group of experimental animals. AOPPs were expressed as μmol/L chloramine-T equivalents.

2.14.2. Lipid Hydroperoxide (LPO) Assay. Lipid hydroperoxide (LPO) was determined according to the method of Esterbauer and Cheeseman [25]. Malondialdehyde (MDA)

was assayed as a marker of lipid peroxidation using colorimetric reaction, which uses 1-methyl-2-phenylindole (MPI) as chromogen. Condensation of one molecule of MDA with two molecules of MPI under acidic condition results in the formation of a chromophore which has maximum absorbance at 586 nm. A total of 150 μL of serum sample was added to 375 μL of MPI (10.3 mM) in acetonitrile and 225 μL HCl (5 M). The mixture was incubated in a water bath at 45°C for 40 minutes. Tetraethoxypropane (TEP) was used as standard solution. After centrifugation at 10,000 ×g for 5 minutes, 200 μL of the reaction mixture was pipetted into a 96-well plate and read at 586 nm in an ELISA reader (UV 1601 spectrophotometer, Shimadzu, Japan). Concentration of MDA was expressed as nM of TEP equivalents. Each of the serum samples was assayed in triplicates, $n = 6$ for each group of experimental animals.

2.15. Statistical Analysis. All values are reported as mean ± standard error mean (S.E.M). Statistical evaluation of the data was done by one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Standardisation and Analyses of Aqueous Extract of G. lucidum. The extraction yield of aqueous extract from the fruiting bodies of *G. lucidum* was 8.98% (w/w) (Table 1). The high performance liquid chromatography (HPLC) revealed that the aqueous extract contained 0.45% and 0.07% (w/w) of ganoderic acid A (Figure 1(a)) and adenosine (Figure 1(b)), respectively. Total polysaccharides were found to be 25.1% by using the phenol-sulphuric acid method. The heavy metal content was determined by using atomic absorption spectrophotometer. Analysis showed that the extract contained <5.0 ppm of arsenic, <10.0 ppm of lead, <0.5 ppm of mercury, and <0.3 ppm of cadmium. The microbial tests showed that the total bacterial count, yeast and mould count, and enterobacteriaceae were not more than 10^5, 10^4, and 10^5 cfu/g, respectively. *Salmonella* spp., *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were not detected in the aqueous extract of *G. lucidum* (Table 1).

3.2. In Vitro Antioxidant Activities of Aqueous Extract of G. lucidum. Cupric reducing antioxidant capacity (CUPRAC) assay was performed in order to have a better understanding of the antioxidative characteristic of the standardised aqueous extract of *G. lucidum*. The assay was based on the measurement of absorbance at 450 nm by the formation of a stable complex between neocuproine and copper(I). The antioxidant activity increased with increasing concentration of the extracts (Table 2). There was no significant difference ($P > 0.05$) in the activity of the aqueous extract of *G. lucidum* at 1.5 mg/mL compared to ascorbic acid (5×10^{-3} g/mL).

3.3. Wound Healing Activity of Treatment Creams on Excision Wounds in Diabetic Rats

3.3.1. Wound Reepithelialisation. Wounds dressed with mushroom extracts as well as Intrasite gel (positive control) showed considerable signs of dermal healing when compared to wounds dressed with aqueous cream only (negative control). Based on Figure 2, the diabetic rats treated with all doses of aqueous extracts of *G. lucidum* healed significantly faster ($P < 0.05$) compared to rats in the negative control group. Notably, treatment with 10% (w/w) of aqueous extract of *G. lucidum* showed the shortest reepithelisation period which was 12.37 ± 0.49 days, then followed by 20%, 5%, and 15% of *Ganoderma* extracts.

3.3.2. Wound Closure. Table 3 shows the percentage of wound closure at various time intervals. On day 8, contraction of the wound in all the experimental rats was observed. The wound closure for diabetic rats receiving Intrasite-gel treatment at day 8 was 136.86 ± 22.8 mm^2 (55.0% of wound closure from day 0). It may be due to dehydration of the necrotic tissue and drying of exudates. However, continuous

Table 1: Specifications of standardised aqueous extract of *Ganoderma lucidum*.

Parameters	Specifications
Appearance and colour	Brown-yellow powder
Marker components	
Total polysaccharides	25.1%
Ganoderic acid A	0.45%
Adenosine	0.069%
Microbial tests	
Total bacterial count	Not more than 10^5 cfu/g
Yeast and mould count	Not more than 10^4 cfu/g
Enterobacteriacea	Not more than 10^3 cfu/g
Salmonella spp.	Absent
E. coli	Absent
S. aureus	Absent
P. aeruginosa	Absent
Heavy metal tests	
Arsenic	Not more than 5.0 ppm
Lead	Not more than 10.0 ppm
Mercury	Not more than 0.5 ppm
Cadmium	Not more than 0.3 ppm

Table 2: Antioxidant activity of standardised aqueous extract of *Ganoderma lucidum* as determined by cupric reducing antioxidant capacity (CUPRAC).

Aqueous extract of *Ganoderma lucidum* (mg/mL)	Absorbance values at 450 nm
0.10	0.15 ± 0.01^a
0.25	0.37 ± 0.01^b
0.50	0.75 ± 0.03^c
0.75	1.10 ± 0.03^d
1.00	1.42 ± 0.03^e
1.50	1.94 ± 0.03^f
Ascorbic acid (5×10^{-3} g/mL)	1.89 ± 0.03^f

Data are expressed as mean ± S.E.M ($n = 3$).
Means with different superscripts were significantly different ($P < 0.05$).

Table 3: The effects of aqueous extract of *G. lucidum* on wound closure in streptozotocin-induced diabetic rats.

Treatment	Day 8		Day 12	
	Wound closure (mm^2)	Percentage of wound closure (%)	Wound closure (mm^2)	Percentage of wound closure (%)
Aqueous cream (negative control)	39.23 ± 6.1^{a}	11	155.81 ± 20.6^{d}	62
Intrasite gel	136.86 ± 22.8^{b}	55	175.68 ± 23.1^{cd}	70
Aqueous cream containing 5% extract of *G. lucidum*	120.98 ± 17.3^{b}	47	217.12 ± 30.1^{c}	83
Aqueous cream containing 10% extract of *G. lucidum*	149.12 ± 16.5^{c}	60	242.08 ± 7.8^{a}	97
Aqueous cream containing 15% extract of *G. lucidum*	110.76 ± 16.0^{d}	44	213.90 ± 16.1^{bc}	86
Aqueous cream containing 20% extract of *G. lucidum*	101.04 ± 23.1^{d}	40	226.62 ± 13.5^{b}	91

Data are expressed as mean ± S.E.M ($n = 6$). Means with different superscripts were significantly different ($P < 0.05$).

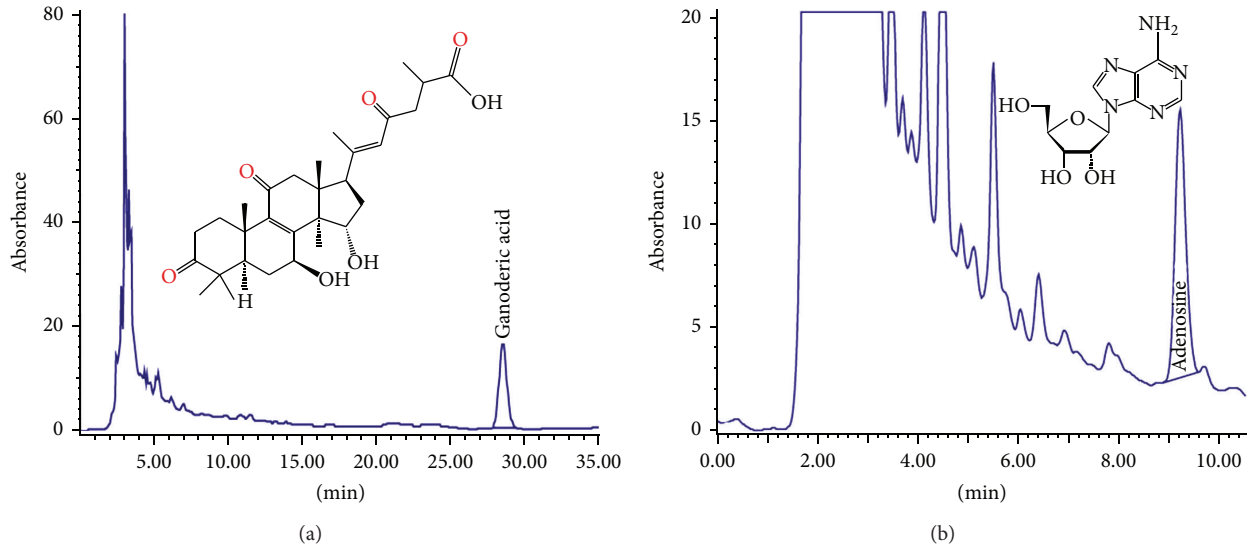


Figure 1: Chromatograms of ganoderic acid A (a) and adenosine (b) from the hot aqueous extract of *Ganoderma lucidum*.

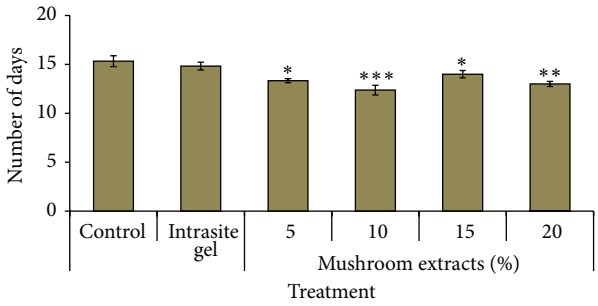
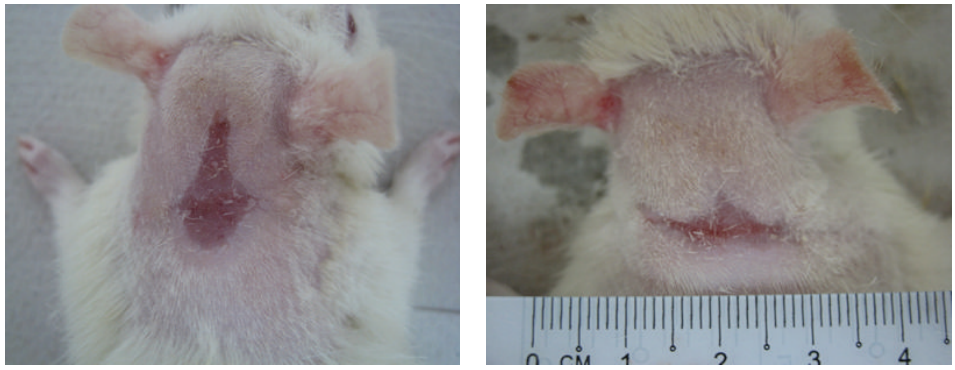


Figure 2: Time required for wound reepithelialisation in diabetic rats by aqueous extract of *G. lucidum*. Values are expressed as mean ± S.E.M. *P* value less than 0.05 is considered as significant difference. $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$ comparison between mushroom extract-treated and control rats.

application of the gel caused excessive drying of the wound tissue and wound healing was delayed at a later stage. Interestingly, diabetic rats receiving treatment of 10% (w/w) of *Ganoderma* extract exhibited a significantly ($P < 0.05$) higher wound closure which was $149.12 \pm 16.5\ mm^2$ (60%) as compared to the Intrasite gel-treated rats on day 8. On day 12, a significant ($P < 0.05$) increase in the percentage of wound closure was noted in all groups. The group treated with 10% (w/w) *Ganoderma* extract showed the highest wound closure which was $242.08 \pm 7.8\ mm^2$ (97%) and this was significantly higher ($P < 0.05$) than that of Intrasite gel ($175.68 \pm 23.10\ mm^2$; 70%).

3.3.3. Macroscopic Analysis of Wound. Macroscopic analysis showed that the scar tissue after being treated with aqueous cream (placebo) showed an irregular healing pattern on day 16 after operation (Figure 3(a)). In contrast, the scar tissue of healed wound treated with 10% (w/w) aqueous extract of *G. lucidum* was comparable to a sutured excision wound (Figure 3(b)). This indicated that aqueous extract of *G. lucidum* had stimulated inflammatory cells, fibroblasts, and keratinocytes to the wound site to induce a more rapid maturation of granulation tissue.

3.3.4. Histological Analysis of Wound. Histological analysis of wound on day 7 after operation revealed that there was more collagen matrix with newly formed capillary vessels below the endothelial cells in rats treated with extracts of *G. lucidum* (Figure 4(a)) compared to control (Figure 4(b)).

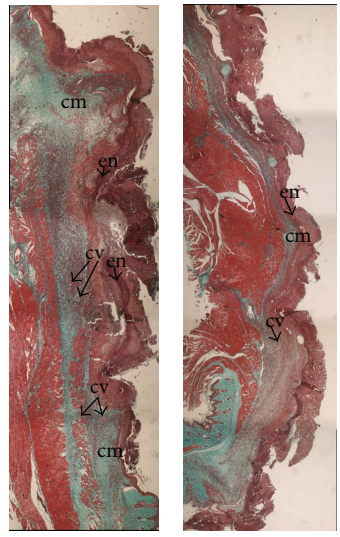


(a) (b)

Figure 3: Gross appearance of the healing of excision wound of diabetic rats at day 16 after operation. (a) Wound dressed with placebo cream—control group. (b) Wound dressed with aqueous cream incorporated with 10% (w/w) of hot aqueous extract of *G. lucidum*.

This could be due to the upregulation of collagen synthesis and angiogenesis at the wound site, with improved blood circulation which provides more oxygen and nutrients essential for the healing process. Meanwhile, histological analysis of healed wound on day 16 after operation revealed that wounds dressed with 10% (w/w) extract of *G. lucidum* showed good epithelisation and well-formed granulation tissue (Figure 5(a)). They contained markedly fewer inflammatory cells and more collagen accompanied with angiogenesis as compared to control (Figure 5(b)). The scar width was smaller at wound closure in the rats treated with extract of *G. lucidum* (Figure 6(a)) as compared to control (Figure 6(b)). The measurements for the scar width of healed wound were 2.56 ± 0.59 mm, 3.42 ± 0.75 mm, and 2.91 ± 1.06 mm for rats treated with extract of *G. lucidum*, placebo, and Intrasite gel, respectively. Meanwhile the blood vessel count for the three treatment groups was 77 ± 23.65, 68.25 ± 12.58, and 51.40 ± 16.88, respectively.

3.4. In Vivo Antioxidant Capacity and Oxidative Damage Assessment. *In vivo* antioxidant capacity and oxidative damages during wound healing were quantified in serum of rats on day 7 after operation. Results showed that antioxidant activity using CUPRAC was significantly higher ($P < 0.05$) in the diabetic rats treated with 15% (w/w) aqueous extract of *G. lucidum* when compared to the negative control and Intrasite-gel-treated rats (Table 4). The AOPP levels in diabetic rats treated with 10%, 15%, and 20% (w/w) aqueous extract of *G. lucidum* were significantly reduced ($P < 0.05$). On the other hand, the LPO levels for all the treatments were reduced with no significant difference ($P > 0.05$) to the negative control.



(a) (b)

Figure 4: Histological analysis of wound at day 7 after operation (4x Masson Trichome Staining). (a) Wound treated with 10% (w/w) extract of *G. lucidum* showed more collagen matrix with newly formed capillary vessels underneath the endothelial cells. (b) Wound treated with placebo. cm: collagen matrix, cv: capillary vessel, en: endothelial cells.

4. Discussion

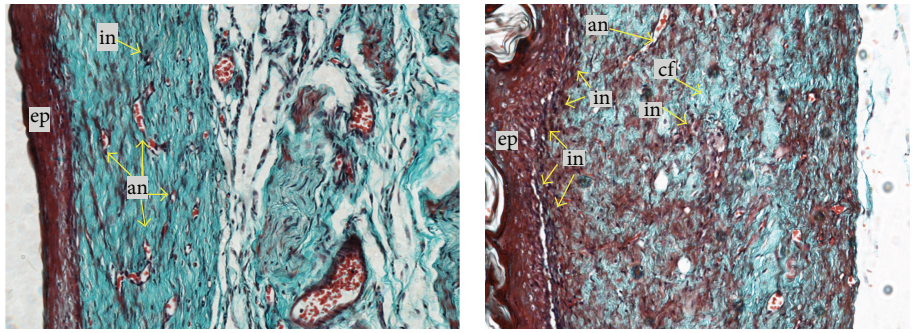
There are three phases in the wound healing process: inflammation, proliferation, and maturation [1, 26]. After initial wounding, blood extravasation causes platelet aggregation and blood clotting. These events initiate inflammation and set the stage for repair processes. During the repair phase, the provisional wound matrix is remodelled and replaced with scar tissue, consisting of new collagen fibres and proteoglycans and elastin fibres, which partially restore the structure and function of the tissue [27]. This is accomplished by the migration, proliferation, and differentiation of epithelial cells, dermal fibroblasts, and vascular endothelial cells from adjacent uninjured tissue to the wound site [28]. Eventually the injured tissue is repaired rather than regenerated [1, 27].

Our preliminary study showed that the ethanol extract of *G. lucidum* contained 0.1% of polysaccharides (unpublished data). In this study the hot aqueous extract had higher polysaccharides (25.1%) as compared to the ethanol extract. Therefore the hot aqueous extract was considered rich in polysaccharides; as compared to the ethanol extract. Bae et al. also reported that the polysaccharides isolated from

Table 4: *In vivo* antioxidant activity and oxidative status of rats during wound healing on day 7 after operation.

Treatment	CUPRAC (A_{450})	AOPP (μmol/L chloramine-T equivalents)	LPO (nM of TEP equivalents)
Aqueous cream (negative control)	0.19 ± 0.03^{a}	627.3 ± 32.6^{a}	22.30 ± 1.60^{a}
Intrasite gel	0.16 ± 0.01^{a}	581.3 ± 71.9^{a}	19.99 ± 2.18^{a}
Aqueous cream containing 5% extract of *G. lucidum*	0.20 ± 0.01^{a}	484.0 ± 67.3^{ab}	20.91 ± 0.47^{a}
Aqueous cream containing 10% extract of *G. lucidum*	0.23 ± 0.04^{ab}	253.5 ± 38.5^{c}	18.46 ± 0.84^{a}
Aqueous cream containing 15% extract of *G. lucidum*	0.30 ± 0.06^{b}	373.0 ± 58.1^{bc}	20.52 ± 1.27^{a}
Aqueous cream containing 20% extract of *G. lucidum*	0.23 ± 0.01^{ab}	353.8 ± 72.0^{bc}	20.02 ± 2.04^{a}

Data are expressed as mean ± SD ($n = 6$). Means with different superscripts were significantly different ($P < 0.05$).

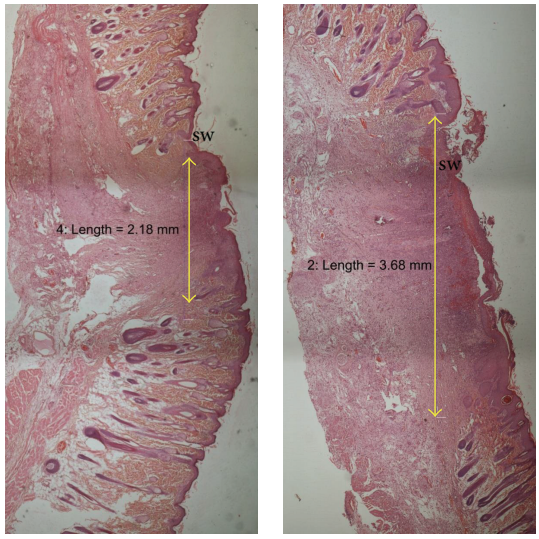


(a) (b)

Figure 5: Histological analysis of healed wound at day 16 after operation (20x Masson Trichome Staining). (a) Wound treated with 10% (w/w) extract of *G. lucidum* showed good epithelisation and well-formed granulation tissue, markedly fewer inflammatory cells, and more collagen accompanied with angiogenesis. (b) Wound treated with placebo. ep: epithelialisation, in: inflammatory cell, cf: collagen fibre, an: angiogenesis.

Phellinus gilvus (Schw.) Patouillard (mustard-yellow polypore) enhanced dermal wound healing in normal [29] and streptozotocin-induced diabetic rats [30]. These results are in agreement with our study that hot water extraction at 100°C is an appropriate method for polysaccharide extraction in mushroom. Kwon et al. [15] also reported that the β-glucan purified from medicinal mushroom *Sparassis crispa* (cauliflower mushroom) increased macrophage infiltration into the wound tissue and enhanced wound healing. Accordingly, the mechanism of β-glucan-induced wound healing was associated with increased types I and III collagen biosynthesis. While the β-glucan was orally administered to the rats in the study by Kwon et al., in this experiment, the wounds of diabetic rats were treated topically with aqueous cream containing varying concentrations of *G. lucidum* extracts. Most recently, polysaccharides purified from *Tremella fuciformis* (white jelly mushroom) and *Auricularia auricula* (wood ear mushroom) were shown to enhance wound healing using the *ex vivo* porcine skin wound healing model [31]. The water-soluble polysaccharide fractions of *G. lucidum* have been reported to have healing effects especially on ulcer lesions [32, 33]. Elsewhere, Sun et al. [34] reported that *G. lucidum* polysaccharides showed healing effects on intestinal epithelium using a nontransformed small-intestinal epithelial cell line, IEC-6 cells. Despite all the beneficial wound healing effects of *G. lucidum*, little attention has been paid to its effects on wounds associated to diabetes. Further, the major chemical components, ganoderic acid and adenosine, may contribute to the wound healing activities of the hot aqueous extracts of *G. lucidum*.

In this study we used streptozotocin to induce diabetes in rats. Streptozotocin-induced rodent is a model widely used in the study of insulin-dependent diabetes mellitus and hyperglycemia [35]. Through our observations, wound healing in diabetic rats was delayed compared to that in normal healthy rats (unpublished data). The mechanism of wound healing in healthy rats is well guided and it is through integration of multiple signals in the form of cytokines and chemokines released by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. However, in diabetic rats, healing impairment is characterised by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis, and decreased collagen and its organisation [36]. The mechanism of this alteration is thought to result from production of high level of reactive oxygen species (ROS) and increased level of apoptosis related to diabetes mellitus, which in turn impairs keratinocyte, endothelial cells, fibroblast, and collagen metabolism. In this study, wound healing on day 12 may be at the most active stage involving inflammation and cell proliferation, where fibroblast proliferated at peak and was responsible for initiating angiogenesis, epithelialisation, and collagen formation. As indicated by the results, aqueous extract of *G. lucidum* stimulated proliferation and migration of fibroblast as well as collagen synthesis in wound healing in diabetic rats.



(a) (b)

Figure 6: Histological section of healed wound showing the scar-width on day 16 after wounding (H&E, 4x). (a) Wound treated with extract of *G. lucidum*. (b) Wound treated with placebo. sw: scar width.

Low levels of antioxidants accompanied by a slight increase in markers of free radical damage played a significant role in diabetic wound healing [37]. The antioxidant defence system in the body consists of endogenous and exogenous antioxidants that work together at the molecular level to protect cell membrane, lipoproteins, and DNA from the damaging effects of free oxygen radicals [38]. Endogenous antioxidants are enzymes that are primarily physiologic in origin while exogenous antioxidants include nutrients that enter the body through the diet. Aqueous extract of *G. lucidum* which exhibited high antioxidant activity and free radicals scavenging activity may reduce oxidative damage to the cell at wound site. Oxidative damage of proteins is one of the modifications leading to severe failure of biological functions and cell death. Prolonged exposure of protein to reactive molecules leads to spontaneous modifications, such as oxidation to form advanced oxidation protein products (AOPPs) [24]. In our experiment, AOPPs in diabetic rats might be due to infection of wound or stress caused by pathogenesis of diabetes. It appears that antioxidant activity as well as immune modulation of polysaccharides may bring about the wound healing effects of this medicinal mushroom. Nevertheless, the underlying mechanism(s) of *G. lucidum* in wound healing effects in diabetic-induced model need to be further investigated.

5. Conclusion

The present study showed that topical application of aqueous cream incorporated with varying concentrations of the hot aqueous extract of *G. lucidum* significantly ($P < 0.05$) enhanced the rate of wound healing in streptozotocin-induced diabetic rats. The polysaccharide-rich (25.1%, w/w) hot aqueous extract of *G. lucidum*, which also had ganoderic acid and adenosine, increased the *in vivo* antioxidant capacity and reduced the oxidative damage during wound healing in diabetic rats. The mechanisms of wound healing contributed by this medicinal mushroom are yet to be elucidated. Furthermore, identification of other active ingredients, if any, in the hot aqueous extract of *G. lucidum* is warranted. To date, scientific investigations had validated the traditional uses of *G. lucidum* in wound management. Therefore, there is a compelling need to develop a complementary and alternative therapy for impaired wound healing, which may help to avoid amputation and improve quality of life in individuals diagnosed with diabetes. Apart from that, *G. lucidum* can be artificially cultivated and abundantly grown in the tropical country. Therefore, it could be a fairly economical therapeutic agent in diabetic wound management.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Lectin from *Crataeva tapia* Bark Improves Tissue Damages and Plasma Hyperglycemia in Alloxan-Induced Diabetic Mice

Amanda Alves da Rocha,[1] Tiago Ferreira da Silva Araújo,[1] Caíque Silveira Martins da Fonseca,[1] Diógenes Luís da Mota,[2] Paloma Lys de Medeiros,[2] Patrícia Maria Guedes Paiva,[1] Luana Cassandra Breitenbach Barroso Coelho,[1] Maria Tereza dos Santos Correia,[1] and Vera Lúcia de Menezes Lima[1]

[1] *Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego S/N, 50670-420 Recife, PE, Brazil*
[2] *Departamento de Histologia e Embriologia, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Avenida Professor Moraes Rego S/N, 50670-420 Recife, PE, Brazil*

Correspondence should be addressed to Maria Tereza dos Santos Correia; mtscorreia@gmail.com and Vera Lúcia de Menezes Lima; lima.vera.ufpe@gmail.com

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Crataeva tapia is a plant popularly used for diabetes treatment, in Brazil. Progressive decline in renal and hepatic functions has been described in patients with diabetes mellitus, and mortality rate is increased in patients with chronic liver and renal disease. This study aimed to evaluate whether *Crataeva tapia* bark lectin (CrataBL) improves hyperglycemia and renal and hepatic damage in diabetic mice. CrataBL was purified by ion exchange chromatography on CM-cellulose, and intraperitoneal administration of CrataBL to alloxan-induced diabetic mice at dose of 10 mg/Kg/day and 20 mg/Kg/day for 10 days significantly reduced serum glucose levels by 14.9% and 55.9%, respectively. Serum urea, creatinine, aspartate aminotransferase, and alanine aminotransferase were also significantly reduced after treatment with both doses of CrataBL. Furthermore, histological analysis of liver, kidney, and pancreas revealed an improvement in the tissue morphology upon treatment with CrataBL. The results suggest that CrataBL has a beneficial hypoglycemic activity and improves the renal and hepatic complications of diabetes. Therefore, this lectin may be a promising agent for the treatment of diabetes, and this might be the basis for its use in the folk medicine as an alternative treatment to manage diabetes-related complications such as hyperglycemia and tissue damage.

1. Introduction

Crataeva tapia (also known as *Crateva tapia*), a plant of Capparidaceae family, is commonly found in Pluvial Tropical Atlantic Forest and Pantanal Tropical Forest in Brazil [1]. *C. tapia* is known by Northeast Brazilian people as "paudalho" or "tapiá" and its bark is largely used in the folk medicine for the treatment of diabetes. Recently, a lectin with a molecular weight of 40 kDa (CrataBL) was purified from the aqueous extract of *Crataeva tapia* bark [2]. Lectins are carbohydrate binding proteins, of nonimmunogenic origin, that bind specifically and reversibly to different types of carbohydrates or glycoproteins and can be obtained from several sources, mainly from vegetal [3]. Several plant lectins have been demonstrated to possess a variety of biological activities including antitumor [4–6], anti-inflammatory [7, 8], antimicrobial [9–11], analgesic [4], antioxidant [3] insecticidal [2, 12–14], anticoagulant [15], and hypoglycemic [16, 17].

Diabetes mellitus is a chronic disease considered to be one of the five leading causes of death in the world, and it is a complex metabolic disease with great development of pathological changes in many tissues [18]. The

disease is characterized by alteration in the carbohydrate metabolism resulting in an increase of the glucose levels [19]. Approximately 360 million of adult people have diabetes, corresponding to 8.3% of the world with diabetes, and this is projected to rise to 552 million by 2030, corresponding to 9.9% of the world population [20]. The hyperglycemia in diabetes produces superoxide anions, which generate hydroxyl radicals, promoting cell membrane damages as a result of lipid peroxidation and protein glycation of membrane [18]. In diabetic individuals the major alterations occur in renal and hepatic tissue and have been associated with functional and morphological damage in these organs [21, 22]. Among the common complications of diabetes the nephropathy is a chronic disease that affects 40% of individuals. Diabetic nephropathy is responsible for 50% of chronic renal failure cases [23]. Furthermore, hepatic dysfunction promoted by diabetes can result in nonalcoholic steatosis, hepatomegaly amongst others [24].

Studies have reported that the doubts about the efficacy and safety of some of the oral hypoglycemic agents have prompted a search for safer and more effective drugs in the treatment of diabetes [25]. Thus, the aim of the present study was to investigate whether CrataBL from *C. tapia* bark is a metabolite with potential antihyperglycemic activity.

2. Material and Methods

2.1. Chemicals. Alloxan monohydrated and CM-cellulose was purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Insulin (Humolin N) was purchased from Lilly, Brazil. All the other chemicals used were in an analytical grade.

2.2. Plant Material. C. tapia barks were collected from the Recife City, PE, Northeast Brazil. The plant was identified by *Instituto Agronômico de Pernambuco* (IPA) and a voucher specimen was deposited (n° 61.415).

2.3. Purification of Crataeva Tapia Bark Lectin. C. tapia bark lectin was obtained through a sequential purification protocol as previously reported by Araújo et al. [2]. Powdered bark (10 g) was suspended in 0.15 M NaCl (100 mL). After homogenization in a magnetic stirrer (16 h at 4°C), followed by filtration through gauze and centrifugation (4,000 ×g, 15 min), the supernatant (crude extract) was taken as starting material. Soluble proteins in crude extract were fractionated with ammonium sulphate and the 30–60% precipitate fraction (30–60 F) was submitted to dialysis (3,500 Da cut-off membrane, 4°C) against distilled water (2 h) followed by 10 mM citrate-phosphate buffer pH 5.5 (2 h). The 30–60 F was applied (11 mg of protein, hemagglutinating activity of 1024) into a CM-cellulose chromatography column (5.2 cm × 1.6 cm) equilibrated with 10 mM citrate-phosphate buffer pH 5.5 at flow rate of 20 mLh^{-1}. The unabsorbed proteins were eluted with the buffer solution until the absorbance at 280 nm was lower than 0.05; CrataBL was eluted with 0.5 M NaCl. Protein concentration was determined according to Lowry et al. [26] using bovine serum albumin as standard.

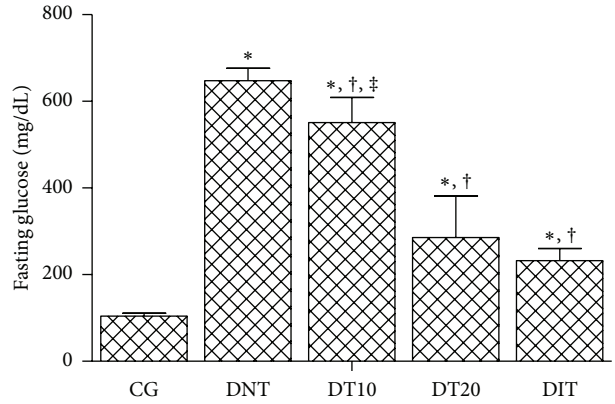

Figure 1: Fasting serum glucose levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT; $^{\ddagger}P < 0.05$ versus DIT.

2.4. Animals. Female albino Swiss mice (*Mus musculus*), six weeks of age, weighing 30 ± 5 g, bred in the Biotherium of *Departamento de Antibióticos*, UFPE, Brazil, were housed in colony cages (six mice per cage) at room temperature of 22 ± 2°C, relative humidity 40–60%, and 12 h light and 12 h dark cycle. The mice were fed standard rodent diet (Labina, Purina Brazil Ltd., Brazil) and water *ad libitum*. The experimental protocol was approved by the Animal Care and Use Committee at the Federal University of Pernambuco, Brazil (CEEA-UFPE-Ofício n° 40/06). All experimental procedures were conducted in accordance with the ethical guidelines for Care and Use of Laboratory Animals.

2.5. Induction of Diabetes in Mice. Experimental diabetes was induced in overnight-fasted mice by a single intraperitoneal injection of freshly prepared alloxan monohydrated (80 mg/kg in 0.9% NaCl solution). After alloxan administration, all animals were relocated to their cages and given free access to food and water. Diabetes was confirmed by measuring the fasting blood glucose levels 72 h after alloxan injection. The mice with serum glucose of >250 mg/dL were considered diabetic and were included in the study.

2.6. Experimental Design. The mice were split into four groups ($n = 6$, for group) as follows:

Group (I)—normoglicemic mice receiving saline solution (0.9%), as control group;

Group (II)—diabetic control mice, named diabetic nontreated;

Group (III)—diabetic mice treated with CrataBL (10 mg/kg/day, intraperitoneally) in saline solution (0.9%) for 10 days, named diabetic treated 10;

Group (IV)—diabetic mice treated with CrataBL (20 mg/kg/day, intraperitoneally) in saline solution (0.9%) for 10 days, named diabetic treated 20;

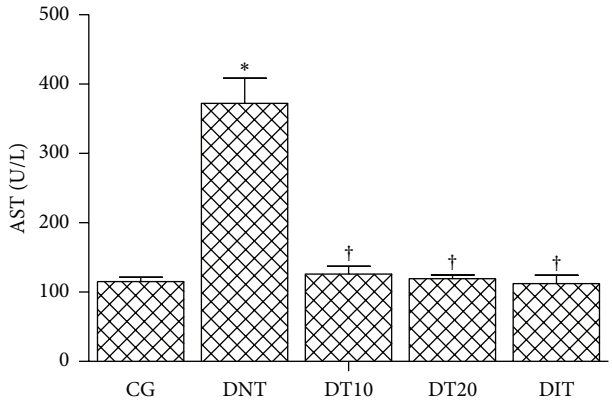

Figure 2: Serum aspartate aminotransferase levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

Group (V)—diabetic mice treated with insulin (10 mg/kg/day, intraperitoneally) for 10 days, named diabetic insulin treated.

Before and at the end of the experimental period, overnight fasting mice were anaesthetized with 2% xylazine hydrochloride (10 mg/kg) and 10% ketamine hydrochloride (115 mg/kg); blood samples were withdrawn with a capillary from mice-cavernous sinus for biochemical parameters determination [27]. The mice were sacrificed by cervical dislocation. Thereafter, pancreas, liver, and kidneys were excised and immediately fixed in 10% neutral buffered formalin for histological analysis.

2.7. Effect of CrataBL on Biochemical Data. Mice blood samples were centrifuged at 2,500 g for 15 min at 4°C (Sorvall RC6, NC, US). Sera were obtained and the levels of the glucose, urea, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by enzymatic colorimetric methods (Labtest Diagnostica, Brazil/SA) in a chemistry autoanalyzer (COBAS 6000, Roche Diagnostics, England).

2.8. Histological Analysis of Pancreas, Kidneys, and Liver. Pancreas, kidney, and liver from all groups were subjected to standard routine tissue processing technique: dehydrated in gradual ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections of 5 μm thickness were cut from each block and stained with haematoxylin-eosin for histological examination. Prepared slides were studied by light microscopy and all sections were evaluated for the tissue injury.

2.9. Statistical Analysis. Values were expressed as the mean ± SD. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. For all analysis the 0.05 level of probability was used as the criterion of significance. The analyses were carried out using software PRISMA (GraphPad Software, Inc., San Diego, CA, version 5.01).

Table 1: Serum urea and creatinine levels in diabetic mice after treatment with CrataBL.

Groups	Urea	Creatinine
CG	34.3 ± 6.8	0.30 ± 0.01
DNT	58.9 ± 5.8*	0.39 ± 0.04*
DT10	46.7 ± 6.6*,†	0.33 ± 0.05†
DT20	44.0 ± 2.9*,†	0.32 ± 0.04†
DIT	43.1 ± 2.6†	0.32 ± 0.02†

CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

3. Results and Discussion

3.1. Effect of CrataBL on Fasting Glucose. Diabetes is a complex metabolic disorder with a characteristic modulation of glucose metabolism. Chronic hyperglycemia promotes tissue damage which can be found in many organs and systems, with consequent often serious disease [28]. Alloxan, a prominent diabetogenic chemical with ability to generate reactive oxygen species formation that induce death of β cell of the pancreas by necrosis [29], is considered a good model for reproducible induction of the metabolic state of this disease in experimental animals [30–33]. Thus, in this study the mice subjected to alloxan injection showed symptoms of severe diabetes such as hyperglycemia. Insulin treatment, as a positive control, validates our model by showing the improvement in diabetes.

In a previous study, the acute toxicity of CrataBL was determined in mice; at the doses from 300 mg/kg to 2,000 mg/kg, mice did not present weight loss or death, and LD_{50} of CrataBL was 2,500 mg/kg [4]. Therefore, CrataBL concentrations used in the present study are considered safe, without problem of toxicity, and indicate that the lectin is a potential pharmaceutical substance.

As demonstrated in Figure 1, CrataBL proved to be an effective hypoglycemic agent after 10 days of treatment and showed significant antihyperglycemic activity in a dose-dependent manner, in alloxan-induced diabetic mice, and at the dose of 20 mg/kg/day it exhibited better glucose reduction (56%) than 10 mg/kg/day (15%), and it was similar to that found by the treatment with the standard drug, insulin (64%), without no significant difference ($P > 0.05$). Studies with soya bean lectin reported a decrease of 17.3% in blood glucose, and it was suggested that this effect is due to an increase in pancreatic growth stimulated by the lectin [34]. Wang et al. [35] demonstrated that *Agaricus bisporus* lectin administration could partially reverse the impaired β-cell growth potential by regulating cell cycle proteins (cyclin D1, cyclin D2, and Cdk4). So, induction of pancreatic β-cell proliferation by lectins suggests the therapeutic potential in decreasing blood glucose and treating experimental diabetes mellitus [34, 35].

Medicinal plants are gaining wide acceptably worldwide because they are the potential sources of bioactive agents in use as pharmaceutics. In a fast changing world, a number of procedures to evaluate hypoglycemia as well as the kidney

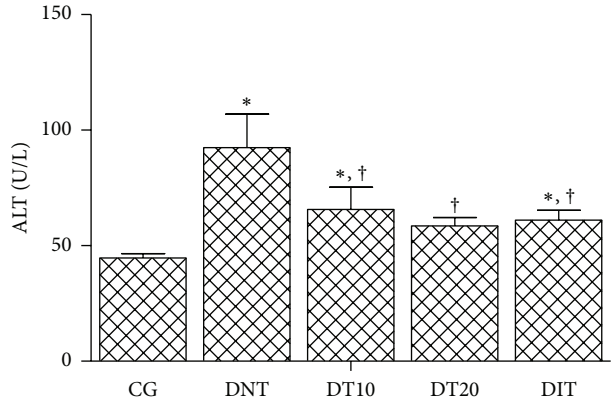

Figure 3: Serum alanine aminotransferase levels in diabetic mice after treatment with CrataBL. CG: control group; DNT: diabetic nontreated; DT10: diabetic treated with CrataBL (10 mg/kg); DT20: diabetic treated with CrataBL (20 mg/kg); DIT: diabetic treated with insulin (10 mg/kg). $^{*}P < 0.05$ versus CG; $^{\dagger}P < 0.05$ versus DNT.

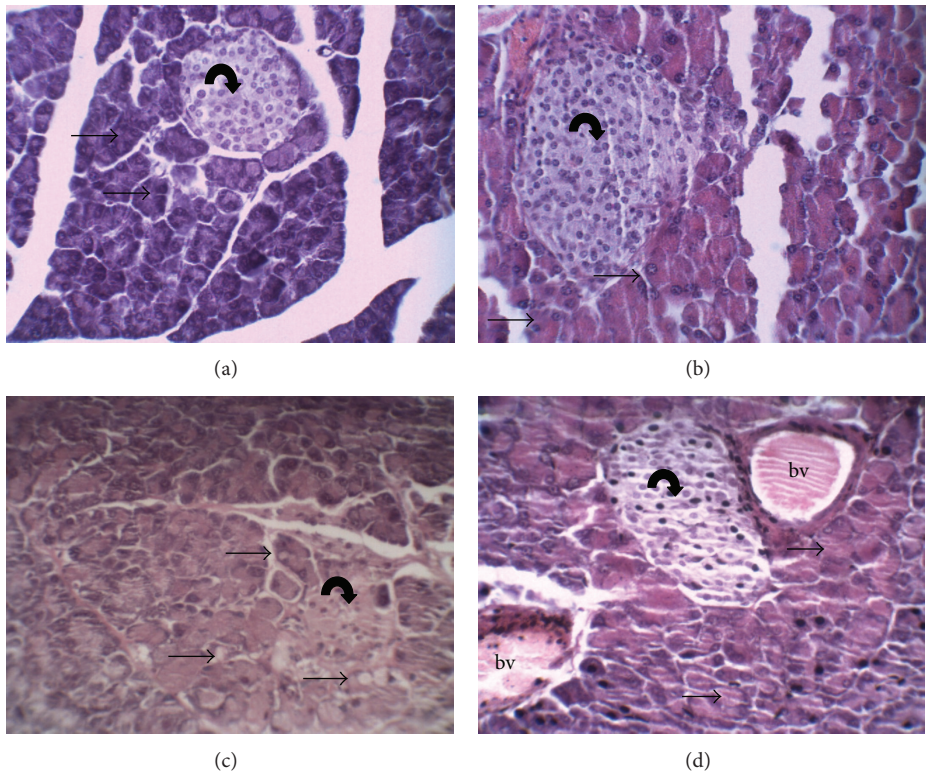

Figure 4: Histopathological changes in pancreatic tissue. (a) Control group—preserved pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows); (b) diabetic nontreated—atrophic pancreatic islet of Langerhans with few cells (curved arrow) and the presence of some vacuoles in the pancreatic acinar cells (straight arrows); (c) diabetic treated with CrataBL (10 mg/kg)—pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows); (d) diabetic treated with CrataBL (20 mg/kg)—preserved pancreatic islet of Langerhans (curved arrow) and preserved pancreatic acinar cells (straight arrows) and blood vessel (bd). Haematoxylin-eosin: 400x.

and liver damage have been used to investigate the effectivity of new natural agents which are explored by experts and clinicians [36–39].

3.2. Effects of CrataBL on Markers of Kidney Damage. As shown in Table 1, levels of urea and creatinine known as kidney function markers were significantly increased in sera of alloxan-induced diabetic mice, in comparison with normal mice. After 10 days of treatment with CrataBL, the levels of urea and creatinine significantly decreased. The diabetic mice treated with CrataBL at doses of 10 and 20 mg/kg reduced serum levels of urea by 20.7% and 25.3%, respectively, and the same doses decreased creatinine concentration by 15.4% and 17.9%, respectively. Insulin, the positive control for treatment,

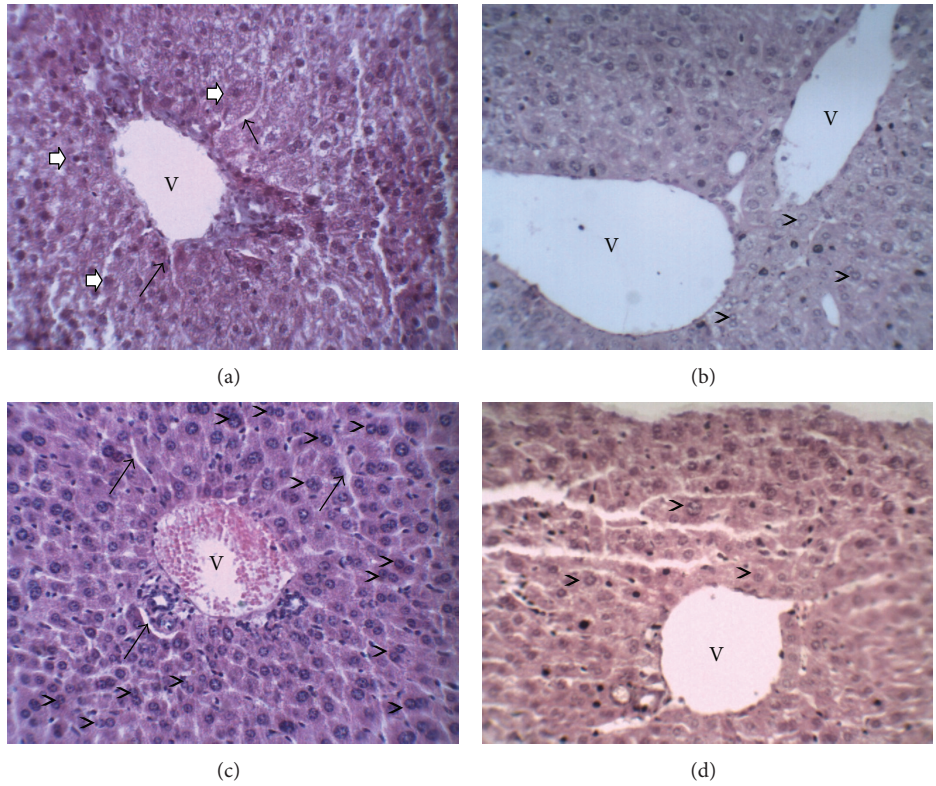

Figure 5: Histopathological changes in hepatic tissue. (a) Control group—centrilobular vein (V), preserved hepatocytes (white arrows), and sinusoidal capillaries (thin arrows); (b) diabetic nontreated—centrilobular vein with many red blood cells (V), intense mitotic activity in hepatocytes (arrowheads), and the presence of sinusoidal capillaries (thin arrows); (c) diabetic treated with CrataBL (10 mg/kg)—centrilobular vein (V) and considerable mitotic activity in hepatocytes (arrowheads); (d) diabetic treated with CrataBL (20 mg/kg)—centrilobular vein (V) and preserved hepatocytes (arrowheads). Haematoxylin-eosin: 400x.

decreased these markers of renal damage by 26.8% and 17.9%. Our results are in agreement with recent reports by Kumar et al. [39], Omara et al. [40], and Yankuzo et al. [41] who demonstrated that renal damage can be ameliorated when the levels of serum urea and creatinine are decreased by treatment with extracts of medicinal plants.

Kidney damage is usually associated with diabetes. In the initial course of disease the presence of hypertrophy of the glomeruli and tubular cells, matrix expansion, and enhanced renal blood flow is common, and these alterations have been postulated to cause loss of renal function [40, 41]. High levels of urea and creatinine are usually reported as one of the most sensitive markers of kidney damage, and it is reported that renal hypertrophy in diabetic animals is caused by an increased formation of advanced glycation end products and accumulation of glycogen granules in distal tubules [42, 43].

Thus, our results clearly indicate that CrataBL possesses an effective potential to improve kidney damage induced by alloxan-diabetes.

3.3. Effects of CrataBL on Markers of Liver Damage. As compared to the control groups, the activities of the markers of liver damage serum AST and ALT were significantly ($P < 0.05$) reduced in alloxan-induced diabetic mice after treatment with 10 or 20 mg/kg of CrataBL; the activity of AST was reduced by 66.2% and 67.9%, respectively (Figure 2) and ALT activity was decreased by 28.9% and 36.6%, respectively (Figure 3). These percentages of reduction were similar to those observed with insulin treatment. Therefore, administration of CrataBL for 10 days reversed the elevated levels of liver marker enzymes, which reflects the capability to conserve the membrane integrity of cellular and mitochondrial membranes of hepatocyte in alloxan-diabetic mice treated with this lectin.

Our results are in agreement with those of Mansour et al. [25] who reported that hepatic damage can be improved by decreasing the levels of serum AST and ALT in alloxan-induced diabetic rats subjected to treatment with herbal bioactive agents. It is well known that liver is the focal organ of oxidative and detoxifying processes [22]. Liver diseases are a high problem of health worldwide and the release of intracellular localized marker enzymes such as AST and ALT into the blood when cell and mitochondria are subjected to injury indicates hepatocytes damage [44, 45]. Furthermore, the elevated serum levels of AST and ALT in nontreated diabetic mice (Figures 2 and 3) indicate that alloxan caused liver damage and loss of the functional integrity of the hepatocyte membranes, as also evidenced in a study reported by Rajesh and Latha [45] about hepatotoxicity of polyherbal formulation.

As indicated by serum levels of AST and ALT CrataBL is able to improve liver damage.

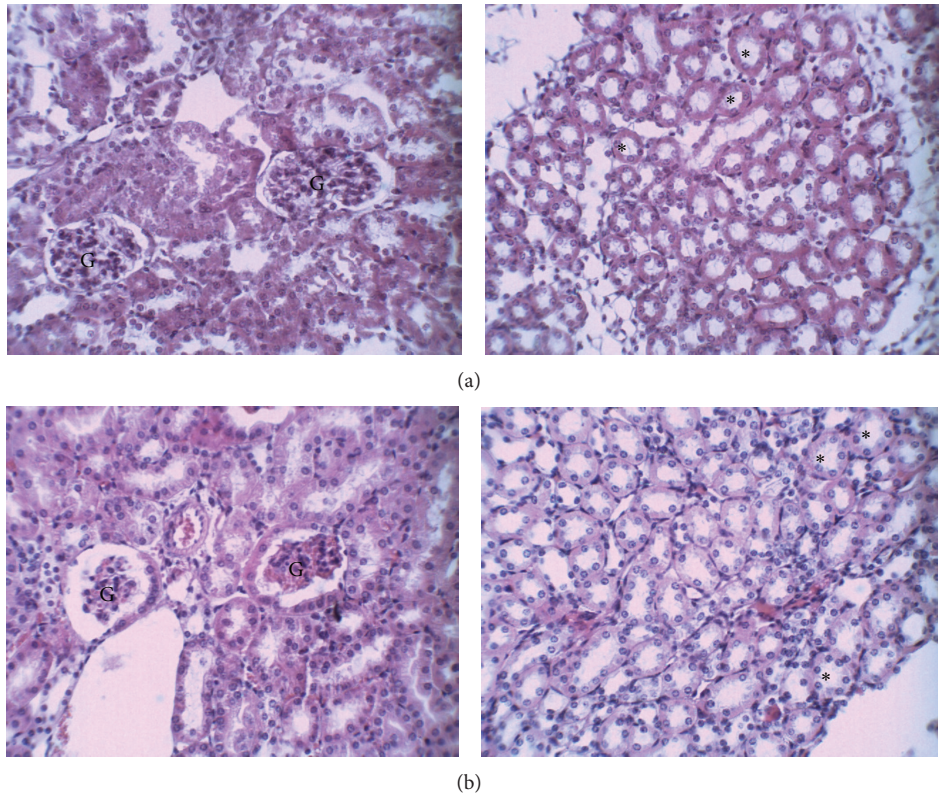

Figure 6: Histopathological changes in renal tissue of the normal and diabetic mice. (a) Control group—renal glomeruli (G) with preserved subcapsular spaces (left) and collecting tubules (stars) without changes in the medullary region (right); (b) diabetic nontreated—retracted glomerular tufts (G) with increased subcapsular space and evident thickening of parietal layer of Bowman's capsule due to have been entirely replaced by the cuboidal cells (left) and preserved collecting tubules (stars) (right). Haematoxylin-eosin: 400x.

3.4. Effects of CrataBL on the Histopathological Changes of the Pancreas, Liver, and Kidneys. The structure of the pancreas of the control and diabetic mice are shown in Figure 4. Pancreas of control group showed normal pancreatic islet of Langerhans and acinar cells (Figure 4(a)). By contrast, in alloxan-induced diabetic mice the acinar cells were altered with presence of vacuoles; furthermore deterioration of pancreatic islets was also observed (Figure 4(b)). CrataBL (10 mg/kg) treatment increased the number of pancreatic islets as compared to that of diabetic nontreated animals (Figure 4(c)). Interestingly, pancreatic section of diabetic mice treated with CrataBL (20 mg/kg) showed pancreatic islet similar to that of the control group (Figure 4(d)).

