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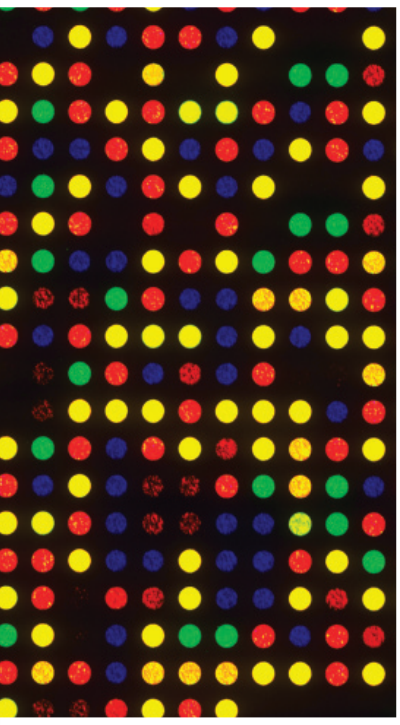
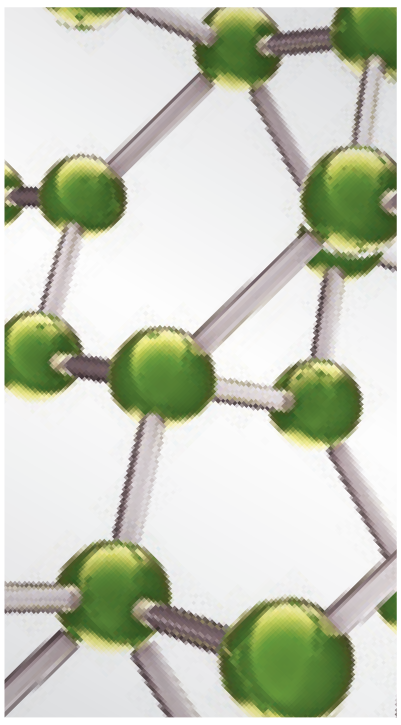
Natural Products for the Treatment of Obesity, Metabolic Syndrome, and Type 2 Diabetes

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



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Editorial Board

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Editorial

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

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Globally, the incidence of obesity, metabolic syndrome, and diabetes (OMD) are continuously on the rise because of rapid changes in human life-style and dietary habits. Herbal extracts are of special interest in treating combination of these diseases because of their multipronged mode of action. The list of potential herbals to control metabolic diseases is ever-expanding. However, because of poor characterization and safety issues, these herbs face limitations for their clinical usage. This special issue is a collection of research and review articles on preclinical and clinical benefits of herbals in controlling OMD. This special issue contains 24 articles accepted from a total of 37 submissions consisting of 20 research articles, 3 review articles, and 1 clinical study. The research articles in this issue can be broadly divided into three disease categories—nonalcoholic steatohepatitis (NASH), obesity, and diabetic complications.

Four articles of this special issue focus on evaluating the protective role of herbal extracts on NASH. The studies by X. R. Yang et al. "*Effect of dietary cocoa tea (Camellia ptilophylla) supplementation on high-fat diet-induced obesity, hepatic Steatosis, and hyperlipidemia in mice*" and H.-Y. Jung et al. "*The Korean mistletoe (Viscum album coloratum) extract has an antiobesity effect and protects against hepatic steatosis in mice with high-fat diet-induced obesity*" report the potential benefits of *Camellia ptilophylla* and *Viscum album coloratum* extracts against HFD-induced NASH. Another two articles evaluated protective effects of biherbal combination (*S. miltiorrhiza*, *G. jasminoides* and Grape Pomace, Omija Fruit) in ameliorating experimental NASH.

This special issue also contains five articles that focus on antiobesity potential of herbal extracts. These detailed studies evaluated the benefits of 10 herbs and their potential mechanisms responsible in controlling obesity using experimental HFD-fed mice/rat *in vivo* and 3T3L1 preadipocyte *in vitro* models. Modulation of PPARγ was a key antiobesity mechanism of *Artemisia iwayomogi*, *Codonopsis lanceolata*, *Populus balsamifera* and its active component (salicortin), and beta-glucan-rich extract from *Pleurotus sajor-caju* (Fr.) Singer. H.-Y. Shin et al. reported an activation of AMP-activated protein kinase by extract of six herbal medicines (OB-1).