The histopathological analysis of pancreas isolated from mice administrated with alloxan alone revealed tissue damage with deterioration of pancreatic islets. In this connection, it may be observed that several authors reported such changes in pancreas tissues of mice exposed to prominent diabetogenic alloxan for its ability to induce reactive oxygen species (ROS) formation, resulting in the selective necrosis of beta cells in pancreatic islets [29, 39, 46, 47]. However, the diabetic animals treated with lectin from *C. tapia* bark showed normal architecture of pancreatic tissue, suggesting the regeneration of pancreatic islet by CrataBL administrations. The ability of lectins to stimulate pancreatic growth has been reported [48]. The regenerative action of CrataBL corroborates with *Agaricus bisporus* lectins (ABL). The ABL administration could partially reverse the impaired β-cell growth potential by induction of pancreatic islet proliferation [35]. Thus, the antidiabetic effect observed by CrataBL administration suggests the therapeutic potential in preventing and/or treating diabetes.

Figure 5 shows the photomicrographs of hepatic tissues of control group and diabetic experimental groups. The section of liver tissue of control mice demonstrates preserved hepatocytes, centrilobular vein, and sinusoidal capillaries (Figure 5(a)). In the alloxan-induced diabetic mice the histopathological analysis of hepatic tissue shows intense mitotic activity in hepatocytes (Figure 5(b)). CrataBL (10 mg/kg) treatment exhibited considerable mitotic activity in hepatocytes (Figure 5(c)). Similar to the control group, diabetic mice treated with CrataBL (20 mg/kg) also revealed an equivalent architecture of hepatic tissue (Figure 5(d)).

The photomicrographs of renal tissues are represented in Figures 6 and 7. Figures 6(a) and 6(b) represent the renal tissues of control group and diabetic nontreated group, respectively. Kidneys of control group show normal architecture of tissue with preserved subcapsular spaces in glomeruli and collecting tubules without change in the medullary region. Differently, the renal tissue of alloxan-induced diabetic mice shows retracted glomerular tufts with increased subcapsular space and evident thickening of Bowman's membrane due

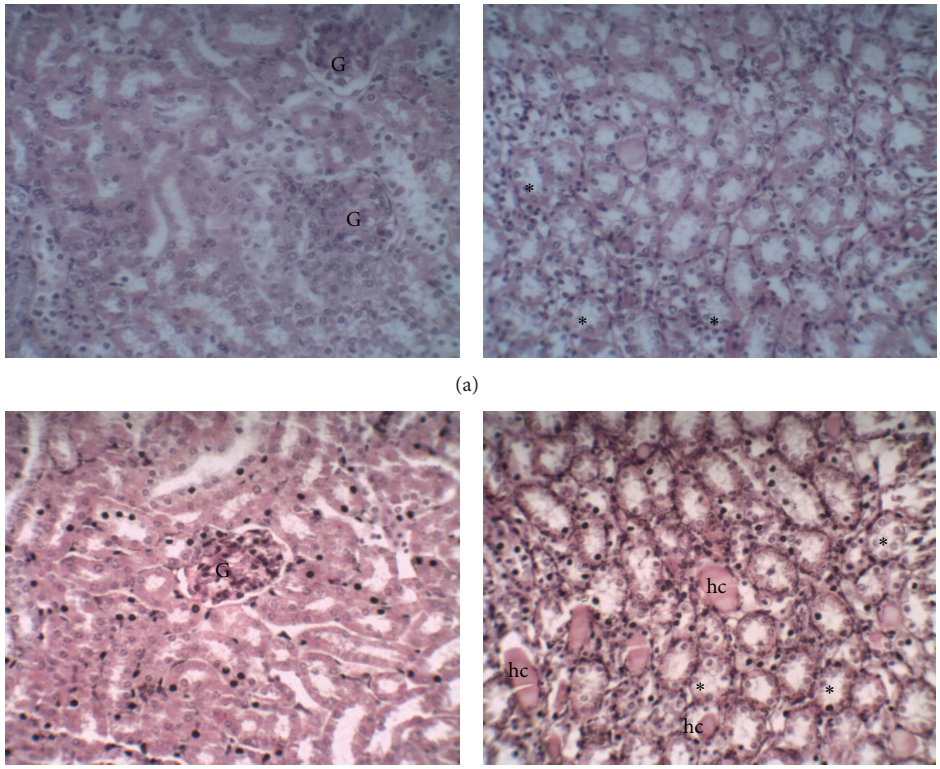

Figure 7: Histopathological changes in renal tissue of the diabetic mice treated with CrataBL. (a) Diabetic treated with CrataBL (10 mg/kg)—renal glomeruli (G) with irregular subcapsular spaces and some distinctly collapsed (left) and collecting tubules with slight swelling of the tubular epithelium (stars) (right); (b) diabetic treated with CrataBL (20 mg/kg)—preserved renal glomeruli (G) (left) and presence in the medullary region of collecting tubules (stars) with evident swelling of the tubular epithelium and hyaline casts (hc) (right). Haematoxylin-eosin: 400x.

to the cuboid appearance of epithelial cells. In kidneys of alloxan-induced mice treated with CrataBL (10 mg/kg) renal glomeruli were evident with irregular subcapsular spaces and some distinctly collapsed (Figure 7(a)). However, renal sections of diabetic mice treated with CrataBL (20 mg/kg) show preserved renal glomeruli and presence in the medullary region of collecting tubules with evident swelling of the tubular epithelium and hyaline casts presence (Figure 7(b)).

The elevated levels of glucose contribute to the generation of ROS in the diabetes, which promotes to the increase of oxidative stress in various organs and tissues [49, 50]. In addition, the hyperglycemia provokes hepatic and renal damage and consequently has been associated with histological and functional alterations and liver and kidneys [51, 52]. In fact, these organs are the focal of important organic functions and damage promoted by diabetes can result in severe complications with nephropathy and nonalcoholic steatosis [23, 24]. The current study demonstrated that CrataBL treatment improves the hepatic and renal histologic damage induced by diabetes. These findings correlated with improved biochemical markers of liver and renal functions by CrataBL. Taken together, these results may contribute to a better understanding of the regenerative effect of CrataBL in pancreas and protective in liver and kidneys, emphasizing the utilization of this lectin in the treatment of complications associated with diabetes mellitus.

4. Conclusion

Our results indicate that CrataBL is a good agent in controlling diabetes induced by alloxan and improves the damage on kidneys and liver tissues. The findings of this study also indicate that the active principle present in *C. tapia* is CrataBL, which is a lectin responsible for the antihyperglycemic activity found in this study and that could explain the basis for its use in the folk medicine as an alternative treatment for diabetes. Therefore, we conclude that CrataBL serves as an excellent candidate for an alternative therapy in the treatment of diabetes mellitus since it revealed an antidiabetic activity and other beneficial effects that ameliorate diabetes and associated complications.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

A Drug-Target Network-Based Approach to Evaluate the Efficacy of Medicinal Plants for Type II Diabetes Mellitus

Jiangyong Gu, Lirong Chen, Gu Yuan, and Xiaojie Xu

Beijing National Laboratory for Molecular Sciences, State Key Lab of Rare Earth Material Chemistry and Applications, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

Correspondence should be addressed to Lirong Chen; lirongc@pku.edu.cn and Xiaojie Xu; xiaojxu@pku.edu.cn

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The use of plants as natural medicines in the treatment of type II diabetes mellitus (T2DM) has long been of special interest. In this work, we developed a docking score-weighted prediction model based on drug-target network to evaluate the efficacy of medicinal plants for T2DM. High throughput virtual screening from chemical library of natural products was adopted to calculate the binding affinity between natural products contained in medicinal plants and 33 T2DM-related proteins. The drug-target network was constructed according to the strength of the binding affinity if the molecular docking score satisfied the threshold. By linking the medicinal plant with T2DM through drug-target network, the model can predict the efficacy of natural products and medicinal plant for T2DM. Eighteen thousand nine hundred ninety-nine natural products and 1669 medicinal plants were predicted to be potentially bioactive.

1. Introduction

Type II Diabetes mellitus (T2DM) has been a major global health problem and affects a large population worldwide [1, 2]. T2DM is a multifactorial and genetically heterogeneous disease caused by various risk factors such as insulin resistance, β-cell dysfunction, and obesity [2–5]. Moreover, T2DM may cause acute cardiovascular disease, retinopathy, nephropathy, neuropathy, and kidney-related complications [5–7]. Therefore, it demands effective drugs with minimal toxicity. The herbal medicines have been used for T2DM for thousands of years and accumulated a great deal of clinical experience. A herbal formula comprises several medicinal plants or animals and thus can affect the biological system through interactions between compounds and cellular targets [3, 8–17]. The main mechanisms of herbal medicines in treating T2DM are that it increases insulin secretion and the sensitivity of insulin, inhibits glucose absorption, and reduces radicals caused by lipid peroxidation [8]. However, the major problem of herbal medicines is lack of scientific and clinical data to evaluate their efficacy and safety.

Network pharmacology proposed by Hopkins is a holistic approach to understand the function and behavior of a biological system at systems level in the context of biological networks and would be the next paradigm for drug discovery [18–20]. Several efforts have been made to explore the mechanism of herbal medicines such as prediction of the active ingredients and potential targets [21–26] and screening synergistic drug combinations [11, 27, 28]. The drug-target network (DTN) which connects drugs and their target proteins is an important biological network and provides an overview of polypharmacology of drugs [29–32]. Since medicinal plants have multiple compounds and a compound would have several target proteins, the DTN may bridge the gap between medicinal plants and diseases. In this work, we developed a computational approach based on DTN to evaluate the efficacy of medicinal plants.

2. Materials and Methods

2.1. Data Collection and Molecular Docking. The pathogenesis of T2DM is concerned with various proteins. We retrieved the information of these proteins from KEGG Pathway database [33] and DrugBank [34] (Figure 1). The pathway of T2DM was downloaded from the KEGG website (http://www.genome.jp/dbget-bin/www_bget?hsa04930), and the information of T2DM-related proteins was collected.

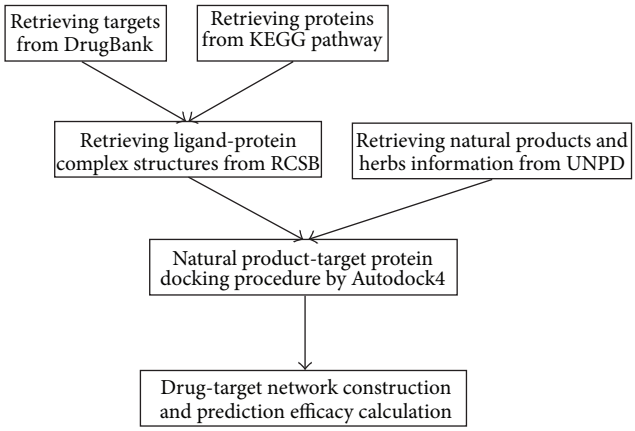

Figure 1: The work flow of this approach.

In DrugBank, we first retrieved the FDA-approved drugs for T2DM and then found the target proteins for each drug. Then we searched the ligand-protein complex structure (x-ray or NMR) for each protein from RCSB protein data bank (http://www.rcsb.org/pdb/home/home.do). Finally, thirty-three proteins and their information were listed in Table 1.

The 3D structures of natural products contained in medicinal plants were retrieved from the Universal Natural Product Database (UNPD) which comprised more than 208 thousands of natural products [54, 55]. The AutoDock 4.0 [56, 57] was adopted to perform the virtual screening, and binding free energy-based docking score (pK_i) was used to evaluate the affinity between each compound and each protein. For each protein, the hetero atoms of the ligand-protein complex structure were deleted and the polar hydrogen atoms were added. The binding site of each protein was defined as a $40 \times 40 \times 40$ Å cube around the original ligand with a spacing of 0.375 Å between the grid points. The center of binding site was located in the center of the original ligand. The molecular docking was conducted according to the protocol described previously [58].

2.2. Drug-Target Network Construction and Analysis. The drug-target network was constructed by linking the compound with target protein if the docking score satisfied the thresholds that were used to determine whether the interaction between compound and protein was strong. According to our previous study, the thresholds were set as follow: the docking score should be greater than 7.00 and the score of original ligand of corresponding protein and the top percentage of rank of docking score should be less than 10% [54]. The edge value was the docking score of corresponding compound and protein. Finally, the DTN consisted of 32 target proteins, 18999 compounds (the UNPD ID, chemical name, formula, molecular weight, and CAS registry number of each compound were listed in Table S1, see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/203614), and 35076 edges (Supplementary Table S2). The glucocorticoid receptor (P04150) did not have any compounds. The compounds were derived from 1669 medicinal plants distinguished by Latin names. The DTN of potentially active compounds and proteins related with T2DM was used as a bridge to build the relationship between compound or medicinal plant and T2DM.

2.3. Chemical Space Analysis. The analysis of the distribution of compounds in the chemical space was conducted by principal component analysis (PCA) module in Discovery Studio. The PCA model was built with 8 descriptors: $A \log P$, molecular weight, number of hydrogen-bond donors, number of hydrogen-bond acceptors, number of rotatable bonds, number of rings, number of aromatic rings, and molecular fractional polar surface area. The variances of PC1, PC2, and PC3 for compounds in Figure 2 were 0.488, 0.186, and 0.145, respectively. The PCA of 25 FDA-approved small-molecule drugs retrieved from DrugBank was performed in the same process as above.

2.4. Prediction Model. Natural products are multitarget agents. The average number of target proteins was 1.84 in the DTN. Therefore, we proposed that the prediction efficacy (PE) of a compound for T2DM was the sum of its all edge values (docking scores) in the DTN:

$$\mathrm{PE}_{\mathrm{compound}} = \sum_{j \in P} \mathrm{score}_j, \tag{1}$$

where P was the set of proteins related to T2DM and score_j was the docking score between this compound and jth protein. The $\mathrm{PE}_{\mathrm{compound}}$ for each compound was listed in Table S3.

Similarly, the prediction efficacy of a medicinal plant was defined as the sum of PE of compounds contained in this plant:

$$\mathrm{PE}_{\mathrm{plant}} = \sum_{i}^{N} \mathrm{PE}_{\mathrm{compound}_i}, \tag{2}$$

where N denoted the number of compounds contained in the medicinal plant. The $\mathrm{PE}_{\mathrm{plant}}$ for each medicinal plant was listed in Table S4.

3. Results and Discussion

3.1. Drug-Likeness of Medicinal Natural Products for T2DM. The natural products contained in medicinal plants for T2DM had good drug-like properties. Lipinski CA and colleagues proposed the "rule of five" (molecular weight (MW) less than 500 Da, the number of hydrogen bond acceptors (HBA) less than 10, the number of hydrogen bond donors (HBD) less than 5, and octanol-water partition coefficient ($A \log P$) less than five) [59, 60] to estimate solubility and permeability of compounds in drug discovery. That is, a compound was unlikely to be a drug if it disobeyed the rules. The mean and median of MW, HBA, HBD, and $A \log P$ of these compounds were 540.43, 494.62; 6.3, 5; 2.5, 2; and 4.94,

Table 1: List of 33 proteins related with T2DM for molecular docking.

Index	UniProt entry	PDB entry	Protein name
1	O43451	3CTT	Maltase-glucoamylase, intestinal
2	P01308	1TYM	Insulin
3	P01375	2AZ5	Tumor necrosis factor alpha
4	P04150	3H52	Glucocorticoid receptor
5	P04746	1XDO	Pancreatic alpha-amylase
6	P05121	3UT3	Plasminogen activator inhibitor 1
7	P06213	3EKN	Insulin receptor
8	P07339	1LYW	Cathepsin D
9	P08069	3I81	Insulin-like growth factor 1 receptor
10	P11474	3K6P	Steroid hormone receptor ERR1
11	P12821	3L3N	Angiotensin-converting enzyme
12	P13569	3GD7	Cystic fibrosis transmembrane conductance regulator
13	P14410	3LPP	Sucrase-isomaltase, intestinal
14	P14618	3BJF	Pyruvate kinase isozymes M1/M2
15	P14735	3E4A	Insulin-degrading enzyme
16	P19367	1DGK	Hexokinase-1
17	P27361	2ZOQ	Mitogen-activated protein kinase 3
18	P27487	3G0D	Dipeptidyl peptidase 4
19	P27986	4A55	Phosphatidylinositol 3-kinase regulatory subunit alpha
20	P28482	3I5Z	Mitogen-activated protein kinase 1
21	P30613	2VGF	Pyruvate kinase isozymes R/L
22	P35557	3IMX	Glucokinase
23	P35568	2Z8C	Insulin receptor substrate 1
24	P37231	3H0A	Peroxisome proliferator-activated receptor gamma
25	P42336	3HHM	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
26	P42345	1FAP	Serine/threonine-protein kinase mTOR
27	P43220	3C59	Glucagon-like peptide 1 receptor
28	P45983	3PZE	Mitogen-activated protein kinase 8
29	P45984	3NPC	Mitogen-activated protein kinase 9
30	P48736	3SD5	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform
31	P53779	3TTI	Mitogen-activated protein kinase 10
32	P62508	2P7A	Estrogen-related receptor gamma
33	Q9BYF1	1R4L	Angiotensin-converting enzyme 2

5.07; respectively. It indicated that most compounds would be drug-like. The wide distribution of natural products in chemical space (Figure 2) showed that there would be vast property (structural and functional) diversity. Moreover, the large overlap between natural products and 25 FDA-approved small-molecule drugs for T2DM demonstrated that natural products contained in these medicinal plants had a hopeful prospect for drug discovery for T2DM.

3.2. Prediction Efficacy of Natural Product and Medicinal Plant. Herb medicines could simultaneously target multiple physiological processes through interactions between multiple compounds and cellular target proteins. For example, there were 105 distinct compounds contained in *Hypericum perforatum*, and 21 compounds existed in DTN. The herbal medicines could influence the biological system through interactions between multi-component and multi-target and thus reverse the biological networks from disease state to health state. Since a group of compounds contained in the herbal medicine could play a therapeutic role, the dosage could be reduced to reduce toxicity and side effects. For example, UNPD43323 (ormojine), UNPD194973 (ormosinin), and UNPD194973 (strychnohexamine) were the top three potential compounds (Supplementary Table S3). ormojine, ormosinin, and strychnohexamine had 27, 24, and 23 targets, respectively. The polypharmacology of natural products was very common.

The predicted efficacy of the top twenty medicinal plants for T2DM was listed in Table 2. There were five plants (*Hypericum perforatum, Ganoderma lucidum, Holarrhena antidysenterica, Celastrus orbiculatus,* and *Murraya euchrestifolia*) where prediction efficacy was higher than 1000. We searched the literatures which reported the anti-T2DM bioactivities of the top twenty medicinal plants (Table 2) and found that 15 medicinal plants had information of definite effectiveness against T2DM. For example, Arokiyaraj and

Table 2: Top twenty potential medicinal plants.

Rank	Latin name	PE_{plant}	Reported bioactivity
1	*Hypericum perforatum*	1777.81	[35, 36]
2	*Ganoderma lucidum*	1560.05	[37]
3	*Holarrhena antidysenterica*	1147.22	[38, 39]
4	*Celastrus orbiculatus*	1089.44	N/A
5	*Murraya euchrestifolia*	1066.97	N/A
6	*Melia azedarach*	980.47	[40]
7	*Datura metel*	894.36	[41, 42]
8	*Ficus microcarpa*	837.65	[43]
9	*Tripterygium wilfordii*	785.30	[44]
10	*Pachysandra terminalis*	740.38	N/A
11	*Calendula officinalis*	729.77	[45]
12	*Vitis vinifera*	719.77	[46]
13	*Melia toosendan*	711.49	N/A
14	*Mangifera indica*	677.08	[47]
15	*Piper nigrum*	667.41	[48]
16	*Solanum dulcamara*	667.12	[49]
17	*Garcinia hanburyi*	641.41	N/A
18	*Momordica charantia*	632.37	[50, 51]
19	*Lantana camara*	625.64	[52]
20	*Ceriops tagal*	623.13	[53]

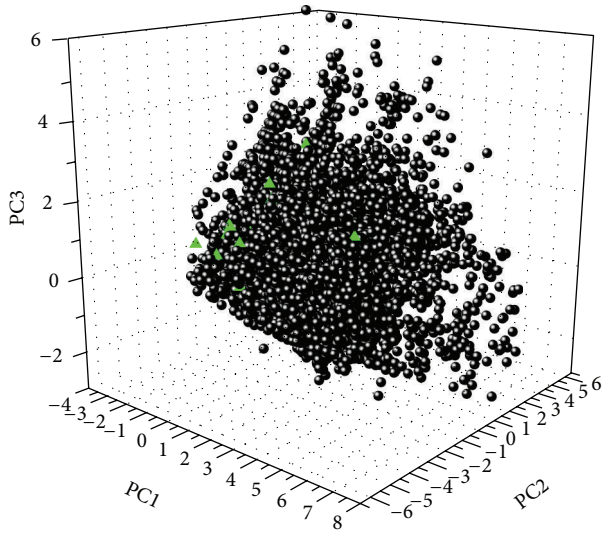

Figure 2: The distribution in chemical space according to PCA of natural products contained in medicinal plants and 25 FDA-approved drugs for T2DM. The black dots and green triangles represent natural products and FDA-approved drugs, respectively.

colleagues evaluated the antihyperglycemic activity of *Hypericum perforatum* in diabetic rats, and it produced significant reduction in plasma glucose level [35].

3.3. Clinical Herbal Formula. Tangminling which was a widely used herbal formula in China to treat T2DM comprised eleven medicinal herbs (*Trichosanthes kirilowii*, *Citrus sinensis*, *Bupleurum chinense*, *Rheum officinale*, *Astragalus membranaceus*, *Pinellia ternata*, *Scutellaria discolor*, *Crataegus pinnatifida var. major*, *Paeonia albiflora*, *Prunus mume*, and *Picrorhiza kurroa*) [3]. The prediction efficacy of each medicinal plant was 493.04, 199.26, 36.06, 29.08, 15.12, 14.80, 7.83, 7.09, 7.07, 7.06, and 7.04, respectively. It indicated that all plants could play a role in the treatment of T2DM. However, the prediction efficacy of eleven herbs differed considerably from each other. It meant that *Trichosanthes kirilowii* and *Citrus sinensis* played major roles (sovereign herbs). Meanwhile, The others worked as assistants which may strengthen the efficacy of sovereign herbs or reduce the toxicity.

4. Conclusions

Medicinal plants are potentially important for novel therapeutic drugs. It is currently estimated that approximately 420,000 plant species exist in nature [61]. However, only 10,000 of all plants have documented medicinal use [62]. Therefore, there are potentially many more important pharmaceutical applications of plants to be exploited. Traditional method (from selecting plants to separating compounds following bioassay) is time-consuming. In this work, we developed a molecular docking score-weighted prediction model based on drug-target network to evaluate the efficacy of natural products and medicinal plants for T2DM. Natural products contained in the medicinal plants would target several cellular target proteins. The prediction efficacy of this model took into account all potential interactions between multicomponents and targets. Therefore, the prediction efficacy was an overall evaluation at systems level. Fifteen out of the top twenty medicinal plants had reported bioactivity

against T2DM in literatures. This approach may promote the research on the use of medicinal plants to treat T2DM and drug discovery from natural products.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Does Oral Ingestion of *Piper sarmentosum* Cause Toxicity in Experimental Animals?

Maizura Mohd Zainudin,[1,2] Zaiton Zakaria,[1] Nor Anita Megat Mohd Nordin,[1] and Faizah Othman[3]

[1] *Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Center, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[2] *Department of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*
[3] *Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Center, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Zaiton Zakaria; zaitonukm@gmail.com

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The prevalence of diabetes mellitus has reached epidemic proportion in Malaysia and worldwide. Scientific studies have shown that herbal plant *Piper sarmentosum* exhibits an antidiabetic property. Despite the extensive usage and studies of this herb as alternative medicine, there is paucity of the literature on the safety information of this plant. Thus, the present study aimed to observe the subacute toxic effects of *Piper sarmentosum* aqueous extract (PSAE) on the haematological profile, liver, and kidney in rats. The extract was administered by oral gavage to 6 male and female *Sprague Dawley* rats in daily dose of 50 mg/kg, 300 mg/kg, and 2000 mg/kg for 28 consecutive days. The control group received normal saline. General behavior of the rats, adverse effects, and mortality were observed for 28 days. The haematological and biochemical parameters were determined at baseline and after the treatment. PSAE did not show abnormality on the body weight and gross observation of internal organs. The haematological, biochemical and histopathological profiles showed minimal changes and variation within normal clinical range except for significant increase in serum potassium level that suggests the need of regular monitoring. Nevertheless, these findings suggested that PSAE up to 2000 mg/kg/day did not show subacute toxicity in *Sprague Dawley* rats.

1. Introduction

Diabetes mellitus (DM) is chronic disease that leads to severe sequelae with multiple organ involvement. The prevalence of DM is currently on the rise in Malaysia and worldwide. WHO has estimated that 366 million people will have DM by the year 2030. The Malaysian National health and Morbidity Survey (NHMS) has shown that the prevalence of DM in individual age of 30 years and above in Malaysia has increased from 6.3% in 1986 to 14.9% in 2006. Various efforts have been done focusing on the advancement of the therapeutic approach to prevent the occurrence of DM, optimization of blood sugar level, minimization of the symptoms and complications, and prolongation of survival rates in patient with DM.

In phytomedicine, various studies have recognized that *Piper sarmentosum* Roxburgh (PS) has high antioxidant activity and also exhibits the antidiabetic property [1, 2]. PS is known as *kaduk* in Malay, a herb that belongs to the Piperacea family. It is widely distributed in tropical countries in southeast Asia, northeast India, and China [3]. It is a creepy terrestrial herb, with an average height of 20 cm, and grows in shaded areas. The leaves are heart-shaped and green in colour. The flower is white in colour and bar-shaped. The flowers will develop into fruits like a berry. PS has been widely used as both cuisine and traditional remedy [2] in the treatment of diabetes mellitus [4], cough, toothache, fungal infection on the skin, asthma, and inflammation of the pleura [5, 6].

Experimental study in the late 1980s had shown that the aqueous extract of PS leaves helps to reduce blood glucose

level in the Alloxan-induced diabetic rabbits. Nevertheless, the extract did not affect the blood sugar level of the normal fasted rabbit [7, 8]. Other studies showed that the water extract of the whole plant of PS exhibits antioxidant effect and hypoglycaemic effect on Streptozotocin-induced diabetic rats [2, 4]. Previous research had shown that PS has cardiovascular protective effects. It showed the ability to increase nitric oxide production in human umbilical vein endothelial cells (HUVECs) [9, 10] and antiatherosclerotic property. In 2012, a study showed that PS aqueous extract was able to remodel ultrastructure stability of the cardiovascular tissue in the Streptozotocin-induced diabetic rats [11]. The other laboratory study showed that PS has a potential as having antiamoebic [12], larvicidal [13], anti-inflammatory, antipyretics [14], antituberculosis [15], and anticarcinogenic effect [16]. It also has the ability to reduce visceral fat, maintain blood glucose level, and reduce 11β-hydroxysteroid dehydrogenase in obese rats [17, 18].

Despite extensive usage and study of this herbaceous plant, no comprehensive study on its toxic effects has been reported. Previous study had shown that the LD_{50} of PS whole plant aqueous extract was more than 10 g/kg per oral in rats [4], while PS leaves' methanol extract LD_{50} was more than 5 g/kg in mice [14]. The present study was carried out to determine the general toxic effects and the dose-related effects specifically focusing on the kidney and liver following subacute oral dosing of PS aqueous extract in rats to provide information on its safety and guidance for selecting a safe dose of PS for its use in medical practice.

2. Materials and Methods

2.1. Plant Material. The leaves of PS were collected from a palm oil farm in Pahang, Malaysia, and authenticated by Mr. Kamaruddin, a plant taxonomist from the Herbarium Unit, Forest Research Institute Malaysia (FRIM). A voucher specimen (PID 240812-17) was deposited in the Department of Physiology, Universiti Kebangsaan Medical Center, Kuala Lumpur, Malaysia.

2.2. Preparation of Aqueous Extract of Piper sarmentosum Leaves. The fresh leaves of PS were washed with tap water and oven-dried at temperature of 50°C for 36 hours. The dried leaves were cut into small pieces. Then, 10 grams of the dried PS leaves was added to 900 mL of distilled water, and it was boiled at 80°C for 3 hours for the extraction process. The water extract was then concentrated, followed by freeze-drying into powder form. The powdered extract was stored at 4°C until usage. In this study, the extract was prepared according to the previous studies protocol [15, 16].

2.3. Experimental Animals. Both male and female *Sprague Dawley* rats weighing 200 g ± 20% body weight, obtained from Animal Unit of Universiti Kebangsaan Malaysia were used in this study. All rats were quarantined for seven days before treatment to allow acclimatization. They were housed in polypropylene cage (one per cage) and kept in temperature of 22°C ± 3°C and humidity constant at 50 to 60% with controlled lighting that provides 12-hour light-dark cycle. All procedures in the experiment were carried out in accordance with the institutional guidelines for animal research of UKM with animal ethics approval number: PP/FISIO/2010/ZAITON/17-MARCH/299-APRIL-2010-DECEMBER-2011. Water and rat chow (Gold Coin, Malaysia) were given ad libitum, and all animal manipulations were carried out in the morning to minimize the effects of circadian rhythm. Treatments of *Piper sarmentosum* aqueous extract (PSAE) were given at constant concentration at different volumes according to the dosage and body weight, respectively. The volume was not more than 2 mL/100 g body weight per dose.

2.4. Toxicological Evaluation of the Piper sarmentosum Aqueous Extract. Forty-eight healthy *Sprague Dawley* rats of both sexes were divided into three treatment groups and a control group consisting of six male and female rats. Pretreatment blood taking was done via retroorbital sinus bleeding method. Sera were collected and sent for haematological and biochemical analysis. Treatment groups were given the extract with different doses of 50, 300, and 2000 mg/kg body weight, while the control groups were given normal saline according to OECD guideline [19]. The body weights were measured and recorded at baseline and then weekly. The water and food intake were determined daily. The extract was administered using a straight, ball-tipped stainless steel feeding needle for 28 consecutive days. At day 28, the rats were anaesthetized with a cocktail of ketamine, xylazil, and zolatil. Then, rats were sacrificed and necropsy was performed. Post treatment blood samples were obtained via intracardiac puncture method for haematological and biochemical profile examination.

Serum was collected from a blood sample that had been centrifuged at 13000 rpm for ten minutes. Blood samples were sent to the Pathology & Clinical Laboratory (M) Sdn. Bhd. for analysis. The haematological parameters including haemoglobin, red blood cell, pack cell volume, and white blood cell were analyzed by auto analysis Sysmex Kx-21 Haematology Cell Counter (SN:A7667)-ISO no. KL/2006/P/0001. The renal and liver biochemical profiles were analyzed by auto analysis machine Advia 2400 Chemistry Analyser (1) (SN:CA12420030)-ISO no. 2005/P/0006. The renal biochemical profile includes the serum urea, creatinine, uric acid, and the electrolytes (sodium, potassium, and chloride), while the liver biochemical profile consists of the total protein, albumin, globulin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), and gamma glutamyl transferase (GGT). The histological sections of the liver and kidney tissues were prepared by haematoxylin and eosin (H&E) staining method at the Department of Anatomy, UKM, Malaysia.

2.5. Statistical Analysis. The results are expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to compare between and within group comparison while Student's *t*-test was used for paired

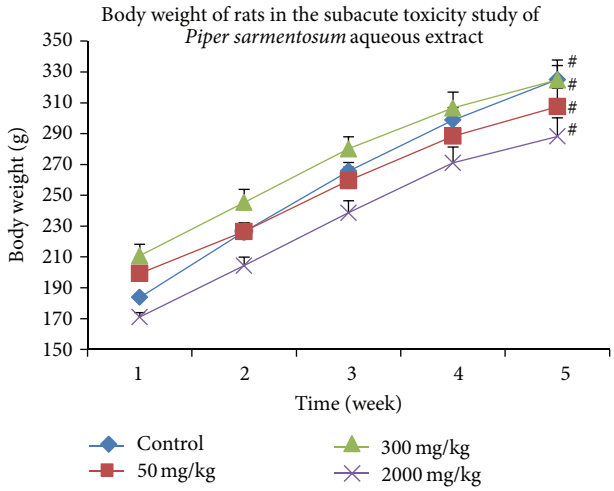

FIGURE 1: Showing changes in the body weight of rats in various groups throughout the study period. Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

comparison. 95% level of significance ($P < 0.05$) was used for the statistical analysis.

3. Results

3.1. Changes in Clinical Observation, Body Weight, Internal Organ Weight, and Water and Food Consumption. There were no PSAE treatment-related mortalities recorded in rats after 28 daily dosing of PSAE. None of them showed any obvious morbidity or clinical symptoms of toxicity such as changes in the skin and fur, eyes, and mucus membrane (nasal), respiratory rate, autonomic effects (salivation, perspiration, and piloerection), and central nervous system (ptosis, drowsiness, abnormal gait, tremors, and convulsion) throughout the experimental period.

The study showed that rats in both control and treated groups attained significant weight gain (35 to 43%, $P < 0.05$) after 28 days of the experiment compared to baseline weight. However, all groups did not show significant difference in weight in each week throughout the study as compared to control and the other treatment groups (Figure 1). The food and water consumption of male and female rats in both control and treated groups was fluctuating within a constant range throughout the study. The results showed no significant differences in treated groups as compared to control or within group (Table 1).

It was found that the mean relative weights of liver for female group treated with PSAE at the dose of 2000 mg/kg/day were higher than the control and the other treated groups but statistically not significant. For the male rats, there were no significant differences in the mean liver weight in all groups. The mean of the kidney weights of the male rats treated with PSAE of 300 mg/kg was lower than the control and the other treatment groups but showed no significant difference. The mean relative weights of the ovary of rats treated with PSAE were lower compared to the control group but did not show significant difference. The other organs such as spleen, lungs, and testis showed no significant difference that is consistent with the body weight variation (Table 2).

3.2. Changes in Haematological Profile. The haemoglobin and pack cell volume (PCV) level in male rats treated with 2000 mg/kg were the lowest at baseline and significant ($P < 0.05$) when compared to control. However, the post treatment levels of both haemoglobin and PCV showed no significant difference when compared to control, baseline, and other groups. The platelets of rats of the female control group were significantly ($P < 0.05$) reduced when compared to baseline. The other parameters such as the red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) showed no significant difference when compared to baseline, control, and other groups. The white bloods cells of male rats administered 50 mg/kg body weight showed a significant increase ($P < 0.05$) when compared with the baseline. However, there was no significant difference in WBC of all the other groups as compared to control, baseline, and the other groups (Table 3).

3.3. Changes in Renal Biochemical Profile. In the male rats, there was a significant increase ($P < 0.05$) in the levels of urea in groups treated with 50 and 300 mg/kg when compared to the baseline level. The rats in the group treated with 2000 mg/kg showed the lowest urea level as compared to the other groups ($P < 0.05$). The serum creatinine level of group 50 mg/kg was significantly higher at baseline ($P < 0.05$) and showed significant reduction after treatment ($P < 0.05$). Besides that, the serum potassium of the control group treated with 300 and 2000 mg/kg, was significantly increased ($P < 0.05$) when compared to the baseline level. However, the serum potassium level of the treated group showed no significant difference when compared to control group. The other parameters such as the uric acid, sodium and chloride levels showed no significant difference when compared to baseline, control, or treated groups. For the female rat's renal biochemical parameters, the urea level of group treated with 300 mg/kg was the lowest at baseline and showed significant increase when compared to other treatment groups. The creatinine level of the group treated with 50 mg/kg was the highest at baseline compared to control and the other groups and significantly reduced ($P < 0.05$) after treatment when compared to baseline. The serum potassium levels of group treated with 50 and 2000 mg/kg increased significantly ($P < 0.05$) when compared to baseline. The other parameters showed no significant changes when compared to the control, baseline, and other groups (Table 4).

3.4. Changes in Liver Biochemical Profile. There was a significant decrease ($P < 0.05$) in the total protein levels in group 50 mg/kg in both male and female rats when compared

Table 1: Table showing changes in the food and water consumption of rats throughout the experimental period.

	Control	*Piper sarmentosum* aqueous extract (mg/kg)		
	Week 1	Week 2	Week 3	Week 4
		Male		
Water consumption (mL)				
Control	31.0 ± 1.2	36.3 ± 0.8	40.4 ± 1.6	44.6 ± 2.4
PSAE 50 mg/kg	32.4 ± 3.5	38.1 ± 4.4	36.4 ± 4.1	38.8 ± 4.0
PSAE 300 mg/kg	35.0 ± 2.8	35.9 ± 2.8	38.0 ± 3.8	35.3 ± 2.8
PSAE 2000 mg/kg	30.8 ± 5.1	35.0 ± 6.3	34.7 ± 6.3	40.2 ± 7.5
Food consumption (g)				
Control	19.5 ± 0.7	21.9 ± 0.8	23.0 ± 0.8	22.6 ± 1.3
PSAE 50 mg/kg	18.8 ± 1.0	21.3 ± 1.2	21.4 ± 1.0	21.1 ± 0.6
PSAE 300 mg/kg	22.6 ± 1.5	22.7 ± 1.3	22.9 ± 1.0	21.1 ± 1.7
PSAE 2000 mg/kg	16.2 ± 0.7	17.1 ± 1.2	17.1 ± 1.2	19.6 ± 1.3
		Female		
Water consumption (mL)				
Control	28.3 ± 3.2	29.7 ± 2.3	32.7 ± 1.8	30.3 ± 1.3
PSAE 50 mg/kg	31.1 ± 3.8	30.5 ± 2.3	33.2 ± 1.8	33.3 ± 0.9
PSAE 300 mg/kg	37.0 ± 3.0	33.8 ± 3.0	38.7 ± 1.9	35.1 ± 0.9
PSAE 2000 mg/kg	29.2 ± 2.8	29.5 ± 2.8	28.9 ± 2.2	29.7 ± 2.0
Food consumption (g)				
Control	14.0 ± 0.7	16.0 ± 0.4	16.1 ± 0.5	15.5 ± 0.6
PSAE 50 mg/kg	12.3 ± 0.4	14.1 ± 0.4	13.6 ± 0.9	13.9 ± 0.3
PSAE 300 mg/kg	15.4 ± 0.7	17.1 ± 0.4	17.1 ± 1.5	18.0 ± 1.0
PSAE 2000 mg/kg	12.6 ± 0.6	15.7 ± 1.8	15.8 ± 0.9	15.7 ± 1.4

Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

Table 2: Table showing changes in the relative organ weight of rats throughout the experimental period.

Relative weight (%)	Control	*Piper sarmentosum* aqueous extract (mg/kg)		
	(normal saline)	50 mg/kg	300 mg/kg	2000 mg/kg
Male				
Liver	3.63 ± 0.13	3.60 ± .0.07	3.51 ± 0.12	3.61 ± 0.13
Left kidney	0.35 ± 0.01	0.36 ± 0.02	0.33 ± 0.01	0.34 ± 0.01
Heart	0.39 ± 0.02	0.36 ± 0.02	0.36 ± 0.01	0.32 ± 0.01
Left lung	0.25 ± 0.01	0.25 ± 0.01	0.23 ± 0.01	0.26 ± 0.01
Spleen	0.19 ± 0.01	0.21 ± 0.01	0.18 ± 0.01	0.18 ± 0.00
Left testis	0.49 ± 0.02	0.51 ± 0.03	0.48 ± 0.02	0.53 ± 0.01
Female				
Liver	3.44 ± 0.07	3.42 ± 0.05	3.34 ± 0.14	3.64 ± 0.13
Left kidney	0.34 ± 0.01	0.34 ± 0.02	0.35 ± 0.01	0.34 ± 0.01
Heart	0.37 ± 0.02	0.35 ± 0.01	0.34 ± 0.01	0.37 ± 0.01
Left lung	0.30 ± 0.01	0.28 ± 0.01	0.29 ± 0.02	0.26 ± 0.01
Spleen	0.24 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.23 ± 0.01
Left ovary	0.04 ± 0.00	0.03 ± 0.001	0.03 ± 0.001	0.03 ± 0.001

Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

Table 3: Table showing changes in the haematological profile of rats throughout the experimental period.

	Piper sarmentosum aqueous extract (mg/kg)							
	Control		50		300		2000	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Male								
RBC ($10^6/\mu$L)	6.7 ± 0.4	7.2 ± 0.3	6.9 ± 0.1	7.4 ± 0.2	6.8 ± 0.3	7.6 ± 0.2	6.1 ± 0.2	7.0 ± 0.3
HB (g/dL)	13.1 ± 0.3	14.0 ± 0.5	14.2 ± 0.2	14.5 ± 0.4	13.4 ± 0.2	14.4 ± 0.2	12.5 ± 0.2$^{\beta}$	13.5 ± 0.6
PCV	42.8 ± 0.9	45.5 ± 1.9	45.3 ± 0.3	46.4 ± 1.6	44.5 ± 0.9	52.3 ± 5.4	40.0 ± 1.8$^{\beta}$	43.2 ± 2.0$^{\#}$
MCV (fL)	67.7 ± 0.7	63.7 ± 0.6	65.7 ± 1.2	63.0 ± 1.0	66.0 ± 1.9	62.0 ± 1.1	66.7 ± 1.2	61.5 ± 0.6
MCH (pg)	20.5 ± 0.3	19.7 ± 0.3	20.7 ± 0.4	19.6 ± 0.4	20.0 ± 0.5	19.0 ± 0.3	20.5 ± 0.4	19.3 ± 0.2
MCHC (g/dL)	30.5 ± 0.2	30.7 ± 0.2	31.7 ± 0.2	31.2 ± 0.2	30.3 ± 0.4	30.5 ± 0.3	31.1 ± 0.6	31.3 ± 0.3
PLT ($\times 10^9/\mu$L)	1028 ± 53	998 ± 149	956 ± 153	1013 ± 116	1122 ± 82	966 ± 106	760 ± 46	723 ± 162
WBC ($\times 10^3/\mu$L)	15.7 ± 1.3	11.4 ± 1.8	16.0 ± 1.3	19.1 ± 3.7$^{\#}$	18.3 ± 1.8	15.3 ± 2.4	14.0 ± 1.5	10.8 ± 2.3
Female								
RBC ($10^6/\mu$L)	6.8 ± .01	6.5 ± 0.2	6.7 ± 0.3	6.9 ± 0.2	7.2 ± 0.1	7.1 ± 0.2	6.6 ± 0.2	6.9 ± 0.1
HB (g/dL)	14.1 ± 0.2	12.9 ± 0.7	13.3 ± 0.4	13.8 ± 0.3	14.3 ± 0.3	13.8 ± 0.4	12.7 ± 0.4	13.4 ± 0.2
PCV	44.3 ± 0.6	40.4 ± 2.4	42.2 ± 1.8	43.5 ± 0.8	44.7 ± 0.7	42.2 ± 1.2	39.8 ± 1.0	41.7 ± 0.4
MCV (fL)	65.0 ± 0.5	61.4 ± 1.1	63.0 ± 1.0	62.8 ± 0.7	62.0 ± 1.3	59.8 ± 0.6	60.8 ± 1.2*	59.8 ± 0.8
MCH (pg)	20.5 ± 0.2	19.8 ± 0.3	19.8 ± 0.4	20.2 ± 0.3	20.0 ± 0.6	19.6 ± 0.2	19.4 ± 0.5	19.3 ± 0.3
MCHC (g/dL)	31.7 ± 0.2	32.2 ± 0.2	31.5 ± 0.2	31.8 ± 0.2	32.0 ± 0.4	32.6 ± 0.4	32.0 ± 0.5	32.3 ± 0.2
PLT ($\times 10^9/\mu$L)	998 ± 68	671 ± 65*	773 ± 128	947 ± 55	1086 ± 132	927 ± 60	858 ± 115	842 ± 116
WBC ($\times 10^3/\mu$L)	19.4 ± 2.3	11.8 ± 2.1	16.4 ± 1.1	10.5 ± 2.5	14.4 ± 2.1	13.6 ± 0.80	15.2 ± 3.0	11.7 ± 2.1

Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

Table 4: Table showing changes in the serum renal biochemical profile of rats throughout the experimental period.

	Piper sarmentosum aqueous extract (mg/kg)							
	Control		50		300		2000	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Male								
Urea (mmol/L)	6.7 ± 0.5	7.6 ± 0.3	5.2 ± 0.1	7.2 ± 0.5$^{\#}$	5.8 ± 0.2	7.8 ± 0.5$^{\#}$	5.4 ± 0.5	6.2 ± 0.2$^{\beta}$
Creatinine (μmol/L)	24.7 ± 4.9	26.7 ± 1.2	46.7 ± 0.8$^{*\beta}$	28.2 ± 1.2$^{\#}$	30.5 ± 6.0	32.0 ± 1.4	22.3 ± 1.1	33.2 ± 1.6
Uric acid (mmol/L)	0.05 ± 0	0.08 ± 0	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.03 ± 0	0.12 ± 0.03
Sodium (mmol/L)	140.8 ± 0.5	139.5 ± 0.7	139.8 ± 0.5	141.3 ± 0.3	140.2 ± 0.8	141.2 ± 0.8	140.7 ± 0.4	140.8 ± 0.4
Potassium (mmol/L)	4.5 ± 0.1	6.3 ± 0.3$^{\#}$	5.2 ± 0.2	5.8 ± 0.3	5.2 ± 0.3	6.2 ± 0.2$^{\#}$	4.2 ± 0.1$^{\beta}$	6.5 ± 0.2$^{\#}$
Chloride (mmol/L)	98.8 ± 0.7	97.5 ± 0.2	101.3 ± 0.6*	100.3 ± 0.4	99.7 ± 0.4	99.3 ± 0.9	99.5 ± 0.3	99.8 ± 0.6
Female								
Urea (mmol/L)	6.6 ± 0.2	8.0 ± 0.4	7.6 ± 0.2	8.2 ± 0.3	6.1 ± 0.3$^{\beta}$	6.8 ± 0.1$^{\beta}$	7.0 ± 0.4	7.7 ± 0.3
Creatinine (μmol/L)	32.5 ± 3.7	28.5 ± 1.7	39.7 ± 1.4$^{*\beta}$	30.2 ± 1.0$^{\#}$	33.8 ± 1.4	31.8 ± 2.3	33.2 ± 2.0	31.3 ± 0.8
Uric acid (mmol/L)	0.10 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.11 ± 0.02	0.04 ± 0.01	0.09 ± 0.01
Sodium (mmol/L)	141.2 ± 1.0	142.5 ± 0.7	140.2 ± 0.9	138.2 ± 0.5	138.0 ± 0.9	141.0 ± 0.9	139.5 ± 1.0	142.5 ± 0.5
Potassium (mmol/L)	4.9 ± 0.3	5.5 ± 0.1	4.8 ± 5.9	5.9 ± 0.2$^{\#}$	4.7 ± 0.2	5.3 ± 0.1	4.2 ± 0.2	5.6 ± 0.3$^{\#}$
Chloride (mmol/L)	102.2 ± 1.1	99.0 ± 1.3	101.0 ± 0.7	100.7 ± 0.8	99.4 ± 0.4	101.1 ± 1.0	98.5 ± 0.7	101.3 ± 0.9

Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

to baseline. The post treatment globulin level of the male rats group treated with 50 mg/kg was the least and showed significant difference when compared to other groups. The baseline level of ALP of the male rats group treated with 50 mg/kg was the lowest while the level of ALT of the female rats was the highest and showed significant difference ($P < 0.05$) when compared to control group. However, there were no significant changes ($P < 0.05$) in total protein, albumin, globulin, total bilirubin, AST, and GGT of the treated groups compared with control in both male and female rats after subacute administration of PSAE (Table 5).

3.5. Histopathological Profile of Liver and Kidney. The histology section of the kidney for the control group by H&E

Table 5: Table showing changes in the serum liver biochemical profile of rats throughout the experimental period.

	Piper sarmentosum aqueous extract (mg/kg)							
	Control		50		300		2000	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Male								
Total protein (g/L)	63 ± 1.1	64.3 ± 1.3	66.7 ± 0.9	63.3 ± 1.5$^{\#}$	64.8 ± 1.2	66.2 ± 1.4	65.3 ± 2.3	65.3 ± 1.9
Albumin (g/L)	34.3 ± 0.4	35.3 ± 0.8	34.5 ± 0.4	36.7 ± 0.8	34.2 ± 0.6	35.2 ± 0.7	35.0 ± 0.6	35.2 ± 0.8
Globulin (g/L)	32.0 ± 1.3	29.0 ± 0.9	32.2 ± 1.2	26.5 ± 0.9$^{\beta}$	30.5 ± 0.7	31.0 ± 1.3	30.3 ± 2.1	32.0 ± 0.9
Bilirubin (μmol/L)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
ALP (iu/L)	531 ± 62	372 ± 44	355 ± 21*	438 ± 39	416 ± 31	332 ± 31	473 ± 17	338 ± 13
AST (iu/L)	131 ± 18	108 ± 7	132 ± 20	147 ± 28	111 ± 8	115 ± 9	105 ± 7	139 ± 19
ALT (iu/L)	60 ± 4	67 ± 3	61 ± 2	84 ± 11	56 ± 4	66 ± 2	63 ± 5	67 ± 4
GGT (iu/L)	0	0	0	0	0	0	0	0
Female								
Total protein (g/L)	74.3 ± 1.3	71.3 ± 1.0	76.2 ± 2.0	66.7 ± 2.1$^{\#}$	70.7 ± 1.3	69.7 ± 1.2	71.7 ± 2.3	71.5 ± 2.0
Albumin (g/L)	38.51.0	36.0 ± 0.5	37.3 ± 1.1	36.2 ± 1.6	35.0 ± 0.6	35.2 ± 0.6	36.5 ± 2.0	38.5 ± 1.3
Globulin (g/L)	34.8 ± 1.0	35.3 ± 0.8	38.8 ± 2.2	30.5 ± 0.6	35.2 ± 0.4	34.7 ± 0.8	35.2 ± 1.0	33.0 ± 0.9
Bilirubin (μmol/L)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
ALP (iu/L)	307 ± 35	291.3 ± 19	301 ± 20	260 ± 23	270 ± 25	265 ± 22	367 ± 46	286 ± 16
AST (iu/L)	171 ± 33	149 ± 9	173 ± 27	135 ± 11	146 ± 9	115 ± 2	129 ± 11	116 ± 9
ALT (iu/L)	61 ± 6	57 ± 3	101 ± 19$^{*\beta}$	56 ± 3	69 ± 8	58 ± 3	63 ± 4	76 ± 4
GGT (iu/L)	0	0	0	0	0	0	0	0

Data were expressed as mean ± SEM. $^{*}P$ value < 0.05 is significant compared to control group; $^{\#}P$ value < 0.05 is significant compared to baseline value; $^{\beta}P$ value < 0.05 is significant compared to other groups.

staining showed the normal renal cell architecture. The cortex consists of the glomeruli, blood vessels, tubules, and interstitium. The glomeruli were symmetrical with regular and thin capillary walls. The cells of the nuclei were not overlapping, and there were no clusters of cells or hypercellularity. There were no cells infiltrations in the lumen of capillaries. The medullary part of the kidney showed the renal tubules that were arranged in a normal architecture with minimal interstitium. The medullary artery also had thin intima and endothelial lining. The histological section of the group treated with extract 50 mg/kg and 300 mg/kg portrayed the same picture as the control group. On the other hand, the group treated with PSAE 2000 mg/kg showed a slight increase in the interstitium that might suggest cellular infiltration; however, the nuclei and the renal cell architecture were clearly seen (Figure 2).

The histopathological examination of the hepatocytes showed that the control group exhibited normal finding where it showed that the portal triads consist of portal veins, hepatic artery, and bile duct situated at the periphery and the central veins with radiating cords of hepatocytes separated by sinusoids. The hepatocytes were of the same size and polygonal in shape with the nucleus at the center and cytoplasm which was regularly distributed. The treated groups also represent the same picture. There was neither loss of radial arrangement nor thickening or congestion of the sinusoids. There were very minimal cells infiltrations around the portal track. There was no obvious area of necrosis around the central vein (Figure 3).

4. Discussion

Herbal plant usage is increasingly becoming more popular as alternative medicine and supplement in the primary health care worldwide [20, 21]. The plan to introduce PS to human should consider the benefits and risks of this herb for the recipient. As PS has been proven to have a tremendous beneficial effect, it is necessary to study its harmful effects before embarking on human studies. In a step to achieve the objective evaluation of the effects of a substance on animals, it is fundamental to look for changes in general behavior, body weight, and haematological profile [22] as such changes are often the first signs of toxicity. Besides, the biochemical profile may also picture the target organ damage. OECD, Guideline 407, has recommended the Repeated Dose 28-day Oral Toxicity Study in Rodents methods to protect animal rights by reducing the number of animals used, reduce suffering, and not to cause death as the endpoint of the study [19].

It is believed that natural plant products are safe, and they has been widely used worldwide for centuries [23]. A previous study showed that PSAE contained high phenolic and flavanoid content in which the main flavonoids are rutin and vitexin [24]. However, it has to be proven safe scientifically before it is used in humans. Another study has showed that the LD_{50} of PS whole plant aqueous extract is more than 10 g/kg per oral in rats [4], while PS leaves' methanol extract LD_{50} was more than 5 g/kg in mice [14]. According to Globally Harmonization System [25], for a substance with

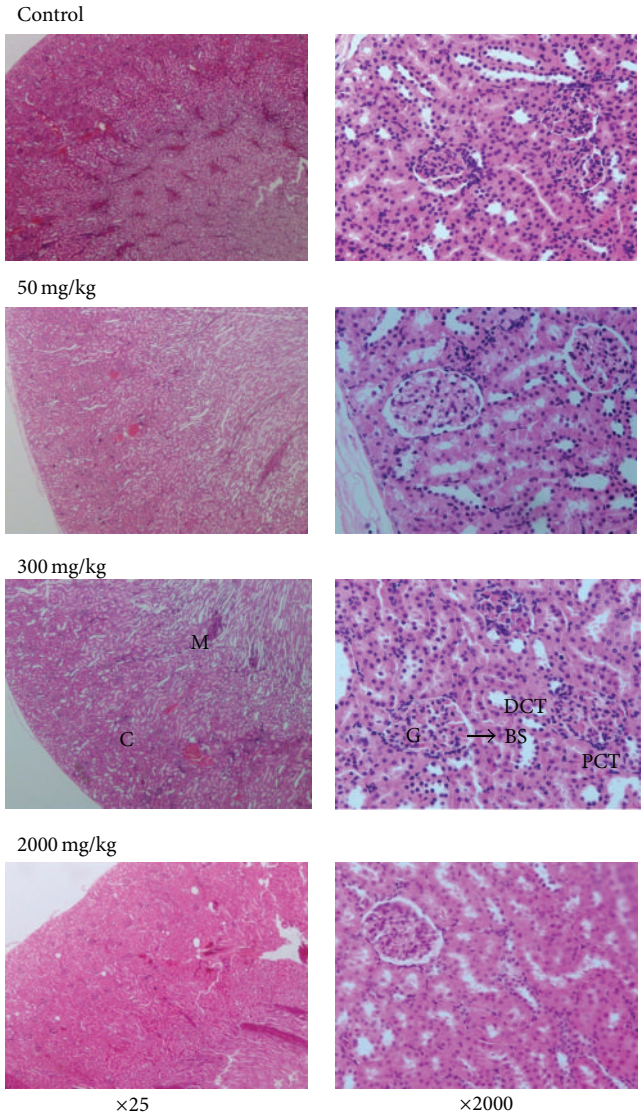

Figure 2: The histology of renal cells with H&E staining method of rats treated with 50 mg/kg, 300 mg/kg, and 2000 mg/kg of *Piper sarmentosum* aqueous extract and normal saline for 28 consecutive days at 25 and 200 times magnification. There were no significant changes in the structure of the kidney cells observed in the histological section of the kidney tissues of the treatment groups. G: glomerulus; BM: basement membrane; PCT: proximal convoluted tubule; DCT: distal convoluted tubule; C: cortex; M: medulla; BS: Bowman's space.

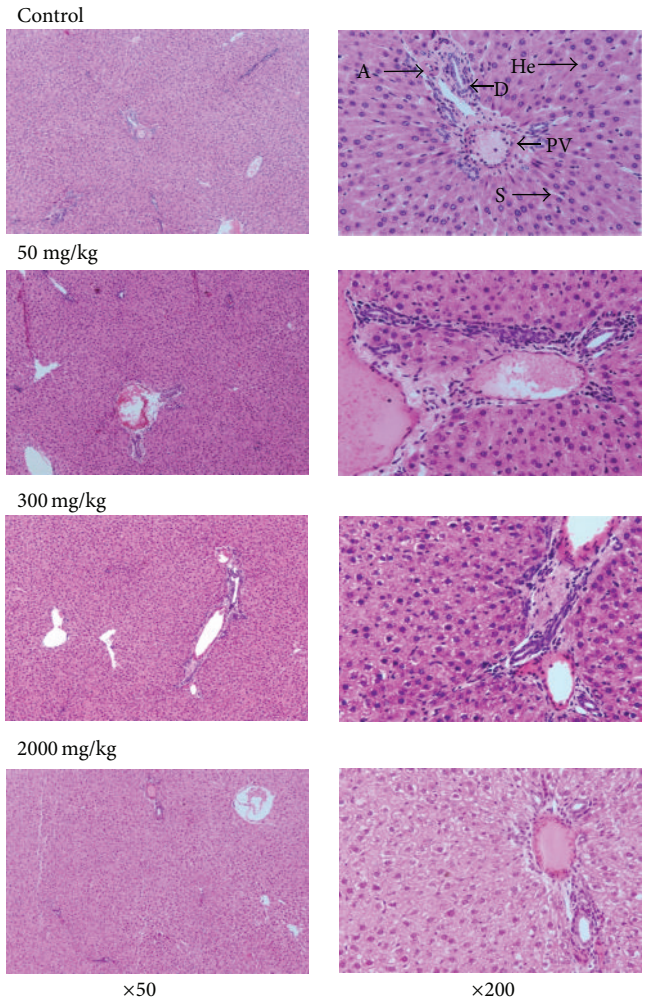

Figure 3: The histology of liver cells with H&E staining method of rats treated with 50 mg/kg, 300 mg/kg, and 2000 mg/kg of *Piper sarmentosum* aqueous extract and normal saline for 28 consecutive days at 50 and 200 times magnification. There were no significant changes showing congestion or destruction of liver cells observed in the histological section of the liver tissues of the treatment groups compared to the control group. PT: portal triad; PV: portal vein; He: hepatocyte; S: sinusoid; A: hepatic artery; D: bile duct.

LD_{50} of more than 5 g/kg the GHS is unclassified. However, to the best of our knowledge, there is no comprehensive toxicity study which has been performed with PS leaves. The leaves are the most commonly used part of this herb as cuisine and traditional remedy. Hence, it may be beneficial to conduct this study for the human usage of PS later.

The present study provides baseline information for the anticipation of the harmful effect of PS in humans. Besides, the information is also useful to predict the nature of the pharmacokinetics and pharmacodynamics. It may also give a clue to the organ or system that might be affected. The aqueous extract of PS leaves was chosen as it is traditionally taken raw as cuisine and supplement and likely to be patterned from aqueous extract as well. Although the ethanol extract showed more antioxidant compounds, nevertheless the aqueous extract of PS also showed high amount of antioxidant activity.

In the present study, the rats did not show any signs of morbidity and mortality after subacute administration of PSAE. Some plant extracts were reported to cause reduced food intake. However, this extract did not cause reduction in rats' body weight, and food and water intake. In addition, a minimal variation of haemoglobin and WBC count which is still in the normal range suggested that PS did not interfere with the haematopoietic system. Despite showing significant

difference statistically, the outcomes of some parameters in the biochemical profile are actually varying within clinical reference range. Important markers for renal impairment such as urea and creatinine did not even double the values similarly with the markers for liver impairment such as ALP, AST, ALT, and GGT [26]. The histopathological examinations also showed very minimal changes that were inconsistent and may be due to technical problems during tissue fixation and processing. The increment of serum potassium level after PSAE administration in this study signifies that a regular monitoring of renal biochemical profile is required upon prolonged intake of PS.

5. Conclusion

The findings in the present study suggested that the subacute administration of PS leaves aqueous extract did not cause subacute toxicity in haematological profile, liver, and kidney in *Sprague Dawley* rats. However, further research on the safety of PS involving the other systems such as the reproductive and the central nervous system may be performed in the future. Besides, the subchronic toxicity study of PS may be performed to obtain a complete guidance for the usage of PS in medical practice in human.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors contributed to and have approved the final paper. Maizura M. Z. performed the work and wrote the paper, Zaiton Z. and Nor Anita M. M. N. designed the project, described the methodology, and edited the paper, and Faizah O. helped in the histopathology preparation and analysis.

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Research Article

Rhinacanthus nasutus Improves the Levels of Liver Carbohydrate, Protein, Glycogen, and Liver Markers in Streptozotocin-Induced Diabetic Rats

Pasupuleti Visweswara Rao,[1,2] K. Madhavi,[3] M. Dhananjaya Naidu,[4] and Siew Hua Gan[2]

[1] *Department of Biotechnology, Sri Venkateswara University, Tirupati, Andhra Pradesh 517502, India*
[2] *Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, 16150 Kubang Kerian, Malaysia*
[3] *Department of Biochemistry, Sri Venkateswara Medical College, Tirupati, Andhra Pradesh 517502, India*
[4] *Department of Zoology, Yogi Vemana University, Kadapa, Andhra Pradesh 516003, India*

Correspondence should be addressed to Pasupuleti Visweswara Rao; visuthebiotech@gmail.com

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The present study was designed to investigate the total carbohydrate, total protein, and glycogen levels in the liver and to measure functional liver markers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in streptozotocin-(STZ-) induced diabetic rats after treatment with methanolic extract of *Rhinacanthus nasutus* (*R. nasutus*). The methanolic extract of *R. nasutus* was orally administered at 200 mg/kg/day while glibenclamide was administered at 50 mg/kg/day. All animals were treated for 30 days before being sacrificed. The amounts of carbohydrate, glycogen, proteins, and liver markers (AST and ALT) were measured in the liver tissue of the experimental animals. The levels of carbohydrate, glycogen, and proteins were significantly reduced in the diabetic rats but were augmented considerably after 30 days of *R. nasutus* treatment. The elevated AST and ALT levels in diabetic rats showed a significant decline after treatment with *R. nasutus* for 30 days. These results show that the administration of *R. nasutus* ameliorates the altered levels of carbohydrate, glycogen, proteins, and AST and ALT observed in diabetic rats and indicate that *R. nasutus* restores overall metabolism and liver function in experimental diabetic rats. In conclusion, the outcomes of the present study support the traditional belief that *R. nasutus* could ameliorate the diabetic state.

1. Introduction

Diabetes is a metabolic disease that is the consequence of a combination of hereditary and environmental factors. This disease causes hyperglycemia and other classical symptoms, especially polyuria, polydipsia, and polyphagia [1]. Diabetes mellitus is a syndrome characterized by the loss of glucose homeostasis as a result of defects in insulin secretion and functionality. The deficiency in insulin causes impaired metabolism of glucose and other energy-yielding fuels such as lipids and proteins [2]. Acute complications include diabetic ketoacidosis, hypoglycemia, hyperosmolar syndrome, and hyperglycemia. Subacute complications include polyuria, lack of energy, thirst, blurred vision, and weight loss. Chronic hyperglycemia leads to the glycation of cellular proteins and may lead to complications affecting the eyes, nerves, kidneys, and arteries [3].

Experimental diabetes in animal models has offered extensive insight into the biochemical and physiological alterations of the diabetic state. Many of these modifications, such as changes in the enzymes of glucose and lipid metabolism, have been characterized in hyperglycemic animals [4]. In many cases, structural alterations are oxidative in nature and are linked to the development of vascular disease [5, 6]. In diabetic rats, augmented lipid peroxidation is also linked to hyperlipidemia [7]. Currently, the available therapies for diabetes include insulin and a variety of oral antihypoglycemic agents such as thiazolidinediones, sulfonylureas, and α-glucosidase inhibitors. These drugs are used either as a monotherapy or in combination to achieve better glycemic

control. All oral hypoglycemic agents are associated with a number of serious, undesirable effects [8]. Plants have played a major role in the introduction of new therapeutic agents [9] and have gained attention as a source of biologically active substances including antioxidants and hypoglycemic and hypolipidemic agents [10–12]. Therefore, scientists have focused on plant sources for new therapeutic agents because plants are natural products and have minimal side effects. For example, the study of *Galega officinalis*, an important medicinal plant, has led to the discovery and synthesis of an important and commonly used antidiabetic drug, metformin [13]. Numerous approaches have been used to investigate the medicinal plants for potential hypoglycemic activities including ethnobotanical survey. The usefulness of plant products is reported to be attributed to the presence of bioactive substances such as flavonoids, alkaloids, essential oils, and phenolic compounds with antioxidant activities [14, 15].

Rhinacanthus nasutus (*R. nasutus*) (Linn) belongs to the Acanthaceae family. It has been used to treat numerous diseases such as eczema, herpes, pulmonary tuberculosis, hepatitis, diabetes, hypertension, and different types of skin diseases [16]. In Thai traditional medicine, the root and whole plant of *R. nasutus* have been used for the treatment of *Tinea versicolor*, ringworm, itching, and skin diseases. The leaves have been used for the treatment of fungal infection, skin diseases [17], allergies [18], cancers, and inflammation [19] because *R. nasutus* is a well-known source of flavonoids, steroids, triterpenoids, anthraquinones, lignans, and especially naphthoquinone analogues [20, 21]. Additionally, the root extract of this plant has been used traditionally as an antidote to snake venom [22].

Previously, we reported that *R. nasutus* has antimicrobial properties acting against several microorganisms in addition to exhibiting antidiabetic effects [23], hypolipidemic activity, significant *in vitro* and *in vivo* antioxidant activities [24], and amelioration of oxidative enzymes [16]. Insulin regulates the metabolism of many substances by converting the uptake and utilization of glucose in target organs such as liver, skeletal muscle, kidney, and adipose tissue and by controlling the amount of various metabolic enzymes. Because the liver plays a major role in glycogen storage and utilization, a partial or total reduction of the insulin level causes a disruption in carbohydrate metabolism that diminishes the activity of a number of key enzymes, protein, and liver markers [25]. The liver is an insulin-dependent tissue that plays a vital role in glucose and lipid homeostasis and is severely affected in diabetes [26]. The enzymes aspartate aminotransferase (AST) and alanine transaminases (ALT) are two of the key markers for liver function [27].

In this study, we investigated the effects of *R. nasutus* on the liver markers AST and ALT and on biochemical parameters (carbohydrate, glycogen, and protein) in experimentally induced diabetic rats to determine if this herb has the potential to be used in the treatment of diabetes.

2. Materials and Methods

2.1. Collection of Plant Material. The fresh leaves of *R. nasutus* were collected from Tirumala Hills, Tirupati, Chittoor districts of Andhra Pradesh from July to October 2009. The plant specimen was verified to be the correct species by Dr. Madhava Setty, a botanist from the Department of Botany, S. V. University, Tirupati, India.

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

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

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

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

The rats were divided into five groups of six animals each as follows.

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

Group II: *R. nasutus*-treated normal rats (200 mg/kg/day) [13, 23].

Group III: diabetic rats (untreated).

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

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

2.6. Acute Toxicity Test. R. nasutus (50–250 mg/kg body weight) was orally administered to rats for acute toxicity studies. Each group was observed individually for signs of toxicity and behavioral changes such as hyperactivity, grooming, convulsions, sedation, or hypothermia. These observations began 1 h after dosing and were continued at least once daily for 14 days. The mortality rate was also calculated.

2.7. Biochemical Measurements. At the end of the study (30 days), after an overnight fasting, the animals were sacrificed by cervical dislocation following anesthesia using isoflurane. The liver tissue was excised and washed with ice-cold saline and was immediately immersed in liquid nitrogen and stored at −80°C for further biochemical analysis. Then, the measurements of liver enzyme activity and biochemical assays were performed.

AST and ALT activities were assayed using the method of Reitman and Frankel [28]. The total carbohydrate content was estimated based on the method established by Carroll et al. [29]. Glycogen content was determined as described by Saifter et al. [30]. The protein content was estimated by the method of Lowry et al. [31] with slight modifications. All enzymatic assays in this study were performed using crude liver homogenate.

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

3. Results

3.1. Toxicity Evaluation of Plant Extract. In the acute toxicity study, the methanolic extract of *R. nasutus* did not lead to mortality even at the highest dose of 250 mg/kg body weight in male rats. At the highest dose, no gross behavioral changes were observed among the rats. These results indicate that the toxicity level of *R. nasutus* is low.

3.2. The Effects of R. nasutus Extract on Total Carbohydrate Levels in Experimental Rats. The effect of oral administration of *R. nasutus* methanolic extract for 30 days on the total carbohydrate content in liver tissues of control and experimental groups of rats is shown in Figure 1. The liver tissues of diabetic rats showed a significant decrease in the content of total carbohydrate. Treatment with *R. nasutus* methanolic extract or with glibenclamide restored the carbohydrate levels. No significant variations in total carbohydrate levels were found in control rats treated with *R. nasutus* methanolic extract alone.

3.3. The Effects of R. nasutus Extract on Glycogen Levels in Experimental Rats. The effects of oral administration of *R. nasutus* methanolic extract for 30 days on glycogen content in liver tissue of control and experimental groups of rats are depicted in Figure 2. The liver tissue of diabetic rats showed a significant decline in glycogen activity. This activity was restored by treatment with *R. nasutus* methanolic extract and by glibenclamide treatment. In contrast, no significant variations were found in the control rats treated with *R. nasutus* methanolic extract alone.

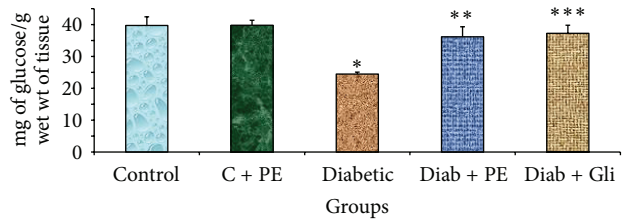

Figure 1: Changes in the total carbohydrate levels in the liver tissue of experimental rats. The bars with different number of asterisks vary significantly at $P < 0.05$. C: control, PE: plant extract, Diab: diabetic, and Gli: glibenclamide.

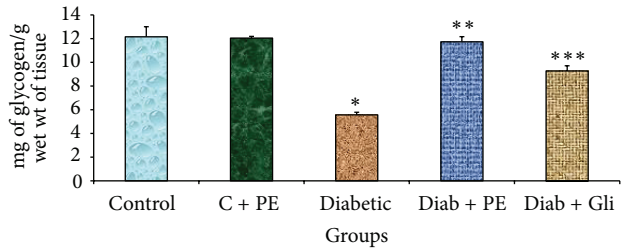

Figure 2: Changes in glycogen levels in the liver tissue of experimental rats. The bars with different number of asterisks vary significantly at $P < 0.05$. C: control, PE: plant extract, Diab: diabetic, and Gli: glibenclamide.

3.4. The Effects of R. nasutus Extract on Total Protein Levels in Experimental Rats. The effects of oral administration of *R. nasutus* methanolic extract for 30 days on the content of total proteins in the liver tissue of control and experimental groups of rats are shown in Figure 3. The liver tissue of diabetic rats showed a significant decline in protein content. The amount of protein was restored by treatment with *R. nasutus* methanolic extract as well as by glibenclamide treatment. No significant variations were found in the control rats that were treated with *R. nasutus* methanolic extract alone.

3.5. The Effects of R. nasutus Extract on AST and ALT Levels of Experimental Rats. The liver tissues of diabetic rats showed elevated AST and ALT levels. The levels of both enzymes were considerably decreased in diabetic rats treated with either *R. nasutus* extract or with glibenclamide. In the control and control treated with plant extract groups, there was no significant change in the AST (Figure 4) and ALT levels (Figure 5).

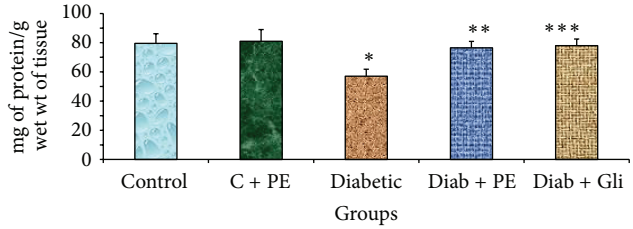

Figure 3: Changes in total protein levels in the liver tissue of experimental rats. The bars with different number of asterisks vary significantly at $P < 0.05$. C: control, PE: plant extract, Diab: diabetic, and Gli: glibenclamide.

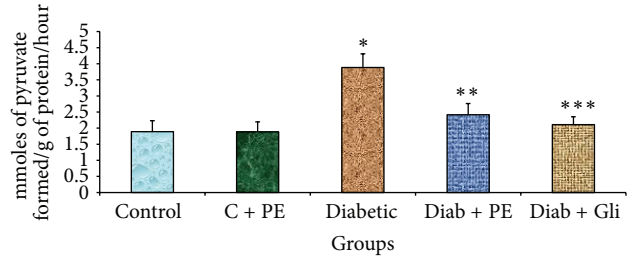

Figure 4: Changes in AST levels in the liver tissue of experimental rats. The bars with different number of asterisks vary significantly at $P < 0.05$. C: control, PE: plant extract, Diab: diabetic, and Gli: glibenclamide.

4. Discussion

Plant-derived products usually do not produce any significant side effects when properly administered [32]. Therefore, plants are good candidates for further investigations aimed at increasing the number of the armamentarium against diabetes mellitus. In the present study, STZ was chosen to induce diabetes in rats rather than alloxan. STZ is recognized for its selective destruction to pancreatic β-cells [33] and is less toxic than alloxan while maintaining a diabetic condition. It also has an irreversible effect on pancreatic beta cells. Our study is the first to show that *R. nasutus* methanolic extract increases the levels of liver carbohydrate, glycogen, total protein and decreases the levels of liver markers (AST and ALT) in STZ-induced diabetic rats.

R. nasutus methanolic extract significantly increases the total carbohydrate content in the liver tissue of diabetic rats to a similar extent as glibenclamide. Glibenclamide stimulates the insulin secretion from the β-cells of the pancreas and is extensively used to treat diabetes mellitus. Glibenclamide mainly acts by inhibiting ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane [33]. The ATP-sensitive channels inhibition leads to membrane depolarization, activation of voltage-gated Ca^{2+} channels, increased Ca^{2+} influx, and a rise in cytosolic (Ca^{2+}) and thereby insulin release. Glibenclamide is extensively used as a standard drug in STZ-induced moderate diabetic model to compare the antidiabetic properties of different types of compounds [34]. The liver is a vital organ that plays an essential role in glycolysis and gluconeogenesis.

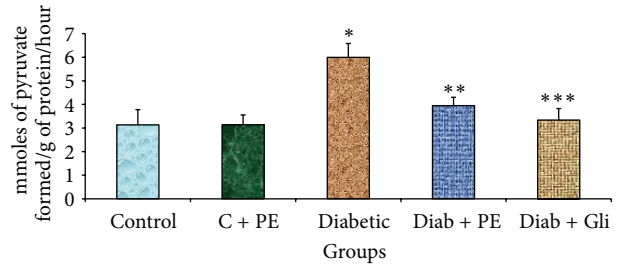

Figure 5: Changes in ALT levels in the liver tissue of experimental rats. The bars with different number of asterisks vary significantly at $P < 0.05$. C: control, PE: plant extract, Diab: diabetic, and Gli: glibenclamide.

The organ is the major site for endogenous glucose production [35] producing glucose by either gluconeogenesis or glycogenolysis. Augmented endogenous glucose production, due to either poor pancreatic function and/or reduced glucose clearance, is associated with diabetes and contributes to hyperglycemia [36, 37]. Insulin controls metabolism by regulating the uptake and consumption of glucose in target organs such as liver, skeletal muscle, kidney, and adipose tissue. This regulation is achieved by controlling the actions of various metabolic enzymes. A partial or total reduction of insulin levels will cause a disruption in carbohydrate metabolism, thereby diminishing the activity of a number of key enzymes including glucokinase, phosphofructokinase, and pyruvate kinase [25]. In this study, we observed the ameliorating effects of *R. nasutus* methanolic extract treatment on the carbohydrate content in the livers of STZ-induced diabetic rats. Similar results were shown in the liver, brain and spleen of experimentally induced diabetic rats treated with other herbs such as *Eugenia jambolana* and *Tinospora cordifolia* [32] indicating that *R. nasutus* may have a similar antihyperglycemic effect as these herbs. The increase in total carbohydrate content could be due to: (1) an increase in insulin levels, (2) an enhancement of insulin activity or sensitivity at the peripheral sites, or (3) a reduction of liver gluconeogenesis. Further work is needed to confirm which of these possibilities causes the total carbohydrate content increase.

Overall, our findings indicate that the use of *R. nasutus* methanolic extract warrants further investigation as an antidiabetic agent or as an adjunct to modern therapies such as glibenclamide. Treatment with *R. nasutus* methanolic extract significantly increased the total glycogen content in the liver tissue of diabetic rats. This effect was similar to the result observed with glibenclamide treatment. The liver preserves normal blood glucose concentrations by storing glucose as glycogen and by generating glucose from glycogen breakdown or from gluconeogenic precursors [38]. Glycogen deposition from glucose is altered in experimentally induced diabetic animals because STZ causes selective damage to pancreatic β-cells which results in the decline in insulin levels. The major storage tissues such as liver, kidney, and skeletal muscle depend on insulin for glucose access [39]. Because glucose synthesis in the rat liver is altered during diabetes [40], glycogen content in the liver is also noticeably

diminished in diabetes [41]. The liver equilibrates the uptake and storage of glucose via glycogenesis and regulates the release of glucose by activating glycogenolysis and gluconeogenesis [42]. The ability of *R. nasutus* to ameliorate glycogen levels indicates that this plant has the potential to be an antidiabetic agent and should be further investigated.

R. nasutus methanolic extract significantly increased the total protein content in the liver tissue of diabetic rats to a similar extent as glibenclamide. The decline in total protein in experimentally induced diabetic rats may be due to microproteinuria, which is a significant systematic indicator of diabetic nephropathy, or to augmented protein catabolism [40, 43, 44]. In diabetic patients with vascular complications, it was also reported that there are major changes in the metabolism of carbohydrates, lipids, and proteins such as augmented lipid peroxidation, dyslipidemia, and other abnormalities [45]. Hyperglycemia is also linked to glucose autooxidation, protein glycation, and the consequent oxidative degradation of glycated proteins that lead to a higher production of reactive oxygen species (ROS) [46]. The increase in total protein content after supplementation with *R. nasutus* indicates that this herb has the potential to lower glucose levels. Similar results were shown in our previous study in which we reported that *R. nasutus* had a significant hypoglycemic effect on STZ-induced diabetic rats and could reduce blood glucose levels [23].

R. nasutus methanolic extract significantly decreased AST and ALT levels in the liver tissue of diabetic rats to a similar extent as glibenclamide. Any abnormality in or stress on the protein or amino acid metabolism will have consequences in the tissue because these changes drive metabolism towards catabolic products such as ammonia. Free amino acids act as the currency through which protein metabolism operates in the cell [47]. Amino acid metabolism is a complex system involving transamination and oxidation. Transamination is a vital step in amino acid metabolism that involves the transfer of an amino group from one amino acid to the α-keto analog of another amino acid. This transfer results in the formation of another amino acid [48, 49]. The enzymes that metabolize the oxidation of the amino acid are known as aminotransferases [50]. Aminotransferase enzymes utilize pyridoxal phosphate, which is a cofactor derived from pyridoxine, as a key component in their catalytic mechanism [51]. Among these enzymes, AST and ALT are extensively distributed in the cells of all animals and provide the link between carbohydrate and protein metabolism by interconverting dynamic substances [52, 53]. AST catalyzes the interconversion of aspartic and α-ketoglutaric acids to oxaloacetic and glutamic acids, while ALT catalyzes the interconversion of alanine and α-ketoglutaric acid to pyruvic and glutamic acids [54]. These enzymes act as a bridge between protein and carbohydrate metabolisms, and the net result is the inclusion of keto acids into the tricarboxylic acid cycle. The increased levels of AST and ALT activity can be regarded as an indicator for gluconeogenesis [48, 55]. Because *R. nasutus* decreases AST and ALT levels, it is plausible that *R. nasutus* reduces gluconeogenesis and can be further investigated for the treatment of diabetes mellitus.

The dose of 200 mg/kg was selected based on the results of our previous study [23] in which we determined that a 200 mg/kg dose gave a similar effect as a 250 mg/kg dose. The toxicity of the extract was reported in earlier studies and no toxic effects were observed when the herb was orally administered at higher doses. Kupradinun et al. [56] reported that no toxic effect was observed when the extract was used at a dose of 500 mg/kg in animals. Furthermore, *R. nasutus* methanolic extract does not affect the levels of total carbohydrate, glycogen, protein, and liver markers (AST and ALT) in control rats. These findings indicate that *R. nasutus* only affects these markers in diseased conditions and suggests that this herb is safe for consumption by healthy subjects.

Recently, a number of experiments have suggested that medicinal plants have potential roles in ameliorating the effects of hyperglycemia and consequences of diabetes mellitus [40, 42, 57]. Our previous studies also indicated that *R. nasutus* had antihyperglycemic [23] and hypolipidemic effects [58] on STZ-induced diabetic rats. The hypoglycemic effects of *R. nasutus* could be related to its capacity to renovate the damage to liver tissue and scavenge free radicals [16, 24]. In this study, we focused on the effects of *R. nasutus* on the levels of tissue glycogen, carbohydrate, protein, and liver markers such as ALT and AST in STZ-induced diabetic rats. We found that treatment with the herb changes the levels of these markers to be similar to the levels found in nondiabetic rats. Future studies should be conducted to measure the active components of *R. nasutus* extract using gas chromatography or high-performance liquid chromatography to further confirm its potential. The effects of *R. nasutus* extract on pancreas function, especially on insulin production and pancreatic cell rescue should also be investigated. The addition of another group of normal rats administered with glibenclamide will also give further information on the effects of glibenclamide on the STZ group.

5. Conclusion

Chronic administration of *R. nasutus* for 30 days resulted in noteworthy improvements in the altered levels of total carbohydrate, total glycogen, total proteins, and AST and ALT activities found in diabetic rats which may contribute to the protective metabolic and hepatic effects of this plant. Overall, our findings indicate that *R. nasutus* may be useful as an antidiabetic drug or as an adjunct to modern antidiabetic therapies such as glibenclamide.

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Research Article

The Effect of Chinese Herbal Medicine on Albuminuria Levels in Patients with Diabetic Nephropathy: A Systematic Review and Meta-Analysis

Ya Xiao,[1,2] Yanyan Liu,[1,2] Keqiang Yu,[1,2] Lin Zhou,[3] Jianlu Bi,[1,2] Jingru Cheng,[1,2] Fei Li,[1,2] Ren Luo,[1,2] and Xiaoshan Zhao[1,2]

[1] *Department of Traditional Chinese Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China*
[2] *School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong 510515, China*
[3] *Endocrinology Department, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China*

Correspondence should be addressed to Ren Luo; luoren41671@aliyun.com and Xiaoshan Zhao; zhaoxs0609@163.com

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To evaluate the effect of Chinese herbal medicine (CHM) on albuminuria levels in patients with diabetic nephropathy (DN), we performed comprehensive searches on Medline database, Cochrane Library, CNKI database, CBM database, Wanfang database, and VIP database up to December 2012. A total of 29 trials including 2440 participants with DN met the selection criteria. CHM was tested to be more effective in reducing urinary albumin excretion rate (UAER) (MD −82.95 μg/min, [−138.64, −27.26]) and proteinuria (MD −565.99 mg/24 h, [−892.41, −239.57]) compared with placebo. CHM had a greater beneficial effect on reduction of UAER (MD −13.41 μg/min, [−20.63, −6.19]) and proteinuria (MD −87.48 mg/24 h, [−142.90, −32.06]) compared with angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB). Combination therapy with CHM and ACEI/ARB showed significant improvement in UAER (MD −28.18 μg/min, [−44.4, −11.97]), urinary albumin-creatinine ratio (MD −347.00, [−410.61, −283.39]), protein-creatinine ratio (MD −2.49, [−4.02, −0.96]), and proteinuria (MD −26.60 mg/24 h, [−26.73, −26.47]) compared with ACEI/ARB alone. No serious adverse events were reported. CHM seems to be an effective and safe therapy option to treat proteinuric patients with DN, suggesting that further study of CHM in the treatment of DN is warranted in rigorously designed, multicentre, large-scale trials with higher quality worldwide.

1. Introduction

Diabetic nephropathy (DN), defined as the presence of micro- or macroalbuminuria in patients with diabetes, is the most common cause of end-stage renal disease (ESRD) across the world [1]. The prevalence of micro- and macroalbuminuria in patients with diabetes is as high as 37–40% in western countries and 57.4–59.8% in Asian countries [2–4]. Albuminuria is a well-established risk factor for cardiovascular disease and is also associated with ESRD [5, 6]. Persistent albuminuria has toxic effect on tubular epithelial cells, causing tubulointerstitial inflammation and subsequent interstitial fibrosis. Angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) have been demonstrated to reduce albuminuria and delay the progression of DN by inhibition of renin-angiotensin system (RAS) and have become the standard of care for albuminuric patients [7, 8]. Despite the renoprotective effects of ACEI and ARB, diabetic nephropathy progresses to ESRD in a large proportion of patients [9]. This indicates that in addition to the RAS, other pathways are involved in the pathogenesis of DN. Chinese herbal medicine (CHM), which can produce a potential effect of multitarget therapy and block these pathways, seems appropriate in the treatment of DN caused by multiple factors [10].

In traditional Chinese medicine, diabetic nephropathy is considered nearly equivalent to the term "Xiao Ke Bing," which has been described in the "Yellow Emperor's Medicine Classic" (Chinese name in pinyin "Huang Di Nei Jing") more than 2000 years ago. Bawei Dihuang wan, originated from the "The Synopsis of Prescriptions of the Golden Chamber" in the Eastern Han Dynasty, is a famous Chinese herbal formula

that has been used for a long time in the treatment of DN. In recent years, more and more herbal products are thought to be effective in reducing urinary protein in patients with DN. A number of randomised controlled trials (RCTs) have suggested that CHM alone or combined with ACEI/ARB has therapeutic potential in the treatment of DN in terms of reducing urinary albumin excretion, ameliorating proteinuria, and symptom improvement [11]. How about the effect of CHM on albuminuria alone or in combination with ACEI/ARB as compared to ACEI/ARB? With a view to answering the question, the systematic review of randomized controlled trials evaluates the effects and safety of CHM on albuminuria in patients with DN.

2. Methods

2.1. Search Strategy. A comprehensive literature search was performed using Medline database (1989 to December 2012), Cochrane Library (1993 to December 2012), CNKI database (1979 to December 2012), Chinese Biomedical Literature database (1990 to December 2012), Wanfang database (1982 to December 2012), and VIP database (1989 to December 2012). Keywords for searching included diabetes or diabetic, nephropathy, kidney disease, traditional Chinese medicine, herbal-medicine, alternative-medicine, complementary-medicine, plants, herbs, and phytotherapy. The search was restricted to studies carried out in humans. No limit was placed on language. Manual searches of conference compilations supplemented electronic searches.

2.2. Study Selection. Studies were considered to be eligible for inclusion if they met all of the following criteria. (i) Patients included in the study were diagnosed with type 2 diabetes mellitus complicated with kidney disease, regardless of the stage of the DN (microalbuminuria defined as urine albumin excretion rate (UAER) of 20–200 μg/min, or macroalbuminuria defined as UAER >200 μg/min). (ii) The study was performed as a randomized controlled trial (RCT) describing a correct randomization procedure. Trials which used a clearly inappropriate method of randomization (e.g., open alternation) were excluded. (iii) The intervention of CHM included extract from herbs, single herbs, Chinese patent medicines, or a compound of herbs that was prescribed (individualized treatment) by Chinese practitioner. The control intervention included placebo or ACEI/ARB. Hypoglycemic therapy was used as a cointervention in both of the arms, including oral hypoglycemic drugs, insulin, and exercise. (iv) Outcomes included at least one of the following: urine albumin excretion rate, proteinuria, urinary albumin-creatinine ratio, or urinary protein-creatinine ratio.

2.3. Data Extraction. Two researchers independently extracted data, including study design, randomization, blinding and subject characteristics (e.g., age, sex, sample size, and albuminuria stage), and duration of treatment. Disagreements were resolved after discussion with other investigators.

2.4. Data Analysis. Meta-analysis was carried out using Review Manager software (version 5.1), provided by the Cochrane Collaboration. The mean change in each study end point from baseline was treated as a continuous variable. Continuous data were presented as mean difference (MD), with 95% confidence interval (CI). The chi-squared test for heterogeneity was performed, and heterogeneity was presented as significant when I^2 is over 50% or $P < 0.1$. Random effect model was used for the meta-analysis if there was significant heterogeneity, and fixed effect model was used when the heterogeneity was not significant.

3. Results

3.1. Search Results. A total of 3937 publications were identified by both computer search and manual search of cited references. Of these, 1343 articles were determined to be duplicated. The remaining 2594 reports were retrieved in full text, of which 1991 were excluded on review of the titles and abstracts. After further reading, we excluded 530 for not describing randomization procedure, 25 non-ACEI/ARB or placebo comparators, 16 no outcome of interest, and 3 duplicated reports. Finally, a total of 29 studies were included in the meta-analysis. Figure 1 is a flow chart of study selection process.

3.2. Characteristics and Methodological Quality of Included Trials. All 29 publications included were of a randomization procedure generated by a random number table or computer [12–40]. Twenty-seven studies were published in Chinese and the other two in English. Numbers of participants of the individual studies varied from 40 to 409 with a total of 2440 participants included in this paper (Table 1). The majority duration of treatment varied from one month to three months.

The Jadad scale is a 5-point scale for assessing the quality of RCTs in which three points or more indicate superior quality [41]. Of the 29 RCTs, 11 trials were of superior quality according to the Jadad score (≥3 points) [12, 15, 17, 21, 24, 25, 32, 34, 38–40]. All studies described a correct randomization procedure, but only one of them mentioned allocation concealment [39]. Three out of 29 studies described blinding of participants [12, 39, 40]. Ten trials reported the dropouts information and mentioned follow-up, but this dropouts were not captured in the analysis [12, 15, 17, 21, 24, 25, 32, 34, 38, 39]. Among all trials, the characteristics of participants in different treatment groups were similar at baseline (age, sex, race, and disease course).

3.3. Analysis of Chinese Herbal Medicine. A total of 84 different kinds of herbs were included in 29 herbal preparations for treatment of DN. In Table 2, we listed the 14 herbs that were included most frequently in the 29 herbal preparations. For example, the herb used most often, *Astragalus membranaceus* (Huang Qi), was used 22 times in 29 different herbal preparations; the herb used second frequently, *Salvia miltiorrhiza* (Dan Shen), was used in 15 of 29 herbal preparations. Each compound prescription contained an average of 9 ingredients (range: 2–14). The formulations of CHM were different and included tablet, capsule, oral liquid, and decoction.

Table 1: Characteristics of the 29 studies included in the meta-analysis.

Author (s), year	Patients included	Men (%)	Age (years)	Albuminuria	Interventions		Treatment duration	Jadad score
					Experimental	Control		
Ma et al., 2011 [12]	409	45	56.6	Microalb Macroalb	Arctiin granule (TID)	Placebo (TID)	8 weeks	5
Chen, 2010 [13]	60	45	60.5	Microalb Macroalb	Anshen yin (TID)	Losartan (50 mg/d, QD)	12 weeks	2
Xu, 2005 [14]	64	62.5	56.2	Microalb	Baoshen tang (TID)	Benazepril (5–10 mg/d, QD)	12 weeks	2
Luo, 2008 [15]	72	54.2	56.8	Microalb	Bushen Huoxue decoction (BID)	Benazepril (10 mg/d, QD)	12 weeks	3
Huang and Xu, 2008 [16]	68	54.4	58.0	Microalb	Tangluo Tongshui decoction (BID)	Losartan (50 mg/d, QD)	8 weeks	2
Ge et al., 2010 [17]	55	56.9	51.5	Macroalb	Tripterygium glycosides (120 mg/d, TID)	Valsartan (160 mg/d, QD)	24 weeks	3
Xue and Bai, 2008 [18]	60	55.0	NA	Microalb	Liuwei Dihuang tang (BID)	Losartan (100 mg/d, QD)	12 weeks	2
Zhang, 2012 [19]	70	54.3	62.4	Microalb	Pishen Shuangbu tang (BID)	Benazepril (10 mg/d, QD)	4 weeks	2
Huang, 2011 [20]	70	52.9	56.0	Microalb	Shen an decoction (BID)	Captopril (37.5 mg/d, TID)	8 weeks	2
Zhang et al., 2011 [21]	227	NA	NA	Microalb Macroalb	Tangshen Kang capsule (TID)	Enalapril (10 mg/d, BID)	8 weeks	3
Huang, 2012 [22]	80	61.3	53.1	Macroalb	Wenshen Jianpi Huoxue tang (BID)	Benazepril (10 mg/d, QD)	8 weeks	2
Dong et al., 2007 [23]	68	57.4	55.0	Microalb	Yiqi Huoxue tang (BID)	Valsartan (80 mg/d, QD)	8 weeks	2
Zhou et al., 2009 [24]	109	38.5	54.8	Microalb	Tangshen decoction (BID)	Losartan (50 mg/d, QD)	12 weeks	3
Wang et al., 2012 [25]	75	51.3	57.2	Microalb	Yiqi Yangyin Xiaozheng Tongluo decoction (BID)	Irbesartan (150 mg/d, QD)	48 weeks	3
Zhong et al., 2012 [26]	100	53.0	48.0	Macroalb	Ziyin Zhuyang Digui tang (BID)	Benazepril (10 mg/d, QD)	12 weeks	2
Chen and Wan, 2011 [27]	62	48.4	61.6	Microalb	Qishen Yiqi drop pill (TID) Enalapril (10 mg/d, QD)	Enalapril (10 mg/d, QD)	8 weeks	2
Wei et al., 2010 [28]	60	55.0	NA	Microalb	Fufang Danpi decoction (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	8 weeks	2
Feng et al., 2005 [29]	60	63.3	54.8	Microalb	Kangshen tang (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	12 weeks	2
Zhu et al., 2004 [30]	42	50.0	54.8	Microalb	Pingxiao Gujing tang (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	8 weeks	2
Li et al., 2006 [31]	81	49.4	50.7	Microalb	Tangshen ling decoction (BID) Telmisartan (80 mg/d, QD)	Telmisartan (80 mg/d, QD)	8 weeks	2
Pan and Xue, 2009 [32]	81	46.6	54.4	Microalb	Tangshen tang (BID) Valsartan (80 mg/d, QD)	Valsartan (80 mg/d, QD)	8 weeks	3
Gong and Wang, 2004 [33]	80	53.8	59.0	Microalb	Yangyin Yiqi decoction (BID) benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	8 weeks	2
Cai et al., 2012 [34]	63	63.5	41.7	Microalb	Yiqi Yangyin Huazhuo Tongluo decoction (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	8 weeks	3
Qu, 2012 [35]	68	55.9	62.4	Microalb	Chunze tang (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	2 weeks	2
Li, 2004 [36]	40	45.0	51.8	Microalb	Modified Liuwei Dihuang tang (BID) Enalapril (10 mg/d, QD)	Enalapril (10 mg/d, QD)	12 weeks	2

Table 1: Continued.

Author (s), year	Patients included	Men (%)	Age (years)	Albuminuria	Interventions		Treatment duration	Jadad score
					Experimental	Control		
Wu and Zhang, 2005 [37]	60	43.3	59.0	Microalb	Tangshen kang (BID) Fosinopril (10 mg/d, QD)	Fosinopril (10 mg/d, QD)	8 weeks	2
Chen and Huang, 2006 [38]	60	NA	NA	Microalb	Wuchong tang (BID) Benazepril (10 mg/d, QD)	Benazepril (10 mg/d, QD)	8 weeks	3
Fallahzadeh et al., 2012 [39]	56	46.7	56.8	Macroalb	Silymarin (520 mg/d, TID) ACEI/ARB	Placebo (TID) ACEI/ARB	12 weeks	5
Khajehdehi et al., 2011 [40]	40	55	52.8	Macroalb	Turmeric (1500 mg/d, TID) ACEI/ARB	Placebo (TID) ACEI/ARB	8 weeks	4

Microalb: microalbuminuria; Macroalb: Macroalbuminuria; QD: once a day; BID: twice a day; TID: three times a day. NA: not applicable.

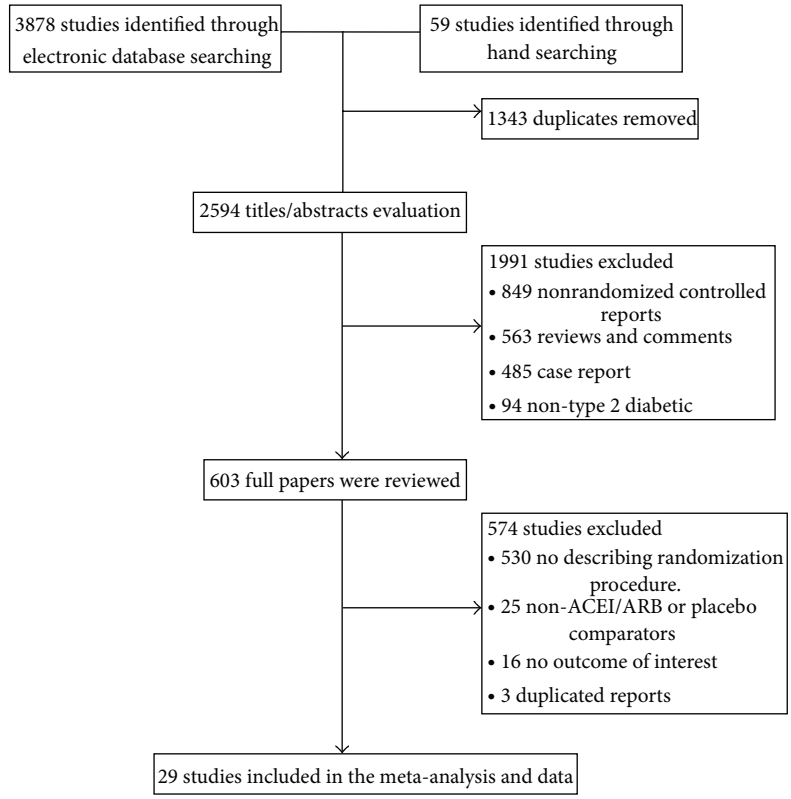


Figure 1: Flow chart of study selection process.

Table 2: The 14 herbs used most often for Chinese herbal preparations in the included 29 RCTs.

English herbal name (Chinese pinyin)	Number of occurrences in 29 herbal preparations	Frequency of use (%)
Astragalus (Huang Qi)	22	75.86
Salvia miltiorrhiza (Dan Shen)	15	51.72
Poria (Fuling)	10	34.48
Rhizoma Dioscoreae Oppositae (Shan Yao)	9	31.03
Rehmannia Root (Sheng Di Huang)	7	24.14
Fructus Macrocarpii (Shan Zhu Yu)	7	24.14
Rhizoma Polygonati Sibirici (Huang Jing)	7	24.14
Rhizoma Alismatis (Ze Xie)	7	24.14
Radix Rehmanniae preparata (Shu Di Huang)	6	20.69
Herba Leonuri Japonici (Yi Mu Cao)	6	20.69
Radix et Rhizoma Rhei Palmati (Da Huang)	6	20.69
Rhizoma Chuanxiong (Chuan Xiong)	5	17.24
Radix Codonopsis (Dang Shen)	5	17.24
Radix Pseudostellariae (Tai Zi Shen)	5	17.24

Frequency of use = number of occurrences/total number of herbal preparations.

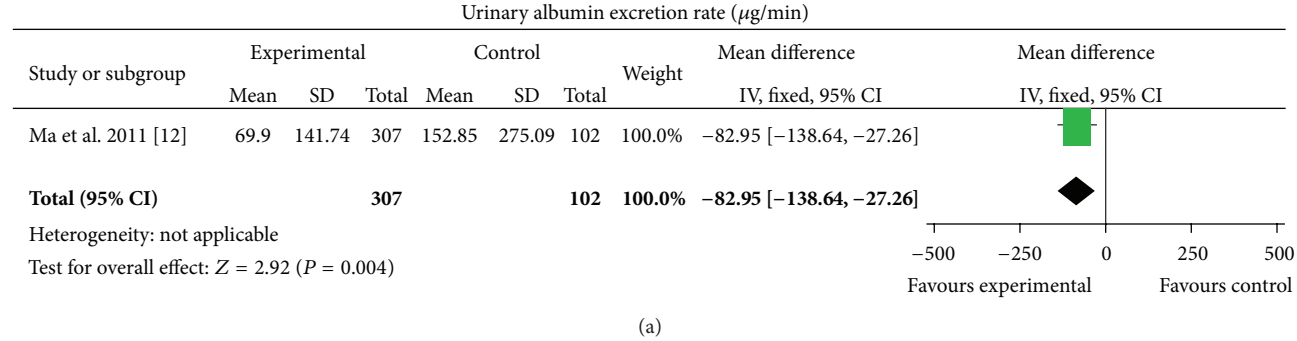
3.4. The Effects of Interventions

3.4.1. CHM versus Placebo. One trial tested Arctiin compared with placebo in patients with DN [12]. Arctiin showed significant improvement in urinary albumin excretion rate (MD −82.95 μg/min, [−138.64, −27.26]) and proteinuria (MD −565.99 mg/24 h, [−892.41, −239.57]) after two months of treatment compared with placebo (Figure 2).

3.4.2. CHM versus ACEI/ARB. 14 different CHM were tested compared with ACEI/ARB [13–26], including one extract from a single herb and 13 self-composed Chinese herbal compound prescriptions. Urinary albumin excretion rate was evaluated in 10 studies and proteinuria in 8 studies. 10 trials reported significant improvement in urinary albumin excretion rate after treatment of CHM compared with ACEI/ARB (MD −13.41 μg/min, [−20.63, −6.19]), with significant heterogeneity between the studies ($\text{Chi}^2 = 81.21$, $I^2 = 89\%$) (Figure 3). CHM showed significant improvement in proteinuria compared with ACEI/ARB in 8 studies (MD −87.48 mg/24 h, [−142.90, −32.06]) and there was significant heterogeneity ($\text{Chi}^2 = 56.78$, $I^2 = 88\%$) (Figure 3).

3.4.3. CHM plus ACEI/ARB versus ACEI/ARB

CHM plus ACEI/ARB versus No Treatment plus ACEI/ARB. One Chinese patent medicine and 11 different self-composed Chinese herbal compound prescriptions were tested [27–38]. Urinary albumin excretion rate was evaluated in 12 studies



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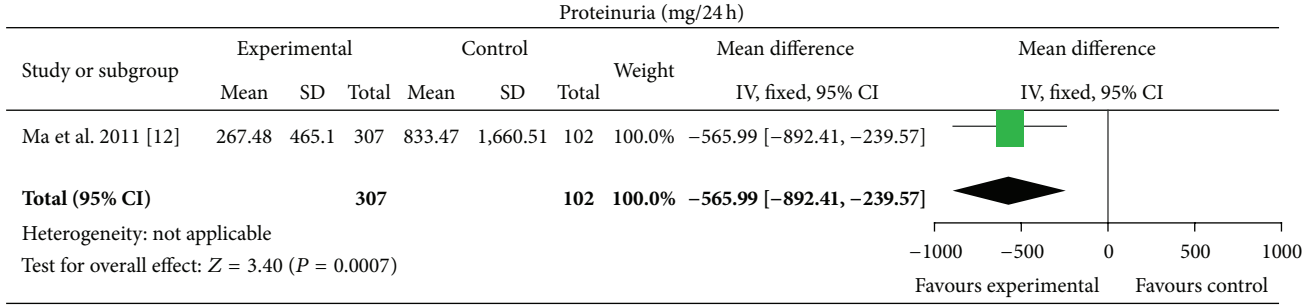


(b)

Figure 2: CHM versus placebo.

and proteinuria in one study. CHM plus ACEI/ARB showed statistically significant improvement in urinary albumin excretion rate (MD −28.18 μg/min, [−44.4, −11.97]), with significant heterogeneity between 12 studies (Chi^2 = 368.41, I^2 = 97%) (Figure 4). One trial reported significant improvement in proteinuria after treatment of CHM plus ACEI/ARB compared with ACEI/ARB (MD −26.60 mg/24 h, [−26.73, −26.47]) (Figure 4).