In this special issue, 11 articles focus on the potential benefits of various herbal extracts/phytocompounds on diabetes-induced insulin resistance, nephropathy, retinopathy, cardiomyopathy, and inflammation. A. l. Al-Malki showed oat extract to be beneficial for diabetic nephropathy and retinopathy by modulating nuclear factor kappa B (NF-kB). Inhibition of aldose reductase activity by scopoletin ameliorated cataractogenesis in galactose-fed rats (J. Kim et al.). *Boehmeria nivea* extract (S. H. Kim et al.) and swertiamarin (T. P. Patel et al.) regulated experimental insulin resistance by modulating PPARγ. In another report, S.

Kadan et al. evaluated effect of eight antidiabetic medicinal plants extracts on GLUT 4 translocation. The benefits of fisetin and an ayurvedic herbal formulation (Kal-1) on diabetes-induced inflammation was also reported. Further, quercetin was shown to preserve β-cell mass and function in fructose-fed hyperinsulinemic rats via modulating pancreatic akt/foxo1 activation. Berberine ameliorated glucose- and insulin-induced cardiomyocyte hypertrophy by modulating PPARα/NO. In an interesting article by S. E. Martinez et al. pharmacometrics of an antidiabetic compound, 3-methoxypterostilbene was reported. 3-Methoxypterostilbene inhibited α-glucosidase and α-amylase activity and exhibited approximately 50% bioavailability.

Three review articles were also incorporated in this special issue. A review by Y. Liu et al. (recent updates on beneficial role of berberine in controlling NASH) provides detailed account on molecular regulation of lipid metabolism and NASH by berberine. In another review by C. D. Lorenzo et al. the use of *in vitro* and clinical approaches to assess the benefits of plant food supplements is critically discussed. The potential benefits of Kampo, a Japanese traditional medicine, in treating obesity was reviewed by J.-i Yamakawa et al. based on basic and clinical evidence.

The only clinical study as a part of this special issue focuses on evaluating body weight lowering effects of herbal extract-THI (target herbal ingredient) on exercising healthy overweight humans following a two-month randomized double-blind, placebo-controlled trial. The study reports a significant reduction in body weight indicating its potential antiobesity effect.

We envisage that this special issue will attract broad interest in the fields of obesity, metabolic syndrome, and type 2 diabetes and encourage the perusal of in-depth molecular and cellular mechanistic investigations into the use of natural products, in particular the herbal therapies for metabolic disorders and their complications.

Acknowledgments

Firstly we express our sincere thanks and gratitude to the Editorial Board of eCAM for their approval on this concept and continuous help in successful publication of this special issue. We would also like to thank contributors of this special issue for their scientifically sound paper. With great pleasure and respect we extend our thanks to the reviewers for critical assessment of each paper, their constructive criticisms, and timely response that made this special issue possible.

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Research Article

Immunomodulatory Role of an Ayurvedic Formulation on Imbalanced Immunometabolics during Inflammatory Responses of Obesity and Prediabetic Disease

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Kal-1 is a polyherbal decoction of seven different natural ingredients, traditionally used in controlling sugar levels, inflammatory conditions particularly regulating metabolic and immunoinflammatory balance which are the major factors involved in obesity and related diseases. In the present study, we aimed to investigate the effect of Kal-1 (an abbreviation derived from the procuring source) on diet-induced obesity and type II diabetes using C57BL/6J mice as a model. The present study was performed with two experimental groups involving obese and prediabetic mice as study animals. In one, the mice were fed on high-fat with increased sucrose diet, and different amounts (5, 20, and 75 μL) of Kal-1 were administered with monitoring of disease progression over a period of 21 weeks whereas in the second group the mice were first put on the same diet for 21 weeks and then treated with the same amounts of Kal-1. A significant reduction in body weight, fat pads, fasting blood glucose levels, insulin levels, biochemical parameters, immunological parameters, and an array of pro- and anticytokines was observed in obese and diabetic mice plus Kal-1 than control (lean) mice fed on normal diet. In conclusion, Kal-1 has immunomodulatory potential for diet-induced obesity and associated metabolic disorders.