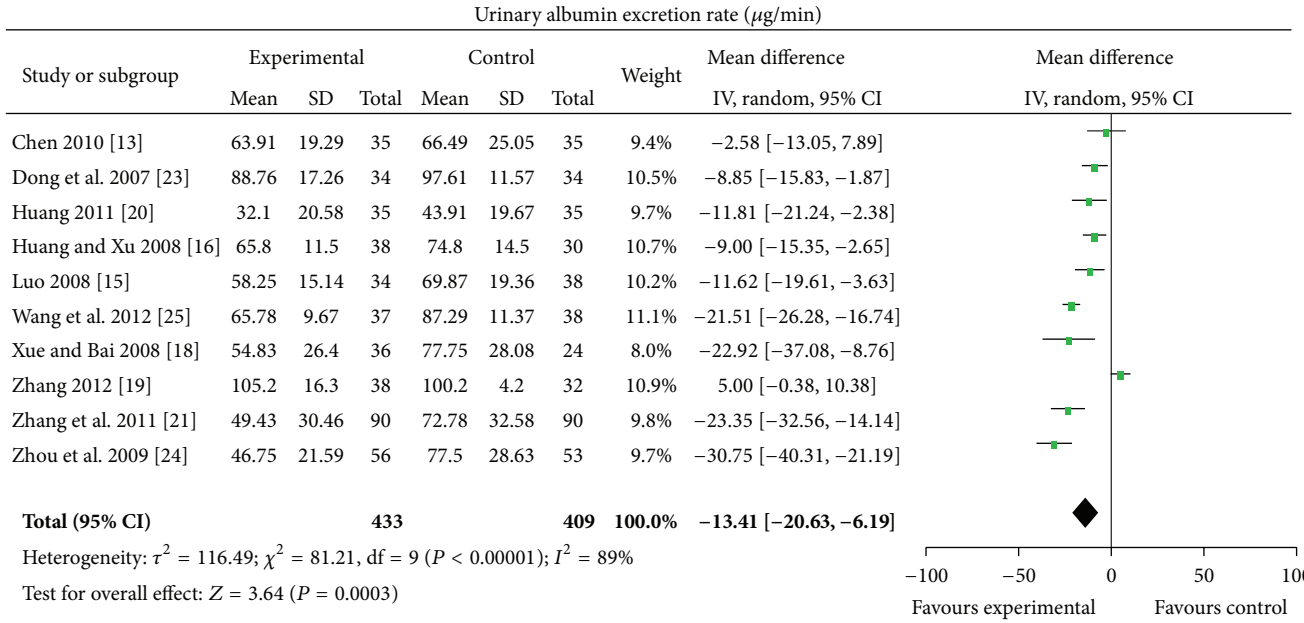
CHM plus ACEI/ARB versus Placebo plus ACEI/ARB. Two different extracts from single herbs were tested [39, 40]. Silymarin plus ACEI/ARB showed significant improvement in the change of urinary albumin-creatinine ratio from baseline (MD −347.00, [−410.61, −283.39]) compared with placebo plus ACEI/ARB (Figure 5). Turmeric plus ACEI/ARB showed significant improvement in the change of protein-creatinine ratio (MD −2.49, [−4.02, −0.96]) and proteinuria (MD −1448.20 mg/24 h, [−2775.35, −121.05]) from baseline compared with placebo plus ACEI/ARB (Figure 5).

3.5. Adverse Events. Fifteen trials out of 29 included trials mentioned the occurrence of adverse events [12, 13, 15, 17, 19, 24, 25, 28, 29, 32, 33, 35, 36, 39, 40]. Seven of these reported no adverse effects during herbal treatment [13, 25, 29, 32, 33, 36, 40]. Eight trials reported nonserious adverse events. Ma et al. reported that 13 out of 307 patients had experienced a variety of symptoms including abdominal pain, diarrhea, and loose stools after taking Arctiin granule [12]. These symptoms could be tolerated by patients. One patient stopped the treatment of Tripterygium glycosides due to leucopenia [17]. Among 38 patients treated with Pishen Shuangbu tang, one patient developed mild diarrhoea, and one developed dizziness [19]. The symptoms were relieved after stopping the treatment. One patient developed mild diarrhea after taking Tangshen fang [24]. Adverse effects in ACEI/ARB treated patients included dry cough, hyperkalemia, and doubling of serum creatinine [15, 17, 19, 28, 35, 39]. There was no significant difference between herbal treatment and ACEI/ARB regarding the incidence of adverse effects. No serious adverse events were reported.

4. Discussion

Based on the meta-analysis of 29 randomized controlled trials, CHM was tested to be more effective in reducing UAER and proteinuria compared with placebo or ACEI/ARB. Combination therapy with CHM and ACEI/ARB showed significant improvement in UAER, urinary albumin-creatinine ratio, protein-creatinine ratio, and proteinuria as compared to ACEI/ARB. It should be noted that there were no reported serious adverse events associated with CHM studied. To summarize, the results revealed that CHM is an effective and safe therapy option to treat albuminuric patients with DN.

In TCM, diabetic nephropathy referred to as an intrinsically deficient but extrinsically excessive syndrome. Deficiency of qi and yin, and excess of stasis and dampness are believed to be the main mechanism responsible for development of DN [42]. Among the included 29 RCTs, 29 different herbal preparations were tested, including four extracts from a single herb, one Chinese patent medicine, and 24 Chinese herbal compound prescriptions. Of the 24 compound prescriptions, Bushen Huoxue decoction, Pishen Shuangbu tang, and modified Liuwei Dihuang tang were prescribed based on Liuwei Dihuang tang, which has the function of nourishing the kidney yin. A total of 84 different



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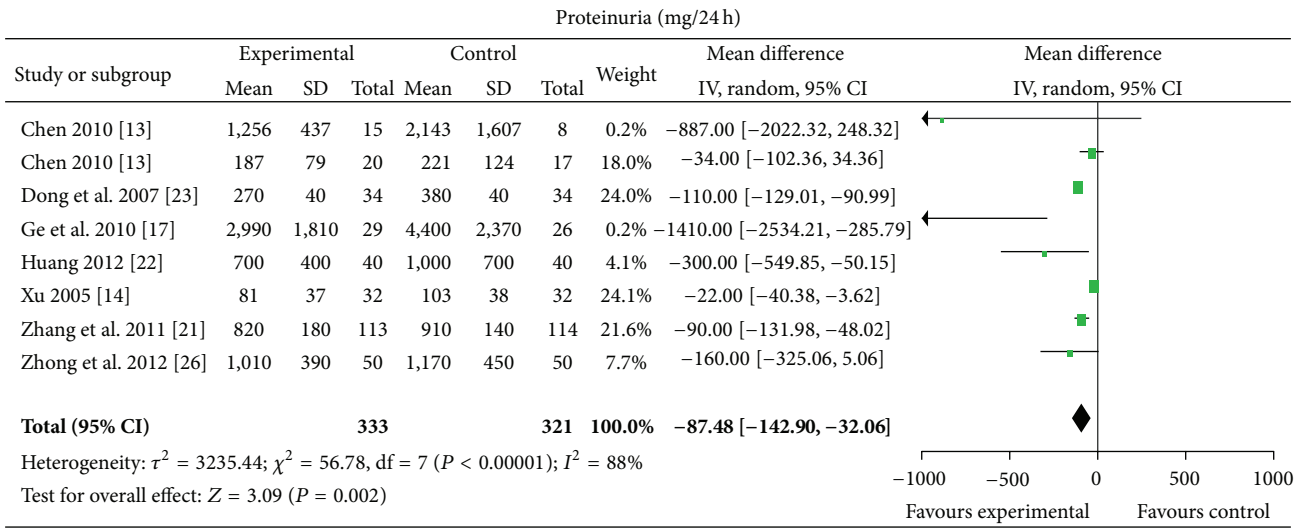
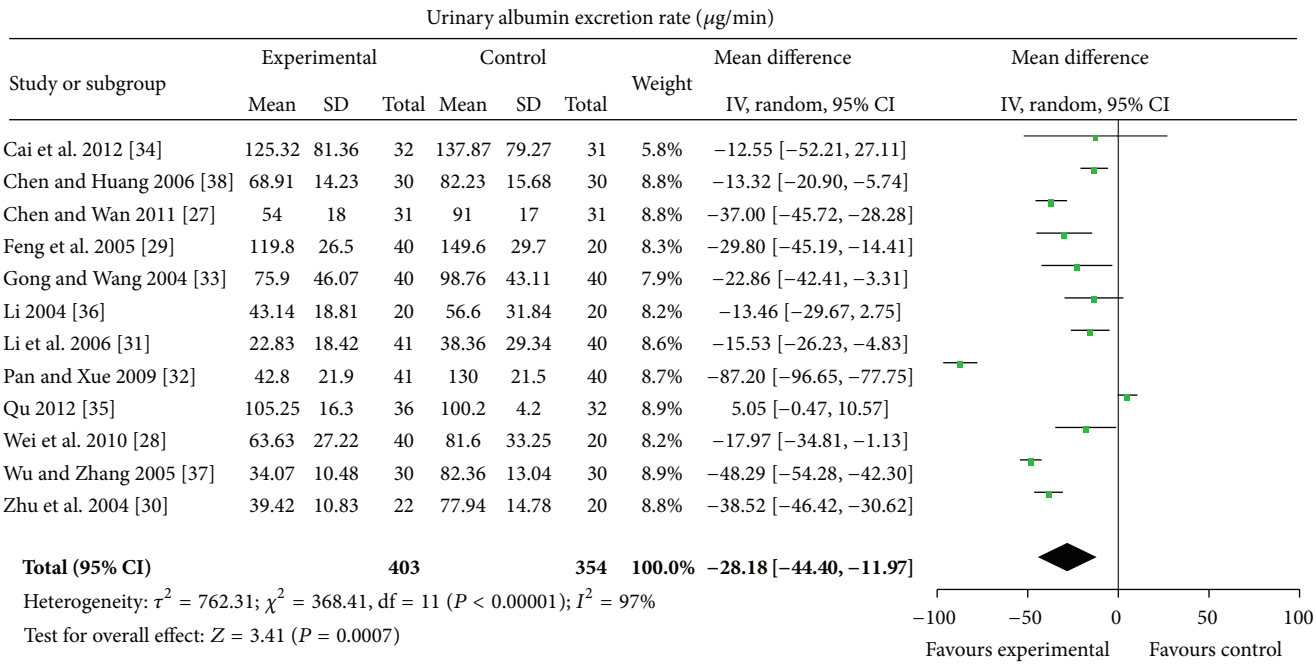


(b)

Figure 3: CHM versus ACEI/ARB.

kinds of herbs were included in 29 herbal preparations for treatment of DN. From the results of frequency distribution of categorized herbs according to their functions, herbs with qi-tonifying and yin-nourishing, blood-activating and stasis-resolving, kidney-replenishing and water-draining appeared to be most frequently prescribed for the treatment of DN.

The pathogenesis of diabetic nephropathy is complex and not yet fully clarified. In addition to the RAS, other pathways such as oxidative stress, inflammation, and excessive production of advanced glycation end products also contribute to the development of DN [43–45]. Therefore, although use of RAS antagonists appears to slow the progression of DN development to ESRD, it does not stop or reverse the pathology. Each herbal product within the TCM formulations could have several different active ingredients to attack a disease process in manifold ways. For example, astragalus polysaccharide has prophylactic and therapeutic effects on the progress of DN by decreasing the mRNA level of NF-κB in renal cortex and increasing IkB mRNA expression in rats [46]. Additionally, the antioxidative effect of *Astragalus membranaceus* as a free radical scavenger implies its protective effect in the early stage of DN [47]. *Salvia miltiorrhiza* could be applicable for the treatment of DN by reducing the serum and kidney levels of transforming growth factor β1 (TGF-β1) and the kidney levels of collagen IV, monocytes/macrophages (ED-1), and the receptor for advanced glycation end-products (RAGE) [48]. Corni Fructus has the potential to protect the animals from diabetic nephropathy by amelioration of oxidative stress and stimulation of PPARγ expression [49]. These studies' results suggest that CHM can produce a potential effect of



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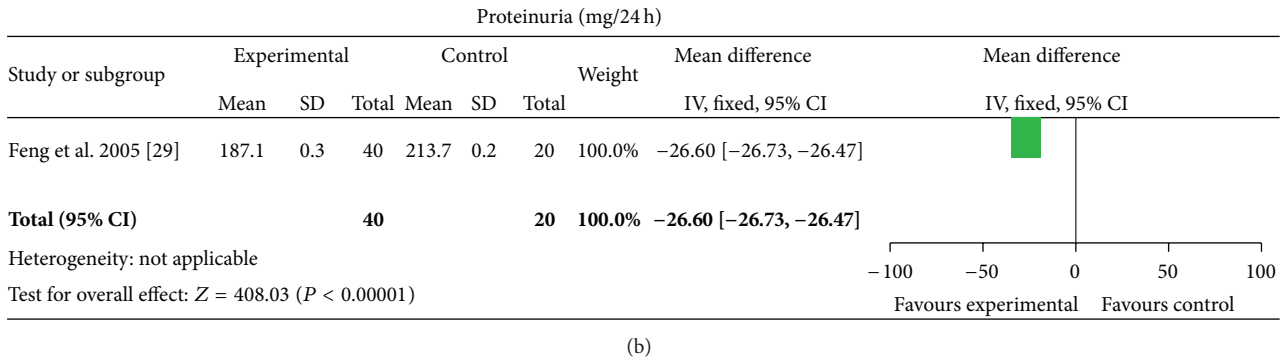

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Figure 4: CHM plus ACEI/ARB versus no treatment plus ACEI/ARB.

multitarget therapy, which seems appropriate in the treatment of DN caused by multiple factors.

It must be acknowledged, however, that the methodological quality of the trials evaluating the effect of CHM on DN was generally not high: 18/29 (62%) of the RCTs included in this review were scored as having mediocre methodological quality [Jadad scores = 2]. No trial was identified as a multicenter, large sample, prospective, double-blinded, controlled randomized trial. Furthermore, most of the studies did not report about allocation concealment process, which may have created potential selection bias. The possibility of publication bias in the reporting of RCTs is always of concern. Although we performed comprehensive searches and tried to avoid bias, since most of the studies were published in Chinese, there remained the possible existence of publication bias.

It is noteworthy that discrepancy in the herbal composition, drug formulation, and dose was observed between the studies, which may be the source of heterogeneity in the included RCTs. TCM formulas were composed of many herbs and the content and biological activities of these herbs can be influenced by many things, including where the herb was grown, and at what season it was harvested. Consequently, CHM for treating DN needs to equip standardized criteria for use to ensure the good reproducibility of the research result in real clinical practices.

The results of the present review provide strong evidence of the efficacy of CHM in reducing UAER, proteinuria, urinary albumin-creatinine ratio, and protein-creatinine ratio, suggesting that CHM can be used as an alternative therapy for the treatment of DN. However, majority of included studies were scored as having mediocre methodological quality. Future clinical trials of CHM on DN need to improve methodological quality and reported well according to the CONSORT statement [50]. Hence, we conclude that further study of CHM in the treatment of DN is warranted in rigorously designed, multicentre, large-scale trials with higher quality worldwide.

Authors' Contribution

Ren Luo and Xiaoshan Zhao contributed in study concept and design: Lin Zhou, Jianlu Bi, Jingru Cheng, and Fei Li

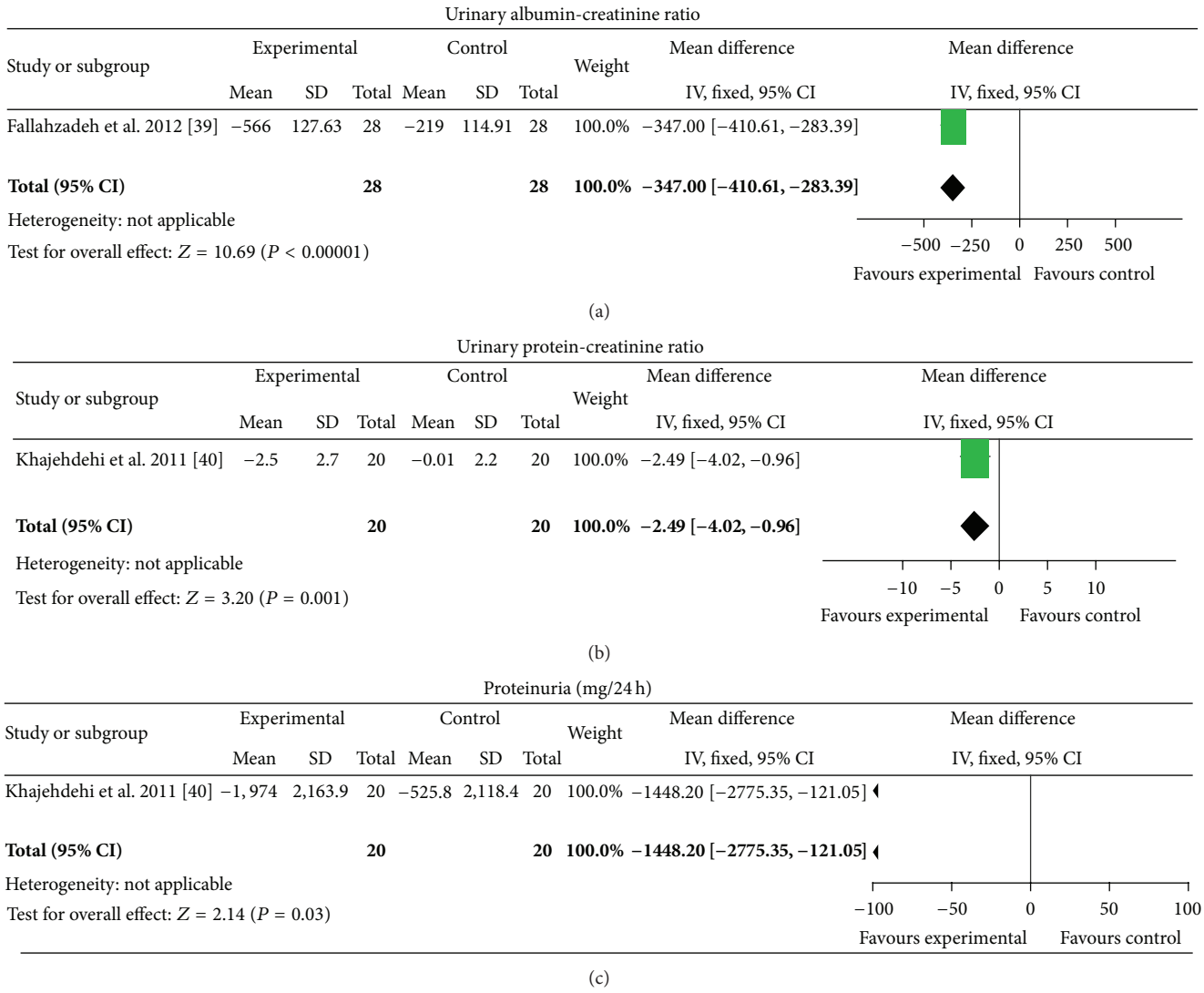

Figure 5: CHM plus ACEI/ARB versus placebo plus ACEI/ARB.

contributed in acquisition of data; Ya Xiao, Yanyan Liu, and Keqian Yu contributed in analysis and interpretation of data; Ya Xiao, Yanyan Liu and Keqian Yu contributed in drafting of the paper for important intellectual content; Ren Luo and Xiaoshan Zhao contributed in study supervision. All authors approved the final version to be published. Ya Xiao, Yanyan Liu, and Keqiang Yu contributed equally to this work.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Renal Protective Role of Xiexin Decoction with Multiple Active Ingredients Involves Inhibition of Inflammation through Downregulation of the Nuclear Factor-κB Pathway in Diabetic Rats

Jia-sheng Wu,[1] Rong Shi,[1] Jie Zhong,[1] Xiong Lu,[2] Bing-liang Ma,[1] Tian-ming Wang,[1] Bin Zan,[1] Yue-Ming Ma,[1] Neng-neng Cheng,[3] and Fu-rong Qiu[4]

[1] *Department of Pharmacology, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*
[2] *Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China*
[3] *Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 201203, China*
[4] *Laboratory of Clinical Pharmacokinetics, Shanghai Shuguang Hospital, Shanghai 201203, China*

Correspondence should be addressed to Yue-Ming Ma; mayueming_117@hotmail.com
and Neng-neng Cheng; nncheng@gmail.com

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In Chinese medicine, Xiexin decoction (XXD) has been used for the clinical treatment of diabetes for at least 1700 years. The present study was conducted to investigate the effective ingredients of XXD and their molecular mechanisms of antidiabetic nephropathy in rats. Rats with diabetes induced by high-fat diet and streptozotocin were treated with XXD extract for 12 weeks. XXD significantly improved the glucolipid metabolism disorder, attenuated albuminuria and renal pathological changes, reduced renal advanced glycation end-products, inhibited receptor for advanced glycation end-product and inflammation factors expression, suppressed renal nuclear factor-κB pathway activity, and downregulated renal transforming growth factor-β1. The concentrations of multiple components in plasma from XXD were determined by liquid chromatography and tandem mass spectrometry. Pharmacokinetic/pharmacodynamic analysis using partial least square regression revealed that 8 ingredients of XXD were responsible for renal protective effects via actions on multiple molecular targets. Our study suggests that the renal protective role of XXD with multiple effective ingredients involves inhibition of inflammation through downregulation of the nuclear factor-κB pathway, reducing renal advanced glycation end-products and receptor for advanced glycation end-product in diabetic rats.

1. Introduction

The number of diabetic patients is increasing rapidly worldwide [1]. Diabetic nephropathy (DN) is one of the main microvascular complications of diabetes, and also the main cause of end-stage renal disease [2]. The pathogenesis of DN is complicated. Hyperglycaemia induces renal injury through multiple pathways, including the polyol pathway [3], the protein kinase C pathway [4], generation of advanced glycation end-products (AGEs) [5], oxidative stress [6], and inflammation [7, 8]. Previous studies have shown that the development of DN is a slow process. Clinical measures currently used to control blood glucose and blood pressure and to inhibit the renin-angiotensin system can delay this process [9–11]. However, the number of patients whose disease has progressed from diabetes mellitus to end-stage renal failure continues to increase, even if these measures have been adopted [12]. Therefore, new drugs must be researched and developed to prevent the occurrence and development of DN more effectively.

Traditional Chinese medicine (TCM) has been used to treat diabetes mellitus for several thousand years [13]. Recent studies have shown that Chinese herbal compounds significantly promote recovery in experimental diabetes and its complications [14–17]. These findings imply that TCM could be useful clinically for the treatment of diabetes mellitus and its complications. The development of new drugs based on classical TCM compounds is an important approach for TCM translational medicine research. Xiexin decoction (XXD) is a classic Chinese herbal preparation containing Radix et Rhizoma Rhei (*Rheum palmatum L.*), Rhizoma Coptidis (*Coptis chinensis* Franch), and Radix Scutellaria (*Scutellaria baicalensis* Georgi) in the ratio of 2 : 1 : 1 (w/w). It has been used for the treatment of diabetic mellitus (called xiaoke disease in TCM) since the Tong Dynasty (6th century C.E.) [18, 19]. Our previous studies showed that XXD had beneficial effects on early-stage DN [20]. However, the molecular mechanism of action of XXD is not yet clear, thereby limiting further research and development. Studies showed that XXD had obvious anti-inflammatory effects [21–23]. The relationship between the anti-DN effect and the anti-inflammatory effects of XXD is not clear. This research aimed to elucidate the molecular mechanism underlying XXD's anti-DN activity, with a focus on its anti-inflammatory effects.

Chinese herbal compounds with multiple ingredients always act on many targets simultaneously to generate a range of actions that manifest as a comprehensive overall effect. Our previous studies [24] showed that 11 ingredients were measurable in rat plasma after oral administration of XXD, including coptisine, jatrorrhizine, berberine, palmatine, baicalin, baicalein, wogonoside, wogonin, rhein, emodin, and aloeemodin (Figure 1). However, the effective ingredients for the anti-DN effect of XXD *in vivo* are currently unclear. Moreover, the relationship between the effective ingredients and their molecular mechanisms is also not clear, thereby limiting further research and development of XXD. A combined pharmacokinetics/pharmacodynamics (PK/PD) approach can be used to identify the effective ingredients in TCM. However, the traditional PK/PD model is not suitable because the time-effect relationship is not always clear after TCM administration; repeated administrations can cause obvious effects; and multiple ingredients simultaneously act on multiple targets to cause different effects [25]. After the administration of TCM, the relationships between the range of ingredients present and their effects on multiple targets *in vivo* are very complicated. The partial least squares (PLS) regression method provides a linear regression model for the analysis of the relationships between multiple dependent, and multiple independent, variables. This method, which has some advantages over traditional regression analysis, can be used to analyse complicated relationships between 2 sets of multiple variables [26]. This approach has been successfully applied in quantitative structure-activity relationship analysis [27], quantitative structure-PK relationship analysis [28], metabolomic analysis [29], and analysis of the relationships between genes and disease [30]. The present study aimed to perform a PK/PD model analysis using the PLS regression method to investigate the relationship between the ingredients of XXD and its anti-DN effect and to explore viable research methods for analysis of the active ingredients of TCM.

Table 1: Contents of the Xiexin decoction ingredients.

Ingredients	Content (mg/g)
Baicalin	32.1 ± 1.3
Berberine	7.2 ± 0.5
Wogonoside	5.8 ± 0.5
Baicalein	3.3 ± 0.1
Coptisine	2.4 ± 0.2
Palmatine	2.2 ± 0.1
Jatrorrhizine	1.8 ± 0.2
Wogonin	1.8 ± 0.3
Rhein	0.26 ± 0.04
Emodin	0.029 ± 0.002
Aloeemodin	0.028 ± 0.003

2. Materials and Methods

2.1. Antibodies. For western blot analysis, polyclonal antibodies of transforming growth factor-β1 (TGF-β1), nuclear factor-κBp65 (NF-κBp65), inhibitor of nuclear factor κB kinase subunit α (IKKα), inhibitor of nuclear factor κB subunit α (IκBα), phospho-NF-κBp65, and phospho-IκBα were obtained from Cell Signaling Technology, USA. Polyclonal antibodies of receptor for AGE (RAGE), intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), and β-actin were obtained from Santa Cruz Biotechnology, USA.

2.2. Preparation and High-Pressure Liquid Chromatography (HPLC) Analysis of XXD. Herbs present in XXD, including Radix et Rhizoma Rhei, Rhizoma Coptidis, and Radix Scutellaria, were purchased from the Shanghai Kang Qiao herbal pieces Co. (Shanghai, China). Authentication of these herbs was performed by Professor Zhi-Li Zhao, Department of Botany, Shanghai University of TCM, China. XXD was prepared as previously described [24]. Simultaneous quantification of 11 typical ingredients of this extract (Table 1) was performed using HPLC methods [31, 32].

2.3. Animals and Diabetic Model. Male Sprague-Dawley rats (90–100 g) were purchased from the Shanghai Slac Laboratory Animal Co. (Shanghai, China). The rats were housed in an air-conditioned room at 22–24°C under a 12-h dark/light cycle and were given food and water at libitum. All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals at Shanghai University of TCM. After 1-week adaptation, animals were divided into a normal control (NC) group fed a standard diet and a high-fat group received a high-fat diet. After 4 weeks, rats on the high-fat diet were treated with streptozotocin (40 mg/kg, intraperitoneal injection). All

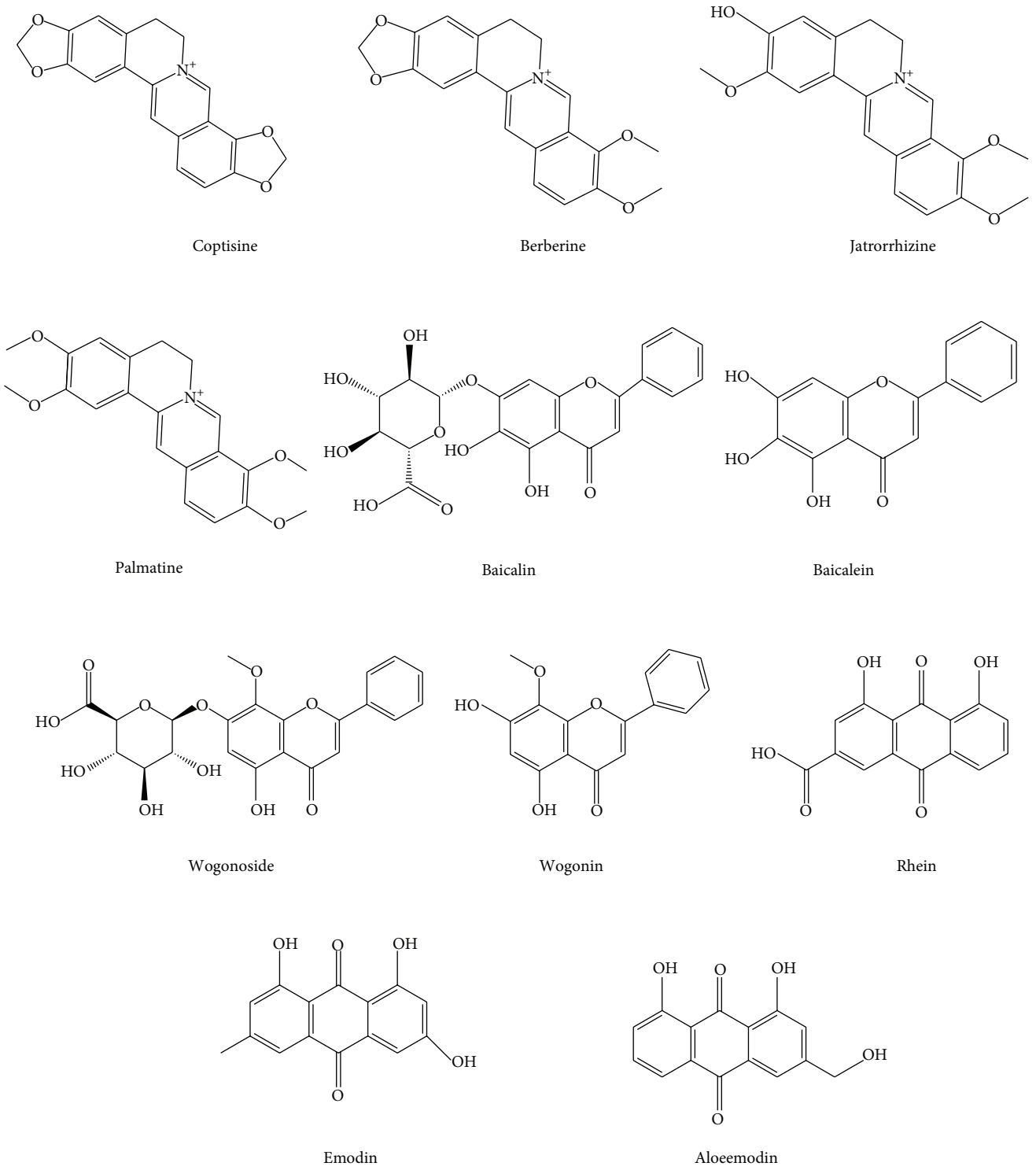

Figure 1: Chemical structures of Xiexin decoction ingredients.

diabetic rats with fasting blood glucose (FBG) levels above 16.7 mmol/L were then randomly divided into 5 groups: diabetic model control (DM); XXD extract 1.25 g/kg (DM + XXDL); XXD extract 2.5 g/kg (DM + XXDH); losartan 10 mg/kg (DM + Losartan); and Metformin 100 mg/kg (DM + metformin). In the clinical practice of TCM, XXD is usually prescribed at a daily dose of 46 g of herbal materials (amount to 12 g extract) for diabetic patient [18]. When this human dose was converted into an animal dose (a person of 60 kg, and a conversion factor of 6.25 between human and rat), it

Table 2: Nucleotide sequence of primers used in real-time PCR.

Gene	Primers	Nucleotide sequence 5′-3′
ICAM-1	Forward; reverse	AGGTATCCATCCATCCCAC; GCCGAGGTTCTCGTCTTC
MCP-1	Forward; reverse	TCTCTTCCTCCACCACTATGCA; GGCTGAGACAGCACGTGGAT
TGF-β1	Forward; reverse	GCTAATGGTGGACCGCAACAAC; TCTGGCACTGCTTCCCGAATG
NF-κBp65	Forward; reverse	GGCAGCACTCCTTATCAA; GGTGTCGTCCCATCGTAG
β-actin	Forward; reverse	TTATCGGCAATGAGCGGTTC; AGCACTGTGTTGGCATAGAG

ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; TGF-β1: transforming growth factor-β1; NF-κBp65: nuclear factor κBp65.

was equivalent to the low dose (1.25 g/kg extract) used in this study. NC and DM were treated with vehicle (normal saline) in a matched volume. All the rats were administered the drugs via intragastric gavage (ig) once a day, for 12 weeks.

2.4. Pharmacokinetics Study. The rats treated with XXD for 12 weeks were fasted with free access to water for 12 h before the PK experiments. Blood samples were collected before dosing and at 0, 0.25, 0.5, 1, 2, 4, 6, 12, and 24 h following administration. A validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [24] was applied to simultaneously determine the concentration of 11 ingredients (coptisine, jatrorrhizine, berberine, palmatine, baicalin, baicalein, wogonoside, wogonin, rhein, emodin, and aloeemodin) in blood plasma [24]. The plasma concentration-time data were analysed by noncompartmental methods with the WinNonLin software package (Pharsight Corporation, Mountain View, CA, USA) to determine PK parameters.

2.5. Urinary Albumin Excretion, Metabolic Parameters, and Renal Function Analysis. At 4, 8, and 12 weeks, 24 h urine of rat was collected for measurements of 24 h urinary albumin excretion (UAE) by radioimmunoassay (Atom High Technology, Beijing, China). At 12 weeks, FBG and area under the blood glucose response curve (GAUC) were measured by glucose oxidase method. HbA_{1c} was determined by HPLC (Biorad, Richmond, CA, USA). Serum creatinine, urine creatinine, serum total cholesterol, and triglyceride levels were measured using an automatic biochemistry analyser (Olympus-2000, Tokyo, Japan). Creatinine clearance was calculated. The kidneys were removed, weighed, and parts of them frozen at −80°C until processing for Western blot and RNA extraction, while other parts were removed for histological examination.

2.6. Optical Microscope. Kidney tissues were fixed in 10% (vol/vol) buffered formalin and embedded in paraffin. Sections (4 μm) were stained with periodic acid-Schiff's reagent (PAS). The ratio of the mesangial matrix area to glomerular area (M/G) was determined by quantitative Image-Pro Plus software (PAX-it; PAXcam, Villa Park, IL, USA). Briefly, 20 glomeruli were randomly selected from each section, and positive signals within the selected glomerulus were highlighted, measured, and quantified as percent positive area of the entire glomerulus [33].

2.7. Electron Microscopy. Kidney samples were fixed in a mixture of 4% (wt./vol.) paraformaldehyde and 0.5% (wt./vol.) glutaraldehyde in PBS, pH 7.4, and prepared as described previously [34]. Ultrathin sections were cut, placed on a nickel grid and then examined under an electron microscope (JEM100CX-α, Japan).

2.8. Quantitative Real-Time PCR Analysis. Total RNA was extracted from renal tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA). First-strand complementary DNA (cDNA) was generated by reverse transcriptase, with random primers (TaKaRa, Otsu, Japan). To evaluate the mRNA expression of ICAM-1, MCP-1, NF-κBp65, and TGF-β1 in the kidney, real-time PCR was performed using a SYBR Green master mix kit and the StepOnePlus Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described [35]. The sequences of the primers are described in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to determine relative amounts of product, and data are presented as fold change, using β-actin as an endogenous control.

2.9. Western Blot Analysis. Kidney tissue was homogenized in radioimmunoprecipitation assay buffer containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L EDTA, and protease inhibitors. Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membrane (Amersham, Little Chalfont, UK). After blocking in 5% nonfat milk for 1 h, membranes were incubated overnight at 4°C with primary antibody. After washing, the membrane was incubated for 1 h at room temperature with horseradish-coupled secondary antibody. The membrane-bound antibody was detected by incubation with chemiluminescent reagent plus (Perkin Elmer Life Sciences, Boston, MA, USA) and the signal captured on X-ray film. Semiquantitative analysis software (FluorChem E; ProteinSimple, CA, USA) was used to evaluate the signal.

2.10. Enzyme-Linked Immunosorbent Assay Analysis (ELISA). Tumor necrosis factor α (TNF-α), IL-6 and AGEs protein levels in renal tissue were measured using commercial ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.11. Statistical Analysis. Results were expressed as mean ± SD. ANOVA was performed to compare multiple groups. When the ANOVA gave a statistically significant difference, Dunnett's test was applied; $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

Table 3: Pharmacokinetic parameters of effective ingredients after intragastric gavage of Xiexin decoction for 12 weeks in diabetic rats.

Constituents	1.25 g/kg		2.5 g/kg	
	C_{max} (μg/mL)	$AUC_{0-24\,h}$ (μg·h/mL)	C_{max} (μg/mL)	$AUC_{0-24\,h}$ (μg·h/mL)
Rhein	0.961 ± 0.430	1.102 ± 0.216	1.753 ± 0.514	2.789 ± 0.937
Emodin	0.013 ± 0.007	0.061 ± 0.008	0.022 ± 0.009	0.119 ± 0.025
Baicalin	1.564 ± 0.570	4.696 ± 1.734	4.309 ± 1.709	16.067 ± 9.597
Wogonoside	1.137 ± 0.321	3.458 ± 0.894	2.240 ± 0.475	8.884 ± 2.992
Wogonin	0.047 ± 0.038	0.154 ± 0.071	0.149 ± 0.086	0.423 ± 0.307
Berberine	0.051 ± 0.020	0.757 ± 0.389	0.118 ± 0.078	1.727 ± 1.037
Palmatine	0.017 ± 0.013	0.113 ± 0.069	0.029 ± 0.018	0.312 ± 0.197
Jatrorrhizine	0.014 ± 0.009	0.172 ± 0.057	0.021 ± 0.013	0.201 ± 0.152

Data are expressed as mean ± SD, $n = 8$ in each group.

2.12. PK/PD Analysis. X is the matrix of the PK parameter (P, i.e., AUC or C_{max}) of multiple ingredients and y is the matrix of every effective indicator E (see formula (1)), where there are 1, 2, 3,. . ., n animals and parameter P (AUC or C_{max}) of 1, 2, 3,. . ., m ingredients:

$$X = \begin{bmatrix} P_{11} & P_{12} & \cdots & P_{1m} \\ P_{21} & P_{22} & \cdots & P_{2m} \\ \cdots & \cdots & \cdots & \cdots \\ P_{n1} & P_{n2} & \cdots & P_{nm} \end{bmatrix}, \qquad y = \begin{bmatrix} E_1 \\ E_2 \\ \cdots \\ E_n \end{bmatrix}. \tag{1}$$

PLS regression model between every effective indicator and the pharmacokinetic parameter of multiple ingredients can be expressed as follows:

$$y = X \times b_{PLS} + A, \tag{2}$$

where y is every effective indicator matrix, A is a residual matrix, b_{PLS} is a regression coefficient matrix, and X is the matrix of the PK parameter of multiple ingredients. The best regression equation was determined by optimizing the cross-validated correlation coefficient (Q^2) using the automatic leave-one-out method to avoid overfitting the data [26]. Q^2 was calculated as follows:

$$Q^2 = 1 - \frac{\sum_{i=1}^{n} (y_{calc} - y_{obs})^2}{\sum_{i=1}^{n} (y_{obs} - y_{mean})^2}, \tag{3}$$

where y_{calc} is the calculated dependent variable, y_{obs} is the observed dependent variable, and y_{mean} is the mean of the observed dependent variable. PLS modelling was performed using Simca-p13 software (Umetrics AB, Umea, Sweden).

In PLS regression, the square of correlation coefficients (R^2), Q^2, ANOVA and the diagnostic plot showing calculated versus observed values of every effective indicator were used for evaluation reliability of PK/PD analysis.

Since the effects used in PK/PD analysis were all inhibitory in this study, ingredients with a negative PK parameter regression coefficient contributed to the effective indicator, whilst ingredients with positive PK regression coefficients showed no contribution to the effective indicator. Therefore, the regression coefficients of ingredient PK parameters were used to assess the relative contributions of each ingredient to every effective indicator.

The relationships between the PK parameters (AUC and C_{max}) of 8 ingredients (berberine, jatrorrhizine, palmatine, baicalin, wogonoside, wogonin, rhein, and emodin) and every quantitative effective indicator with dose-dependence were analysed using PLS regression. Either AUC or C_{max} (the parameter with the larger R^2 and Q^2 values) was selected to explain the relationship between the PK parameters of 8 ingredients and the effective indicator.

3. Results

3.1. Pharmacokinetics of Effective XXD Ingredients in Diabetic Rats. After ig administration of XXD for 12 weeks, 11 ingredients (coptisine, jatrorrhizine, berberine, palmatine, baicalin, baicalein, wogonoside, wogonin, rhein, emodin, and aloeemodin) were determined in diabetic rat plasma. The absorption of the most active components was relatively rapid, with peak concentrations occurring at 10 min for rhein, baicalin, wogonoside, and wogonin, and at 30 min for emodin. The concentrations of coptisine, baicalein, and aloeemodin were slightly higher than the lower limit of quantification (LLOQ) at about 0.5–2 h and below the LLOQ at other times. The concentration-time curves of berberine, jatrorrhizine, palmatine, baicalin, and wogonoside exhibited double peaks in the plasma concentrations. The main PK parameters of 8 ingredients (rhein, emodin, baicalin, wogonoside, wogonin, berberine, palmatine, and jatrorrhizine) from XXD are shown in Table 3. The PK parameters of coptisine, baicalein, and aloeemodin could not be calculated because there were too few time points with detectable concentrations.

3.2. Effect of XXD on Metabolic Parameters in Diabetic Rats. After 12 weeks of diabetes, the levels of FBG and GAUC, HbA_{1c}, serum total cholesterol, and triglyceride were significantly higher in DM than in the NC group. Compared with DM, treatment with XXD at high dose

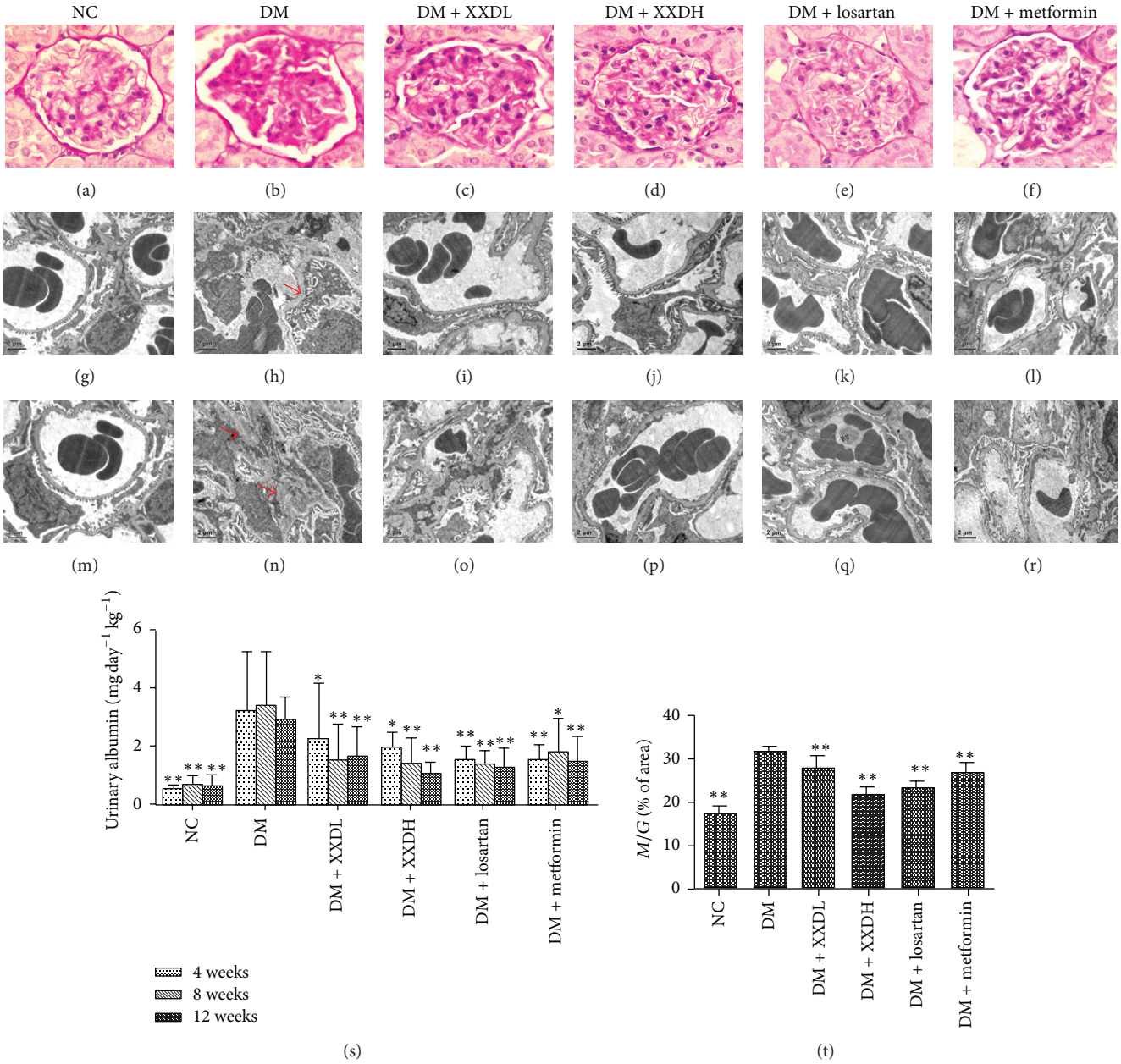

FIGURE 2: Renal pathology and urinary albumin excretion from diabetic rats treated with Xiexin decoction. (a)–(f) Periodic acid-Schiff's reagent (PAS) stain. Original magnification (a)–(f) × 400. (g)–(r) Electron microscopy (EM) analysis. Representative images of glomerular basement membrane thickening (g)–(l) and mesangial matrix expansion (m)–(r), scale bars 2 μm, original magnification electron microscopy × 4,700. (s) Urinary albumin excretion at 4, 8, and 12 weeks. (t) Ratio of mesangial matrix area to glomerular area (M/G) in PAS staining. NC: normal control; DM: diabetic model control; XXDL: XXD extract 1.25 g/kg; XXDH: XXD extract 2.5 g/kg; losartan 10 mg/kg and metformin 100 mg/kg. NC and DM were treated with normal saline. All the rats were administered intragastric gavage once a day for 12 weeks. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with DM.

markedly lowered the FBG and serum cholesterol (Table 4). In addition, treatments with XXD at both doses significantly decreased the HbA_{1c} and serum triglyceride and improved glucose tolerance (Table 4). Similarly, significantly decreased levels of FBG, GAUC, and HbA_{1c} were also noted in animals treated with metformin, but not in those treated with losartan (Table 4).

3.3. Effect of XXD on Urinary Albumin Excretion and Renal Function in Diabetic Rats. UAE was significantly increased at 4, 8, and 12 weeks, and creatinine clearance and the kidney weight to body weight ratio were also markedly increased at 12 weeks in the DM group, as compared with the NC group. In contrast, XXD and or losartan treatments significantly reduced UAE (Figure 2(s)), creatinine clearance, and kidney

Table 4: Effect of Xiexin decoction on metabolic parameters and renal function in diabetic rats.

Groups	NC	DM	DM + XXDL	DM + XXDH	DM + losartan	DM + metformin
FBG (mmol/L)	$3.0 \pm 0.5^{**}$	22.3 ± 2.8	21.0 ± 3.3	$17.2 \pm 2.9^{*}$	21.6 ± 4.4	$9.4 \pm 2.5^{**}$
HbA_{1c} (%)	$4.0 \pm 0.1^{**}$	7.2 ± 0.3	$6.6 \pm 0.9^{*}$	$6.6 \pm 0.2^{*}$	6.9 ± 0.2	$6.6 \pm 0.5^{**}$
GAUC (mmol/L × h)	$28.6 \pm 4.7^{**}$	117.1 ± 6.4	$89.9 \pm 6.4^{**}$	$77.4 \pm 10.7^{**}$	111.8 ± 11.1	$70.9 \pm 9.5^{**}$
Serum triglyceride (mmol/L)	$0.6 \pm 0.2^{**}$	1.1 ± 0.6	$0.6 \pm 0.2^{*}$	$0.5 \pm 0.1^{**}$	0.7 ± 0.3	1.0 ± 0.3
Serum cholesterol (mmol/L)	$1.1 \pm 0.2^{**}$	1.5 ± 0.2	1.2 ± 0.2	$1.1 \pm 0.2^{**}$	1.3 ± 0.2	1.2 ± 0.4
Creatinine clearance ($mL\,min^{-1}kg^{-1}$)	$3.1 \pm 1.5^{**}$	7.5 ± 3.9	$3.6 \pm 0.9^{**}$	$3.7 \pm 1.9^{**}$	$4.8 \pm 3.6^{*}$	$4.6 \pm 2.7^{*}$
Kidney weight/body weight (%)	$0.58 \pm 0.05^{**}$	1.18 ± 0.06	1.12 ± 0.16	$1.04 \pm 0.10^{*}$	$1.00 \pm 0.05^{*}$	1.10 ± 0.04

NC: normal control; DM: diabetic model control; XXDL: XXD extract 1.25 g/kg; XXDH: XXD extract 2.5 g/kg; losartan 10 mg/kg and metformin 100 mg/kg. NC and DM were treated with normal saline. All the rats were administered via intragastric gavage once time each day for 12 weeks. FBG: fasting blood glucose; GAUC: area under the blood glucose response curve. Data are expressed as mean ± SD; $n = 8$–10. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with DM.

weight to body weight ratio, as compared with the DM group (Table 4). In addition, the diabetic rats treated with metformin for 12 weeks also exhibited a significant reduction in UAE (Figure 2(s)) and creatinine clearance (Table 4).

3.4. Effect of XXD on Renal Histopathology and Ultra-Structural Pathology in Diabetic Rats. After 12 weeks of diabetes, light microscopy revealed glomerular hypertrophy, mesangial matrix expansion, and an increased M/G, as compared with the NC group, in PAS-stained kidney sections (Figures 2(a)–2(f), and 2(t)). In addition, electron microscopy of glomerular ultrastructure also revealed glomerular basement membrane thickening (Figure 2(h)) and mesangial expansion, mesangial matrix deposition (Figure 2(n)) in the DM group. However, compared with DM group, these changes were ameliorated in XXD, losartan and metformin groups (Figure 2).

3.5. Effect of XXD on AGEs and RAGE Expression in Diabetic Rat Kidneys. Kidney levels of AGEs and protein expression of RAGE increased in DM rats, compared with the NC group. However, treatment with XXD or metformin significantly reduced the total renal AGEs content and downregulated RAGE expression (Figure 3).

3.6. Effect of XXD on Renal Inflammation Factor and TGF-β1 Expression in Diabetic Rats. After 12 weeks of diabetes, renal protein and mRNA MCP-1 and ICAM-1 expression, and levels of TNF-α and IL-6, were markedly increased in the DM group, as compared with NC rats. Renal TGF-β1 protein and mRNA expression were also significantly increased in DM. XXD and losartan treatment significantly downregulated these changes (Figure 4). In addition, the diabetic rats treated with metformin also exhibited a significant reduction in MCP-1 and ICAM-1 expression and TNF-α level (Figure 4). Collectively, these data indicated that XXD could suppress the renal inflammation induced by diabetes.

3.7. Effect of XXD on Renal NF-κB Signaling Pathway in Diabetic Rats. After 12 weeks of diabetes, increased protein expression of renal IKKα, phospho-IκBα, phospho-NF-κBp65, and NF-κBp65, with decreased IκBα expression, was observed in DM rats, compared with the NC group. XXD and losartan treatments significantly ameliorated these changes. In addition, the increased renal NF-κBp65 mRNA expression in diabetic rats was downregulated by XXD and losartan treatment (Figure 5). These findings suggested that XXD treatment could suppress activation of the renal NF-κB signalling pathway in diabetic rats.

3.8. PK/PD Relationships. Using PLS models analysis, R^2, Q^2, ANOVA P values, and a diagnostic plot showing the calculated effect values from the PK parameters of 8 ingredients, versus the observed effect values for each of 10 quantitative effective indicators, are summarized in Figure 6. The relationships all appeared to show reasonable correlations (R^2 range 0.509–0.816), evaluation performances (Q^2 range 0.404–0.788), and significant ANOVA ($P < 0.01$). The differences between the R^2 and Q^2 values (<0.11) were moderate, indicating sufficient model reliability. Good agreement for all models was observed. From the regression coefficients of PK parameters of 8 ingredients (Figure 6), we found that 8 XXD constituents (berberine, jatrorrhizine, palmatine, baicalin, wogonoside, wogonin, rhein and emodin) made significant contributions to the renal protection (reduced UAE, M/G, renal TNF-α, and IL-6 level and inhibited MCP-1, ICAM-1, TGF-β1 and NF-κB p65 expression) observed in diabetic rats. Seven constituents (berberine, jatrorrhizine, palmatine, baicalin, wogonoside, rhein, and emodin) were found to make significant contributions to the improvement of glucose tolerance, and 6 constituents (berberine, baicalin, wogonoside, wogonin, rhein, and emodin) made significant contributions to the decrease in renal AGEs in diabetic rat kidneys.