1. Introduction

Globally, around 1.5 billion of the world's population are obese due to energy imbalance between calories consumed and calories expended [1]. Obesity is an inflammatory state particularly affecting the endocrine tissues mainly adipose tissues and is referred to as subclinical chronic mild inflammation, which is distinctive of clinical classic acute inflammation [2]. Fat tissue comprises a number of cell types primarily adipocytes, vascular endothelial cells, fibroblasts, and macrophages. Adipose is largely found in areas enriched for loose connective tissue. The urge to better understand the adipose architecture has provided answers to a lot of intriguing questions. The inflammation embarks on adipocytes, which are major endocrine cells that have specialization in lipid storage, and with the expansion of adipocytes mass (both hypertrophy and hyperplasia), inflammation increases. The chronic low-grade inflammation activates the innate immune system that subsequently leads to insulin resistance and further leads to type II diabetes mellitus. The mechanism and association of complete molecular and cellular inflammation between obesity and insulin resistance are just beginning to be revealed [3].

An important source of expansion or accumulation of fat in adipose tissue is consumption of high-fat high-sugar diet (HFHSD). Fat accumulation is closely associated with qualitative changes in lipoproteins (low- and high-density), cholesterol, and triglycerides. It is also suggested that free fatty acid production is increased during adipocyte mass expansion in obese state, and it is being implicated that it could be playing an important role in blocking the insulin signal transduction [4].

Adipose tissue is of two different types: the white (WAT) and brown (BAT) adipose tissues; both can be clearly distinguished at morphological and functional level. BAT is known for heat production by thermogenesis, whereas WAT is considered as important endocrine tissue and contributes to the pathogenesis of insulin resistance and regulation of

metabolic inflammation [5]. WAT (subcutaneous and epididymal) is a known site for storing calories as triglycerides and main site of inflammation related to obesity [6]. Along with inflammatory modulators such as leptin, resistin, and adiponectin, a number of pro- (IL-1β, IL-6, IL-10, and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) are also secreted by WAT. These adipokines dynamically affect metabolism as their production is considered to be regulated by nutritional state [7]. Anomalous production of mentioned adipocytokines and activation of inflammatory signaling pathways, namely, Jun N-terminal kinase (JNK) and inhibitor of NF-κB kinase (IKK), are closely associated with chronic low-grade inflammation [8].

Though both WAT and BAT have unique specific roles in the body, WAT depending upon the location in the body can serve different functions [9]. The latter indicates that the variations in fat distribution in humans are correlated with metabolic disorders. In fact, now it is well appreciated that differentiated brown and white adipocytes have significant transcriptional, secretory, and morphological differences [10].

It is clear that the myriad of roles that adipose tissue plays in the body, together with an increasing relevance of understanding adipose tissue as it relates to obesity, calls for a need and importance of better understanding this tissue. A recent study done in mice using known stem cell surface markers has shown the importance of a set of markers which could be useful in enriching cells in fat tissue likely to be a white adipocyte precursor population [11]. However, there still remains lack of useful unique markers of white adipocyte precursors or adipocytes available for fat research [12]. These observations not only amplify the importance of understanding the histoarchitectural features of adipose tissue which can be clearly used as one of the significant markers underlying obesity.

In modern era of pharmaceutical, most of the antiobesity and antidiabetic drugs have been found to be inconsistently effective and also have their associated side effects. As an alternative form of medicine, herbal or ayurvedic (a typical antique and religious type of medicine predominantly practiced in Asia) formulations are now being increasingly considered worldwide because of their utmost least toxic nature and side effects compared to synthetic drugs. These herbal formulations are well known to treat metabolic disorders including obesity and diabetes. For instance, Shao et al. [13] described the role of curcumin as an antiobesity and diabetes herbal medicine in an organized manner. Still, there are very limited systematic studies on the effect of herbal formulations on metabolic immunobalance in obese and diabetic individuals.