4. Discussion

This research showed that rats where diabetes was induced by high-fat diet and streptozotocin for 12 weeks exhibited a number of characteristics of early DN, including glucolipid metabolism disorder, increased UAE, high glomerular

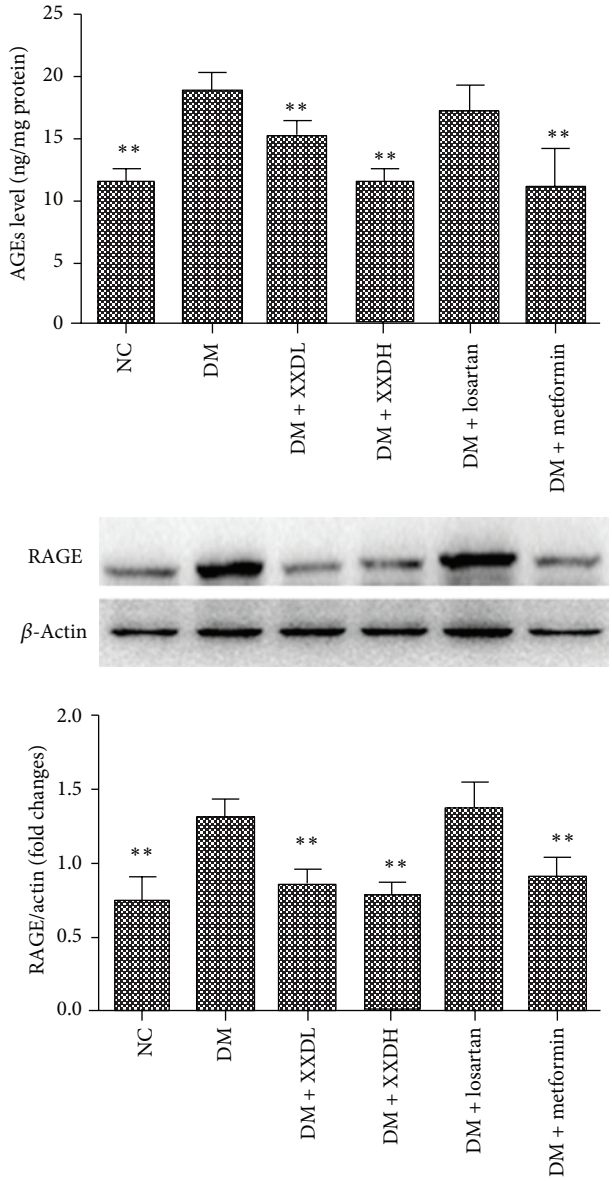

Figure 3: Levels of renal advanced glycation end-products (AGEs) and receptor for AGEs (RAGE) expression in diabetic rats treated with Xiexin decoction. NC: normal control; DM: diabetic model control; XXDL: XXD extract 1.25 g/kg; XXDH: XXD extract 2.5 g/kg; losartan 10 mg/kg and metformin 100 mg/kg. NC and DM were treated with normal saline. All the rats were administered via intragastric gavage once time each day for 12 weeks. Data are presented as means ± S.D, $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with DM.

filtration, glomerular mesangial matrix proliferation, and basement membrane thickening. XXD exhibited an anti-early DN effect, as it improved the above changes.

Our data indicated that in diabetic rat kidneys, renal AGEs and RAGE increased. This would be predicted to activate the downstream IκB kinase, promoting IκB phosphorylation and IκB degradation and allowing NF-κBp65 to be released and phosphorylated. The phosphorylated NF-κBp65 would upregulate target gene expression, such as inflammatory cytokines and cell adhesion molecules, including IL-6, TNF-α, MCP-1, and ICAM-1. The resulting increase in kidney inflammation could further promote renal TGF-β1 expression, which enhanced the accumulation of glomerular mesangial extracellular matrix and mesangial expansion, resulting in the development of DN (Figure 7). These results were similar to the pathogenesis of DN reported in the literature [6, 8], whereby the long-term hyperglycaemia found in the diabetic state could induce AGEs accumulation in the kidney, activating RAGE and subsequently the NF-κB inflammatory pathway. Moreover, the resulting kidney

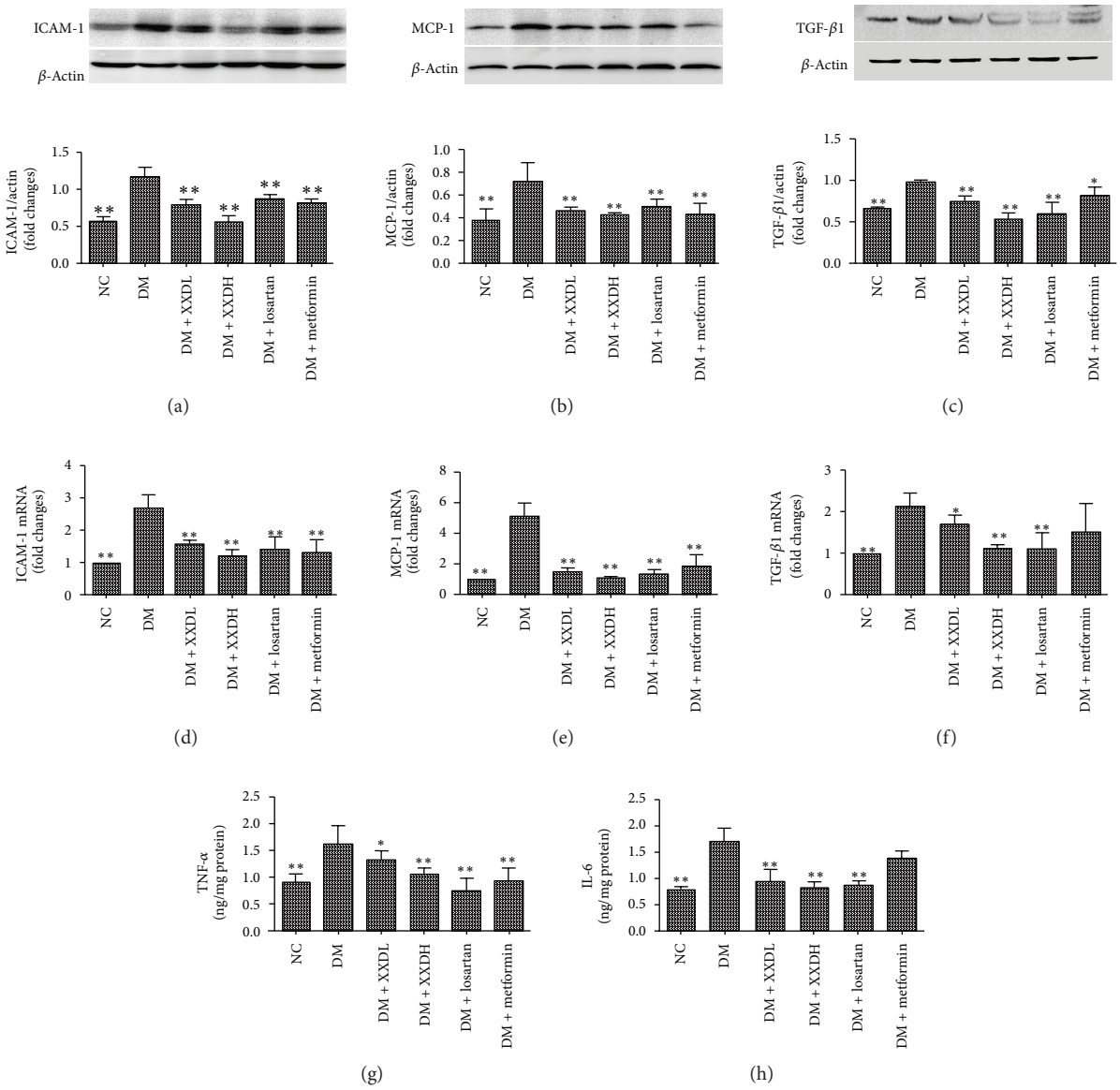

Figure 4: Effects of Xiexin decoction on renal inflammation factor and transforming growth factor β1 (TGF-β1) expression in diabetic rats. (a)–(c) Western blot analysis of protein levels; (d)–(f) Real-time PCR analysis of mRNA levels; (g)–(h) Quantification by ELISA. NC: normal control; DM: diabetic model control; XXDL: XXD extract 1.25 g/kg; XXDH: XXD extract 2.5 g/kg; losartan 10 mg/kg and metformin 100 mg/kg. NC and DM were treated with normal saline. All the rats were administered via intragastric gavage once time each day for 12 weeks. ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6. Data are expressed as mean ± SD $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with DM.

inflammation can promote DN progression [8, 33]. The results of the present study, therefore, indicated that the molecular mechanism underlying XXD's anti-DN activity related to its ability to decrease renal AGEs, downregulate RAGE expression, inhibit NF-κB pathway activation, inflammatory factor formation, and TGF-β1 expression, thus preventing kidney injury (Figure 7).

Because DN is a complicated disease, it has proved difficult to treat using a single compound acting on a single target. The present study found, through combined PK/PD analysis of the relationships between the PK parameters of XXD ingredients and their anti-DN effects, that multiple active ingredients of XXD acted on multiple targets *in vivo* to produce an overall comprehensive anti-DN effect. In recent years, network pharmacology and multipharmacology research studies have shown that multiple active ingredients in TCM may act on multiple targets within the diabetic network to generate an overall comprehensive effect [36]. However, *in vivo* studies are essential to determine whether the active ingredients predicted by computer are correct

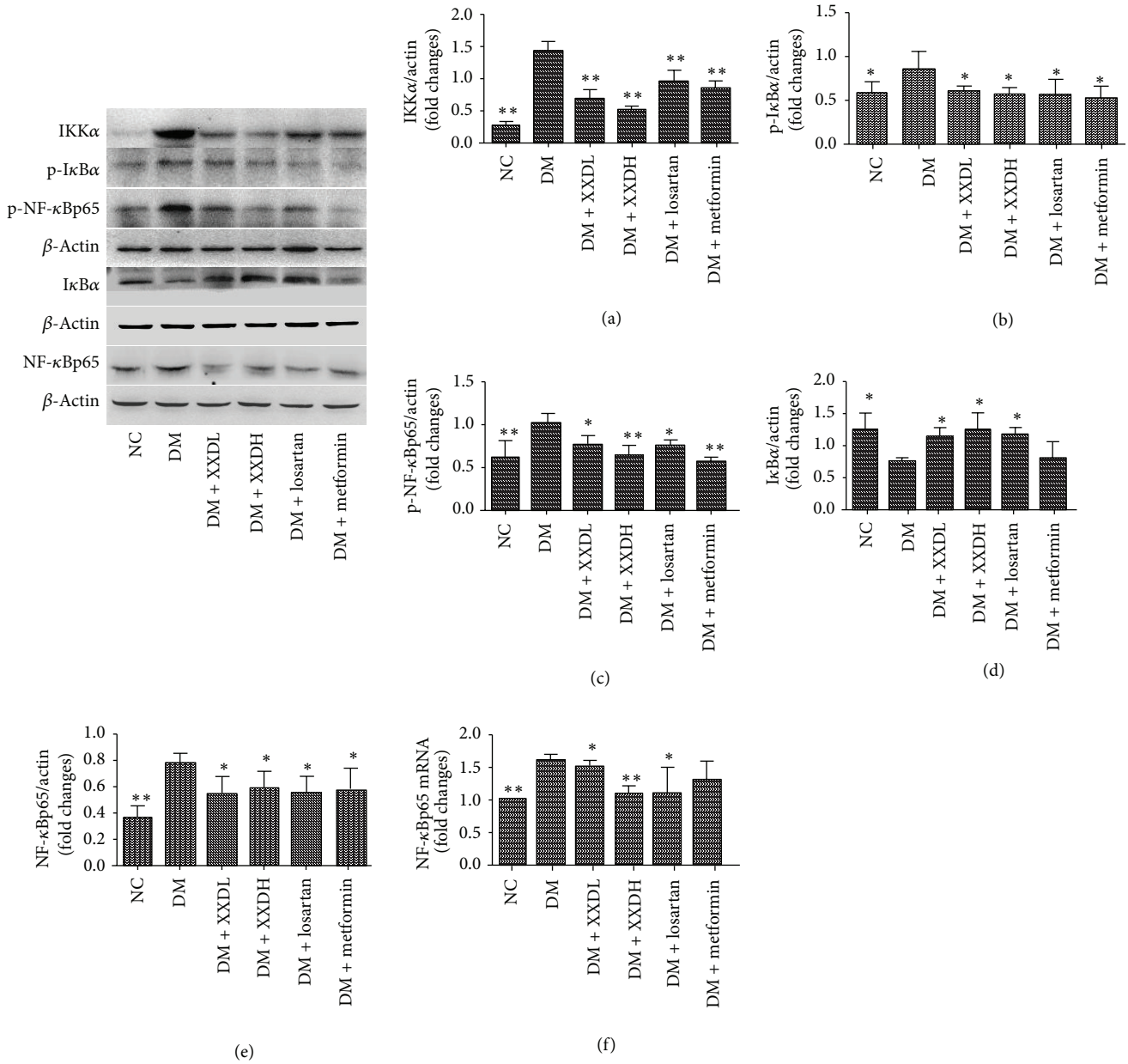

Figure 5: Effect of Xiexin decoction on renal nuclear factor-κB (NF-κB) signalling pathway in diabetic rats. (a)–(e) Western blot analysis of protein levels. (g) Real-time PCR analysis of mRNA levels. NC: normal control; DM: diabetic model control; XXDL: XXD extract 1.25 g/kg; XXDH: XXD extract 2.5 g/kg; Losartan 10 mg/kg and metformin 100 mg/kg. NC and DM were treated with normal saline. All the rats were administered via intragastric gavage one time each day for 12 weeks. IKKα: inhibitor of nuclear factor-κB kinase subunit α; IκBα, inhibitor of nuclear factor-κB subunit α; p-IκBα: phospho-IκBα; NF-κBp65: nuclear factor-κBp65; p-NF-κBp65: phospho-NF-κBp65. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with DM.

because their pharmacological properties can be affected by the concentrations achieved *in vivo* and interactions between ingredients. This study provided an appropriate research method for analysis of the active ingredients in TCM and their mechanisms of action, through combined PK/PD analysis using PLS regression *in vivo*.

Several studies have reported that rhein [37], emodin [38], baicalin [39], and berberine [40, 41] exhibited anti-DN effects. Rhein and baicalin could downregulate renal TGF-β1 protein expression [37, 39]. Berberine increased IKBα and decreased NF-κBp65 protein levels in diabetic mouse kidney, as well as inhibiting renal AGE generation and downregulating TGF-β1, ICAM-1, and VCAM-1 protein expression [40, 41]. However, the mechanisms of action of these TCM ingredients and the concentrations achieved *in vivo* were unclear prior to the present study. In addition, our results showed that wogonoside, wogonin, palmatine, and jatrorrhizine also exhibited anti-DN activity and illustrated

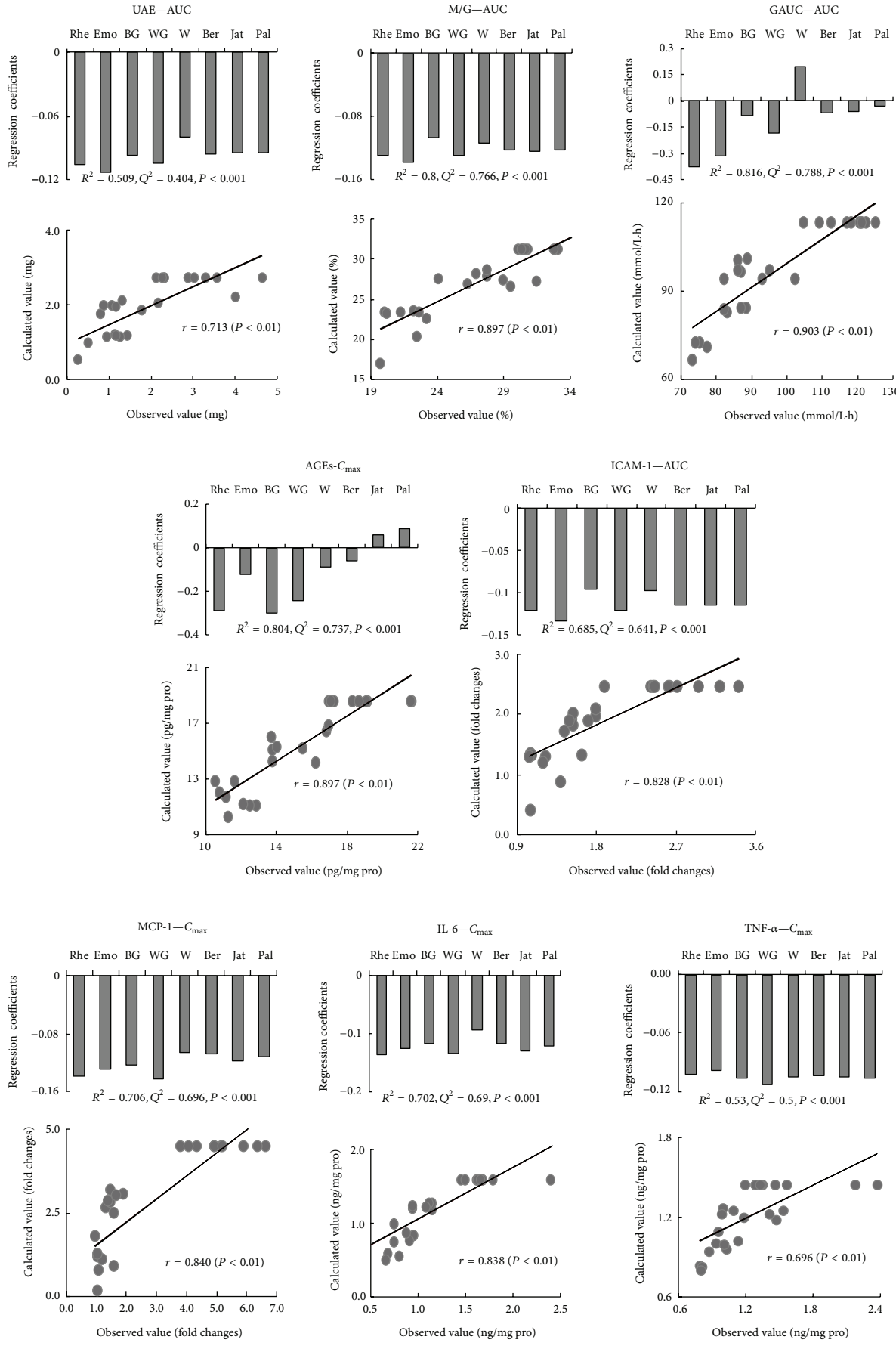

Figure 6: Continued.

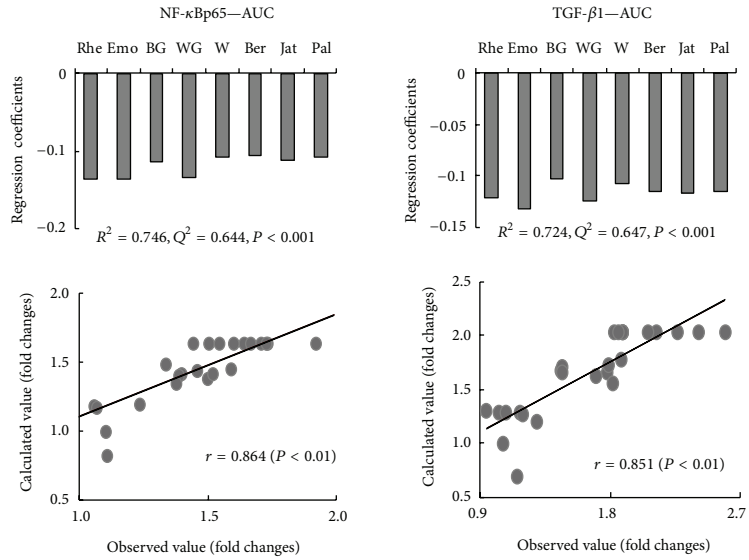

Figure 6: Reliability evaluation for the pharmacokinetics/pharmacodynamics analysis using partial least squares models and regression coefficients between pharmacokinetic parameter of ingredients with effective indicators in diabetic nephropathy rats treated with XXD for 12 weeks. R^2: square of correlation coefficients; Q^2: the cross-validated correlation coefficients, P: significance using ANOVA. Rhe: Rhein; Emo: emodin; BG: baicalin; WG: wogonoside; W: wogonin; Ber: berberine; Jat: jatrorrhizine; Pal: palmatine. C_{max}: the maximum plasma concentration; AUC: the area under the curve of plasma concentration of ingredient; UAE: Urinary albumin excretion; M/G: ratio of mesangial matrix area to glomerular area; GAUC: area under the blood glucose response curve; AGEs: advanced glycation end-products; ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; NF-κBp65: nuclear factor-κBp65; TGF-β1: transforming growth factor β1. ICAM-1, MCP-1, TGF-1, and NF-κB panels show mRNA expression.

their mechanisms of action, indicating that these ingredients are worthy of further study.

In the present PLS analysis, we found that the C_{max} or AUC of 8 ingredients had a poor correlation with the observed effect indicators, which lacked dose dependency, such as FBG, HbA_{1c}, serum triglyceride, serum cholesterol, creatinine clearance and kidney weight/body weight. Among these indicators, reduced blood lipid levels may relate to local effects of XXD ingredients on the gut, because it was reported that *Rhizoma coptidis* and berberine reduced blood lipid levels by regulation of gut microbes [42, 43]. The other reasons call for further studies. This phenomenon also showed that alternative approaches need to be developed for PK/PD analysis of effect indicators without dose dependency.

In the present study, we used metformin and losartan as two positive control drugs to evaluate the reliability of the DN model. Metformin has a hypoglycaemic effect and losartan has a renal protective effect. Our data indicated that both exhibited an anti-DN effect, losartan via inhibition of NF-κB signalling activity and reduction in levels of inflammatory factors, and metformin via improving the glucolipid metabolism disorder, decreasing AGEs, and suppressing expression of RAGE and inflammatory molecules. These results are in agreement with those of previous studies [44–48].

5. Conclusion

In conclusion, XXD exhibited an anti-DN effect via inhibition of renal inflammation, mediated via NF-κB signalling as well as inhibition of renal AGEs accumulation and expression of its receptor. Based on the combined PK/PD analysis using PLS regression, XXD was found to act on multiple targets to generate an overall anti-DN effect. This study provides a foundation for further research and development of XXD. Furthermore, this study demonstrated an effective experimental approach to analysis of the active ingredients in herbal compounds.

Abbreviations

AGE:	Advanced glycation end-product
C_{max}:	The maximum plasma concentration
DN:	Diabetic nephropathy
FBG:	Fasting blood glucose
GAUC:	Area under the blood glucose response curve
ICAM-1:	Intercellular adhesion molecule-1
IκBα:	Inhibitor of nuclear factor κB subunit α
IKKα:	Inhibitor of nuclear factor κB kinase subunit α
LC/MS/MS:	Liquid chromatography and tandem mass spectrometry
MCP-1:	Monocyte chemotactic protein-1
M/G:	Ratio of the mesangial matrix area to glomerular area
NF-κB:	Nuclear factor κB
PK/PD:	Pharmacokinetic/pharmacodynamic
PLS:	Partial least squares
PAS:	Periodic acid-Schiff's reagent

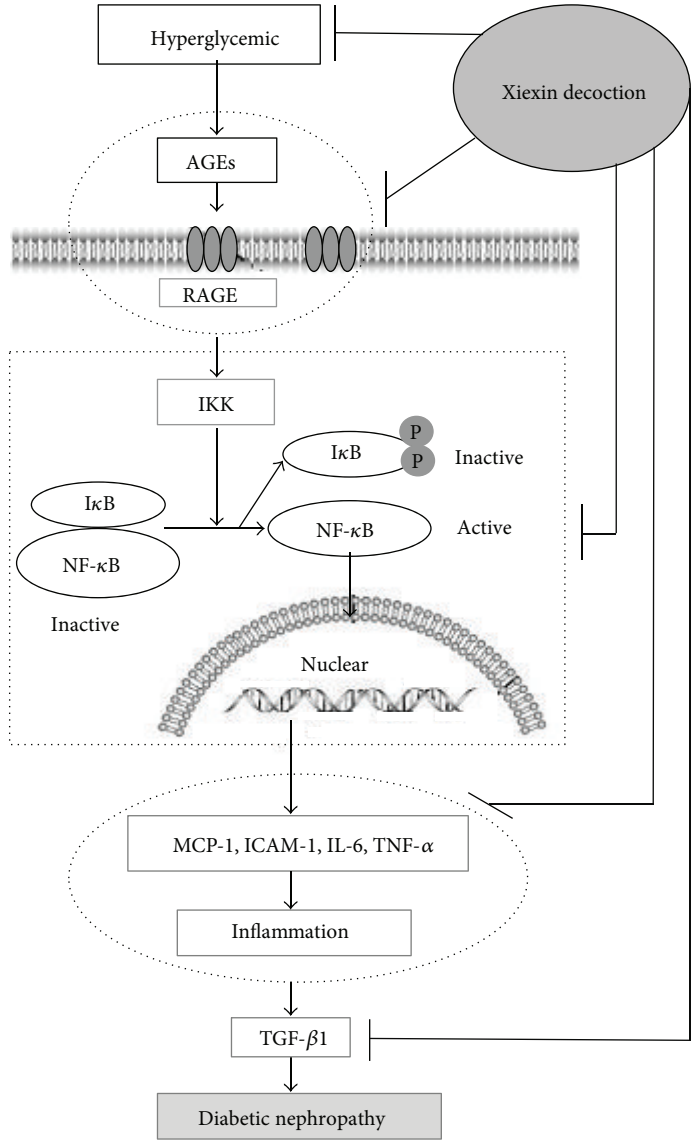

Figure 7: Proposed molecular mechanisms underlying the renal protective role of Xiexin decoction in diabetic nephropathy rats. AGEs: advanced glycation end-products; RAGE: receptor for AGEs; IKK: inhibitor of nuclear factor-κB kinase; IκB: inhibitor of nuclear factor-κB; NF-κB: nuclear factor-κB; P: phosphorylation; ICAM-1: intercellular adhesion molecule-1; MCP-1: monocyte chemotactic protein-1; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; TGF-β1: transforming growth factor β1. $\vdash$: inhibition.

Q^2: The cross-validated correlation coefficient
RAGE: Receptor for advanced glycation end-product
R^2: Square of correlation coefficients
TCM: Traditional Chinese medicine
TNF-α: Tumor necrosis factor α
TGF-β1: Transforming growth factor-β1
UAE: Urinary albumin excretion
XXD: Xiexin decoction.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Hu-Lu-Ba-Wan Attenuates Diabetic Nephropathy in Type 2 Diabetic Rats through PKC-α/NADPH Oxidase Signaling Pathway

Lishan Zhou,[1,2] Hui Dong,[1] Yi Huang,[3] Lijun Xu,[1] Xin Zou,[1] Kaifu Wang,[1] Guang Chen,[4] and Fuer Lu[1]

[1] *Institute of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China*
[2] *Department of Integrated Traditional Chinese and Western Medicine, Wuhan Children's Hospital, Wuhan, Hubei 430016, China*
[3] *Department of Nephrology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China*
[4] *Department of Integrated Traditional Chinese and Western Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, China*

Correspondence should be addressed to
Guang Chen; guangchen8086@126.com and Fuer Lu; felu@tjh.tjmu.edu.cn

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Hu-Lu-Ba-Wan (HLBW) is a Chinese herbal prescription used to treat kidney deficiency. The aim of this study was to explore the effect and mechanism of HLBW on diabetic nephropathy (DN) in type 2 diabetic rats. The rat model of DN was established by being fed a high-fat diet and intravenous injection of streptozotocin. Then, HLBW decoction was administered for 16 weeks. Blood glucose level, lipid profile, renal function, 24-hour total urinary protein, and albumin content were examined. Renal morphology and superoxide anion levels were evaluated. The activity of nicotinamide-adenine dinucleotide phosphate (NADPH) and protein kinase C-alpha (PKC-α) related genes expression in renal tissue were also determined. Our data demonstrated that HLBW significantly improved hyperglycemia, hyperlipidemia, and proteinuria in diabetic rats compared with those of control group. HLBW also alleviated glomerular expansion and fibrosis, extracellular matrix accumulation and effacement of the foot processes. Additionally, HLBW reduced superoxide anion level, NADPH oxidase activity, the protein and mRNA expressions of $p47^{phox}$, and the protein expression of phosphorylated PKC-α in renal tissue. These results suggest that HLBW is effective in the treatment of DN in rats. The underlying mechanism may be related to the attenuation of renal oxidative stress via PKC-α/NADPH oxidase signaling pathway.

1. Introduction

The epidemic of diabetes mellitus (DM) is rapidly becoming a severe public health problem, especially in China. It is reported that the prevalence of diabetes has reached 9.7%, accounting for 92.4 million adults with diabetes [1]. The morbidity and mortality associated with this disease derives primarily from its microvascular and macrovascular complications caused by persistent hyperglycemia [2]. Diabetic nephropathy (DN), with hidden symptoms in early stage and lack of effective therapy, is the most common microvascular complication of diabetes [3]. Accumulating evidence has demonstrated that hyperglycemia and the resulting oxidative stress play important roles in the development of DN [4–6]. Therefore, therapies designed to improve hyperglycemia and reduce oxidase stress may be promising in the treatment of DN.

Plant-based medicines are widely used to treat diabetes and its complications in the local clinics of China. In Chinese medicine, DN is referred to kidney deficiency disease. Therefore, prescriptions reinforcing kidney may have the potential to treat DN. Among effective prescriptions, Hu-Lu-Ba-Wan

Table 1: Real-time PCR primer sequences.

Gene	Forward ($5' \rightarrow 3'$)	Reverse ($5' \rightarrow 3'$)
β-Actin	5′-GGAGATTACTGCCCTGGCTCCTA-3′	5′-GACTCATCGTACTCCTGCTTGCTG-3′
$p47^{phox}$	5′-GGACACCTTCATTCGCCACA-3′	5′-GTCCTGCCACTTAACCAGGAACA-3′
PKC-α	5′-TCCAGGATGACGACGTGGAG-3′	5′-CGTTGACGTATTCCATGACGAAG-3′

Table 2: The effect of HLBW on OGTT in rats with DN.

Group	FBG (mmol/L)	PG-1 h (mmol/L)	PG-2 h (mmol/L)
Control	5.84 ± 0.55	6.15 ± 0.37	6.05 ± 0.60
Diabetic	$15.81 \pm 1.99^{\triangle\triangle}$	$27.10 \pm 4.52^{\triangle\triangle}$	$23.29 \pm 3.77^{\triangle\triangle}$
TFG	$5.76 \pm 0.44^{\blacktriangle\blacktriangle}$	$10.11 \pm 2.97^{\blacktriangle\blacktriangle}$	$6.51 \pm 1.32^{\blacktriangle\blacktriangle}$
PC	$5.75 \pm 1.30^{\blacktriangle\blacktriangle}$	$9.81 \pm 3.71^{\blacktriangle\blacktriangle}$	$6.26 \pm 1.13^{\blacktriangle\blacktriangle}$
HLBW	$5.73 \pm 1.01^{\blacktriangle\blacktriangle}$	$8.08 \pm 0.91^{\blacktriangle\blacktriangle}$	$6.01 \pm 0.91^{\blacktriangle\blacktriangle}$
Captopril	$8.50 \pm 1.58^{\blacktriangle\blacktriangle}$	$14.90 \pm 5.78^{\blacktriangle\blacktriangle}$	$9.84 \pm 1.64^{\blacktriangle\blacktriangle}$

Values are mean ± SD ($n = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group. FBG: fasting blood glucose; PG-1 h: postprandial blood glucose at 1 hour after glucose loading; PG-2 h: postprandial blood glucose at 2 hours after glucose loading.

(HLBW), which consisted of *Trigonella foenum-graecum* L. (TFG) and *Psoralea corylifolia* L. (PC), attracts much attention for their novel kidney reinforcing efficacy. Many researches have shown the beneficial effect of TFG and PC on improving hyperglycemia and alleviating inflammation [7–13]. In a recent, their antioxidative activity and renal protective effect have also been documented [14–19]. However, these individual studies just included incomplete description in this aspect. The effect of HLBW, which is the combination of TFG and PC, on DN is rarely reported. Therefore, the aim of this study was to explore the effect of HLBW on DN in type 2 diabetic rats and investigate its possible oxidative stress against mechanism.

2. Materials and Methods

2.1. Animals. Male Wistar rats weighting 180~200 g, obtained from Hubei province Center for Disease Control and Prevention, were used in this study. The rats were maintained at ambient temperature (22°C ± 1°C) with a 12 : 12 h light-dark cycle and free access to water and food. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the Chinese guidelines.

2.2. Preparation of HLBW. TFG and PC were purchased from Traditional Chinese Medicine Company in Hubei Province (Wuhan, China) and authenticated by the Department of Pharmacognscy, Hubei University of Chinese Medicine (Wuhan, China). The rat doses of TFG and PC were obtained by the conversion of human doses (Chinese Pharmacopeia, 2010) to rat equivalent doses based on body surface areas. The weight ratio of TFG to PC is 1 : 1. HLBW preparation process was as follows. The dry seeds were crushed into powder and soaked overnight before boiled. After cooling down, the decoction was deposited in 95% ethanol (1 : 1 v/v) overnight at room temperature. The precipitate from the decoction was separated by a filter. Then, the alcoholic filtrate was distilled to remove the ethanol and was concentrated by Rotavapor (BUCHI, Flawil, Switzerland). The final liquid extracted from 1 g HLBW (which contains 0.5 g TFG and 0.5 g PC), 1 g TFG, and 1 g PC was 1.32 mL, 0.63 mL, and 0.66 mL, respectively.

2.3. Animal Modeling, Grouping, and Treatment. After adaptive feeding with standard rat diet (containing 35% flour, 20% soy meal, 20% corn meal, 15.5% bran, 0.5% bean oil, 5% fish meal, 2.5% bone meal, 1% dusty yeast, and 0.5% salt) for one week, eight rats were selected randomly as the normal control group (control). The rats in the control group continued their standard diet, while the remaining rats were fed with a high-fat diet (containing 67.5% standard laboratory rat chow, 15% lard, 15% sugar, 2% cholesterol, and 0.5% bile salts) for 4 weeks. Then, they were injected with streptozotocin (STZ, Sigma Chemical Co. MO, USA, 30 mg/kg) into the tail vein after an overnight fast. One week later, oral glucose tolerance test (OGTT) was performed. The 95% range of confidence was calculated according to the plasma glucose levels of normal rats. Rats with impaired glucose tolerance (IGT) (Plasma glucose levels of rats at two time points were higher than the upper limit at two time points, or 20% higher than the upper limit at one time point.) were selected. Then the IGT rats were randomized into the untreated diabetic control group (Diabetic), TFG treated group (TFG), PC treated group (PC), HLBW treated group (HLBW), and Captopril (Sino-American Shanghai Squibb Pharmaceuticals Ltd., Shanghai, China) treated group (Captopril). Rats in the previous mentioned treatment groups were administered with corresponding therapy, TFG (9 g/kg/d), PC (9 g/kg/d), HLBW (18 g/kg/d), or Captopril (10 mg/kg/d), intragastrically for 16 weeks. Oral gavage was performed once a day between 8:00 and 10:00 a.m. Rats in untreated diabetic and normal control group were administered with the same volume of distilled water. Doses were adjusted to the body weight recorded once a week.

2.4. Sampling. All the animals were sacrificed at the end of the 16th week. Urine samples of 24 hours were collected by metabolic cages the day before sacrifice. Blood samples were obtained by aorta abdominalis puncture at the time of sacrifice. After centrifuging at 3000 r/min for 30 min at 4°C, the specimen serum was collected and stored at −20°C until analysis. Meanwhile, kidneys were quickly taken out by laparotomy and flushed with normal saline on ice. Then, the entire kidney mass was scaled. Part of the left renal samples was, respectively, fixed in 4% paraformaldehyde solution for paraffin embedding or fixed in 2.5% glutaraldehyde solution

Table 3: The effect of HLBW on plasma lipid profiles in rats with DN.

Group	TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)
Control	1.04 ± 0.16	2.09 ± 0.29	0.51 ± 0.15	1.50 ± 0.24
Diabetic	3.48 ± 1.14$^{\triangle\triangle}$	7.91 ± 1.53$^{\triangle\triangle}$	4.46 ± 1.57$^{\triangle\triangle}$	1.13 ± 0.18
TFG	1.23 ± 0.38$^{\blacktriangle\blacktriangle}$	4.73 ± 0.74$^{\blacktriangle\blacktriangle}$	1.29 ± 0.36$^{\blacktriangle\blacktriangle}$	1.40 ± 0.36
PC	1.49 ± 0.19$^{\blacktriangle}$	5.06 ± 1.05$^{\blacktriangle}$	1.71 ± 0.51$^{\blacktriangle}$	1.35 ± 0.35
HLBW	1.18 ± 0.23$^{\blacktriangle\blacktriangle}$	4.33 ± 0.59$^{\blacktriangle\blacktriangle}$	1.20 ± 0.28$^{\blacktriangle\blacktriangle}$	1.46 ± 0.29
Captopril	1.96 ± 0.80	7.23 ± 1.62	2.84 ± 0.76	1.28 ± 0.16

Values are mean ± SD ($N = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group. TG: triglyceride; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol.

Table 4: The effect of HLBW on renal function and proteinuria of rats with DN.

Group	Kidney/body weight (%)	BUN (mmol/L)	SCr (μmol/L)	Urinary total protein (μg/24 h)	Urinary albumin (μg/24 h)
Control	0.42 ± 0.04	3.96 ± 0.81	146.86 ± 12.39	6.71 ± 2.39	0.08 ± 0.03
Diabetic	0.64 ± 0.12$^{\triangle\triangle}$	11.46 ± 1.50$^{\triangle\triangle}$	229.61 ± 30.10$^{\triangle\triangle}$	50.79 ± 7.41$^{\triangle\triangle}$	3.54 ± 1.19$^{\triangle\triangle}$
TFG	0.48 ± 0.08$^{\blacktriangle\blacktriangle}$	5.68 ± 1.53$^{\blacktriangle\blacktriangle}$	184.89 ± 15.72$^{\blacktriangle\blacktriangle}$	13.40 ± 5.22$^{\blacktriangle\blacktriangle}$	0.41 ± 0.35$^{\blacktriangle\blacktriangle}$
PC	0.43 ± 0.06$^{\blacktriangle\blacktriangle}$	5.70 ± 1.02$^{\blacktriangle\blacktriangle}$	183.34 ± 23.61$^{\blacktriangle\blacktriangle}$	17.30 ± 8.30$^{\blacktriangle\blacktriangle}$	0.54 ± 0.37$^{\blacktriangle\blacktriangle}$
HLBW	0.42 ± 0.04$^{\blacktriangle\blacktriangle}$	4.88 ± 0.93$^{\blacktriangle\blacktriangle}$	165.16 ± 16.56$^{\blacktriangle\blacktriangle}$	10.74 ± 6.62$^{\blacktriangle\blacktriangle}$	0.31 ± 0.21$^{\blacktriangle\blacktriangle}$
Captopril	0.45 ± 0.06$^{\blacktriangle\blacktriangle}$	6.69 ± 1.78$^{\blacktriangle\blacktriangle}$	190.13 ± 21.62$^{\blacktriangle\blacktriangle}$	14.00 ± 4.08$^{\blacktriangle\blacktriangle}$	0.40 ± 0.28$^{\blacktriangle\blacktriangle}$

Values are mean ± SD ($n = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group. BUN: blood urea nitrogen; SCr: serum creatinine.

for transmission electron microscope (TEM), while the other part was prepared for frozen sections. The right renal samples were preserved at −80°C until use.

2.5. OGTT and Biochemical Analysis. After the rats fasted for 12 hours, 50% glucose at a dose of 2 g/kg was orally administered. Then, blood samples were collected from tail veins at 0 (prior to glucose loading), 60 and 120 minutes (after glucose loading). Blood glucose level was examined by glucose-oxidase method using a glucose monitor (LifeScan Inc., J&J Company, Milpitas, CA, USA). Serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), blood urea nitrogen (BUN), and serum creatinine (SCr) were determined using commercial reagents (Jiancheng Bioengineering Institute, Nanjing, China). Urinary total protein and albumin concentrations were measured by the immunoturbidimetric method using a biochemical analyzer (Roche, Basel, Switzerland).

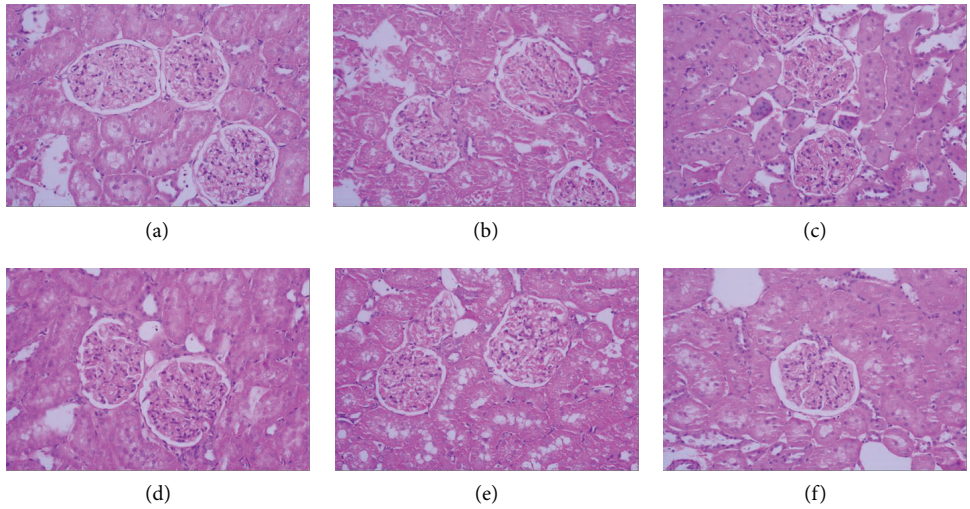
2.6. Renal Histological Studies. The paraffin slides were stained with hematoxylin and eosin (HE) to evaluate the histology of glomerulus, periodic acid-schiff (PAS) to evaluate the thickening of glomerular basement membrane and hyperplasia of mesangium, and Masson's trichrome stain (Masson) to evaluate the fibrosis of glomerulus, which were all observed under optical microscope. The following criteria were used for scoring renal histology. A semiquantitative score (sclerosis index (SI)) was used to evaluated the degree of glomerulosclerosis [20]. Severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0 represents no lesion, 1+ represents sclerosis of <25% of the glomerulus, while 2+, 3+, and 4+ represent sclerosis of 25% to 50%, >50% to 75%, and >75% of the glomerulus. The whole kidney average sclerosis index on one section was obtained by averaging scores from all glomeruli. Renal ultrastructure was also observed under a transmission electron microscope (FEI Tecnai $G^2$12, Holland).

2.7. Detection of Renal Superoxide Anion Levels. Dihydroethidium (DHE), an oxidant-sensitive probe, is widely used for detection of reactive oxygen species (ROS). Two products of DHE oxidation, ethidium and 2-hydroxyethidium, can bind to the nuclear DNA, thereby forming a strong red fluorescent complex [21]. Frozen sections of the kidney (8 μm) were placed on glass slides and incubated with DHE (10 mmol/L, Beyotime Institute of Biotechnology, Shanghai, China) in a dark container at 37°C for 30 min. After rinsing in PBS three times, the sections were viewed with an inverted microscope (Nikon, Tokyo, Japan) [22].

2.8. Measurement of Renal NADPH Activity. Renocortical tissues were homogenated and lyzed in mammal tissue protein extraction reagent. Then, the extracted protein was supplemented with protease inhibitor cocktail and phenylmethylsulfonylfluoride (PMSF) (Guge shengwu Technology Co., Wuhan, China). After centrifuged at 12000 r/min for 30 min at 4°C, the supernatant was collected to quantify the protein concentration with BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Renal NADPH activity was measured using an NADPH Activity Quantification Kit (Genmed Scientifics Inc., Shanghai, China).

2.9. Western Blot Analysis. Renocortical extracts (100 μg protein) were mixed with sample buffer, boiled for 10 min, and subjected to 10% SDS-PAGE gel (100 v, 2 h). Separated

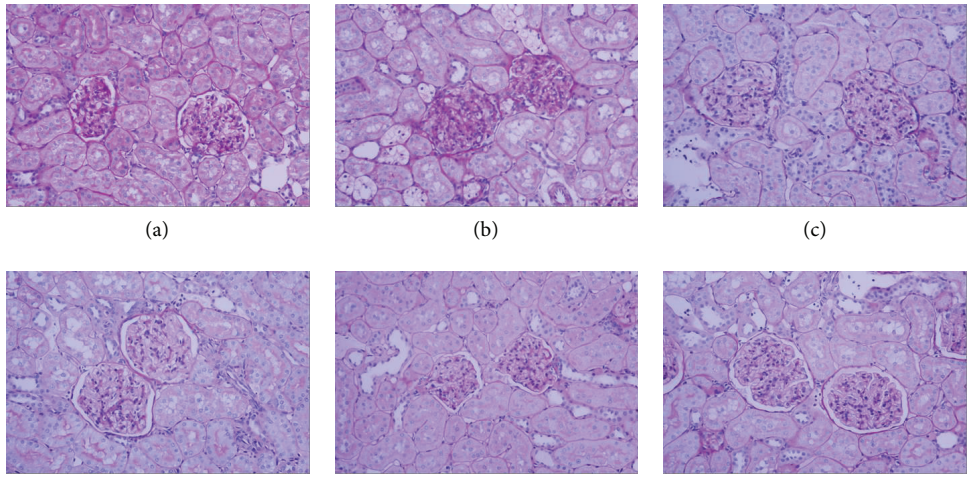
Hematoxylin and eosin staining (HE)



(a) (b) (c)

(d) (e) (f)

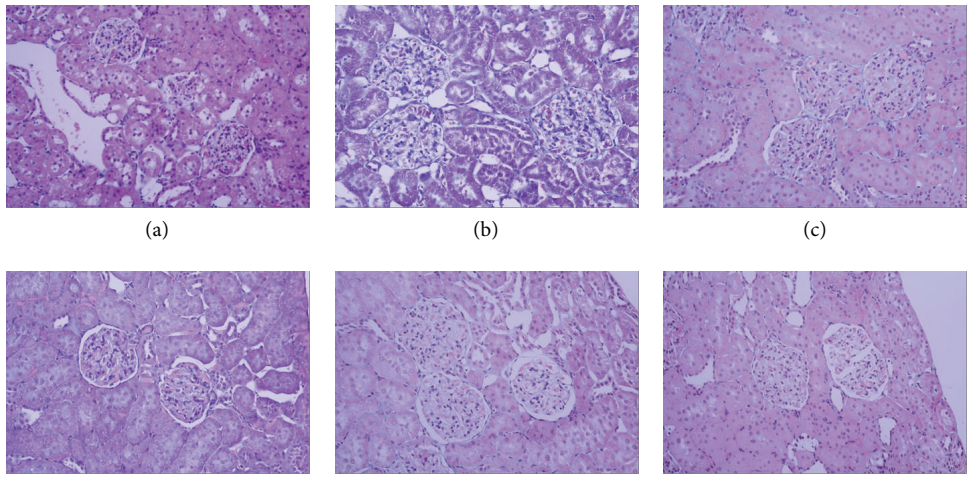
Periodic acid-schiff staining (PAS)



(a) (b) (c)

(d) (e) (f)

Masson's trichrome staining (Masson)



(a) (b) (c)

(d) (e) (f)

Figure 1: Continued.

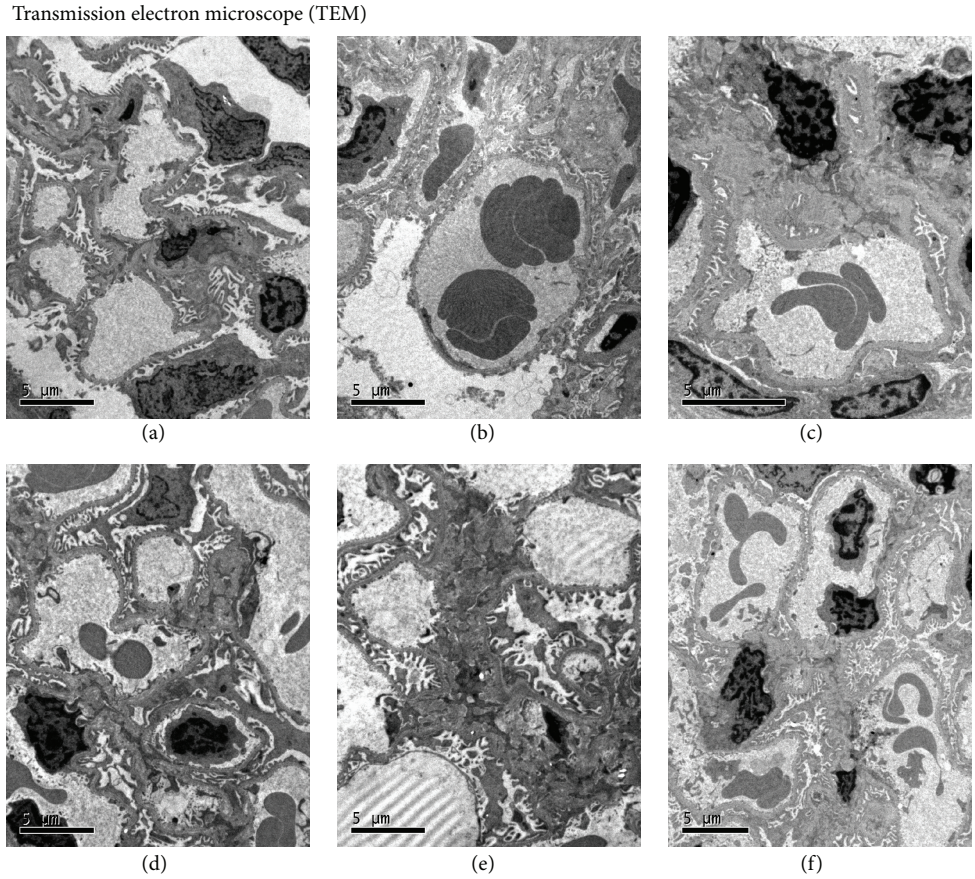


Figure 1: Morphological pictures of the glomerulus. (a) Control group; (b) Diabetic group; (c) TFG group; (d) PC group; (e) HLBW group; (f) Captopril group.

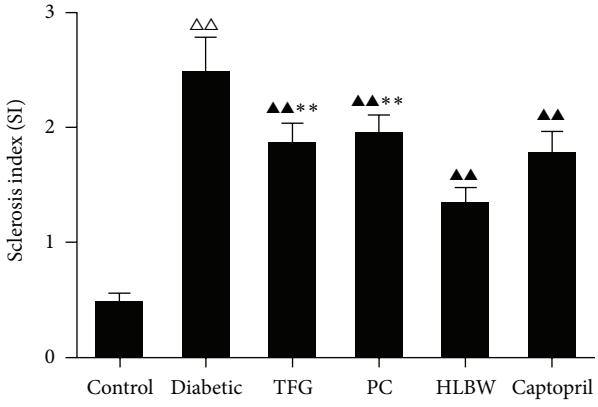


Figure 2: The effect of HLBW on glomerular sclerosis index (SI) of rats with DN. Values are mean ± SD ($n = 5$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group, and $^{**}P < 0.01$ versus HLBW group.

proteins on the gel were transferred to nitrocellulose membranes. The membranes were then blocked with 5% fat-free dry milk in TBST or 0.5% bovine serum albumin (BSA) for 2 h at room temperature, followed by overnight incubation at 4°C with antibodies ($p47^{phox}$, phosphorylated PKC-α, fibronectin, and β-actin) (Abcam, Hong kong, China). After washed by TBST three times, the membranes were lucifugally incubated with the dylight 800-labeled antibody to rabbit IgG (H+L) (KPL Company, Hongkong, China) at room temperature for 1 h. Then, the membranes were lucifugally washed with TBST three times. Immunoreactive proteins were detected by near infrared double color laser imaging system (Odyssey, Lincoln, USA). Band densities were determined by Bio-Rad Quantity One software and quantified as the ratio between OD value of target band to OD value of β-actin.

2.10. Quantitative RT-PCR Analysis. Total RNA was extracted from the renocortical tissue with Trizol reagent according to the manufacturer's instructions. RNA purity and concentration were measured by a Nucleic Acid/Protein Analyzer (Thermo, Rockford, USA). The extracted total RNA (1 μg) was reverse transcribed with PrimeScript RT reagent Kit (TaKaRa Company, Dalian, China) on a Mastercycler gradient PCR apparatus (Eppendorf Company, Hamburg, Germany). The cDNA was kept at −20°C prior to PCR amplification. Real-time PCR reactions were performed in 48-well optical PCR plates using SYBR Premix Ex Taq (TaKaRa Company, Dalian, China) on an Applied Biosystems StepOne Real-Time PCR System (Stepone, Foster City, USA). A $2^{-\Delta\Delta CT}$ was used for analyzing the data. Primer sequences are listed in Table 1.

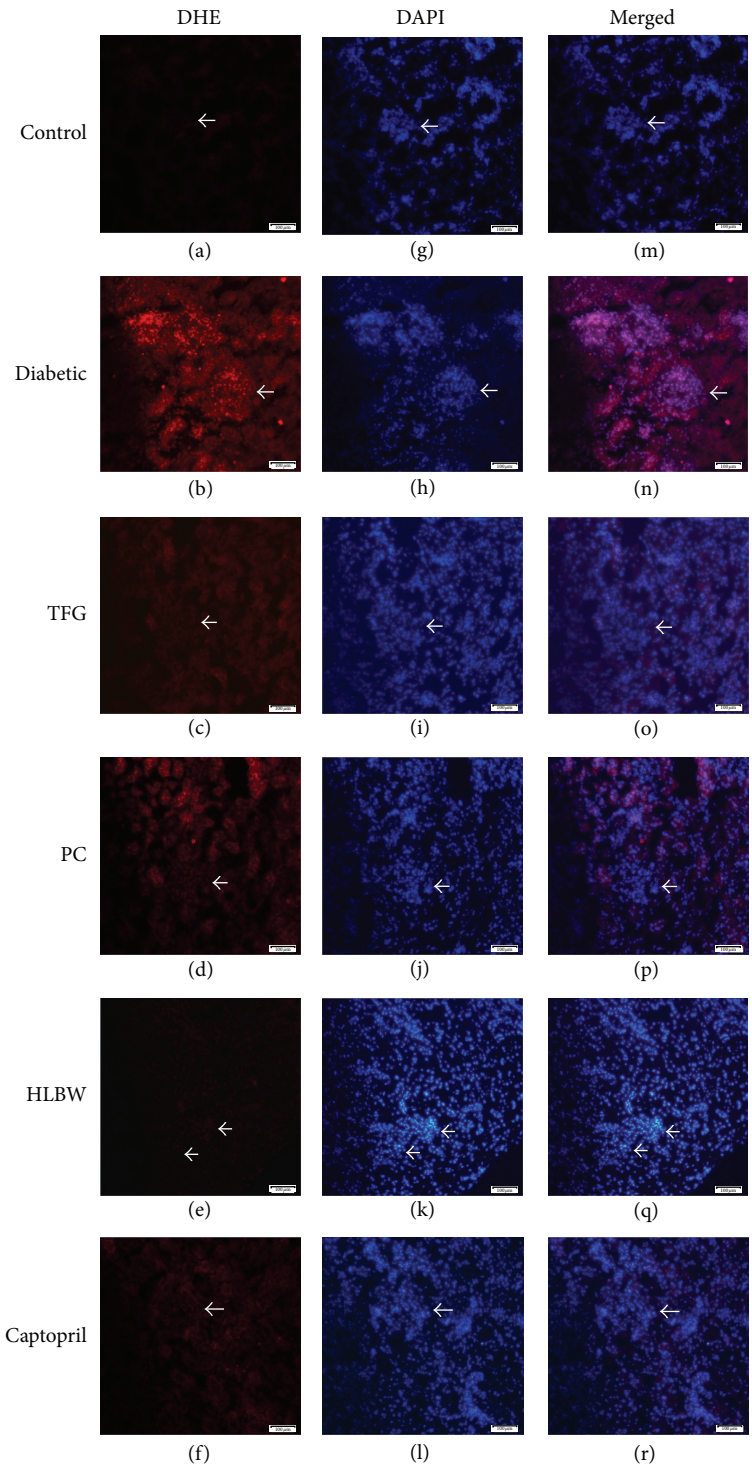


Figure 3: Dihydroethidium staining in the glomeruli from the rats in different groups. (original magnification: ×100). (a)–(f) Visualization of ROS in the glomeruli using DHE stains. (g)–(l) Visualization of nucleus in the glomeruli using DAPI stains. (m)–(r) The superimposed pictures of different groups. (Arrows are pointing at the glomerulus.)

2.11. Statistical Analysis. All data are presented as mean ± standard deviation (SD) and analyzed by SPSS19.0 Statistical Software. Statistical significance was determined by one-way analysis of variance (ANOVA). Data with equal variances were not assumed followed by Dunnett's T3 test, while data with equal variances assumed followed by LSD test. A probability of less than 0.05 was considered to be statistically significant.

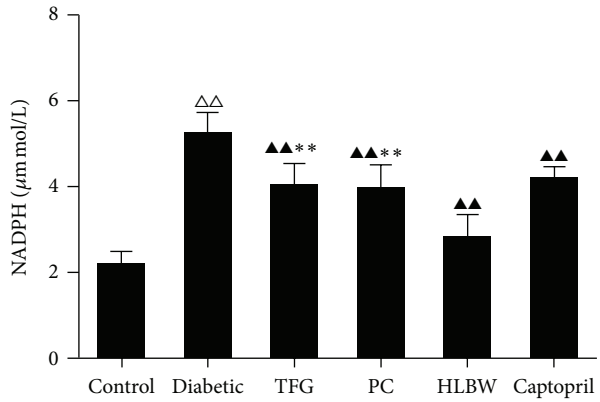


Figure 4: The effect of HLBW on renal NADPH activity of rats with DN. Values are mean ± SD ($n = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group, and $^{**}P < 0.01$ versus HLBW group.

3. Results

3.1. HLBW Improved the Glucose Tolerance of Rats with DN. Rats with untreated DN showed severe hyperglycemia characterized by elevated fasting and postprandial plasma glucose levels ($P < 0.01$). However, treatment with HLBW and its single components, as well as Captopril, significantly decreased plasma glucose levels compared with those of untreated diabetic rats ($P < 0.01$) (Table 2).

3.2. HLBW Improved Plasma Lipid Profiles of Rats with DN. As shown in Table 3, rats with untreated DN showed severe dyslipidemia. The serum TC, TG, and LDL-C levels increased compared with those of control rats ($P < 0.01$). Treatment with HLBW and its single components markedly alleviated hyperlipidemia in rats with DN ($P < 0.01$, $P < 0.05$, resp.). However, Captopril did not show any beneficial effect on dyslipidemia when compared with diabetic rats.

3.3. HLBW Improved Renal Function and Proteinuria of Rats with DN. As shown in Table 4, rats with untreated DN exhibited an elevation in the term of ratio of kidney to body weight, as well as severe renal dysfunction and proteinuria. The BUN, SCr, urinary total protein, and albumin concentrations increased significantly in comparison to those of control rats ($P < 0.01$). However, treatment with HLBW and its single components, as well as Captopril, significantly reduced the ratio of kidney to body weight and reversed renal dysfunction and proteinuria ($P < 0.01$).

3.4. HLBW Improved Renal Morphology Changes of Rats with DN. As shown in Figure 1, renal tissues of rats with untreated DN showed remarkable glomerular hypertrophy and fibrosis, hyperplasia of mesangial area, and effacement of the podocyte foot processes. As shown in Figure 2, SI of rats with untreated DN elevated significantly. However, treatment with HLBW restored these morphology changes and SI level. Treatment with either TFG or PC also attenuated glomerular hypertrophy and fibrosis, mesangial hyperplasia, podocyte foot processes effacement, and glomerular SI level.

3.5. HLBW Decreased Renal Superoxide Anion Production of Rats with DN. As shown in Figure 3, a high level of DHE fluorescence, indicating the increased superoxide anion production [23, 24], was observed in renal tissues of DN rats. However, treatment with HLBW and its single components, as well as Captopril, significantly reduced the level of DHE fluorescence in renal tissues of rats with DN.

3.6. HLBW Decreased Renal NADPH Activity of Rats with DN. As shown in Figure 4, the activity of renal NADPH was much higher in DN rats than that in control rats ($P < 0.01$). After the treatment with HLBW, TFG, PC, or Captopril, the activity of renal NADPH was significantly decreased in rats with DN ($P < 0.01$). Furthermore, a significant difference in renal NADPH activity was identified between HLBW and its single components, indicating a better renal NADPH decreasing activity of HLBW than that of TFG and PC ($P < 0.01$).

3.7. HLBW Decreased Renal PKC-α, Phosphorylated PKC-α, $p47^{phox}$, and Fibronectin Gene Expressions of Rats with DN. As shown in Figures 5 and 6, renal $p47^{phox}$ protein level and mRNA concentration significantly increased in DN rats compared with those in control rats ($P < 0.01$). However, there was a significant reduction in the expression of $p47^{phox}$ protein and mRNA in all the treatment groups ($P < 0.01$). Moreover, a marked reduction in the expression of $p47^{phox}$ protein and mRNA was identified after HLBW treatment in contrast to TFG or PC treatment alone ($P < 0.01$, $P < 0.05$).

As shown in Figures 5 and 6, renal phosphorylated PKC-α protein level increased significantly in rats with untreated DN ($P < 0.01$). HLBW, TFG, PC and Captopril treatment significantly reduced the expression of phosphorylated PKC-α protein ($P < 0.01$). Additionally, HLBW, better than TFG or PC alone, significantly decreased phosphorylated PKC-α protein expression ($P < 0.01$). However, in terms of PKC-α mRNA expression, no significant difference was identified between the groups.

With regard to Fibronectin, which is a marker of renal fibrosis, rats with untreated DN exhibited a significant increase of renal Fibronectin protein expression ($P < 0.01$). HLBW, treatment with TFG, PC, and Captopril significantly reduced the expression of renal fibronectin protein ($P < 0.01$). HLBW also better than TFG or PC alone, significantly decreased renal expression of fibronectin protein ($P < 0.01$).

4. Discussion

HLBW is a Chinese herbal prescription that is just composed of two herbs, TFG and PC. Previous evidence from animal and clinical studies has suggested that TFG has the potential to treat hyperglycemia, hyperlipidemia, and renal disease [7–10, 18]. However, the comparisons between the effect of HLBW and its single components on DN have rarely been reported.

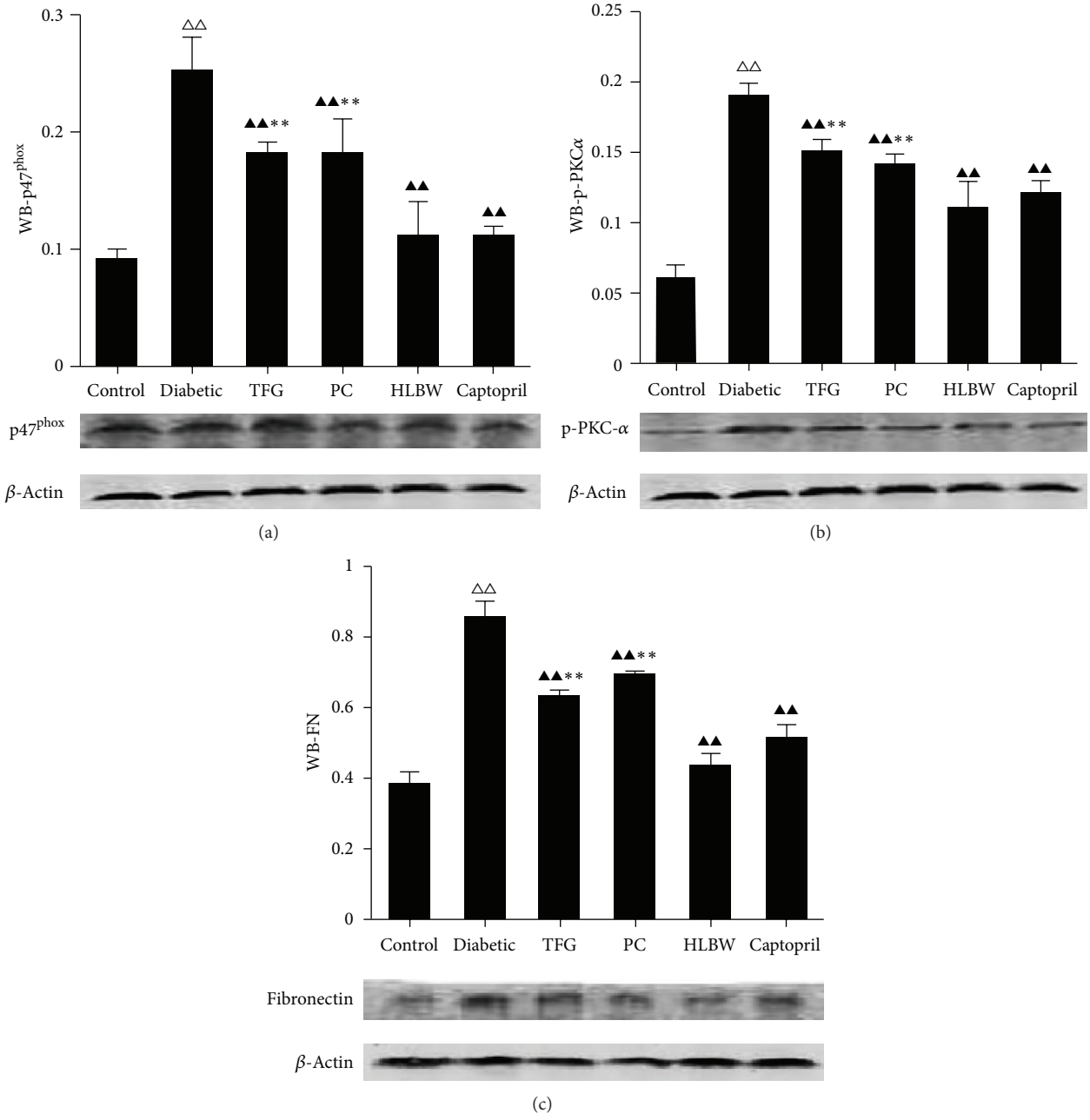


Figure 5: The effect of HLBW on the expression of renal proteins: (a) $p47^{phox}$; (b) p-PKC-α; (c) fibronectin. Values are mean ± SD ($n = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group, and $^{**}P < 0.01$ versus HLBW group.

In our study, the rat model of type 2 diabetes was successfully established by a high-fat diet accompanied by intravenous injection of relatively small doses of STZ. The animals manifested the characteristics of hyperglycemia and hyperlipidemia. BUN, SCr, 24-hour urinary total protein, and albumin concentrations significantly increased at the same time, which indicated the development of DN. HLBW showed hypoglycemia, hypolipidemia, and renal protection against DN. Meanwhile, such the beneficial outcomes were also confirmed in DN rats treated with TFG or PC alone. With regard to renal morphology changes, the untreated diabetic rats were characteristized of remarkable glomerular expansion and fibrosis, extracellular matrix (ECM) accumulation, and effacement of the foot processes. HLBW, also similar to TFG and PC, attenuated these histopathological abnormalities in renal tissue in diabetic rats. However, HLBW, better than TFG and PC, reduced the expression of renal fibronectin protein and the level of SI. Since fibronectin is a predominant matrix protein representing the degree of renal fibrosis and SI represents the extent of glomerular sclerosis, this result indicates that HLBW may be promising at ameliorating renal fibrosis.

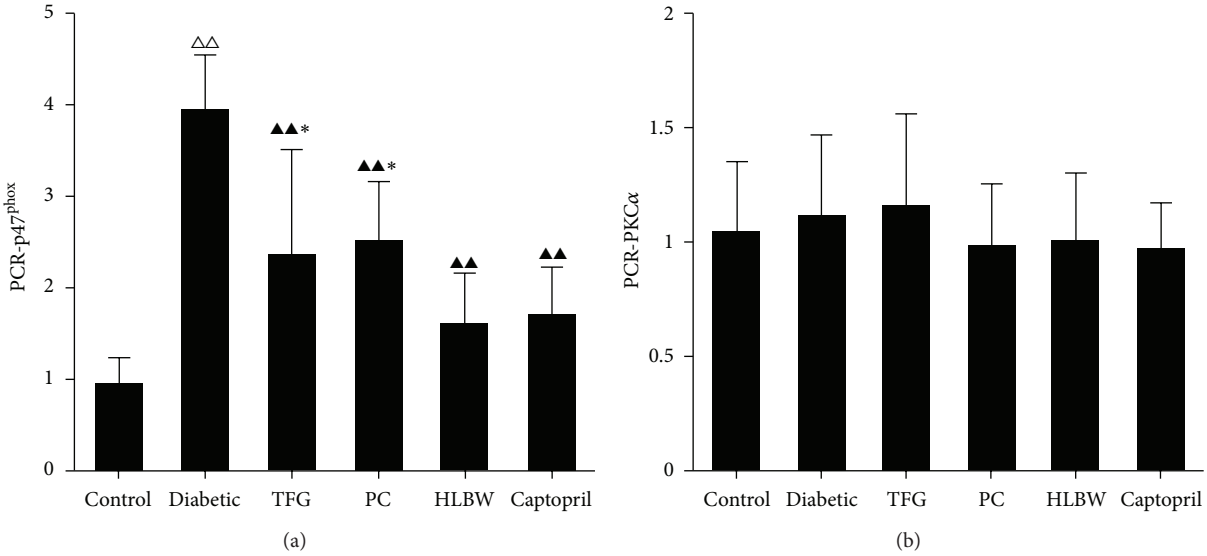


FIGURE 6: The effect of HLBW on the expression of renal $p47^{phox}$ (a) and PKC-α (b) mRNA. Values are mean ± SD ($n = 8$). $^{\triangle\triangle}P < 0.01$ versus the control group, $^{\blacktriangle\blacktriangle}P < 0.01$ versus the untreated diabetic group, and $^{*}P < 0.05$ versus HLBW group.

In diabetic patients, hyperglycemia and hyperlipidemia enhance the oxidative stress which is involved in the mechanism of diabetic vasculopathy. The oxidative stress can also aggravate the glucolipid metabolism disorder, thereby forming a vicious circle [5, 6, 25]. Excessive reactive oxygen species (ROS) generated by oxidative stress not only induces oxidative damage via peroxidation of the biomacromolecule but also interferes with cell signal transduction via serving as a second messenger [26]. To evaluate superoxide production in the kidneys, dihydroethidium (DHE) staining is often performed. In the presence of superoxide, DHE is changed into ethidium bromide, which binds to DNA and exhibits red fluorescence in the nucleus [27–29]. In our study, a massive increase in superoxide anion generation was identified in the diabetic renal tissues. It was attenuated by HLBW and its single components. However, HLBW treatment, compared with TFG or PC, showed the maximum decreasing of superoxide production, indicating the efficient oxidative stress protection in DN.

In order to elucidate the protection mechanisms of HLBW against oxidative stress in renal tissues, we further evaluated the gene expressions involved in the production of ROS. Among the multiple sources in the diabetic kidney, ROS derived from NADPH oxidase is crucial to the development of DN [30, 31]. Accordingly, we further examined the activity of NADPH oxidase and the gene expressions of its upstream regulator, protein kinase C-α (PKC-α), and $p47^{phox}$, which are the regulatory subunit of NADPH oxidase [26]. The results showed that HLBW significantly inhibited NADPH oxidase activity and $p47^{phox}$ gene expression in renal tissues of diabetic rats. HLBW also decreased phosphorylated PKC-α protein expression. These effects were also superior to that observed in DN rats treated with TFG or PC alone. However, no significant difference was identified between the groups regarding the expression of PKC-α mRNA. It might give a hint that the effect of HLBW on PKC-α is posttranscription. Therefore, our study firstly demonstrated the inhibitory effect of HLBW on PKC-α/NADPH oxidase signaling pathway, which may attribute to the reduction of ROS production in renal tissues.

In summary, our study demonstrates that HLBW and its single components, TFG and PC, improve renal function and ameliorate renal histopathological alterations in type 2 diabetic rats. The mechanism may be related to reducing oxidative stress via PKC-α/NADPH oxidase signaling pathway. Moreover, HLBW exhibits a better efficacy than TFG or PC alone on protecting against oxidative stress in DN, which indicates a theory of prescription compatibility in traditional Chinese medicine.

Conflict of Interests

The authors declare that they have no conflict of interests to disclose.

Authors' Contribution

Lishan Zhou and Hui Dong contributed equally to this work.

Acknowledgment

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Research Article

Evaluation of Antidiabetic Activity and Associated Toxicity of *Artemisia afra* Aqueous Extract in Wistar Rats

Taofik O. Sunmonu and Anthony J. Afolayan

Research Center for Phytomedicine, Department of Botany, University of Fort Hare, Alice 5700, South Africa

Correspondence should be addressed to Anthony J. Afolayan; aafolayan@ufh.ac.za

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Artemisia afra Jacq. ex Willd. is a widely used medicinal plant in South Africa for the treatment of diabetes. This study aimed to evaluate the hypoglycemic activity and possible toxicity effect of aqueous leaf extract of the herb administered at different dosages for 15 days in streptozotocin-induced diabetic rats. Administration of the extract at 50, 100, and 200 mg/kg body weight significantly ($P < 0.05$) increased body weight, decreased blood glucose levels, increased glucose tolerance, and improved imbalance in lipid metabolism in diabetic rats. These are indications of antidiabetic property of *A. afra* with 200 mg/kg body weight of the extract showing the best hypoglycemic action by comparing favourably well with glibenclamide, a standard hypoglycemic drug. The extract at all dosages tested also restored liver function indices and haematological parameters to normal control levels in the diabetic rats, whereas the kidney function indices were only normalized in the diabetic animals administered with 50 mg/kg body weight of the extract. This investigation clearly showed that in addition to its hypoglycemic activity, *A. afra* may also protect the liver and blood against impairment due to diabetes. However, some kidney functions may be compromised at high dosages of the extract.

1. Introduction

Diabetes mellitus is a major endocrine disorder and growing health problem in most countries. It is a metabolic disease as old as mankind; and its incidence is considered high all over the world [1]. Increase in sedentary lifestyle, consumption of energy-rich diets, and obesity are some of the factors causing the rise in the number of diabetics. The World Health Organization (WHO) estimated diabetes in adults to be around 173 million, and about two-thirds of these patients live in developing countries [2]. The prevalence of diabetes is on the increase worldwide including South Africa; and it is still expected to increase by 5.4% in 2025 [3]. WHO further reports that over 4.8 million annual deaths (9% of global total) are attributable to either diabetes or its complications [4].

Despite these alarming statistics, there is no specific and definite therapy currently for diabetes. However, a few chemotherapeutic drugs have been in use to manage the disease since the accidental discovery of the hypoglycemic action of sulfonamides [5]. The thrust of such management measures is to achieve an effective blood glucose control or utilization, with a view to delaying or averting the onset of complications. The application of these measures is, however, limited due to their high cost and associated side effects. Consequently, attention is being focused on the use of herbal medicines for the treatment of diabetes.

In traditional African societies, phytotherapy is highly valued and widely utilized. South Africa, particularly, has remarkable biodiversity and rich cultural traditions of plant use. Hence, it is not surprising why the majority of the population in South Africa use plant materials as their source of primary healthcare and as an alternative or supplement to visiting western healthcare practitioners [6]. This is particularly true for the treatment of diabetes, and WHO has also authenticated phytotherapeutic approach to the treatment of the disease [7, 8]. The use of natural remedies for diabetes treatment is also strengthened due to the belief that herbs can provide some benefits over allopathic medicine and allows users to feel that they have some control in their choice of medication [9]. However, their general acceptability has been limited by lack of dose regimen and adequate data

on their toxicity. Traditional medical practitioners and users of medicinal plants also believe that herbs are safe simply because they are natural in origin. It is, therefore, pertinent to provide information on the effective dose and toxicity risk associated with the use of these medicinal plants for the treatment of ailments.

Artemisia afra Jacq. ex Willd. (Asteraceae) is known as *Umhlonyane* in Xhosa and African wormwood in English. It is one of the widely used medicinal plants in South Africa because of its acclaimed healing properties against many ailments including diabetes. It is an erect, shrubby, and perennial plant growing up to 2 m tall with a leafy and hairy stem. The leaf shape is narrowly ovate, feathery, and finely divided, which grows up to 8 cm long and 4 cm wide. It is widespread in all the provinces of South Africa except the Northern Cape, and it is easily identifiable by its characteristic aromatic smell [6]. Despite the widespread abundance and traditional use of this indigenous plant in South Africa, no systematic study has been done to substantiate its acclaimed anti-diabetic property. The present study was designed to evaluate the antidiabetic activity and the safety/toxicity risk associated with the use of aqueous leaf extract of *A. afra* in streptozotocin-induced diabetic rats. The efficacy was compared with glibenclamide, a standard hypoglycemic drug.

2. Materials and Methods

2.1. Chemicals. Streptozotocin (STZ) was procured from Sigma Chemical Co., St. Louis, MO, USA while Glibenclamide was a product of Taj Pharmaceuticals Ltd., India. The assay kits used for biochemical assays were products of Randox Laboratories Limited, Ardmore, Co Antrim, UK. All other chemicals and reagents used were of analytical grade.

2.2. Plant Material and Authentication. Freshly picked *A. afra* comprising mature leaves and stems were collected from the University of Fort Hare, Alice (Eastern Cape Province), South Africa, in June 2009. The plant was authenticated by Professor D.S. Grierson, a botanist in the Department of Botany at the University of Fort Hare, and a voucher specimen (Sunmed. 2009/01) was prepared and deposited at the Giffen Herbarium of the university.

2.3. Preparation of Aqueous Extract. The aqueous extract of the plant was prepared in a manner that mimicked the traditional method of preparation. Briefly, the leaves of *A. afra* plucked from the stalks were rinsed with distilled water, dried in the oven at 30°C, and slightly crushed by hand. The dried leaves (100 g) were suspended in 1 L distilled water and the mixture boiled for 30 min. The decoction obtained was cooled, filtered, frozen at −70°C, and then freeze-dried (Virtis benchtop K, Virtis Company, Gardiner, NY, USA) to give a yield of 17.4 g. This was reconstituted separately in distilled water to give the required doses for the experiment.

2.4. Animals Used. Male albino rats of Wistar strain with a mean weight of 152 ± 5.32 g were obtained from the Experimental Animal House of the Agricultural and Rural Development Research Institute (ARDRI), University of Fort Hare, Alice, South Africa. The animals were housed in clean metabolic cages placed in a well-ventilated house with optimum condition (temperature: 23 ± 1°C; photoperiod: 12 h natural light and 12 h dark; and humidity: 45–50%). They were acclimatized to animal house conditions and allowed free access to commercial pelleted rat chow (Pioneer Foods (Pty) Ltd., Huguenot, South Africa) and water. The cleaning of the cages was done on a daily basis. This study was carried out following the approval from the Ethical Committee on the Use and Care of Animals of the University of Fort Hare, South Africa, and an ethical clearance number (AFO011) was assigned for the project.

2.5. Induction of Diabetes in Rats. The rats were fasted for 18 h, and diabetes was induced by a single intravenous injection of freshly prepared solution of STZ (55 mg/kg of body weight) in 0.1 M citrate buffer (pH 4.5) [10]. The animals were allowed to drink 5% glucose solution to protect them against the diabetogenic action of STZ and subsequently kept fasting in order to avoid excessive accumulation of feeding glucose which may antagonize STZ effect. Control rats were injected with citrate buffer alone. After 24 h of injection, fasting blood glucose level was checked, and animals with levels above 13.9 mmol/L were considered diabetic [10].

2.6. Experimental Design. The rats were divided into two sets, each comprising six groups ($n = 6$ in each group): one for antidiabetic/toxicity studies and the other for the evaluation of glucose tolerance. Group 1 (normal control) and Group 2 (diabetic control) were administered with 0.5 mL of distilled water. Groups 3 to 5 are diabetic rats treated with 50, 100, and 200 mg/kg body weight/day of *A. afra* extract, respectively, while Group 6 comprised diabetic animals administered with glibenclamide (600 μg/kg body weight/day).

2.7. Collection of Blood Sample and Isolation of Organs. After 15 days of extract administration, the rats were humanely sacrificed by anaesthetization and the neck area was quickly cleared of fur before the jugular vein was sharply cut with sterile surgical blade. An aliquot (2 mL) of blood was collected into ethylene diamine tetra-acetic acid (EDTA) embedded sample bottles for haematological analysis. Another 5 mL of the blood was collected and centrifuged at 1282 g × 5 min, and the serum was carefully aspirated with a Pasteur pipette into sample bottles for the various biochemical assays. The rats were quickly dissected and the whole liver and two kidneys were excised, freed of fat, blotted with clean tissue paper, and then weighed. The organ-to-body weight ratio was determined by comparing the weight of each organ with the final body weight of each rat.

2.8. Water, Feed, Weight, and Blood Glucose Monitoring. The water intake, feed intake, and body weight gain of all the rats were monitored during the 15-day experimental period. At an interval of 5 days, blood samples were collected from the

tail vein of the experimental animals after overnight fasting for the estimation of blood glucose level using a glucometer (Accu-Chek, Roche Products (Pty) Ltd., South Africa).

2.9. Analysis of Lipid Profile and Total Protein. The serum concentrations of total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol were determined by automatic analyser technique (Beckman Coulter Inc., Ireland). Total protein in the serum was estimated using bovine serum albumin as standard [11].

2.10. Liver and Kidney Function Tests. The concentrations of creatinine [12], urea [13], calcium [14], uric acid [15], total bilirubin [16], albumin, and globulin [17] as well as the activities of alkaline phosphatase (ALP) [18], gamma glutamyl transferase (GGT) [19], and aspartate and alanine transaminases (AST and ALT) [20] were determined in the serum using Randox Assay kits.

2.11. Determination of Haematological Parameters. Using Horiba ABX 80 Diagnostics (ABX Pentra Montpellier, France), the following analyses were carried out: red blood count (RBC), haemoglobin (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet (PLT), white blood cells (WBCs), and white blood cell differential counts.

2.12. Oral Glucose Tolerance Test. On day 15, the rats in groups 1 to 6 (from the second set) were given glucose (2 g/kg body weight; p.o.) 30 min after administration of the extract/drug [21]. Blood samples were collected from the tail vein prior to glucose administration and at 30, 60, and 90 min after glucose loading for immediate measurement of blood glucose levels.

2.13. Statistical Analysis. Data were expressed as mean ± SE of six replicates and subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine significant differences in all the parameters. Values were considered statistically significant at $P < 0.05$.

3. Results

3.1. Water/Feed Intake and Weight Gain. While water consumption increased in the untreated diabetic rats (Group 2), the administration of aqueous extract of *A. afra* significantly reduced ($P < 0.05$) the quantity of water and feed intake in diabetic animals (Table 1). Similarly, the untreated diabetic rats showed polyphagic condition and consumed higher quantity of feed compared to the control and treatment groups. There was a significant reduction ($P < 0.05$) in the weight gained by the untreated diabetic rats when compared with the control and treatment groups. Generally, the effect of treatment with 200 mg/kg body weight of *A. afra* compared favourably well with that of glibenclamide which is a known standard drug for diabetes.

3.2. Blood Glucose Level. The continuous administration of aqueous extract of *A. afra* was found to significantly reduce ($P < 0.05$) the blood glucose level in diabetic rats at the end of the experiment (Table 2). Again, the effect was more pronounced in the rats treated with 200 mg/kg body weight of the extract and it compared favourably well with glibenclamide-treated rats.

3.3. Serum Lipid Profile and Total Protein. There was a significant elevation ($P < 0.05$) in the levels of serum cholesterol, triglycerides, and LDL and reduced HDL and protein concentrations in diabetic rats when compared with the control group (Table 3). The aqueous extract of *A. afra* and glibenclamide significantly reduced ($P < 0.05$) the levels of serum cholesterol, triglycerides, and LDL and increased HDL and protein concentration to near normalcy as observed in the control after 15 days of treatment.

3.4. Oral Glucose Tolerance Test. Table 4 shows the blood glucose levels of the rats after oral administration of glucose. The level in the control rats rose to the peak 30 min after glucose load and decreased to near normal levels at 90 min. In the untreated diabetic rats, the peak increase in blood glucose concentration was observed after 30 min and remained high over the next 60 min. *A. afra*- and glibenclamide-treated diabetic rats showed significant decrease ($P < 0.05$) in blood glucose concentration at 60 and 90 min compared with diabetic control rats.

3.5. Liver Function Parameters. The untreated diabetic rats exhibited significant increase ($P < 0.05$) in serum activities of ALP, GGT, ALT, AST, liver-to-body weight ratio, and bilirubin; as well as reduced albumin and globulin concentrations when compared with the control (Table 5). Continuous administration of aqueous extract of *A. afra* to diabetic rats for 15 days was able to restore all the liver function indices back to normalcy.

3.6. Kidney Function Parameters. A significant increase ($P < 0.05$) was observed in all the kidney function indices examined in the untreated diabetic rats when compared with the control (Table 6). The aqueous extract of this herb had a positive impact on the kidney function indices of diabetic rats by significantly reducing ($P < 0.05$) kidney-to-body weight ratio as well as the serum concentrations of calcium ion, creatinine, urea, and uric acid. The positive impact is, however, more pronounced in the rats treated with 50 mg/kg body weight extract.

3.7. Haematological Parameters. In addition, the diabetic rats exhibited significantly reduced levels ($P < 0.05$) in all the haematological parameters with the exception of white blood cell count and lymphocytes which were significantly increased (Table 7). Oral administration of aqueous extract of *A. afra* in diabetic rats for 15 days, however, restored the haematological parameters to normalcy with the exception of platelets and neutrophils which were significantly increased but not to the control levels.

Table 1: Effect of oral administration of aqueous extract of *Artemisia afra* on water intake, feed intake, and body weight in diabetic rats ($n = 6$, mean ± SE).

Groups	Water intake (mL/day)	Feed intake (g/day)	Weight gain (g)
Control	19.08 ± 1.07^{a}	14.07 ± 1.47^{a}	31.30 ± 4.50^{a}
Diabetic control	91.89 ± 2.13^{b}	33.90 ± 3.14^{b}	3.17 ± 0.96^{b}
Diabetic + 50 mg/kg *A. afra*	83.84 ± 1.88^{c}	27.18 ± 1.09^{c}	6.76 ± 1.12^{c}
Diabetic + 100 mg/kg *A. afra*	77.55 ± 1.53^{d}	22.23 ± 1.06^{d}	11.60 ± 1.41^{d}
Diabetic + 200 mg/kg *A. afra*	44.94 ± 1.31^{e}	19.03 ± 1.17^{e}	19.82 ± 2.14^{e}
Diabetic + glibenclamide	47.03 ± 1.47^{e}	20.97 ± 1.01^{e}	16.20 ± 1.17^{e}

Values with different superscripts along the same column indicate statistically significant difference at $P < 0.05$.

Table 2: Effect of oral administration of aqueous extract of *Artemisia afra* on blood glucose levels in diabetic rats ($n = 6$, mean ± SE).

Groups	Blood glucose (mmol/L)			
	Initial	Day 5	Day 10	Day 15
Control	5.24 ± 0.69^{a}	5.26 ± 0.72^{a}	5.26 ± 0.61^{a}	5.24 ± 0.85^{a}
Diabetic control	5.26 ± 0.61^{a}	24.90 ± 1.22^{b}	27.44 ± 1.36^{b}	21.60 ± 0.24^{b}
Diabetic + 50 mg/kg *A. afra*	5.26 ± 0.72^{a}	24.80 ± 1.12^{b}	17.70 ± 1.22^{c}	7.80 ± 0.04^{c}
Diabetic + 100 mg/kg *A. afra*	5.24 ± 0.68^{a}	24.28 ± 1.19^{b}	16.70 ± 1.36^{c}	7.10 ± 0.05^{c}
Diabetic + 200 mg/kg *A. afra*	5.25 ± 0.71^{a}	24.20 ± 1.00^{b}	10.58 ± 1.33^{d}	5.47 ± 0.26^{a}
Diabetic + glibenclamide	5.25 ± 0.69^{a}	24.30 ± 1.19^{b}	10.70 ± 1.31^{d}	5.40 ± 0.06^{a}

Values with different superscripts along the same column indicate statistically significant difference at $P < 0.05$.

4. Discussion

The observation of higher consumption of water and food accompanied by high blood glucose levels and urine output is an indication of diabetic state in the animals resulting from STZ administration. The present study demonstrated that aqueous extract of *Artemisia afra* has antidiabetic activity; and the efficacy is comparable to glibenclamide, a standard hypoglycemic drug.

Administration of the plant extract was effective in preventing polydipsia and polyphagia conditions. Similar observation was reported by Shetty et al. [22] using *Momordica charantia* in diabetic rats. Despite the high feed and water intake in the untreated diabetic rats, the gain in body weight was very minimal compared to the extract treated groups. The enhancement of body weight in the *A. afra*-treated rats could be attributed to the increase in metabolic activity of their body systems. This clearly indicates that the plant extract increased glucose metabolism which enhanced body weight in the rats. Again, this observation was reported by Ravi et al. [10]. According to these authors, *Eugenia jambolana* seed kernels increased body weight of diabetic rats. Of particular interest is the fact that the effect of *A. afra* at the dose of 200 mg/kg body weight compared favourably well with glibenclamide.

The increase in blood glucose concentration is an important characteristic feature of diabetic state. *A. afra* extract produced significant hypoglycemic effect on diabetic rats, and by day 15, the glucose levels tended towards normalcy as was found in the control rats. Microchemical analyses of *A. afra* have indicated the presence of saponins [23], which have been reported to possess hypoglycemic activity in diabetic rabbits [24]. Therefore, the hypoglycemic activity of *A. afra* observed in this study could be attributed to the presence of saponins which might be acting as a stimulant for the release of insulin following the repair of pancreatic beta cells by the extract [25].

Abnormalities in lipid profile are very common in the diabetic state [26]. Although lipoprotein alteration is an intrinsic part of diabetic mellitus, such alterations are also induced by diabetes-associated complications such as obesity or renal disease [27]. In the present study, aqueous extract of *A. afra* was able to bring down the levels of cholesterol, triglycerides, and LDL but increased the levels of HDL in diabetic rats to near normal levels when compared to the untreated diabetic group. The serum level of cholesterol is usually increased in diabetes, and such an elevation is a risk factor for coronary heart disease. The abnormal high concentration of cholesterol in the blood during diabetes is mainly due to the increase in the mobilization of free fatty acids from the peripheral depots, since insulin inhibits the hormone-sensitive lipase [28]. Administration of *A. afra* to diabetic rats significantly decreased the plasma cholesterol level to near normalcy and therefore reduced the risk of cardiovascular disease [29]. An increase in the concentrations of total cholesterol and LDL-cholesterol and reduced HDL-cholesterol as observed during diabetes are associated with raised risk of myocardial infarction [30]. Treatment of diabetic rats with *A. afra* extract elevated HDL-cholesterol and reduced LDL-cholesterol levels, which are indications of reduced risk of myocardial infarction. There is a growing body of evidence from epidemiologic, clinical, and laboratory data indicating that elevated triglyceride level is an independent risk factor for cardiovascular disease [31]. Hypertriglyceridemia is a

Table 3: Effect of oral administration of aqueous extract of *Artemisia afra* on serum lipid profile and total protein in diabetic rats (n = 6, mean ± SE).

Groups	Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Total protein (g/L)
Control	50.43 ± 2.70^{a}	95.73 ± 2.50^{a}	16.92 ± 1.98^{a}	35.19 ± 2.01^{a}	78.60 ± 1.69^{a}
Diabetic control	76.62 ± 1.22^{b}	154.27 ± 4.31^{b}	10.22 ± 1.89^{b}	58.10 ± 1.12^{b}	63.41 ± 2.72^{b}
Diabetic + 50 mg/kg *A. afra*	56.97 ± 2.57^{a}	102.28 ± 3.80^{a}	14.33 ± 0.97^{a}	43.12 ± 2.80^{a}	73.92 ± 2.18^{a}
Diabetic + 100 mg/kg *A. afra*	56.85 ± 2.40^{a}	101.88 ± 3.44^{a}	14.28 ± 0.98^{a}	42.32 ± 3.62^{a}	76.92 ± 2.66^{a}
Diabetic + 200 mg/kg *A. afra*	54.40 ± 1.22^{a}	98.30 ± 3.56^{a}	13.36 ± 1.00^{a}	40.66 ± 3.50^{a}	74.20 ± 3.84^{a}
Diabetic + glibenclamide	53.95 ± 2.80^{a}	96.19 ± 3.68^{a}	13.01 ± 0.99^{a}	38.63 ± 1.98^{a}	73.08 ± 3.78^{a}

Values with different superscripts along the same column indicate statistically significant difference at $P < 0.05$.

Table 4: Effect of oral administration of aqueous extract of *Artemisia afra* on blood sugar levels in glucose-loaded diabetic rats (n = 6, mean ± SE).

Groups	Blood glucose (mmol/L)			
	Fasting	30 minutes	60 minutes	90 minutes
Control	4.43 ± 0.22^{a}	5.87 ± 0.26^{a}	5.03 ± 0.26^{a}	4.53 ± 0.25^{a}
Diabetic control	21.08 ± 1.26^{b}	36.90 ± 1.22^{b}	30.93 ± 1.28^{b}	22.90 ± 1.23^{b}
Diabetic + 50 mg/kg *A. afra*	6.10 ± 0.84^{c}	15.20 ± 0.85^{c}	12.10 ± 0.94^{c}	9.10 ± 0.85^{c}
Diabetic + 100 mg/kg *A. afra*	5.90 ± 0.83^{c}	14.50 ± 0.83^{c}	10.05 ± 0.83^{c}	8.05 ± 0.84^{c}
Diabetic + 200 mg/kg *A. afra*	5.40 ± 0.86^{c}	8.67 ± 0.35^{d}	7.01 ± 0.33^{d}	5.07 ± 0.36^{a}
Diabetic + glibenclamide	5.30 ± 0.35^{c}	8.90 ± 0.34^{d}	7.50 ± 0.33^{d}	5.01 ± 0.33^{a}

Values with different superscripts along the same column indicate statistically significant difference at $P < 0.05$.

Table 5: Effect of aqueous extract of *Artemisia afra* on some liver function parameters of diabetic rats (n = 6 ± SE).

	Control	Diabetic	Diabetic + *Artemisia afra* (mg/kg body weight)		
			50	100	200
Liver-to-body weight ratio (%)	2.41 ± 0.19^{a}	3.96 ± 0.24^{b}	2.41 ± 0.20^{a}	2.50 ± 0.46^{a}	2.60 ± 0.18^{a}
Total bilirubin (μmol/L)	0.50 ± 0.07^{a}	1.89 ± 0.11^{b}	0.58 ± 0.03^{a}	0.63 ± 0.08^{a}	0.63 ± 0.07^{a}
Albumin (g/L)	24.94 ± 1.22^{a}	19.16 ± 0.98^{b}	24.52 ± 1.22^{a}	24.20 ± 1.00^{a}	23.90 ± 1.27^{a}
Globulin (g/L)	53.66 ± 3.40^{a}	44.25 ± 0.99^{b}	52.40 ± 1.80^{a}	49.72 ± 1.08^{a}	49.18 ± 1.07^{a}
Serum alkaline phosphatase (U/L)	12.34 ± 1.70^{a}	30.16 ± 2.01^{b}	13.71 ± 1.20^{a}	14.53 ± 1.25^{a}	15.08 ± 1.80^{a}
Serum γ-glutamyl transferase (U/L)	3.32 ± 0.02^{a}	8.11 ± 0.08^{b}	3.47 ± 0.06^{a}	3.48 ± 0.06^{a}	3.48 ± 0.08^{a}
Serum alanine transaminase (U/L)	16.71 ± 1.98^{a}	37.54 ± 1.23^{b}	19.21 ± 2.47^{a}	20.19 ± 1.90^{a}	20.44 ± 1.93^{a}
Serum aspartate transaminase (U/L)	11.29 ± 1.53^{a}	22.30 ± 1.23^{b}	11.75 ± 1.30^{a}	12.62 ± 1.00^{a}	13.49 ± 1.02^{a}

Values carrying different superscripts from the control for each parameter are significantly different ($P < 0.05$).

Table 6: Effect of aqueous extract of *Artemisia afra* on some kidney function parameters of diabetic rats (n = 6 ± SE).

	Control	Diabetic	Diabetic + *Artemisia afra* (mg/kg body weight)		
			50	100	200
Kidney-to-body weight ratio (%)	5.32 ± 0.35^{a}	9.91 ± 0.14^{b}	5.87 ± 0.46^{a}	7.84 ± 0.21^{c}	8.57 ± 0.19^{d}
Calcium (mmol/L)	1.49 ± 0.04^{a}	2.99 ± 0.08^{b}	1.56 ± 0.03^{a}	1.87 ± 0.05^{c}	1.98 ± 0.04^{c}
Creatinine (mg/dL)	31.52 ± 0.84^{a}	59.25 ± 0.50^{b}	33.49 ± 1.00^{a}	43.34 ± 0.48^{c}	47.28 ± 0.38^{d}
Urea (mg/dL)	36.42 ± 3.50^{a}	163.32 ± 5.31^{b}	41.72 ± 1.36^{a}	70.60 ± 2.08^{c}	81.29 ± 2.29^{d}
Uric acid (mg/dL)	5.03 ± 0.21^{a}	9.74 ± 0.16^{b}	5.29 ± 0.18^{a}	6.92 ± 0.09^{c}	6.97 ± 0.08^{c}

Values carrying different superscripts from the control for each parameter are significantly different ($P < 0.05$).

Table 7: Effect of aqueous extract of *Artemisia afra* on some haematological parameters of diabetic rats ($n = 6 \pm$ SE).

	Control	Diabetic	Diabetic + *Artemisia afra* (mg/kg body weight)		
			50	100	200
White blood cells ($\times 10^9$/L)	7.76 ± 0.95^a	13.15 ± 1.04^b	8.62 ± 0.67^a	8.71 ± 0.99^a	8.79 ± 0.90^a
Red blood cells ($\times 10^{12}$/L)	8.28 ± 0.27^a	6.56 ± 0.10^b	8.07 ± 0.37^a	7.94 ± 0.37^a	7.92 ± 0.26^a
Haemoglobin (g/dL)	15.43 ± 0.29^a	12.57 ± 0.35^b	15.35 ± 0.45^a	15.30 ± 0.42^a	15.08 ± 0.46^a
Packed cell volume (L/L)	0.48 ± 0.02^a	0.24 ± 0.02^b	0.47 ± 0.02^a	0.46 ± 0.03^a	0.46 ± 0.02^a
Mean corpuscular volume (fL)	62.43 ± 1.09^a	53.55 ± 1.24^b	60.18 ± 1.22^a	59.90 ± 1.53^a	59.85 ± 1.54^a
Mean corpuscular haemoglobin (pg)	19.70 ± 0.65^a	15.03 ± 0.62^b	19.27 ± 0.46^a	19.10 ± 0.26^a	19.00 ± 0.41^a
Mean corp. haemoglobin conc. (g/dL)	33.20 ± 0.51^a	26.77 ± 0.21^b	32.30 ± 0.59^a	32.28 ± 0.61^a	32.13 ± 0.58^a
Platelets ($\times 10^9$/L)	924.00 ± 11.36^a	639.75 ± 12.84^b	765.75 ± 14.10^c	746.33 ± 15.77^c	746.00 ± 13.16^c
Neutrophils (%)	13.20 ± 0.28^a	4.53 ± 0.22^b	8.10 ± 0.41^c	7.85 ± 0.35^c	7.85 ± 0.31^c
Lymphocytes (%)	60.13 ± 1.93^a	68.67 ± 1.08^b	61.33 ± 1.44^a	62.34 ± 1.46^a	62.45 ± 1.25^a
Eosinophils (%)	3.03 ± 0.50^a	1.15 ± 0.18^b	2.67 ± 0.46^a	2.50 ± 0.18^a	2.47 ± 0.32^a

Values carrying different superscripts from the control for each parameter are significantly different ($P < 0.05$).

characteristic condition observed in diabetics. In this study, treatment with *A. afra* extract has prevented the elevation of triglycerides, signifying that the myocardial membrane is intact and not damaged.

During diabetes, there is increased protein catabolism with flow of amino acids into the liver, which feeds gluconeogenesis [32]. These authors reported that accelerated proteolysis of uncontrolled diabetes occurs as a result of deranged glucagon-mediated regulation of cyclic AMP formation in insulin deficiency. This might have accounted for the observed decrease in the total protein content in STZ-induced untreated diabetic rats. Administration of aqueous extract of *A. afra* to diabetic rats significantly inhibited proteolysis caused by insulin deficiency and thus increased the level of plasma proteins to near normalcy.

In this study, *A. afra* extract enhanced glucose utilization by significantly reducing blood sugar level in the glucose-loaded rats. The possible mechanism by which the extract achieved this may be by increasing pancreatic secretion of insulin from beta cells of pancreas [25].

One major problem associated with the use of herbs for treating ailments is the choice of dosage. Most of the herbal preparations are administered without any standard dosage which may have some toxicological implications on vital organs in the body. The increase in liver-to-body weight ratio observed in the untreated diabetic rats in this study may be an indication of liver inflammation [33] which probably accounted for the increase in serum levels of bilirubin and marker enzymes; as well as reduced albumin and globulin concentrations.

Alkaline phosphatase is a liver marker enzyme often employed to assess the integrity of plasma membrane and endoplasmic reticulum [34], while GGT is a membrane-localized enzyme that plays a major role in glutathione metabolism in the liver [35]. Damage to structural integrity of the liver is reflected by an increase in the activity of these two enzymes in the serum, probably as a result of leakage from altered cell membrane structure. Therefore, the increase in serum ALP and GGT activities in the untreated diabetic rats confirms damage to the plasma membrane, leading to a compromise of membranal integrity [36]. The transaminases (AST and ALT) are well-known enzymes used as biomarkers to predict possible toxicity to the liver [37]. Elevation in serum activities of both transaminases as observed in diabetic rats suggested damage to the liver cells as well [38].

Oral administration of aqueous extract of *A. afra* attenuated the elevated activities of all investigated enzymes in diabetic rats comparable to the control. This may be an indication of nontoxic nature and protective action of the extract in reversing liver damage due to diabetes. Similar observation was also reported by Ravi et al. [10] using *Eugenia jambolana* seed kernels in STZ-induced diabetic rats.

Albumin and globulin are mixtures of protein molecules that are used to assess the health status of the liver. Albumin, which is manufactured in the liver, is a major carrier protein that circulates in the bloodstream while globulins are larger proteins responsible for immunologic responses [39]. Low serum albumin and globulin concentrations suggest chronic damage to the liver as a result of infection [40]. Therefore, the reduction in serum albumin and globulin levels in the untreated diabetic rats is an indication of diminished synthetic function of the liver. Oral administration of *A. afra* extract, however, restored the albumin and globulin levels to normalcy. This further confirmed conferment of protection to the liver of diabetic rats.

Bilirubin is the major product that results from the breakdown and destruction of old red blood cells. It is an important metabolic breakdown product of blood with biological and diagnostic values [39]. It is removed from the body by the liver; hence, it is a good indication of the health status of the liver. Elevated serum level of bilirubin in diabetic rats as observed in the present study may be a result of reduced uptake arising from liver disease. Treatment with *A. afra* extract was able to reverse this condition in diabetic rats, thereby lowering the bilirubin level to normalcy. All the data obtained with respect to liver function indices indicated absence of any significant liver damage as a result of treatment with aqueous extract of *A. afra* in diabetic rats.

As observed in the liver of untreated diabetic rats, the significant increase in kidney-to-body weight ratio may also

be a result of inflammation [33]. This is an indication of kidney damage which probably accounted for the reduced functional capacity as reflected by the increased serum levels of calcium ion, creatinine, urea, and uric acid.

Glucose excretion in urine by diabetics imposes an osmotic diuresis [41], with the consequence of electrolyte loss and dehydration. An attempt by the kidney to buffer the urine decreases electrolytes such as calcium in the serum [42]. Treatment with *A. afra* extract significantly reduced the serum calcium levels when compared with untreated diabetic rats. The reduction is more pronounced in rats treated with 50 mg/kg body weight of the extract as the serum calcium level tended towards normalcy in this group. This is an indication that the extract at this dosage could restore the osmotic regulatory functions of the kidney.

The increase in serum levels of urea, creatinine, and uric acid in the untreated diabetic rats as observed in the present study is expected. Deficiency of insulin and consequent inability of glucose to reach the extrahepatic tissues stimulate gluconeogenesis as an alternative route of glucose supply [5]. This route is sustained by increased proteolysis which releases free glucogenic amino acids into the plasma that are deaminated in the liver with the consequence of increased urea in the blood. Creatinine is a metabolite of muscle creatine, and the concentration in serum is proportional to the body muscle mass. The amount of creatinine is usually constant; hence, elevated levels indicate diminished renal function only, since it is easily excreted by the kidneys [41]. Uric acid is the major metabolic product of purine metabolism, and its elevated level in the serum signifies kidney impairment. Administration of aqueous extract of *A. afra*, however, produced a significant reduction in the levels of these three metabolites, thereby conferring protection against impairment due to diabetes. Similar observations have been reported using *Picrorhiza kurroa* and *Vernonia amygdalina* extracts in diabetic rats [21, 43]. Of particular interest is the fact that the effect of *A. afra* at the dosage of 50 mg/kg body weight compared favourably well with the control.