This present study was conducted to test one such formulation, Kal-1, a polyherbal decoction of seven different natural ingredients (see Supplementary Table 1 in Supplementary Materials available online at http://dx.doi.org/10.1155/2013/795072), for its effect on the metabolic and immunoinflammatory balance in mouse model of diet-induced obesity and diabetes. Although Kal-1 is a proprietary product of Kerala Ayurveda, Kerala, India, the procuring source has authorized both (1) the use of Kal-1 name in the paper and (2) the listing of specific ingredients of Kal-1 in Supplementary Table 1. We evaluated the efficacy of Kal-1 as antiobesity and antidiabetic agent, in addition to its utility in controlling low-grade systemic inflammation and the overall energy equilibrium. We report here that, in addition to ameliorating the symptoms both of obesity and diabetes, Kal-1 administration also restored the normal balance of pro- versus anti-inflammatory cytokines, thereby skewing the immune response to more of anti-inflammatory type. Importantly, this activity was evident in regimens that probed possible potential value of Kal-1 to be explored further for supplementing it from the nutrient or food perspective to control imbalanced immune responses and resulting metabolic disease entities.

2. Materials and Methods

2.1. Preparation of Kal-1. Kal-1 formulation is essentially a concoction of seven different ingredients implicated to play a protective role in inflammation. It is prepared using a methodology prescribed by the ancient ayurvedic texts. Briefly, the different ingredients are washed, cleaned, dried, and sieved to get a coarse powder. The latter is then steamed and boiled till the starting volume reduces to one-eighth. This is followed by filtration and boiling till the decoction reaches one-fourth of the volume from the 1st filtrate (Supplementary Figure 9). The detailed protocol belongs to Kerala Ayurveda whose proprietary product is Kal-1. The resulting filtrate is then used as Kal-1, which is a dark brown liquid with a peculiar rotten leaf-like smell. Kal-1 dose to be administered in mice was calculated using human to mice dose conversion formula as described elsewhere [14].

2.2. Animals, Diets, and Experimental Setup. All animal studies were carried out at BIONEEDS Laboratory Animals & Preclinical Services, Bangalore, India, and approved by institutional animal ethics committee (IAEC). All experimental protocols were done as per applicable national and international guidelines. BIONEEDS is approved by committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Forests and Environments, Government of India. Briefly, 3-4-week-old male C57BL/6J mice (7–9 gm) were housed (3–5 animals per cage) under standard conditions. All animals were initially put on two different diets (normal diet (LFD, D12492) containing 10% kcal fat and high fat with increased sugar diet (HFHSD, D03062301) containing 60% kcal fat in pellet forms, procured from Research Diet, NJ, USA). The first two weeks without the formulation were considered as "acclimatization phase" wherein mice were given their respective diets only before starting the formulation. The administration of Kal-1 was done in two different disease rescue (up to 21 weeks) and treatment (up to 30 weeks) experiments in which mice were divided into five groups—LFD control group, HFHSD control group, and three different amounts of Kal-1 (5, 20, and 75 μL) supplemented with high-fat high-sugar diet (HFHSD + Kal-1) test groups. In rescue experiments, all three amounts of Kal-1 were administered for next 19 weeks after acclimatization period, whereas Kal-1 with the same

amounts were administered only for 8 weeks after 22 weeks in treatment experiments. The feed intake was monitored daily, which included residual feed quantification as well, and body weights were recorded twice a week.

2.3. Tissues Isolation and Blood Collection. At an interval of three weeks, that is, weeks 3, 6, 9, 12, 15, 18, and 21 over the entire experimental period, mice were kept on fasting for a period of 5-6 hours prior to blood collection and then anesthetized with ether in rescue experiment, whereas the same procedure was followed only at weeks 26 and 30 in treatment experiment. White adipose tissues (epididymal and subcutaneous) fat depots were removed carefully in both the cases at mentioned time points, thoroughly rinsed with phosphate buffer saline, and weighed.