Changes in hematological profile are very common in the diabetic state [44]. The observed reduction in the concentrations of RBC, HGB, MCH, MCHC, MCV, PCV, PLT, neutrophils, and eosinophils as well as increased concentrations of WBC and lymphocytes in the diabetic rats indicated impairment in hematological function. Administration of *A. afra* extract reversed these abnormal situations and restored normalcy. This again suggested hematoprotective ability of the extract. Similar observation was also reported with *Solanum lycocarpum* and *Phellinus igniarius* on some hematological parameters in diabetic rats [44, 45].

5. Conclusions

Oral administration of aqueous extract of *Artemisia afra* showed hypoglycemic activity in STZ-induced diabetes in experimental Wistar rats. The results also revealed the beneficial effects of this herb in improving the imbalance in lipid metabolism experienced during diabetes. It can, therefore, be concluded from this study that the aqueous leaf extract of *A. afra*, besides its hypoglycemic action, could protect the liver, kidney, and blood against impairment due to diabetes. However, some renal functions may be compromised at higher dosages of the extract.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Antidiabetic and Hypolipidemic Activities of *Curculigo latifolia* Fruit:Root Extract in High Fat Fed Diet and Low Dose STZ Induced Diabetic Rats

Nur Akmal Ishak,[1] Maznah Ismail,[1,2] Muhajir Hamid,[3] Zalinah Ahmad,[4] and Siti Aisyah Abd Ghafar[1]

[1] *Nutricosmeceutical and Nutrigenomic Programme, Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[2] *Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[3] *Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[4] *Chemical Pathology Unit, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*

Correspondence should be addressed to Maznah Ismail; maznah@medic.upm.edu.my

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Curculigo latifolia fruit is used as alternative sweetener while root is used as alternative treatment for diuretic and urinary problems. The antidiabetic and hypolipidemic activities of *C. latifolia* fruit:root aqueous extract in high fat diet (HFD) and 40 mg streptozotocin (STZ) induced diabetic rats through expression of genes involved in glucose and lipid metabolisms were investigated. Diabetic rats were treated with *C. latifolia* fruit:root extract for 4 weeks. Plasma glucose, insulin, adiponectin, lipid profiles, alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), urea, and creatinine levels were measured before and after treatments. Regulations of selected genes involved in glucose and lipid metabolisms were determined. Results showed the significant ($P < 0.05$) increase in body weight, high density lipoprotein (HDL), insulin, and adiponectin levels and decreased glucose, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), urea, creatinine, ALT, and GGT levels in diabetic rats after 4 weeks treatment. Furthermore, *C. latifolia* fruit:root extract significantly increased the expression of *IRS-1*, *IGF-1*, *GLUT4*, *PPARα*, *PPARγ*, *AdipoR1*, *AdipoR2*, *leptin*, *LPL*, and *lipase* genes in adipose and muscle tissues in diabetic rats. These results suggest that *C. latifolia* fruit:root extract exerts antidiabetic and hypolipidemic effects through altering regulation genes in glucose and lipid metabolisms in diabetic rats.

1. Introduction

Type 2 diabetes mellitus is a metabolic disorder which causes hyperglycemia [1] due to defect in insulin secretion and insulin resistance [2]. It results from failure of pancreatic β-cell to secrete insulin sufficiently in response to elevate blood glucose [3]. In early stage of type 2 diabetes mellitus, peripheral tissues such as liver, muscle, and adipose tissues are not sensitive towards insulin [4] and they cause common symptoms such as increased thirsty, frequent urinary, ketonuria and ketonemia in diabetic patients [5]. In a long-term of insulin resistance period, it will lead to chronic hyperglycemia and hyperlipidemia in diabetic patients [6]. Therefore, if no prevention action is taken, diabetes can lead

to several complications such as heart attack, nephropathy, retinopathy, and neuropathy [7].

Till now, numerous types of diabetic models are used for screening antidiabetic properties of plants. These diabetic models are developed through several methods either through genetic or chemically induced diabete [8]. There are genetic models of diabetes known as db/db mouse and Zucker diabetic fatty (ZDF) rats which develop similar features as in human type 2 diabetes [9]. However, the development of diabetes in these rats is due to genetic and this is unlikely in humans. Besides, these rats are expensive to be used as diabetic models for pharmacological screening [10]. Meanwhile, development of diabetic rats following streptozotocin (STZ) injection also presents hyperglycemia similar to human type 2 diabetes mellitus [11]. However, this method only develops insulin deficiency rather than insulin resistance in the model [12]. Despite that, the pattern of disease progress did not appear to be similar to diabetic situation in human type 2 diabetes mellitus. Thus, several researchers have been investigating to find a better diabetic model for type 2 diabetes mellitus by modifying the existing method. Recently, many studies reported that rats induced with high fat diet with combination of (STZ) have developed similar situation as type 2 diabetes progress in humans. Diets containing high fat will cause insulin resistance in peripheral tissues due to lipotoxicity [13]. Meanwhile, low dose of STZ has known to induce mild defect in insulin secretion, which is similar to the characteristics of the later stage of type 2 diabetes [14]. Combination of high fat diet with low dose STZ has successfully mimicked natural progress of diabetes development as well as metabolic features in human type 2 diabetes [15, 16]. Apart from that, these models are also cheaper, easy to develop, and practical for pharmacological screening [17].

Currently, there are five major classes of therapeutic drugs that have been used to treat diabetes through several target sides: sulfonylureas, biguanides, thiazolidinediones, meglitinide, and α-glucosidase inhibitor. However, according to Bastaki (2005), combination of two drugs such as metformin and sulfonylurea can increase the hypoglycemic activity where it treats diabetes through two different modes of action [18]. In spite of antidiabetic drugs effectiveness, prolonged usage of it will cause adverse effect. Sulfonylureas have been reported to cause hypoglycemia, increase in body weight, gastrointestinal (GI) disturbance, and headache to the user [19]. Besides, metformin could cause abdominal pain, diarrhea, nausea, and lactic acidosis to the diabetic patient [20]. In other diabetic drug that causes adverse side effects is thiazolidinediones as it causes hepatotoxicity after long-time usage [21]. Apart from adverse side effect, antidiabetic drug also has limited mode of action. Available diabetic drugs only show single mode of action in treating diabetes and then need to combine with another class of antidiabetic drug to make these drugs more efficient such as combination of metformin and sulfonylurea [18].

Although there was a plenty amount of antidiabetic drugs available, the numbers of type 2 diabetes mellitus patients are still increased constantly. The incidence of T2DM has become worldwide epidemic and there are approximately 246 million people who are suffering from this disease [22]. The highest rate of diabetic population is in India with current figure 40.9 million and followed by China with 39.8 million. Furthermore, Pakistan, Japan, USA, Russia, and Germany are also widely affected with diabetes [22]. This number is continually increasing and it might be due to antidiabetic drug adverse side effect and also due to drug limitation action.

There is a dramatic revival of interest in using natural sources in treating diabetes due to side effects of prolonged consumption of therapeutic drugs. Asian countries such as India and China are already known for their contributions toward the usage of plant medicine in preventing and overcoming diabetes problems [23, 24]. More than 4000 plants have been studied and identified to have hypoglycemic effect through several mechanisms of antidiabetic activity. Some plants acts through either secretagogues or insulin mimetic properties such as *Momordica charantia*, *Aloe vera,* and *Allium sativum* [25–27]. Studies have reported that these plants have the ability to reduce blood glucose and improve insulin secretion [28]. Meanwhile, according to Malviya et al. (2010), secondary metabolites from plants such as phenolic, alkaloids, and glycosides are the ones which are implicated as having antidiabetic effect [26]. Among those secondary metabolites, phenolic compounds are the ones which are abundantly present in plants and are demonstrated to have antioxidant, antidiabetic, and antiobesitiy properties [29].

Curculigo latifolia (Dryand, ex W. T.Aiton) is a shrub tree that mainly grows under rubber tree and it is also known as Lemba among local community in Malaysia [30]. This plant belongs to the Hypoxidaceae family. To date, there are about 20 species of *Curculigo* that have been identified and *C. latifolia* and *C. capitulate* are mostly distributed in Malaysia [31]. This shrub tree consists of berry-like fruit and this fruit exhibits both sweet tasting and taste modifying activities [32]. Curculin and neoculin have been identified as proteins that possess those activities [33]. Despite *C. latifolia* is sweet and can be used as alternative sweetener for diabetic patient, and there is no scientific study on *C. latifolia* as antidiabetic agent. Preliminary study that has been conducted in our laboratory showed that *C. latifolia* fruit and root extracts have the highest antioxidant activity where the IC_{50} for both of the fruit and root extracts is 1.0 mg/mL. In spite of high antioxidant, fruit and root also consist of high phenolic content, 95 mg GAE/100 g extract and 90 mg GAE/100 g extract. Both data revealed that there is positive correlation between total phenolic content and antioxidant activity in fruit and root. Besides, an *in vitro* study that has been conducted in our laboratory indicates that *C. latifolia* fruit and root extracts have antidiabetic activity by increased insulin and adiponectin secretion in cell lines. *C. latifolia* fruit and root extracts also significantly increased glucose uptake activity in 3T3 adipocytes and L6 myotubes cell lines (patent pending). The present study was performed to determine antidiabetic and hypolipidemic activities of *C. latifolia* fruit:root extract in HFD and low dose STZ induced diabetic rats by evaluating the potential of this plant to regulate expression of genes involved in glucose and lipid metabolisms.

Table 1: Nutrient composition of NPD and HFD.

Nutrient	% in 100 g of NPD	% in 100 g of HFD
Fat	4	34
Fiber	5	3
Protein	14	16
Carbohydrate	72	42
Mineral and vitamin mix	5	5
Total calories (kcal/100 g)	380	538

2. Materials and Methods

2.1. Preparation of C. Latifolia fruit:root Extract. *C. latifolia* plant was collected from Beranang, Selangor, Malaysia. It was identified by the taxonomist from the Biodiversity Unit in the Institute of Bioscience, Universiti Putra Malaysia with voucher number SK 1709/09. The fruits were plucked at the apex of *C. latifolia* stem, cleaned with tap water, blotted with tissue paper, and stored at −20°C until further use. Roots were cleaned with tap water and immediately dried for overnight in an air oven (Memmert, Schwabach, Germany) at 40°C. Dried roots were grounded to fine powder using electric grinder (Philips, Malaysia). Powdered root was sealed in plastic bags and kept at 4°C until further use.

2.1.1. Extraction of C. Latifolia Fruits:Root. Fifty grams of fresh *C. latifolia* fruits was mashed using mortar and pestle. Mashed fruits were extracted with 2000 mL of distilled water. Meanwhile, 50 g of *C. latifolia* root powder was soaked in 2000 mL of distilled water. Both extractions were extracted 24 h with continuous stirring at room temperature. This extract was filtered through Whatman number 1 filter paper and the filtrate was collected and lyophilized. The lyophilized sample was kept at −80°C until further use. In this study, fruit and root extracts were mixed at 1 : 1 ratio for rats treatment.

2.2. Preparation of High Fat Diet (HFD). The normal pallet diet from Miba Mansura (Malaysia) consists of 46% of cornstarch, 26% of palm kernel meal, 4% of soybean oil, 3.5% of minerals mixture, 1% of vitamins mixture, 0.25% of choline bitartrate, and 0.18% of L-cystine. The nutrient composition is shown as Table 1. The HF diet was formulated based on the composition provided by Levin et al. (1989) [34]. It will be prepared from a mixture of 50% normal rat chow pellet, 24% of corn oil (Mazola brand), 20% of full-cream milk powder (NESPRAY brand from Nestlé), and 6% sugar.

2.3. Animal Study. Forty-two male Sprague-Dawley rats weighing 160–180 g each were housed individually in polypropylene cages and maintained under controlled room temperature (22 ± 2°C) and humidity (55 ± 5%) with 12:12 h light-dark cycle. All experimental protocols for animal care and use were approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (project approval number UPM/FPSK/PADS/BR-UUH/0030). Upon receipt, rats were acclimatized for a week with free access of water and normal pellet diet. After acclimatized, normal rats ($n = 6$) were maintained on normal pellet diet while remaining rats were given high fat diet to induce obesity. This treatment was conducted for 4 weeks. After 4 weeks, obese rats were anesthetized with diethyl ether after being fasted overnight and injected with 40 mg/kg bw of STZ via intravenous to induce type 2 diabetes. Diabetic rats (fasting blood glucose level > 170 mg/dL after 7 days of STZ injected) were randomly divided into 5 groups (Groups 3, 4, 5, 6, and 7) and each group consists of 6 rats. Below are the lists of the rat groups for this study.

Group 1: normal (normal pellet diet, untreated) rats.

Group 2: obese (high fat-fed diet, obese, untreated) rats.

Group 3: diabetic control (high fat-fed diet, induced with STZ, diabetic, untreated) rats.

Group 4: diabetic test rats (high fat-fed diet, induced with STZ, diabetic) treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts.

Group 5: diabetic test rats (high fat-fed diet, induced with STZ, diabetic), treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts.

Group 6: diabetic test rats (high fat-fed diet, induced with STZ, diabetic), treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts.

Group 7: diabetes test rats (high fat-fed diet, induced with STZ, diabetic) treated with 10 mg/kg bw of glibenclamide.

Treatment on diabetic rats was done for 4 weeks. Body weight of each rat was recorded before (0 week) and after 4 weeks of treatment. At the end of the experimental period, all rats were fasted for 15 h prior to sacrifice. Blood samples were collected by cardiac puncture. Meanwhile, adipose and muscle tissues were excised and stored at −80°C prior used.

2.4. Biochemical Parameter Analysis of Blood. Blood samples were collected using K_2EDTA blood collection tube (BD Diagnostics, Franklin Lakes, NJ, USA). Plasma was collected after blood was centrifuged at 3000 rpm for 10 minutes [35]. Biological assay such as glucose, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), urea, creatinine, alanine amino-transferase (ALT), and γ-glutamyltransferase (GGT) was measured using Selectra XL clinical chemistry analyzer (Vital Scientific, the Netherlands). Insulin level was measured using rat insulin ELISA kit (Mercodia AB, Uppsala, Sweeden) with rat insulin as a standard. Adiponectin level was measured using BioVision rat adiponectin ELISA assay (BioVision Inc., Mountain View Milpitas, CA, USA).

2.5. Quantification of Different Expression Genes between Group Treatments Using GenomeLab. The white adipose tissues (retroperitoneal, subcutaneous, and epididymal) and muscle tissues were harvested from rats. Frozen tissues were thawed and homogenized and total RNA was extracted

Table 2: Sequence of primers used.

Accession number	Left sequence w/universals	Right sequence w/universals
NM_012969 (IRS1)	AGGTGACACTATAGAATAACCCT AGACCCACTGCCTTT	GTACGACTCACTATAGGGATGGAGGAAGCA AGCAGAAAT
NM_013124 (PPARγ)	AGGTGACACTATAGAATAGATCCTCCTG TTGACCCAGA	GTACGACTCACTATAGGGATCAAAGGAATGGG AGTGGTC
NM_178866 (IGF)	AGGTGACACTATAGAATACCGCTGAAGCC TACAAAGTC	GTACGACTCACTATAGGGAGCTCAAGCAGCAA AGGATCT
NM_012751 (GLUT4)	AGGTGACACTATAGAATAAATGACTGAGGGG CAAAATG	GTACGACTCACTATAGGGAGGGTAAGAGGAA GGCAGGAC
NM_207587 (AdipoR1)	AGGTGACACTATAGAATAGGACTTGGCTTG AGTGGTGT	GTACGACTCACTATAGGGACGGAATTCCTGTA GGTTGGA
Kan^r	AGGTGACACTATAGAATAATCATCAGCA TTGCATTCGATTCCTGTTTG	GTACGACTCACTATAGGGAATTCCGACTCGT CCAACATC
NM_013196 (PPARα)	AGGTGACACTATAGAATACTCGTGCAGGTCA TCAAGAA	GTACGACTCACTATAGGGAGCCTCTGATCACC ACCATTT
NM_013076 (Leptin)	AGGTGACACTATAGAATACAAAACGTGC TGCAGATAGC	GTACGACTCACTATAGGGACATTCAGGGCTA AGGTCCAA
NM_012598 (LPL)	AGGTGACACTATAGAATAACTCGCTCTCA GATGCCCTA	GTACGACTCACTATAGGGACTGACCAGCGGA AGTAGGAG
NM_012859 (Lipase)	AGGTGACACTATAGAATACCTTCGGGGAA CACTACAAA	GTACGACTCACTATAGGGACCAAGGGAGGTG AGATGGTA
NM_023964 (GAPDH)	AGGTGACACTATAGAATAATCAATGGATTT GGACGCAT	GTACGACTCACTATAGGGAAGCTCCAGGGGA TTTCCTTA
BC168964	AGGTGACACTATAGAATACGGAAGAAGGCT CTTGAAAA	GTACGACTCACTATAGGGACGCCACCCTCTT CATCTCTA
NM_001037979 (AdipoR2)	AGGTGACACTATAGAATACGGTGTACTGCCA CTCAGAA	GTACGACTCACTATAGGGAGCAAGGTAGGGAT GATTCCA

using RiboPure isolation of high quality total RNA (Ambion, USA) according to manufacturer's instructions. Reverse transcription and PCR procedures were performed according to GenomLab GeXP kit protocol (Beckman Coulter, USA) using XP Thermal Cycler (Bioer Technology, Germany). The amplicons from PCR reaction were used for quantification of different expression genes between group treatments using GenomeLab GeXP Start kit. Samples were prepared and were added to the appropriate wells of 96-well sample microplate. All the samples were run in triplicates. Besides, all the data were analyzed using Express analysis software where fragment data is easily identified. Multiplex genes were normalized with 18S by dividing the peak area of each gene by peak area of 18S gene. The expression level was calculated according to the following formula:

$$\text{Fold change} = \frac{\text{Normalized data of the gene from treated samples}}{\text{Normalized data of the gene from untreated samples}}. \quad (1)$$

Primer sequences for all rat genes were designed using eXpress designer module of the GenomeLab eXpress Profiler software based on gene sequences from GeneBank database (Table 2).

2.6. Statistical Analyses. All results are expressed as the mean ± standard deviation. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Level of significance was set at $P < 0.05$.

3. Results

3.1. Energy Contributed from HFD. In the present study, diabetic rats were developed using high fat diet. The high fat diet was formulated according to Levin et al.'s [34] method. The nutrient composition of NPD and HFD used in this experiment was mentioned in Table 1. Meanwhile, Table 3 shows the energy contributed from NPD and HFD. Energy contributed from fat sources in HFD diet was 56.9% and it indicates that there was 47.4% increase in fat composition in HFD compared to NPD. In spite of fat, energy contributed by protein and carbohydrate was 11.9% and 31.2%.

3.2. Body Weight. Body weight from week 0 to week 4 of normal, obese, untreated diabetic and treated diabetic rats increased significantly ($P < 0.05$). In normal rats (Group 1), body weight increased by 20% followed by 30% in obese rats (Group 2). Meanwhile, body weight of diabetic control rats (Group 3) increased by 6%. However, body weight of diabeticrats treated with *C. latifolia* fruit:root extract increased by 12% (Group 4), 9% (Group 5), and 7% (Group 6) after 4 weeks of treatment. Body weight of diabetic rats in

Table 3: Energy contributed from NPD and HFD.

Nutrient	% in 100 g of NPD	% energy in NPD	% in 100 g of HFD	% energy in HFD
Fat	4	9.5	34	56.9
Protein	14	14.7	16	11.9
Carbohydrate	72	75.8	42	31.2

Percentage (%) of energy was measured by kilocalories.

Table 4: The plasma glucose level and percentage of plasma glucose changes.

Plasma glucose level (mmol/L) and percentage of glucose level (%)	Rat group						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Plasma glucose level at week 0 (mmol/L)	5.21 ± 2.18	9.52 ± 4.31	21.61 ± 5.56	20.41 ± 5.81	19.75 ± 5.18	21.55 ± 4.64	22.26 ± 6.12
Plasma glucose level at week 4 (mmol/L)	5.34 ± 3.57	11.69 ± 5.31	25.47 ± 4.38*	13.5 ± 21.13**	12.70 ± 15.72**	13.10 ± 19.27**	16.8 ± 17.22**
Plasma glucose changes from week 0 to week 4	2.4	18.6	15.2	−51.6	−54.3	−64.5	−32.4

Data are means ± SD for plasma glucose level of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts (Group 5), diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7) at weeks 0 and 4.
* Are significantly different at $P < 0.05$ compared with diabetic control, that is, Group 3.
** Are significantly different at $P < 0.001$ compared with diabetic control, that is, Group 3.
− Indicates the reduction in plasma glucose.

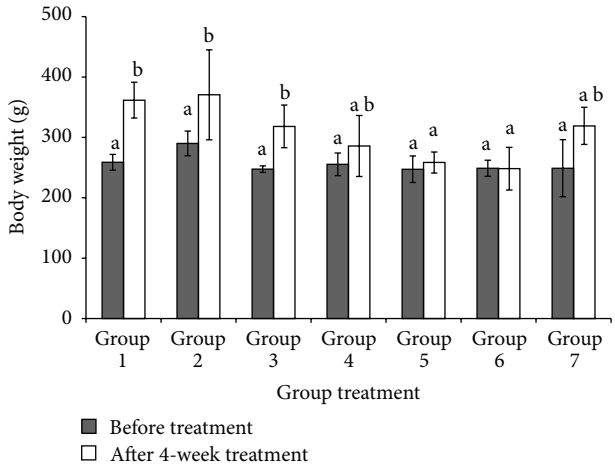

Figure 1: Rats body weight before (0 week) and after treatment (4 week). Body weight of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root (Group 5), diabetic rats treated with 200 mg/kg of *C. latifolia* fruit:root (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7). Columns represent the mean ± S.D. ($n = 6$). [a,b] significantly different at $P < 0.05$.

group 7 which has been treated with glibenclamide increased by 19% (Figure 1).

3.3. Plasma Glucose Level. After 4 weeks of intervention, glucose level in normal group was maintained in normal range throughout the experiment although it increased slightly by 2.4%. Meanwhile, glucose level in obese (Group 2) and diabetic control (Group 3) rats was significantly ($P < 0.05$) increased by 18.6% and 15.2% by the end of the study. However, glucose level was decreased in treated diabetic rats. The most significant ($P < 0.05$) reduction was showed in diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6) followed by 100 (Group 5) and 50 (Group 4), 64.5% > 54.3% > 51.6%. Meanwhile, diabetic rats treated with 10 mg/kg bw of glibenclamide show 32.4% reduction in glucose level (Table 4).

3.4. Plasma Lipid Profiles. Lipid profiles in normal rats were in normal range. However, plasma TC, LDL, and TG levels in obese rats (Group 2) were significantly higher ($P < 0.05$) while HDL level was lower than that normal rats (Group 1) after 4 weeks of study (Table 5). Diabetic control rats (Group 3) also showed a similar pattern to obese rats where plasma TC, TG, and LDL levels were increased by 56.2%, 52.6%, and 75.4% compared to normal rats. However, plasma HDL level in diabetic control rats was decreased by 51%. The higher level of lipid in diabetic control rats can be seen in plasma. Plasma lipids in diabetic control rats were higher and plasma colour has turned to opaque colour instead of clear (Figure 2). The posttreatment levels of TC, TG, and LDL of treated groups were significantly decreased compared to pretreatment levels. Diabetic rats in Groups 4, 5, and 6 showed a significant ($P < 0.05$) reduction in plasma TC, TG, and LDL levels compared to diabetic rats treated with glibenclamide. Besides, HDL level also increased after 4 weeks of treatment in diabetic rats in Groups 4, 5, and 6.

Thus, hypocholesterolemia effect has been found the be higher in 200 mg then followed by 100 mg and 50 mg of *C. latifolia* fruit:root extracts. The most striking result emerging from this study is that lipid content in plasma was ameliorated

Table 5: Lipid profiles of rats group.

Group	Total cholesterol (mmol/L)		TG (mmol/L)		LDL (mmol/L)		HDL (mmol/L)	
	Before	After	Before	After	Before	After	Before	After
1	1.73 ± 0.29	1.62 ± 0.18	0.48 ± 0.26	0.57 ± 0.11	0.54 ± 0.31	0.57 ± 0.42	0.57 ± 0.22	0.53 ± 0.24
2	2.65 ± 0.11	3.38 ± 0.16*	0.43 ± 0.13	1.17 ± 0.36*	0.77 ± 0.11	0.92 ± 0.26	0.54 ± 0.36	0.26 ± 0.09
3	2.17 ± 0.12	2.53 ± 0.24	0.60 ± 0.16	0.87 ± 0.20	0.70 ± 0.06	1.00 ± 0.10*	0.52 ± 0.05	0.26 ± 0.15
4	3.43 ± 0.15*	1.49 ± 0.22*	0.53 ± 0.09	0.64 ± 0.30	1.34 ± 0.42*	0.85 ± 0.06	0.40 ± 0.15	0.75 ± 0.21*
5	2.20 ± 0.23	1.21 ± 0.16*	0.52 ± 0.03	0.67 ± 0.13*	0.66 ± 0.16	0.59 ± 0.38*	0.37 ± 0.29*	0.82 ± 0.19*
6	3.24 ± 0.10*	1.17 ± 0.28*	0.60 ± 0.24	0.69 ± 0.19*	1.07 ± 0.15*	0.56 ± 0.13*	0.34 ± 0.15*	0.69 ± 0.20*
7	2.96 ± 0.14	1.35 ± 0.16*	0.45 ± 0.24	0.59 ± 0.13*	0.68 ± 0.17	0.76 ± 0.24	0.38 ± 0.11*	0.77 ± 0.27*

Data are means ± SD for lipid profiles of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts (Group 5), diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7) at weeks 0 (before) and 4 (after).
*Are significantly different at $P < 0.05$ compared with diabetic control, that is, Group 3.

Figure 2: Picture of plasma lipid in diabetic control and normal rats. Diabetic control rats are marked with (#) and normal rats with (∗).

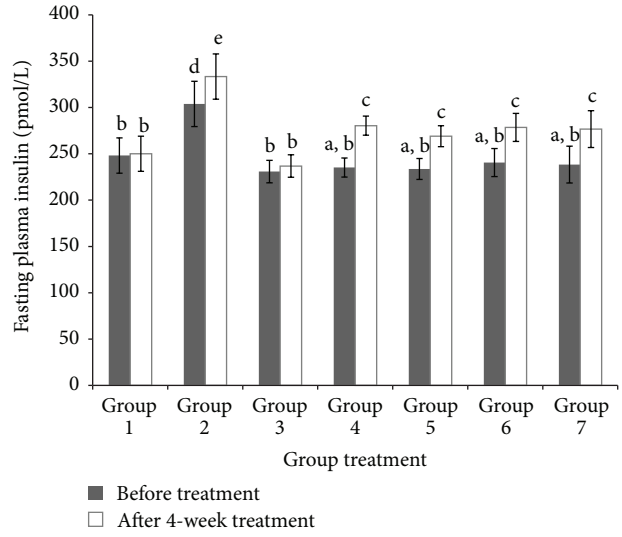

Figure 3: Fasting plasma insulin before (0 week) and after treatment (4 week). Insulin level of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root (Group 5), diabetic rats treated with 200 mg/kg of *C. latifolia* fruit:root (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7). Columns represent the mean ± SD ($n = 6$). [a,b,c]significantly different at $P < 0.05$.

when treated with *C. latifolia* extracts. As consequence, the opaque colour in plasma turned into clear in plasma sample in Groups 4, 5, and 6.

3.5. Plasma Insulin and Adiponectin. The changes in insulin level after 4-week intervention are shown in Figure 3. There was no significant ($P < 0.05$) change in plasma insulin level in normal and diabetic control rats before and after 4 weeks of study. However, plasma insulin level in obese rats significantly increased. Four weeks of treatment with *C. latifolia* fruit:root extract and glibenclamide had increased insulin levels in diabetic rats. There was 16% of insulin increasing in rats in Group 4 followed by 13% in Group 5, 12% in Group 6, and 11% in Group 7.

There are remarkable changes in adiponectin level in obese and diabetic control rats compared to normal rats (Figure 4). Adiponectin level in obese and diabetic control rats was lower than that normal rats. However, *C. latifolia* fruit:root extract prevents further decrease in adiponectin in diabetic rats. Adiponectin level was significantly ($P < 0.05$) increased in diabetic rats treated by *C. latifolia* fruit:root extract. Group 5 increased adiponectin level by 56% followed by Group 4 (48%) and Group 6 (41%). Besides, in Group 7 there is no significant difference in adiponectin level after 4 weeks of intervention.

3.6. Plasma ALT, GGT, Urea, and Creatinine. Results in Table 6 indicate that ALT, GGT, urea, and creatinine levels significantly ($P < 0.05$) increased in obese and diabetic control rats when compared to normal rats. However, there is no significant difference ($P < 0.05$) in ALT, GGT, urea, and creatinine level in diabetic control (Group 3) rats when compared to obese rats. Four weeks of intervention with *C. latifolia* fruit:root extract have reduced ALT, GGT, urea, and creatinine levels towards normalcy in diabetic rats.

3.7. Gene Expression Study Using GeXP Analyzer. Current study was conducted to determine the mechanism of *C. latifolia* fruit:root extract in alleviating insulin resistance

Table 6: Effect of *C. latifolia* extracts on ALT, GGT, urea, and creatinine levels in HFD and low dose STZ induced diabetic rats.

Group	ALT (U/L)		GGT (U/L)		Urea (mmol/L)		Creatinine (mmol/L)	
	Before	After	Before	After	Before	After	Before	After
1	75.12 ± 2.34	109.24 ± 3.42	6.73 ± 1.19	5.04 ± 0.69	5.51 ± 0.33	5.89 ± 0.51	31.30 ± 1.11	30.97 ± 2.52
2	88.24 ± 1.55	114.81 ± 5.10	8.65 ± 2.12	10.32 ± 0.52*	5.07 ± 0.14	5.82 ± 0.37	49.40 ± 2.54	62.90 ± 3.46*
3	89.12 ± 3.42	149.65 ± 4.26*	13.11 ± 1.31*	27.88 ± 2.37*	4.53 ± 0.35	6.83 ± 0.40	52.85 ± 2.68	72.10 ± 2.58*
4	77.50 ± 2.27	76.49 ± 1.33*	9.53 ± 1.34	5.39 ± 0.89*	9.61 ± 0.16*	6.66 ± 0.25	58.80 ± 2.34	47.80 ± 1.19
5	76.21 ± 3.17	84.77 ± 2.50*	9.07 ± 1.28	3.18 ± 1.41	12.55 ± 0.42	5.66 ± 0.26*	59.13 ± 1.19	51.40 ± 0.77
6	80.50 ± 1.24	78.10 ± 2.46*	9.25 ± 1.31	5.10 ± 0.17*	9.15 ± 0.36	6.65 ± 0.41	51.93 ± 0.76	42.60 ± 0.15
7	85.74 ± 5.31	83.10 ± 3.26*	8.90 ± 1.19	5.84 ± 0.21*	14.62 ± 0.54*	4.98 ± 0.33	57.63 ± 0.50	56.21 ± 0.32

Data are means ± SD for ALT, GGT, urea and creatinine levels of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts (Group 5), diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7).
Significant difference is at $^{*}P < 0.05$ when compared with diabetic control rats (Group 3).

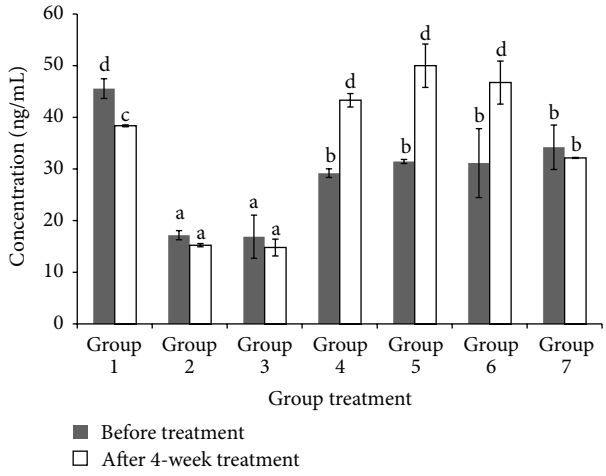

Figure 4: Fasting plasma adiponectin before (0 week) and after treatment (4 week). Adiponectin level of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root (Group 5), diabetic rats treated with 200 mg/kg of *C. latifolia* fruit:root (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7). Columns represent the mean ± SD. ($n = 6$). ^{a,b,c}significantly different at $P < 0.05$.

through altering expression of genes involved in glucose and lipid metabolisms. In muscle tissue, the expression of *IRS1, IGF, GLUT4, PPARα, PPARγ, AdipoR1, AdipoR2*, *leptin, LPL,* and *lipase* genes in obese and diabetic control rats was significantly ($P < 0.05$) lower than that in normal rats either in muscle or adipose tissues. However, *C. latifolia* fruit:root extract significantly ($P < 0.05$) increased the mRNA expression of these genes in diabetic rats as compared to diabetic control rats in Group 3 (Tables 7 and 8).

4. Discussion

In order to find the similarity in the development of diabetes in humans, induction of HFD with low dose STZ is the preferred method. However, the rats with HFD will increase their energy expenditure and then lead towards the progression of insulin resistant in the organs. Moreover, low dose STZ induced hyperglycemia in rats where it defects the secretion of insulin [5]. This progressive development of type 2 DM is similar to humans where several researchers agree [36]. In this study, high fat diet was formulated according to Levin et al. (1989) and this diet contains 34% of fat out of 100 g of total HFD diet [34]. There was 88% increase in fat compared to normal diet. Study done by Warwick et al. (2002) has reported that the amount of fat needed in high fat diet must be in the range 30% to 60% out of total diet and that is because this amount allows the changes of body weight composition, endocrine secretion and metabolic [37]. Thus, this indicates that, the findings of our HFD are in agreement's with Warwick et al. findings. The current result clearly demonstrated that body weight of obese rats (Group 2) increased over normal rats and this is due to high fat intake. Meanwhile, body weights among diabetic control rats were lower compared to obese rats due to STZ injection. Chatterjee and Shinde (2002) mentioned in their report that STZ causes reduction in body weight due to the loss of tissue protein and increased muscle wasting [38–40]. However, *C. latifolia* fruit:root extract caused weight loss in diabetic rats in Groups 4, 5, and 6 compared to diabetic control rats. This could be due to lipid lowering activity by *C. latifolia* fruit:root extract [41]. Besides, body weight among diabetic rats treated with glibenclamide increased compared to diabetic control rats and this indicates that *C. latifolia* fruit:root extract is better than glibenclamide in order to prevent body weight gain in diabetic models.

Diabetic rats induced by high fat diet with combination of low dose STZ have closely mimicked the natural process of the diabetic occurrence and metabolic disturbance in human diagnosis as type 2 diabetes [42]. In the present study, diabetic model that has been developed using high fat diet with combination of 40 mg of STZ also shows the same symptoms as reported by Unger et al. (2010) [13]. Blood glucose level in diabetic rats was increased and this finding supported the findings of Poitout and Robertson (2002), where they have mentioned that STZ causes destruction of pancreatic β-cells and it makes the cells less active to be sensitive enough towards insulin for glucose uptake and

Table 7: Expression of candidate genes in muscle tissue.

Group	IRS1	IGF	GLUT4	PPARγ	PPARα	AdipoR1	AdipoR2	Leptin	LPL	Lipase	Gapdh
Group 1	1.8260	1.9954	0.9704	1.5499	1.3345	1.4130	1.2861	1.3915	0.5263	0.5804	0.7407
Group 2	0.8634	0.9465	0.5567	0.4557	0.7075	0.5208	0.4300	2.5701*	0.9524	0.9544	0.9787
Group 3	0.5530	0.6140*	0.5163	0.3232	0.5192	0.4040	0.3334	0.6173*	0.7185	0.7652	1.0191
Group 4	1.3284*	5.2166*	1.3854*	2.0186*	3.0955*	1.4058*	1.5691*	2.1324*	0.8530	1.9529*	1.1178
Group 5	1.4668*	5.3301*	1.9030*	2.4303*	3.2608*	1.4815*	1.5556*	2.2548*	0.7183	1.6386*	1.2593*
Group 6	1.7949*	5.6444*	2.8137*	3.5724*	3.8732*	2.5713*	2.8885*	3.2222*	0.5769	2.0382*	1.3244*
Group 7	1.2346*	4.0180*	1.0819*	0.3098	0.2155	0.8305	1.3058*	0.3030	1.1089*	0.9428	1.2346*

Data are means ± SD for expression of candidate genes in muscle tissue of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts (Group 5), diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7). Significant difference is at $^*P < 0.05$ when compared with diabetic control rats (Group 3).

Table 8: Expression of candidate genes in adipose tissue.

Group	IRS1	IGF	GLUT4	PPARγ	PPARα	AdipoR1	AdipoR2	Leptin	LPL	Lipase	Gapdh
Group 1	1.5371	1.8971	1.4174	1.5781	1.7697	1.6875	1.2268	0.9548	0.5681	0.6158	0.9851
Group 2	0.4879	0.7495	0.5275	0.2414	0.4568	0.5967	0.5692	3.7523*	0.8751	0.7681	0.8562
Group 3	0.4378	0.5644	0.3194	0.2089	0.3699	0.4298	0.4692	0.5689	0.7521	0.5821	1.5240
Group 4	1.3280*	1.7462*	2.0911*	2.9885*	1.5538*	2.8792*	2.1732*	1.5982*	3.5418*	1.5556*	1.4338
Group 5	1.7888*	2.3779*	1.4028*	1.3769*	0.8992*	2.0781*	2.3976*	2.8826*	2.4351*	1.9261*	1.3561
Group 6	1.6971*	2.5811*	1.0247*	1.5571*	1.6982*	2.1169*	3.4691*	2.9871*	2.9984*	2.4861*	1.5983
Group 7	1.1555*	0.7924	1.3333*	1.8634*	0.6282*	1.7295*	1.8059*	1.055	0.8564	0.7539	1.2374

Data are means ± SD for expression of candidate genes in adipose tissue of normal rats (Group 1), obese rats (Group 2), diabetic control rats (Group 3), diabetic rats treated with 50 mg/kg bw of *C. latifolia* fruit:root extracts (Group 4), diabetic rats treated with 100 mg/kg bw of *C. latifolia* fruit:root extracts (Group 5), diabetic rats treated with 200 mg/kg bw of *C. latifolia* fruit:root extracts (Group 6), and diabetic rats treated with 10 mg/kg bw of glibenclamide (Group 7).
*Indicates $P < 0.05$ compared with diabetic control rats (Group 3).

this will cause high glucose concentration in blood [43]. Besides, Bansal et al. (2012) Insulin-mediated glucose uptake mentioned that HFD fed with STZ combination causes hyperglycemia in rats [44]. However, aqueous extract of the *C. latifolia* fruit:root exhibited a hypoglycemic effect and significantly ($P < 0.05$) decreased the glucose level. Extract at 200 mg/kg bw has showed a higher decrease compared to other concentrations. It has decreased 64.5% inblood glucose level and followed by 100 mg/kg bw and 50 mg/kg bw. The ability of *C. latifolia* fruit:root extract in reducing glucose level was in agreement with previous finding in an *in vitro* study (patent pending). *C. latifolia* increased glucose uptake activity in adipocyte and myotube cells at basal and through insulin-mediated glucose/STZ is being uptake : glucose/STZ is being translocation. Besides, *C. latifolia* also possesses sensitize and insulin mimicking actions in order to stimulate glucose uptake activity. In insulin presence, *C. latifolia* extracts might sensitizing insulin signaling cascade and stimulates translocation of glucose transporter GLUT4 into plasma membrane and then glucose is being uptake into adipose and muscle through phosphatidylinositol 3-kinase (PI3K) pathway [45].

The abnormality in lipid metabolism in type 2 diabetes mellitus has caused hyperlipidemia in diabetic patient. Thus, diabetic rats that have been induced by HFD with combination of low dose STZ also showed similar situation, hyperlipidemia. This finding further support the idea of Lombardo and Chicco (2006) where it is shown that those rats administrated with HFD cause dyslipidemia and other syndromes in diabetices [46]. Besides, defect in insulin secretion due to STZ also causes defect in lipogenic activity. Insulin plays an important role in stimulating lipogenesis in mammals, by low secretions of insulin it implicates of high level of lipid in plasma [47–49]. Furthermore, plasma colour has turned to opaque due to lipid presence. However, after 4 weeks of treatment with 200 mg/kg bw of *C. latifolia* fruit:root extracts, TC, TG, and LDL levels were significantly ($P < 0.05$) decreased and HDL level was increased compared to pretreatment levels. In spite of that, opaque colour in plasma was turned into clear in plasma sample in Groups 4, 5, and 6.

Disruption of pancreatic β-cells by STZ in diabetic rats has caused insufficient insulin secretion in blood [42]. Thus, this STZ is being uptake into pancreatic cells by GLUT2 and it causes DNA damage via reactive oxygen species generation [42]. However, *C. latifolia* fruit:root extracts in all concentration have prevented further disruption of cells. It indicates that antioxidant properties of *C. latifolia* have scavenged free radicals which cause oxidative stress in cells. Moreover, adiponectin secretion was also decreased in diabetic control rats. This finding is in agreement with Yang et al.'s (2006) finding which showed that rats treated with HFD for 4 weeks showed significantly decreased in adiponectin [50]. However, after 4 weeks of treatment with *C. latifolia* fruit:root extracts, adiponectin levels were increased. The possible mechanism of *C. latifolia* towards this increasing is due to

improvement of insulin secretion [41]. Besides, *C. latifolia* extracts also possess insulin mimicking properties which trigger the adiponectin secretion in adipose tissues. These results suggest that amelioration of insulin and adiponectin secretions by *C. latifolia* fruit:root extract may be a key to decrease glucose and lipid levels in diabetic rats.

Hepatotoxicity and nephropathy are complications from T2DM. Hepatocytes damaged due to hepatotoxicity cause ALT and GGT enzymes leaking out into the blood circulation. Present findings showed that those enzymes were significantly higher in diabetic rats in Groups 2 and 3. These findings are consistent with those of Bolkent et al. (2004) who found that high cholesterol level could cause damage to the liver [51]. Since in our study diabetic rats also showed high cholesterol level, so it supports the idea that high cholesterol level leads to liver damage. Meanwhile, result also shown that creatinine and urea levels were significantly high ($P < 0.05$) in diabetic rats. This finding is similar to that of Sugano et al. (2006) where they had developed nephropathy in T2DM rats model using the same method as in our study and it indicates that HFD and low dose STZ can produce naturally nephropathy symptom similar in humans [52]. However, ALT, GGT, creatinine, and urea levels in diabetic rats treated with *C. latifolia* fruit:root extract were lower than diabetic control rats. This finding showed that no lethality or toxicity was observed during 4 weeks of intervention with *C. latifolia* fruit:root extracts on diabetic rats. Besides, it indicates that *C. latifolia* fruit:root extract prevents further defect in kidney and liver functions.

High fat diet influences human health status by changing cellular function during transcription process [53]. According to Rakhshandehroo et al. (2010), high fat diet may downregulate several transcription factors such as nuclear receptor (PPAR) and sterol regulatory binding proteins (GLUT) [53]. IRS-1, GLUT4, and IGF-1 are responsible of glucose metabolisms. Binding of insulin on IRS initiates PI3K substrates and triggers the activation of glucose transporter (GLUT4) vesicles to plasma membrane for glucose uptake [54]. Meanwhile, IGF-1 also has direct effect in order to trigger insulin sensitivity and regulate glucose uptake similar to insulin. Although insulin and IGF has similar mechanism in inducing glucose uptake, several studies showed that IGF is more efficient than insulin [55]. Thus, our finding showed that *IRS-1, IGF-1,* and *GLUT4* genes have been downregulated in obese and diabetic control rats. This finding was associated with the occurrence of defect insulin secretion and insulin resistance in obese and diabetic control rats [56]. However, *C. latifolia* fruit:root extract increased *IRS-1, IGF-1,* and *GLUT4* genes expression in diabetic rats. The augmented expression of these genes is due to the ability of *C. latifolia* fruit:root extract to increase insulin secretion and sufficient insulin will trigger the expression of *IRS-1* gene. Moreover, expression of *IGF-1* gene was higher compared to IRS-1. This indicates that *C. latifolia* fruit:root extract is more potent to improve glucose metabolism through IGF-1 action than IRS-1. Besides, it also indicates that *C. latifolia* fruit : root extract could act in two different situations, early and late phase of type 2 diabetes where pancreatic cells could not keep up with demand. However, further study needs to be done to study the mechanism involved.

Meanwhile, *PPARγ* and *PPARα* were also downregulated in obese and diabetic control rats. Research done by Petersson et al. (2009) has also found that the occurrence of insulin resistance, hyperglycemia, and dyslipidemia in diabetes subjects is because of downregulation of several genes such as *IGF-1, PPAR,* and *GLUT* families [57, 58]. Four weeks of intervention with *C. latifolia* fruit:root extract caused upregulation of *PPARγ* gene. According to Pita et al. (2012), the upregulation of *PPARγ* will stimulate *IRS-1, IGF-1, GLUT4, AdipoR1, AdipoR2, LPL, PPARα, leptin,* and *lipase* genes expression in the treated diabetic rats [59] and their findings support our results where the same genes are also being upregulated. Besides, *C. latifolia* fruit:root extract might have similar action to thiazolidinedione (TZD) which possess PPARγ ligand-binding activity and stimulate PPARγ transcription [60].

On the other hand, *AdipoR1, AdipoR2, leptin, LPL,* and *lipase* genes were also downregulated in diabetic control rats either in muscle and adipose tissues. According to Kadowaki and Yamauchi (2005), AdipoR1 and AdipoR2 ameliorate defect in glucose and lipid metabolism through different pathways [61]. AdipoR1 activates the AMPK pathway in order to reduce hepatic glucose production and increase FA oxidation. Meanwhile, AdipoR2 activates PPARα pathway [61]. In the present study, *C. latifolia* fruit:root extract increased regulation of *AdipoR1* and *AdipoR2* genes. These genes might be upregulated during adipocyte differentiation or through other genes expression. According to Pita et al. (2012), the enhancement of adiponectin receptors expression is correlated with increasing AMPK activity which is necessary for adiponectin action [59]. Our finding is parallel to their finding where *C. latifolia* fruit:root extract augmented glucose metabolism genes such as *IRS-1, IGF-1,* and *GLUT4* and it might increase AMPK activity in diabetic rats and lead to upregulation of *AdipoR1* and *AdipoR2* genes.

Several studies have indicated the importance of PPARα in lipid and glucose metabolisms [62]. According to Gross and Staels (2007), activation of PPARα may express other genes involved in lipid and lipoprotein metabolism [62]. Besides, PPARα is also responsible of glucose homeostasis and it directly regulates gluconeogenesis due to pyruvate dehydrogenase kinase 4 expression [63]. Apart from that, PPARα has the ability to prevent insulin resistance in diabetic due to the increase of fatty acids oxidation in pancreas, muscle and liver [64]. In spite of that, it also improves insulin secretion by preventing lipotoxicity and glucotoxicity in pancreatic cells [65]. In the present study, *C. latifolia* fruit:root extracts upregulate PPARα transcription in both muscle and adipose tissues of diabetic rats. The activation of PPARα will lead to high level of HDL and low level of TG in diabetic model [53]. This finding is in agreement with the present data where TG level was decreased and HDL level was increased in treated diabetic rats after 4 weeks of intervention with *C. latifolia* fruit:root extracts. Moreover, the upregulation of PPARα will initiate the regulation of genes involved in lipoprotein metabolism such as LPL [52]. Thus, it compliments with the recent study where *C. latifolia*

fruit:root extracts increased LPL expression in diabetic rats. The possible mechanism that can be drawn from this finding is that *C. latifolia* fruit:root extract indirectly increased LPL expression via activation of PPARα through adipocytokine signaling pathway. Apart from that, LPL expression might be another possible explanation on how opaque colour in plasma turned into clear. Lipid content in plasma will be hydrolyzed by LPL and converted into TG for storage [66]. Besides, slight increase in body weight among treated diabetic rats was due to upregulation of leptin expression.

5. Conclusion

The present study demonstrates that *C. latifolia* fruit:root extract reduced glucose and lipid levels in diabetic rats. Meanwhile, insulin and adiponectinlevels were increased. The extract exerts antidiabetic and hypolipidemic effects by stimulating PPARγ, IRS-1, IGF-1, GLUT4, AdipoR1, AdipoR2, LPL, PPARα, leptin, and lipase expressions in adipose and muscle tissues. The antidiabetic and hypolipidemic activities of *C. latifolia* fruit:root support its potential as a therapeutic option for diabetes and its complication.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Evaluation of the Hypoglycemic Properties of *Anacardium humile* Aqueous Extract

Márcio A. Urzêda,[1] Silvana Marcussi,[2] Luciana L. Silva Pereira,[2] Suzelei C. França,[1] Ana Maria S. Pereira,[1] Paulo S. Pereira,[1] Saulo L. da Silva,[3] César L. S. Guimarães,[4,5] Leonardo A. Calderon,[4] Rodrigo G. Stábeli,[4] Andreimar M. Soares,[4] and Lucélio B. Couto[1]

[1] *Unidade de Biotecnologia, Universidade de Ribeirão Preto, UNAERP, Ribeirão Preto, SP, Brazil*
[2] *Departamento de Química, Universidade Federal de Lavras, UFLA, Lavras, MG, Brazil*
[3] *Universidade Federal de São João Del Rei, UFSJ, Campus Alto Paraopeba, Ouro Branco, MG, Brazil*
[4] *Centro de Estudos de Biomoléculas Aplicadas à Saúde, CEBio, Fundação Oswaldo Cruz, FIOCRUZ Rondônia e Departamento de Medicina, Universidade Federal de Rondônia, UNIR, Porto Velho, RO, Brazil*
[5] *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, IBAMA, Porto Velho, RO, Brazil*

Correspondence should be addressed to Andreimar M. Soares; andreimar@fiocruz.br

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The antihyperglycemic effects of several plant extracts and herbal formulations which are used as antidiabetic formulations have been described and confirmed to date. The main objective of this work was to evaluate the hypoglycemic activity of the aqueous extract of *Anacardium humile*. Although the treatment of diabetic animals with *A. humile* did not alter body weight significantly, a reduction of the other evaluated parameters was observed. Animals treated with *A. humile* did not show variation of insulin levels, possibly triggered by a mechanism of blood glucose reduction. Levels of ALT (alanine aminotransferase) decreased in treated animals, suggesting a protective effect on liver. Levels of cholesterol were also reduced, indicating the efficacy of the extract in reestablishing the balance of nutrients. Moreover, a kidney protection may have been achieved due to the partial reestablishment of blood glucose homeostasis, while no nephrotoxicity could be detected for *A. humile*. The obtained results demonstrate the effectiveness of *A. humile* extracts in the treatment of alloxan-induced diabetic rats. Therefore, *A. humile* aqueous extract, popularly known and used by diabetic patients, induced an improvement in the biochemical parameters evaluated during and following treatment of diabetic rats. Thus, a better characterization of the medicinal potential of this plant will be able to provide a better understanding of its mechanisms of action in these pathological processes.

1. Introduction

Plants have been used for years as a source of traditional medicine to treat various diseases. Many of these medicinal plants are also excellent sources for phytochemicals, many of which contain potent antioxidant activities [1]. The study of medicinal plants as therapeutic agents is a huge approach regarding health problems of indigenous communities and third world countries. In Brazil, the influence of indigenous, African, and European cultures regarding the use of these plants is widely reported, evidencing the knowledge and use of these plants by indigenous communities until now [2]. Several medicinal plants have been used in traditional medicine for the treatment of diabetic patients in different ethnic societies of Africa, Asia, and South America [3]. The antihyperglycemic effects of several plant extracts and herbal formulations which are used as antidiabetic formulations have been confirmed [4].

Although other researchers had already noticed the presence of sugar in the urine of diabetics, this was demonstrated by Dobson only in 1755 [4]. *Diabetes mellitus* characterizes the most common endocrine disease worldwide, reaching about 173 million people, and it is estimated that this number will more than double over the next 25 years. According to Funke

and Melzig [5], this disorder is characterized by chronic hyperglycemia with disturbances of metabolism resulting from lack of insulin secretion and insufficient cellular production of insulin.

Diabetes is now the fourth or fifth leading cause of death in most developed countries, and with people suffering from the disease worldwide, its incidence is approaching epidemic proportions [6].

Diabetes is significantly (30% to 50%) related to diseases as ischemic cardiopathy, heart failure, cerebral vascular accident and systemic arterial hypertension [7]. The incidence (25%) of cardiovascular diseases (acute infarct) and mortality are increased in diabetic patients [8]. *Diabetes mellitus* is an apprehensive disease due to its diffused characteristics that, progressively, induce various pathologies with severe consequences.