Blood was collected from retroorbital sinus for serum separation. Blood glucose levels were measured by using a glucometer (Roche Diagnostics GmbH, Germany) at the above-mentioned time periods.

2.4. Biochemical Analysis. The serum concentrations of low-density lipids (LDL), high-density lipids (HDL), total cholesterol, and triglycerides were assayed enzymatically by using an automatic analyzer (ERBA, automated random access clinical chemistry analyzer, EM260, Mannheim, Germany) with their respective kits. The serum insulin levels were measured using commercially available ELISA kit (ALPCO ultrasensitive mouse insulin kit, Salem, NH, USA) for mouse. Leptin and resistin levels were measured using commercially available radioimmunoassay kits (Quantikine, mouse leptin and mouse resistin, immunoassay, R&D, Minneapolis, MN, USA) and high-molecular-weight adiponectin (ALPCO, adiponectin mouse total, HMW, Salem, NH, USA).

2.5.Cytokine Measurement. An array of seven cytokines (pro- and anti-inflammatory) was measured in serum of the experimental groups. Briefly, sera from the blood were used to detect the following cytokines, namely, IL-1α, IL-1β, IL-4, IL-6, IL-10, TNF-α, and MCP-1, using the Luminex system (Liquichip 200, Luminex xMAP Technology, Valencia, CA, USA) as per the manufacturer's protocols. Kits for multiplex analysis were obtained from Millipore, Billerica, MA, USA. All samples were run in triplicates.

2.6. Statistics. To check the significant difference between LF and HF control groups, we performed Student's t-tests whereas one-way ANOVA followed by Tukey's multiple comparison test was used to compare between control groups (LF and HFHS) and test groups (HFHS supplemented with different amounts of Kal-1 doses). P value less than 0.05 was considered to be statistically significant. All the data was expressed as mean ± SEM ($n = 5$, each group). Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Simultaneous Kal-1 Administration Mitigates the Effects of HFHSD on Systemic Inflammation and Metabolic Dysfunction in Mice. Here, we evaluated the effect of LFD, HFHSD, and Kal-1 supplemented HFHSD group in C57BL/6J mouse model up to 21 weeks. We monitored body weights, metabolic biochemical parameters, and immunological readouts like hormones and cytokines.

To assess the potential effects of KAL-1 on body weight regulation, we observed body weights of animals fed on LFD and HFHSD for a period of twenty-one weeks (a period including fifteen days of acclimatization). Wherein, we screened several amounts of Kal-1 ranging from very low (0.04 μL) to high amounts (300 μL). A total of 275 mice were grouped (5 mice/group) into eleven different groups, namely, LF control, HFHS control, and HFHS supplemented with nine different test amounts Kal-1. A clear-cut dose-dependent effect of Kal-1 was observed over the entire experimental phase (week 3 to week 21) at all amounts of Kal-1. However, this trend excluded the two higher amounts of 150 and 300 μL (that could be potentially toxic). Body weights at Kal-1 amounts of 5, 20, 38, and 75 μL were observed to be closely comparable to LF control group (Supplementary Figure 1). Therefore, for all further experiments, we mainly focused on three Kal-1 amounts of 5, 20, and 75 μL.

Additionally, a significant difference in mean body weights of LF and HFHS control groups was observed (8.8 gm or 26.3%, $P < 0.0005$; Figure 1(a)); HFHSD-fed animals were heavier than LFD-fed animals at week 21. The closer group to LF control group was HFHS + 20 μL Kal-1 with the difference of 1.1 gm or 3%. A difference in body weights of HFHS + 5 μL Kal-1 and LF control group was 2.4 gm or 7.1%. Though, HFHS + 75 μL Kal-1 treatment group showed a different profile (lower than LF control group) with differences in body weights being 7.2 gm or 21.5% (Figure 1(a)). However, all the differences among experimental and control groups were found to be statistically nonsignificant ($P > 0.05$) when analyzed by ANOVA followed by Tukey's multiple comparison test.

Further, in order to ensure that any changes or effects observed were true effects of Kal-1, gavage control alone was also put (wherein the mice were administered same volume of distilled water). There was no difference observed in the weight of the animals between the control groups and gavage control group (Supplementary Figure 2).

Furthermore, similar effect of Kal-1 was also observed on the weights of fat pads, that is, WAT. At week 21, the mean of relative weights of WAT, namely, epididymal and subcutaneous fat depots, was significantly higher in HFHS control animals than LF control animals (4 gm and 5.6 gm, resp.). Similarly, epididymal fat pads on analysis using one-way ANOVA followed by Tukey's multiple comparison test for experimental (HFHS + KAL-1 20 μL and 75 μL treated animals) and control group (LFD) were found to be different but not significant. Differences among experimental (HFHS + KAL-1 5 μL, 20 μL, and 75 μL treated animals) and control groups (LFD) on analysis using one-way ANOVA

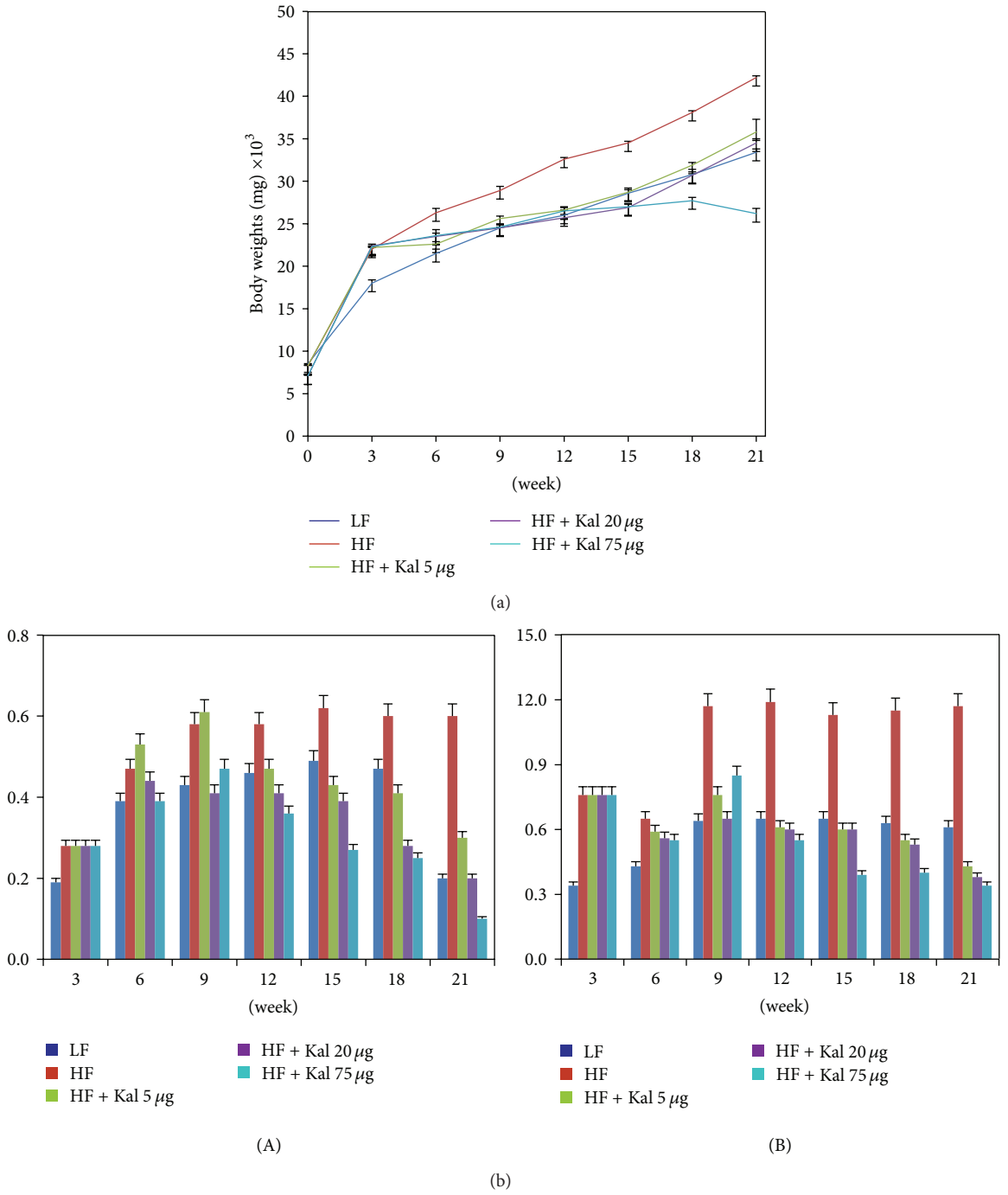

Figure 1: Body and tissue weights in normal diet fed control were higher than high-fat high-sugar diet fed control, and Kal-1 doses rescue mice fed on high-fat high-sugar diets from being obese. (a) Weekwise effect of Kal-1 on body weights in high-fat high-sugar fed mice. (b) Effect of Kal-1 on tissue weights in high-fat high-sugar fed mice at week 21. (A) Epididymal fat; (B) subcutaneous fat. All doses (5, 20, and 75 μL) of Kal-1 were supplemented along with HFHSD. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

followed by Tukey's multiple comparison test for weight of subcutaneous fat pads were also found to be nonsignificant (Figure 1(b)).

3.2. Amount of Feed Taken Does Not Affect the Weight of Animals as Confirmed by Pair Fed Experiments. Since gain in body weight was significantly higher in mice receiving HFHSD as compared to the group being fed on LFD and the HFHSD + Kal-1 treated group was almost comparable with LFD control group, we measured the feed intake and body weights up to 21 weeks in all the groups including pair-fed group to demonstrate that the difference between LFD and