Diabetes therapies are associated with the control of the signs and symptoms of the pathologies without, however, promoting the disease cure. The currently available treatment options for hyperglycemia, apart from lifestyle changes and weight reduction, are oral hypoglycemic agents (OHAs) with various modes of action, and different types of insulin [9]. Many classes of medicines are used for the treatment of diabetes, but all of them show adverse effects. Several approaches are described to reduce the hyperglycemia, such as treatment by sulfonylureas, which stimulates pancreatic islet cells to secrete insulin and metformin, reducing the hepatic glucose production [10]. All these therapies have limited efficacy and various side effects, and thus searching for new classes of compounds is essential to overcome these problems. Thus, it has increased the demand for low-cost alternatives to aid in controlling blood glucose levels, to prevent or delay the onset of complications related to diabetes, based on pharmaceutical ethnobotany [11].

Many plants have been reported to present hypoglycemic activity in the last years. In Brazil, the popular use of medicinal plants to control diabetes is very common. *Anacardium humile* St.-Hil., a species from the Anacardiaceae family, native to the Brazilian cerrado, has been popularly acclaimed against diabetes. This plant is a shrub (30 cm tall), with very long roots, small flowers, a greenish calyx, and red petals with stripes [12]. *Anacardium* species are known for their anti-inflammatory and astringent effects, activity against cancer cells, and their beneficial effects on gastrointestinal ulcers [12–14]. The aim of this work was to evaluate the hypoglycemic properties of the aqueous extract of *A. humile* on alloxan-induced diabetic rats.

2. Materials and Methods

2.1. Plant Material. Phloem of *A. humile* was collected in the Brazilian cerrado, region of Araxá, Minas Gerais, Brazil, $19^{o}43'04,0''$, W $46^{o}53'54,0''$ and 949 m of altitude WGS84 datum, coordinates measured by Garmin, model Legend global positioning system. Plant material was dried in a circulating air oven at 50°C for 24 h and then ground in a knife mill until total pulverization. The stems from *A. humile* were washed and dried to constant weight in a forced air circulating drier at 40 ± 5°C. Dried stems were ground and stored at ~4°C until use. To make an aqueous extract, the ground dried stems (100 g) were placed in a beaker containing deionized water (1 : 5 w/v) and held in a water bath at 60°C for 60 min and then hot-filtered through a gauze funnel. After cooling, the solution was frozen, lyophilized, and stored at −20°C, thereby enabling the weighing and dilution in deionized water. Before use, the freeze-dried aqueous extracts were weighed, dissolved in saline, and centrifuged at 10,000 ×g for 10 min, and the supernatants (stock solutions at 250 μg/μL) were stored at −20°C.

2.2. Animals and Diets. Male Wistar rats (*Rattus norvegicus,* 8 weeks old; 200 ± 20 g) were grown in the Animal Facilities of the University of Ribeirão Preto-UNAERP. The animals were housed in polypropylene cages at room temperature (20–25°C) under 12 h light-dark photoperiod. During the experiment, the rats received *ad libitum* water and standard pellet diet (Nuvilab) composed of (wet weight) 66% carbohydrates, 16% proteins, 8% fat, and 10% micronutrients-vitamins-mineral salts for six days. After diabetes induction, the animals were isolated in metabolic cages with daily replacement of food (45 g) and water (200 mL) at 8:00 AM. The experiments described were approved by Institutional Committee for Ethics in Animal Experimentation and were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.3. Induction of the Experimental Diabetes. Prior to diabetes induction, the animals were kept fasting for 12 hours. The inducing agent used was alloxan, dissolved in saline solution and injected intraperitoneally (100 mg/kg) within five minutes after dissolution [15, 16]. Considering that glucose administration protects the animals against the diabetogenic action of alloxan [17], the animals were kept fasting in order to avoid excessive accumulation of feeding glucose, which would antagonize the alloxan effects [18]. The volume of alloxan injection was calculated according to the animal body weight, observing the maximum volume limit of 0.5 mL, and the control group received equal volume of saline solution. Two hours after diabetes induction, the animals received *ad libitum* diet. Forty-eight hours after alloxan administration, diabetes was verified by blood glucose (manual glucometer Advantage II) and animals that showed glucose concentration higher than 250 mg/dL were selected.

After the isolation period, the animals ($N = 18$) were distributed in three groups of 6 rats each: Group (1) non-diabetic control rats (C) and group (2) untreated diabetic rats (D) and Group (3) diabetic rats treated with the aqueous extract of *A. humile* (T). Clinical parameters as the ingestion of water and food, excretion of urine, body weight and the biochemical parameters as glucose, urea, creatinine and urinary proteins were evaluated on day 2. The glycemic parameter was checked weekly using a manual glucometer (Advantage II). Total cholesterol, alanine aminotransferase (ALT) and insulin were measured on the 28th day.

2.4. Administration of the A. humile Aqueous Extract. After 14 days, blood glucose was measured to exclude animals with

possible spontaneous reversions of *diabetes mellitus* induced earlier and the animals were divided into three groups for the experiment. Rats were treated by gavage, using stainless steel tube and syringes, twice a day at 8:00 am and 8:00 pm during 28 days, by the same handler, respecting the maximum volume of 1 mL per animal. The animals of the T group received the dose of 175 mg/kg of aqueous extract of *A. humile* in variable volumes according to the animal weight. Diabetic rats of the D group as well as the rats of the C group received only saline solution.

2.5. Sampling and Tests. Urine was collected in plastic flasks every day, discarding the samples of the first 24 hours. The urinary volumes of the samples were measured, homogenized, placed in 10 mL tubes, and centrifuged at 11,200 ×g for 5 minutes. Analyses to determine the levels of glucose, urea, creatinine and protein were accomplished one hour after centrifugation. Glycemia tests were carried out using a glucometer. For the determination of plasma glucose levels, the tip of the rat tail was cut and the vein blood was collected weekly from all groups (between 08:30 and 09:00 h). At the end of the experiment (28 days), the animals were anesthetized (40 mg/kg, i.p.) with sodium thiopental (Tiopental). When rats were under deep anesthesia, a ventral midline incision was made, and 3 mL of blood was collected from the abdominal aorta and gently placed in Vacutainer tubes, containing a coagulation activator to avoid hemolysis. After coagulation, the material was centrifuged at 1,550 ×g for 5 minutes to separate the serum from the fibrin mesh. Biochemical analyses were performed within one hour after sampling.

2.6. Analytical Procedures. The laboratorial analyses were carried out at the Laboratory of Clinical Analysis of the University of Ribeirão Preto (UNAERP). Glucose levels in serum and urine were measured using the hexokinase method; urea in urine and total cholesterol were determined by the enzymatic method; urinary proteins were evaluated by a colorimetric method. All analyses were performed using Labtest kit (Bayer). The quantitative determination of ALT was performed employing the method optimized by the International Federation of Clinical Chemistry (IFCC) using the Biosystems test kit. Insulin was determined by the chemiluminescence method using the Kit Ultrasensitive insulin 33410-Beckman.

2.7. Statistical Analysis. All collected data represent the average ± standard deviations (S.D.). Comparisons between the different groups were carried out by analysis of variance followed by the Tukey test. Differences were considered statistically significant when $P < 0.05$.

3. Results and Discussion

Alloxan was able to produce diabetes in rats, with the development of clinical and laboratory definition, including elevation of water intake (Figure 1(a)), and urine output (Figure 1(d)), blood glucose levels above 400 mg/dL (Figure 2(a)) and urinary glucose greater than 7000 mg in 24 h (Figure 2(b)).

The present study demonstrated that the acute hyperglycemia induced by alloxan in rats was reduced by the administration of *A. humile* aqueous extract (175 mg/kg) by gavage twice a day during 28 days (Figures 2(a) and 2(b)). A similar effect was reported by Kamtchouing et al. [19] using the same dosage of *A. occidentale* aqueous extract. The effects of *A. humile* on blood glucose levels were very relevant, since blood collection was not performed during fasting, but with animals being fed *ad libitum*.

Figueroa-Valverde et al. [20] attributed the hypoglycemic effect of *Cnidoscolus chayamansa* to the presence of amentoflavone. Additionally, a study accomplished by Ferreira [21] with the extract and fractions obtained from *A. humile* showed that the majority of substances present in this species of plant are flavonoids derived from quercetin, an amentoflavone biflavonoid, derived from gallic acid and tannins. Therefore, flavonoids like amentoflavone may be related to the hypoglycemic effect observed in this study.

The treatment of diabetes with *A. humile* promoted a reduction of 32% in food intake (Figure 1(b)), comparing rats at the first and last days of treatments when the animals presented almost normal glucose levels, probably as a consequence of the consumption of available glucose in the blood [22]. Also, the synthesis of proteins and lipids may have contributed to the decrease of food ingestion, considering that the high levels of creatinine, urea, and urinary proteins found for the diabetic control group indicate a lower protein synthesis and a higher protein excretion in these animals compared to the treated group (Figures 2(c), 2(d), and 2(e)). The accentuated and rapid loss of weight is one of the first reasons that lead an individual to search for medical care [23]. In our study, significant alterations in body weight were observed in treated animals. However, an enhanced weight gain (32%) was observed (Figure 1(c)) in control rats, which may be related to the effective production of insulin in response to nourishment. According to Cha et al. [24], the significance of insulin for the physical development functioning is associated with the growth hormone (GH). Diabetic animals treated with *A. humile* presented a low gain of weight, suggesting a possible secondary effect of this plant in reducing or inhibiting growth. It is possible that *A. humile* causes an inhibition of α-amylase secretion into the intestine, improving the metabolism of glucose and triglyceride, as observed by Chen et al. [25] using *Rhizoma polygonati odorati* extracts. In this work lower gain of weight was observed in the diabetic animals treated with the aqueous extract, which presented a reduction of 2.37% in weight gain. This hypothesis may be confirmed by the lack of alterations in the insulin synthesis, which also acts as a growth hormone [26]; however, additional studies are necessary.

In the present work it was verified that the administration of *A. humile* significantly reduced water ingestion from the twelfth day to the end of the treatment (Figure 1(a)). It was observed that the ingestion of water by control rats did not achieve significant variation, probably because blood glucose did not return to normal levels. At the end of the experiments, diabetic treated rats reduced by 61.23% the ingestion of water

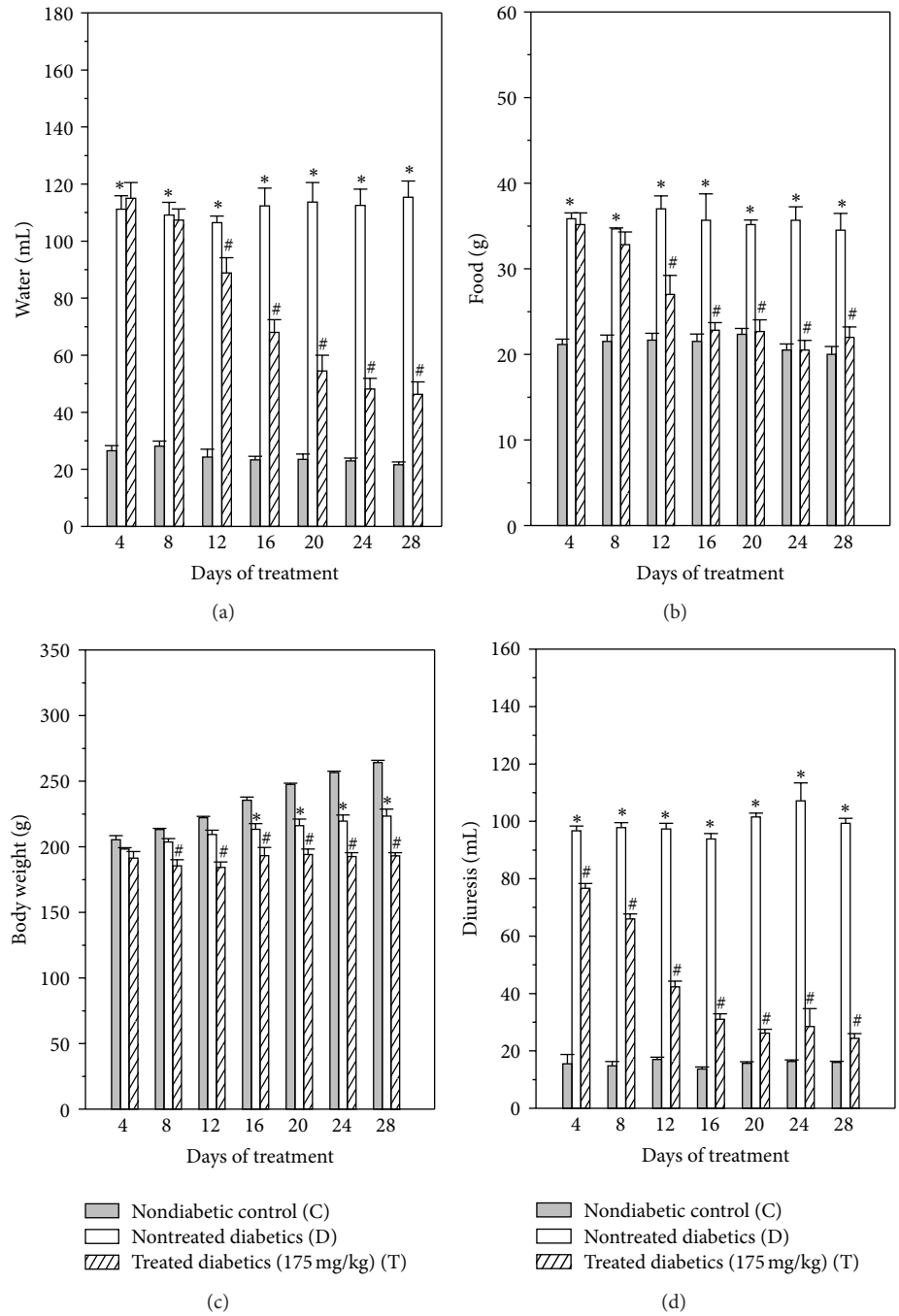

Figure 1: Evaluation of clinical parameters: average ± SD. (a) Water ingestion; (b) food ingestion; (c) body weight; (d) diuresis (mL). $^{*}P < 0.05$ between groups C and D; $^{\#}P > 0.05$ between groups D and T.

compared to the fourth day of treatment. The effect of *A. humile* on diuresis seems to be related to its effectiveness in reducing blood glucose. However, the decrease in glucose in the treated group causes an improvement in the characteristic symptoms of hyperglycemia, polyphagia among them, resulting in a lower food intake.

Treated rats showed decreased diuresis with a reduction of 68.33% being observed in the urinary volume at the end of the treatment (Figure 1(d)). The polydipsia present in diabetic animals is due to blood hyperosmolarity, due to the high levels of circulating glucose, which causes the movement of water of the extracellular to the intracellular space, in order to maintain osmotic equilibrium. The intracellular dehydration is sensed by osmoreceptors in the brain, triggering intense thirst. The improvement in clinical status due to lower blood glucose in the treated group may be evidenced by the significant decrease in polyuria and polydipsia at the end of the experiment.

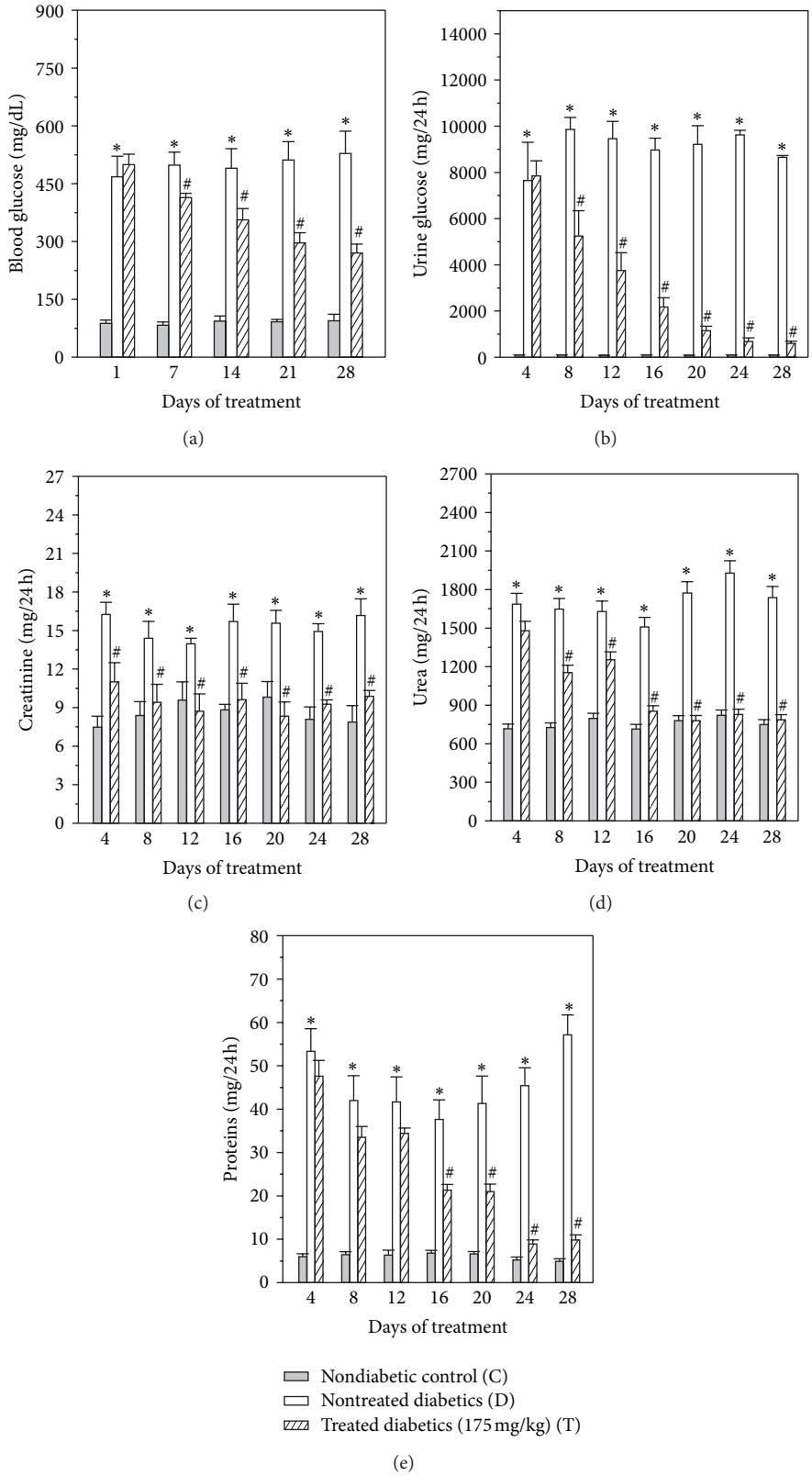

FIGURE 2: Evaluation of biochemical parameters: average ± SD. (a) Blood glucose; (b) urine glucose; (c) urinary creatinine; (d) urinary urea; (e) urinary proteins. $^{*}P < 0.05$ between groups C and D; $^{\#}P > 0.05$ between groups D and T.

Table 1: Total cholesterol, insulin, and ALT levels on the 28th day of treatment.

Group	N	Treatment	Cholesterol (mg/dL)	Insulin (μUI/mL)	ALT (UI/L)
C	6	Saline	53.83 ± 0.79^{b}	0.0800 ± 0.0044^{a}	64 ± 23.11^{b}
D	6	Saline	70.66 ± 1.64^{a}	0.0383 ± 0.0044^{b}	165.3 ± 22.01^{a}
T	6	*A. humile* (175 mg/kg)	59.33 ± 2.18^{b}	0.0383 ± 0.0044^{b}	146 ± 22.11^{ab}

Mean values with different letters are significantly different at $P < 0.05$ (average ± SD).
C: control
D: diabetic rats
T: treated rats.

The administration of *A. humile* was showed to significantly reduce the urine proteins from the 16th day of treatment in (Figure 2(e)), suggesting some kind of protective effect of this extract on the nephropathy induced by diabetes. However, simple microalbuminuria laboratory tests may not be effective in predicting a diabetic nephropathy, which makes additional biochemical analysis necessary, as for urine creatinine levels [27].

Dosing urine proteins is of great help for evaluating diabetes treatment, since it may indicate diabetic nephropathy, which may be preceded by a period of increased urinary albumin excretion (UAE; 20–300 mg/24 h), defined as microalbuminuria [28]. Urine protein dosage is also effective in evaluating hypoglycemic activity of plant extracts and in determining the risk of renal lesions by plant active principles [29]. Grindley et al. [30] reported an increase of proteinuria (nephropathy) after administration of Yam (*Dioscorea cayenensis*), considered a hypoglycemiant plant.

Dornas et al. [31] reported that nephrotoxicity induced by gentamicin can be attenuated by gallic acid, one of the major components among the phenolic compounds present in the extract of *A. humile*. According to these authors, the phenolic compounds participate in increasing the antioxidant function, and due to their action as metal chelators and enzyme modulators, they may attenuate renal damage caused by oxidative changes. In some cases this possible protection of renal function is consistent with the increase in urine creatinine levels; however, it was not observed in the present study (Figure 2(c)). Creatinine produced by creatine is a chemical waste molecule that is, generated from muscle metabolism and excreted entirely by the kidneys. Bwititi et al. [32] showed an absence of nephrotoxicity in diabetic rats and confirmed a renal protection of *Opuntia megacantha* treated animals, which presented decreases in glomerular filtration rate. In spite of the revealed protection, additional analysis, as kidney histopathology, is necessary to discard the inexistence of any renal lesions [33].

Results showed that the administration of *A. humile* aqueous extract decreased (41.75%) the excretion of urine urea along the treatment (Figure 2(d)). Moreover, it was observed that, after the twelfth day of treatment, the urine urea levels were similar for both the control and treated rats, emphasizing the effectiveness of the extract in reducing rates of urea excretion. According to Pepato et al. [34], the decrease of blood glucose causes a reduction of urea renal excretion. Our data suggest a partial glucose homeostasis by the administration of *A. humile* aqueous extract.

High levels of glucose are a major symptom of diabetes and the detection of glucose in the urine is relevant in selecting the treatment against this pathology, once there is a direct correlation between glucose concentration in the urine and in the blood [35]. The administration of *A. humile* extract in diabetic rats significantly reduced the levels of glucose excretion in the urine compared with results obtained for untreated rats (Figure 2(b)). Glucose in urine of T group animals was similar to the animals of the C group, from the twentieth day of administration until the end of experiments.

The production of cholesterol, the major constituent of the lipoproteins, may be enhanced by diabetes [36]. Our results showed that the cholesterol levels between the nondiabetic control group and treated group were statistically equal. An increase was observed in the level of cholesterol in the diabetic control. However, studies report that average values observed for the HDL cholesterol were significantly higher in diabetic rats. This could possibly provide protection against the development of atherosclerotic macrovascular disease in diabetic animals, contrary to what is observed in humans. The genesis of these findings has not yet been elucidated [22].

A. humile extract reduced the levels of total cholesterol and no significant difference was observed in either treated or control rats (Table 1). El-Missiry and El Gindy [15] and Benwahhoud et al. [37] obtained similar results using *Suaeda fruticosa* extract, while the administration of *Spergularia purpurea* aqueous extract was ineffective in reducing cholesterol levels [38]. Both cholesterol increase and reduction have been reported after alloxan administration in rats [38].

The administration of the *A. humile* extract decreased by 21% the rates of alanine aminotransferase (ALT) in the diabetic rats, when compared to control animals at the end of the treatment, suggesting a hepatic protection of *A. humile* against the damaging effects of diabetes (Table 1). Also, results showed that *A. humile* extract did not change the pancreatic insulin levels during the experimental period (Table 1), suggesting no side effects of this treatment.

Indeed, among the factors related to the effects of administration of *A. humile*, the decrease in insulin degradation by the inhibition of the enzyme insulinase [39] or the increase in the outlying sensibility of the existent insulin receivers in the β-cell [25] or even direct activation of these same receptors [40] may be considered. In addition, the hyperglycemia reduction observed in the present study could be related to a possible decrease of glucose absorption due to the inhibition of the α-amylase secretion responsible for the increase of both glucose and triglycerides in the metabolism [41]. Finally,

the possibility that the bioactive metabolites act on modulating the synthesis and secretion of insulin should not be excluded [42].

4. Conclusions

In conclusion, the use of aqueous extracts and isolated natural products, exclusively derived from plants, in alternative therapies for antidiabetes drugs has been increasing. Obtained results demonstrate that the administration of *Anacardium humile* aqueous extract to alloxan-induced diabetic rats is effective in regulating the levels of blood glucose and other related parameters. However, it does not seem to alter insulin secretion into the blood stream and shows no hepatic or renal toxicity. Analysis of the different bioactive compounds of *A. humile* aqueous extract, as well as the preclinical and clinical toxicity evaluation followed by the determination of therapeutic intervals, should insure the efficacy and safety of *A. humile* in the treatment of diabetes in humans. The potential use of this extract or its isolated compounds as alternative treatment and/or as molecular models for the development of new therapeutic agents in the treatment of diabetes or other diseases needs to be evaluated in future studies.

Conflict of Interests

The authors report no conflict of interests.

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Research Article

Decrease of Plasma Glucose by *Hibiscus taiwanensis* in Type-1-Like Diabetic Rats

Lin-Yu Wang,[1,2] Hsien-Hui Chung,[3] and Juei-Tang Cheng[4,5]

[1] *Department of Pediatrics, Chi-Mei Medical Center, Yong Kang, Tainan City 73101, Taiwan*
[2] *The Center of General Education, Chia Nan University of Pharmacy & Science, Rende, Tainan City 71710, Taiwan*
[3] *Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan City 70101, Taiwan*
[4] *Graduate Institute of Medical Sciences, Chang Jung Christian University, Quei-Jen, Tainan City 71101, Taiwan*
[5] *Department of Medical Research, Chi-Mei Medical Center, Yong Kang, Tainan City 73101, Taiwan*

Correspondence should be addressed to Juei-Tang Cheng; jtcheng@mail.ncku.edu.tw

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Hibiscus taiwanensis (Malvaceae) is widely used as an alternative herb to treat disorders in Taiwan. In the present study, it is used to screen the effect on diabetic hyperglycemia in streptozotocin-induced diabetic rats (STZ-diabetic rats). The extract of *Hibiscus taiwanensis* showed a significant plasma glucose-lowering action in STZ-diabetic rats. Stems of *Hibiscus taiwanensis* are more effective than other parts to decrease the plasma glucose in a dose-dependent manner. Oral administration of *Hibiscus taiwanensis* three times daily for 3 days into STZ-diabetic rats increased the sensitivity to exogenous insulin showing an increase in insulin sensitivity. Moreover, similar repeated administration of *Hibiscus taiwanensis* for 3 days in STZ-diabetic rats produced a marked reduction of phosphoenolpyruvate carboxykinase (PEPCK) expression in liver and an increased expression of glucose transporter subtype 4 (GLUT 4) in skeletal muscle. Taken together, our results suggest that *Hibiscus taiwanensis* has the ability to lower plasma glucose through an increase in glucose utilization via elevation of skeletal GLUT 4 and decrease of hepatic PEPCK in STZ-diabetic rats.

1. Introduction

Diabetes is a well-known metabolic disease and often leads to many physiological complications, including cardiovascular diseases, renal diseases, and retinal damage [1–3]. Recently, the management of diabetic hyperglycemia has attracted much attention in alternative aspect [4, 5]. Some agents from herbal plants are thought to improve diabetic hyperglycemia [6–8]. Thus, alternative medicine and other herbal supplements for handling of diabetic disorders are necessary.

Hibiscus taiwanensis S. Y. Hu (Malvaceae) is native to Taiwan and widely distributed throughout the island. Recently, the stems and roots of *Hibiscus taiwanensis* have been used as anti-inflammatory, antifungal, antipyretic, and anthelmintic agents in traditional Chinese medicine [9, 10]. *Hibiscus taiwanensis* had anti-inflammatory action in vitro and in vivo via increasing the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [11]. Many active principles have been isolated from the stems of *Hibiscus taiwanensis*, and some of them showed cytotoxic activity against human carcinoma, including breast and lung cancer cells [12, 13]. Antioxidant-like substances are known to produce antidiabetic action [14, 15]. In recent, an active principle isolated from *Hibiscus taiwanensis* named syringaldehyde showed a plasma glucose-lowering action in streptozotocin-induced diabetic rats (STZ-diabetic rats) [16]. However, the effect of *Hibiscus taiwanensis* on diabetic disorders remained obscure.

In the present study, we thus employed STZ-diabetic rats as an animal model of type-1-like diabetic disorders to screen the changes in plasma glucose and clarify the potential mechanism for this action. The main aim is going to provide a new insight of alternative medicine into the improvement of diabetic hyperglycemia.

2. Materials and Methods

2.1. Plant Materials. The extract with 60% aqueous acetone of *Hibiscus taiwanensis* was provided by Hercet Co. Ltd. (Kaohsiung, Taiwan). The plant material was identified by Professor M. I. Wu (Kaohsiung Committee of Chinese Medicine; Kaohsiung City, Taiwan). A voucher specimen (BT-H-00151) was deposited in the herbarium of the Agricultural Research Institute (Taichung, Taiwan).

2.2. Preparation of Plant Extracts. In the present study, the plant parts were used to compare their plasma glucose-lowering action, including leaf, stem, and fruit. Dried each part of *Hibiscus taiwanensis* (1.5 kg) was extracted with 7 L of aqueous acetone solution (60%) by maceration at room temperature for three days. The extraction process was repeated three times. The extract was concentrated under reduced pressure at 40°C, yielding 1.5 L of an aqueous extract, and it was diluted to the desired concentration when used.

2.3. Animal Models. Ten-week-old male Wistar rats weighing 250 to 300 g were obtained from the Animal Center of National Cheng Kung University Medical College. The diet of the animals used for the study was standard laboratory diet. The number of animals for each group of experiment is eight. STZ-diabetic rats were induced by intravenous injection (IV) of STZ (65 mg/kg) into Wistar rats according to the previous method [17]. Animals were considered to be diabetic if they had plasma glucose concentrations of 320 mg/dL or greater in addition to polyuria and other diabetic features. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. In our preliminary data, the effective dose of the plant extract in reducing the plasma glucose in type-1 diabetic rats was 500 mg/kg, and the volume of the extract was administered according to the body weight of the rat (mL/kg). Also, the treated dose of each part was 500 mg/kg.

2.4. Laboratory Determinations. The determination of plasma glucose was conducted according to the previous study [18]. The concentration of plasma glucose was measured by the glucose oxidase method using an analyzer (Quik-Lab, Ames; Miles Inc., Elkhart, IN, USA).

2.5. Measurement of Insulin Sensitivity in Rats. Because the STZ-induced diabetic rats used in the present study were negligible for endogenous insulin, the plasma glucose-lowering action depended on the action of exogenous insulin in STZ-induced diabetic rats. The obtained results can thus be used to indicate the insulin sensitivity. These rats received an injection of long-acting human insulin at 1 IU/kg once daily to normalize the insulin sensitivity. Then, three days later, the STZ-diabetic rats were divided into two groups. One group received the oral treatment of *Hibiscus taiwanensis* at 500 mg/kg dissolving in saline solution, three times daily (t.i.d.), and another group received similar treatment with the same volume of saline. After three days of treatment, all rats were used to challenge with exogenous insulin. According to a previous method [16], an intravenous insulin challenge test was performed by giving 0.1 to 1.0 IU/kg of short-acting human insulin into these STZ-diabetic rats. Blood samples (0.2 mL) from the femoral vein were drawn at 30 min following the intravenous insulin challenge test for the measurement of plasma glucose concentrations.

2.6. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from liver and soleus muscle tissue samples using Trizol reagent (Invitrogen). Two microgram of total RNA was used for the reverse transcription reaction, along with Superscriptase II (Invitrogen), oligo-dT, and random primers. The web-based assay design software from the Universal Probe Library Assay Design Center was used to design the TaqMan primer pairs and to select the appropriate hybridization probes. The reactions were performed in 20 μL of a mixture consisting of 13.4 μL of PCR buffer, 0.2 μL of each probe (20 μmol/L), 4 μL of LightCycler TaqMan (Roche Diagnostics GmbH), and 2 μL of template cDNA. A LightCycler Detection System (Roche Applied Science) was used for amplification and detection. The PCR reaction was carried out as follows: one cycle of 95°C for 10 min, 45 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 1 s. The crossing point for each amplification curve was determined using the second derivative maximum method. The concentration of each gene was calculated with the aid of the LightCycler software using the respective standard curve as reference. Relative gene expression was expressed as a ratio of the target gene concentration to the housekeeping gene 36B4 concentration.

2.7. Western Blotting Analysis. Western blotting analysis was carried out as previously described [19] and quantification was obtained from three individual experiments. After homogenization of liver and soleus muscle using a glass/Teflon homogenizer, the homogenates (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and western blot analysis was performed using either an anti-rat GLUT 4 antibody purchased from (Abcam, Cambridge, UK) in soleus muscle or an anti-rat PEPCK antibody from Santa Cruz Biotechnology, CA, USA, in liver. The blots were probed with a goat polyclonal actin antibody from (Millipore, Billerica, MA, USA) to ensure that the amount of protein loaded into each lane of the gel was constant. Blots were incubated with the appropriate peroxidase-conjugated secondary antibodies. After removal of the secondary antibodies, the blots were washed and developed using the ECL-Western blotting system. Densities of the obtained immunoblots at 45 KDa for GLUT 4, 69.5 KDa for PEPCK, and 43 KDa for actin were quantified using laser densitometer.

2.8. Statistical Analysis. The plasma glucose-lowering activity of *Hibiscus taiwanensis* was calculated as the percentage

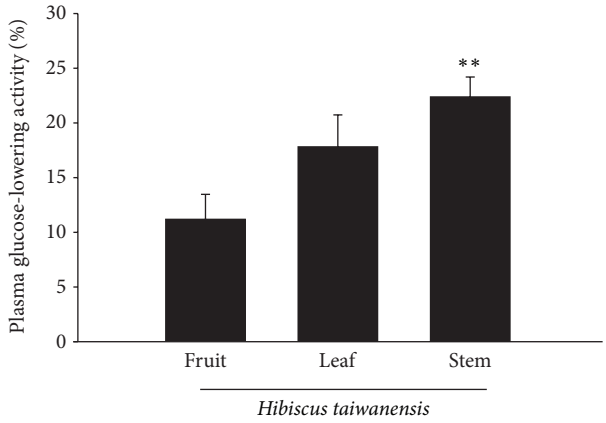

Figure 1: Comparison of the glucose-lowering activity between the extracts from fruit, leaf, or stem of *Hibiscus taiwanensis*. Values of mean and bar of S.E.M. were obtained from each group of eight rats. Vehicle only (0.9% saline) was given at the same volume. $^{**}P < 0.01$ versus data from animals treated with vehicle (0).

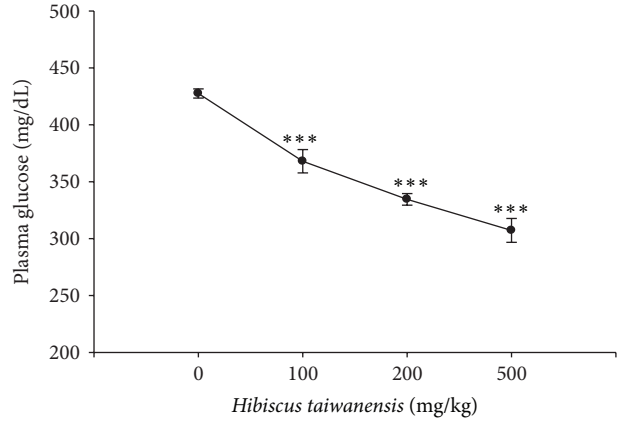

Figure 2: The plasma glucose-lowering activity produced by an oral intake of the stem extract from *Hibiscus taiwanensis* in STZ-diabetic rats. Values of mean and bar of S.E.M. were obtained from each group of eight rats. Vehicle only (0.9% saline) was given at the same volume. $^{***}P < 0.001$ versus data from animals treated with vehicle (0).

decrease of the initial glucose value according to the following formula: $(G_i - G_t)/G_i \times 100\%$, where G_i is the initial glucose concentration and G_t is the plasma glucose concentration after treatment of *Hibiscus taiwanensis*. Data are expressed as the mean ± S.E.M. for the number (n) of animals in the group as indicated in tables and figures. Differences among groups were analyzed by one-way ANOVA. The Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. A P value of 0.05 or less was considered statistically significant.

3. Results

3.1. Comparison of Effects of Various Parts Prepared from Hibiscus taiwanensis on Plasma Glucose Concentration in STZ-Diabetic Rats. As shown in Figure 1, the plasma glucose-lowering activities were 11.2 ± 2.3%, 17.8 ± 2.9%, and 22.4 ± 1.8% in STZ-diabetic rats receiving oral intake of extract (500 mg/kg) prepared from fruit, leaf, and stem of *Hibiscus taiwanensis*, respectively ($n = 8$). The stems show a better plasma-lowering action than other parts. Thus, the following experiments were performed using the extract of stems from *Hibiscus taiwanensis*.

3.2. Dose-Dependent Action of Hibiscus taiwanensis to Lower Plasma Glucose in STZ-Diabetic Rats. Ninety minutes after treatment, the plasma glucose-lowering activities were 15.1 ± 2.0%, 22.1 ± 2.4%, and 28.9 ± 1.9% in STZ-diabetic rats receiving oral intake of *Hibiscus taiwanensis* at 100 mg/kg, 200 mg/kg and 500 mg/kg, respectively ($n = 8$). As shown in Figure 2, a dose-dependent reduction of plasma glucose by *Hibiscus taiwanensis* was observed and it significantly decreased the plasma glucose concentration to 307.2 ± 10.5 mg/dL ($P < 0.001$; $n = 8$) at 500 mg/kg. As the positive control, treatment with metformin in various doses (50 mg/kg, 75 mg/kg, and 100 mg/kg) attenuated the plasma glucose from 431.8 ± 5.6 mg/dL to 356.6 ± 3.4 mg/dL, 326.6 ± 2.9 mg/dL, and 294.9 ± 3.7 mg/dL ($n = 8$) showing 17.3 ± 1.3%, 24.2 ± 1.4%, and 31.6 ± 1.3% plasma glucose-lowering activities, respectively.

3.3. Effect of Hibiscus taiwanensis on Insulin Sensitivity in STZ-Induced Diabetic Rats. The change of insulin sensitivity was investigated in STZ-induced diabetic rats. The basal plasma glucose concentration in STZ-diabetic rats was 334.9 ± 7.2 mg/dL. The plasma glucose-lowering activity of short-acting human insulin (exogenous insulin) at doses from 0.1 to 1.0 IU/kg in STZ-diabetic rats receiving oral intake of *Hibiscus taiwanensis* (500 mg/kg, three times daily) for 3 days was markedly higher than that in the control group receiving same volume of vehicle (Figure 3). The plasma glucose-lowering activity of exogenous insulin in the *Hibiscus taiwanensis*-treated group (500 mg/kg) was about 34.0 ± 2.8% at 0.5 IU/kg and more markedly 42.0 ± 3.1% at the dose of 1.0 IU/kg (Figure 3). An increase in insulin sensitivity by *Hibiscus taiwanensis* can be identified.

3.4. Effect of Hibiscus taiwanensis on Changes of GLUT 4 in Skeletal Muscle of STZ-Diabetic Rats. Treatment of STZ-diabetic rats with oral intake of *Hibiscus taiwanensis* (500 mg/kg) three times daily for 3 days resulted in an elevation of GLUT 4 mRNA level in skeletal muscle (Figure 4(a)). Western blot analysis showed a similar effect of *Hibiscus taiwanensis* (500 mg/kg) on the changes of GLUT 4 protein level in skeletal muscle (Figure 4(b)).

3.5. Effect of Hibiscus taiwanensis on Changes of Hepatic PEPCK in STZ-Diabetic Rats. In the present study, the mRNA level of PEPCK in liver of STZ-diabetic rats was raised to about 4.4-folds of that in nondiabetic rats. The reduction of PEPCK mRNA level by *Hibiscus taiwanensis*

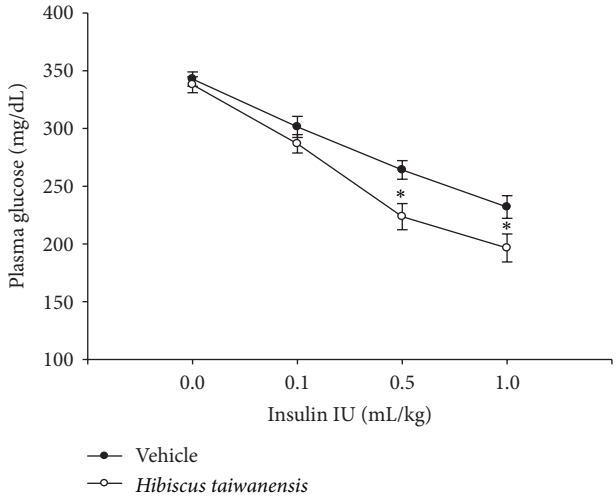

Figure 3: Effect of *Hibiscus taiwanensis* on insulin sensitivity in STZ-diabetic rats. *Hibiscus taiwanensis* at 500 mg/kg was treated orally into STZ-diabetic rats three times daily for three days. Then, the animals were injected intravenously with exogenous insulin at the indicated dose to show the changes in plasma glucose as open circles. Changes of plasma glucose in another group of STZ-diabetic rats receiving a similar treatment with vehicle at the same volume are shown as closed circles. Values (means ± SE) were obtained from each group of eight animals. $^{*}P < 0.05$ as compared with values from vehicle-treated group (closed circles) at the same dose of insulin.

in diabetic rats was observed (Figure 5(a)). Similarly, the protein level of PEPCK in liver of STZ-diabetic rats was raised to approximately 4.0-folds of that in nondiabetic rats. The protein level of PEPCK in diabetic rats was also reversed by *Hibiscus taiwanensis* to normal level (Figure 5(b)).

4. Discussion

In the present study, we found that the extract of herb named *Hibiscus taiwanensis* at 500 mg/kg has an ability to improve diabetic hyperglycemia in animal. Also, the stems show more effective plasma glucose-lowering action than fruit and leaf. This is consistent with previous report showing actions of stems [12, 13] in a dose-dependent manner. In addition, *Hibiscus taiwanensis* increased the insulin sensitivity to improve diabetic hyperglycemia via the regulation of peripheral glucose utilization and hepatic glucose output in STZ-diabetic rats.

In the present study, we employed STZ-diabetic rats to screen the effectiveness of herbal extract. This model belongs to type-1-like diabetic disorders due to insulin deficiency as described previously [20]. Although we did not check the plasma insulin level, the higher plasma glucose supports the success of this model similar to our reports [21, 22]. Diabetic hyperglycemia is resulted mainly from the dysfunction in glucose homeostasis while insulin sensitivity plays a critical role in the regulation of diabetic hyperglycemia [23]. Some agents useful in the improvement of diabetic hyperglycemia are believed to increase insulin sensitivity [24–26]. According to the previous method [16], *Hibiscus taiwanensis* was orally administered into diabetic rats at 500 mg/kg three times per day for three days. Then, the responses to exogenous insulin were compared with the vehicle-treated group. As shown in Figure 3, responses to exogenous insulin were markedly increased by treatment with *Hibiscus taiwanensis* in STZ-diabetic rats. It means that *Hibiscus taiwanensis* elevated the sensitivity of peripheral tissues to insulin directly because type-1-like diabetic rats lack endogenous insulin. An increase of insulin sensitivity by *Hibiscus taiwanensis* can thus be considered. This is useful to apply in insulin resistance and/or type-2 diabetic disorders widely observed in clinics [27]. However, the molecular mechanism for *Hibiscus taiwanensis* to increase insulin sensitivity shall be investigated in the future.

In addition to insulin deficiency, diabetic hyperglycemia is thought to be the consequence of increased hepatic glucose output and reduced peripheral glucose utilization [28, 29]. Many related metabolic proteins were involved in the diabetic hyperglycemia, including AMP Kinase (AMPK), GLUT 4, PEPCK, acetyl CoA carboxylase, glycogen synthase kinase-3, and glycogen synthase [30]. Among them, GLUT 4 and PEPCK were commonly used to investigate diabetic disorders [31], and plasma glucose-lowering activities of some agents were associated with an increase in the glucose utilization in peripheral tissues and a reduction in hepatic gluconeogenesis [19, 32]. Glucose transport, which depends on insulin-stimulated translocation of glucose carriers to the cell membrane, is the rate-limiting step in the glucose metabolism of skeletal muscle [33]. The decreased expression of skeletal muscle GLUT 4 was previously proposed to cause the reduction of insulin-mediated glucose utilization in diabetic skeletal muscle [34–36]. Hepatic gluconeogenesis has an important influence on glucose metabolism [37]. Additionally, insulin deficiency is closely correlated with hepatic glucose output via increased expression of PEPCK that is a key enzyme of hepatic carbohydrate metabolism in diabetic hyperglycemia [28, 38, 39]. Liver-specific inhibition of PEPCK with RNAi improved diabetic hyperglycemia [40]. It is worthwhile to investigate whether *Hibiscus taiwanensis* exerted its antihyperglycemic action in diabetic rats by overturning the diabetes-dependent reduction of GLUT 4 expression and increased PEPCK expression. To provide ample time for alterations in gene expression, STZ-diabetic rats received repeated *Hibiscus taiwanensis* treatments for 3 days. As shown in Figures 4(a) and 5(a), the mRNA level of GLUT 4 was raised and the mRNA level of PEPCK was reduced by this herbal extract at dose sufficient to produce plasma glucose-lowering action; this change is similar to the action of effective agent showed in previous studies [41, 42]. Moreover, the lowered GLUT 4 protein level due to diabetes was also elevated by treatment with *Hibiscus taiwanensis* (Figure 4(b)). Similarly, the increased hepatic PEPCK protein by diabetes was attenuated by *Hibiscus taiwanensis* (Figure 5(b)). Thus, under an insulin-independent condition, *Hibiscus taiwanensis* modulated the gene expressions of muscle GLUT 4 and hepatic PEPCK of both mRNA and protein levels.

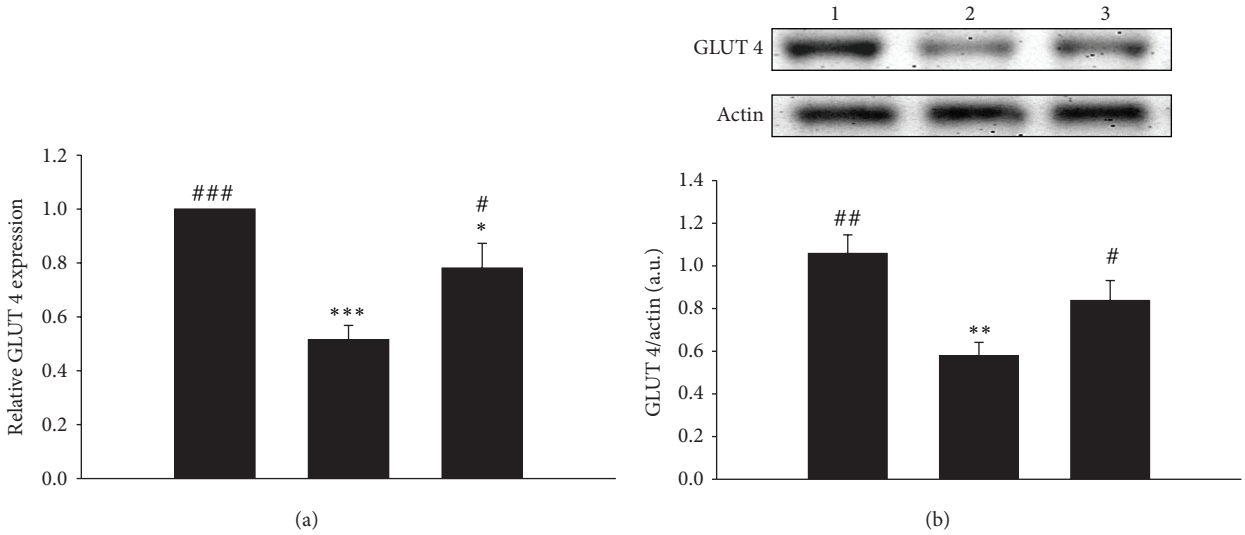

Figure 4: The relative GLUT 4 expression in skeletal muscle isolated from STZ-diabetic rats received oral intake with *Hibiscus taiwanensis* (500 mg/kg) three times daily for 3 days. (a) Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, *Hibiscus taiwanensis* (500 mg/kg)-treated STZ-diabetic rats. The samples were then collected for qRT-PCR. Data expressed as mean with standard error (SE) ($n = 6$ per group) is indicated in each column. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with data obtained from lane 1. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared with data obtained from lane 2. (b) Upper panel shows the representative response of protein level for GLUT 4 or actin in skeletal muscle isolated from STZ-diabetic rats receiving treatment with *Hibiscus taiwanensis* three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, *Hibiscus taiwanensis* (500 mg/kg)-treated STZ-diabetic rats. Quantification of protein level using GLUT 4/actin expressed as mean with standard error (SE) ($n = 6$ per group) in each column is indicated in the lower panel. $^{**}P < 0.01$ compared with data obtained from lane 1. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with data obtained from lane 2.

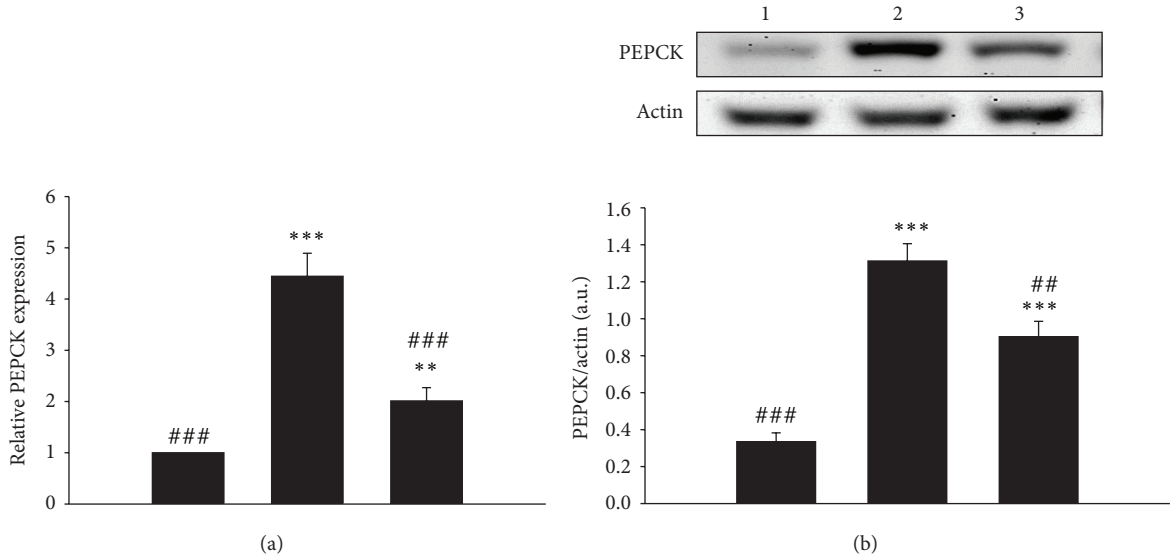

Figure 5: The relative PEPCK expression in liver isolated from STZ-diabetic rats received oral intake with *Hibiscus taiwanensis* (500 mg/kg) three times daily for 3 days. (a) Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, *Hibiscus taiwanensis* (500 mg/kg)-treated STZ-diabetic rats. The samples were then collected for qRT-PCR. Data expressed as mean with standard error (SE) ($n = 6$ per group) is indicated in each column. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with data obtained from lane 1. $^{\#\#\#}P < 0.001$ compared with data obtained from lane 2. (b) Upper panel shows the representative response of protein level for PEPCK or actin in liver isolated from STZ-diabetic rats receiving treatment with *Hibiscus taiwanensis* three times daily for 3 days. Lane 1, vehicle-treated Wistar rats; lane 2, vehicle-treated STZ-diabetic rats; lane 3, *Hibiscus taiwanensis* (500 mg/kg)-treated STZ-diabetic rats. Quantification of protein level using PEPCK/actin expressed as mean with standard error (SE) ($n = 6$ per group) in each column is indicated in the lower panel. $^{***}P < 0.001$ compared with data obtained from lane 1. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with data obtained from lane 2.

5. Conclusions

In conclusion, our results suggest that *Hibiscus taiwanensis* is merit for diabetic hyperglycemia mainly mediated by the enhancement of GLUT 4 gene expression and/or the amelioration of hepatic PEPCK gene expression. Therefore, *Hibiscus taiwanensis* can be used as the alternative agent for improvement of diabetic hyperglycemia.

Abbreviations

GLUT 4: Glucose transporter subtype 4
PEPCK: Phosphoenolpyruvate carboxykinase
STZ: Streptozotocin.

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