HFHSD control groups was due to high carbohydrate with increased sucrose content in HFHSD, not due to more eating of HFHSD (Supplementary Figures 3 and 4). Furthermore, to verify the effect of Kal-1 on body weights of mice, we monitored the *ad libitum* feed intake in HFHSD fed control mice and restricted the amount of feed to HFHSD + pair-fed control and HFHSD + pair-fed + Kal-1 test groups. Supplementary Figure 4 shows that body weights of HFHSD + pair-fed control and Kal-1 supplemented test groups gained weight (47.0 and 35.1 gm, resp.) almost similar to HFHSD control group and LFD control groups (47.9 and 35.7 gm, resp.) at week 21. These findings indicate that HFHSD diet promoted increase in animal body mass, and KAL-1 is effective in reducing body weight gain.

Furthermore, there was no observable change in the core body temperature in either control or Kal-1 supplemented test group. Rectal temperature of all the animals from pair-feeding experiment was also recorded for all four groups from week 15 to week 21 and it was ranging from 94.2°F to 98.2°F (Supplementary Figure 5), which was again normal.

3.3. Kal-1 Rectifies the Metabolic Imbalance in HFHSD Fed Mice

3.3.1. Fasting Blood Glucose and Insulin Profiles. To determine that high-calorie diet results in a shift in immunobalance which leads to symptoms towards development of diabetes, blood glucose levels were also recorded. This was done after 5-6 hours of fasting for all 275 animals which were further grouped (11 groups) in the same manner as mentioned earlier (Supplementary Figure 6).

In comparison to LFD fed animals, blood glucose levels were significantly elevated ($P < 0.005$) in HFHSD fed animals. Differences among experimental (HFHS + KAL-1 5 μL and 75 μL treated animals) and control group (LFD) were found to be statistically significant ($P < 0.05$) whereas HFHS + KAL-1 20 μL test animals were found to be nonsignificant though comparable on analysis using one-way ANOVA followed by Tukey's multiple comparison test (Figure 2(a)).

Fasting serum insulin levels were also measured for LF, HFHS, HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL test groups. At week 21, serum insulin levels were 0.2 ng/dL, 0.3 ng/dL, and 0.4 ng/dL higher in HFHS + Kal-1 20 μL, HFHS + Kal-1 5 μL and HFHS groups, respectively, than LF animals. On analysis of differences among HFHS and HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups using one-way ANOVA followed by Tukey's multiple comparison test, and it was found to be significant only for HFHS and HFHS + Kal-1 75 μL groups ($P < 0.05$).

3.3.2. Effect on HDL, LDL, Cholesterol, and Triglycerides Levels. At week 15, nonsignificant elevated levels (12%, 45.3%, 31.6%, and 19.8%) of fasting serum in HFHS control animals were noticed for HDL, LDL, cholesterol, and triglycerides, respectively, as compared to LF group. Similar to body weight and insulin profile, dose-dependent effects of Kal-1 were seen in HDL, cholesterol, triglycerides, and LDL levels. However, differences among LF and HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups were found to be significant with HFHS + Kal-1 5 μL for cholesterol whereas both groups HFHS + Kal-1 5 μL and HFHS + Kal-1 20 μL were found to be significant ($P < 0.05$) for LDL levels and nonsignificant for HDL and triglycerides levels on analysis using one-way ANOVA followed by Tukey's multiple comparison test.

3.4. Kal-1 Corrects Immunological Readouts in HFHSD Fed Mice. HFHSD altered all the immunological readouts, namely, hormones and cytokines pattern in HFHSD group in comparison to LFD group. These altered patterns came back to the normal when Kal-1 was administered in the similar groups of HFHSD, and these parameters were tracked over the same time phase as done earlier for body weights and biochemical readouts.

3.5. Hormone and Cytokine Production is Affected by HFHSD Intake and Kal-1 Administration. We noticed dose-dependent effect of Kal-1 for all three hormones, namely, resistin, leptin, and HMW adiponectin at week 15. The differences between LFD and HFHSD control groups were 50% ($P < 0.005$), 56% ($P < 0.0005$), and 38% ($P < 0.0001$) for resistin, leptin, and HMW adiponectin, respectively, which were statistically significant (Figures 3(a), 3(b), and 3(c)). Regulation for both leptin and resistin was exhibited at Kal-1 dose of 20 μL which was in concordance with other previous observations. However, comparison between LF and HFHS + Kal-1 5 μL; HFHS + Kal-1 20 μL and HFHS + Kal-1 75 μL groups was found to be significant with HFHS + Kal-1 75 μL ($P < 0.05$) only on analysis using one-way ANOVA followed by Tukey's multiple comparison test for leptin. Differences among experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control groups (HFD) on analysis using one-way ANOVA followed by Tukey's multiple comparison test were found to be statistically significant ($P < 0.05$) with HFHS + Kal-1 5 μL and HFHS + Kal-1 20 μL for adiponectin.

Two panels of cytokines, namely, pro- (IL-1α, IL-1β, IL-6, MCP-1, and TNF-α) and anti-inflammatory (IL-4 and IL-10), were analyzed in serum at week 15. Statistically significant differences were observed in LFD and HFHSD groups (33 pg/mL, $P < 0.0001$ and 38 pg/mL, $P < 0.0001$) for IL-4 and IL-10 concentrations, respectively. These results suggest that increased body weight and related metabolic disorders due to HFHSD also affected the concentrations of anti-inflammatory cytokines.

Similar to IL-4 and IL-10, t-test showed that all studied proinflammatory cytokines were significantly different between LFD and HFHSD fed animals. The highly significant difference in values ranged between 57 pg/mL ($P < 0.00001$), 161 pg/mL ($P < 0.00001$), 112 pg/mL ($P < 0.00001$), 67 pg/mL ($P < 0.000008$), and 226 pg/mL ($P < 0.000005$), respectively for IL-1α, IL-1β, IL-6, MCP-1, and TNF-α.

Differences among experimental group (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control group (LFD) on analysis using one-way ANOVA followed by Tukey's multiple comparison test were found to be statistically significant ($P < 0.05$) for all with IL-4 and

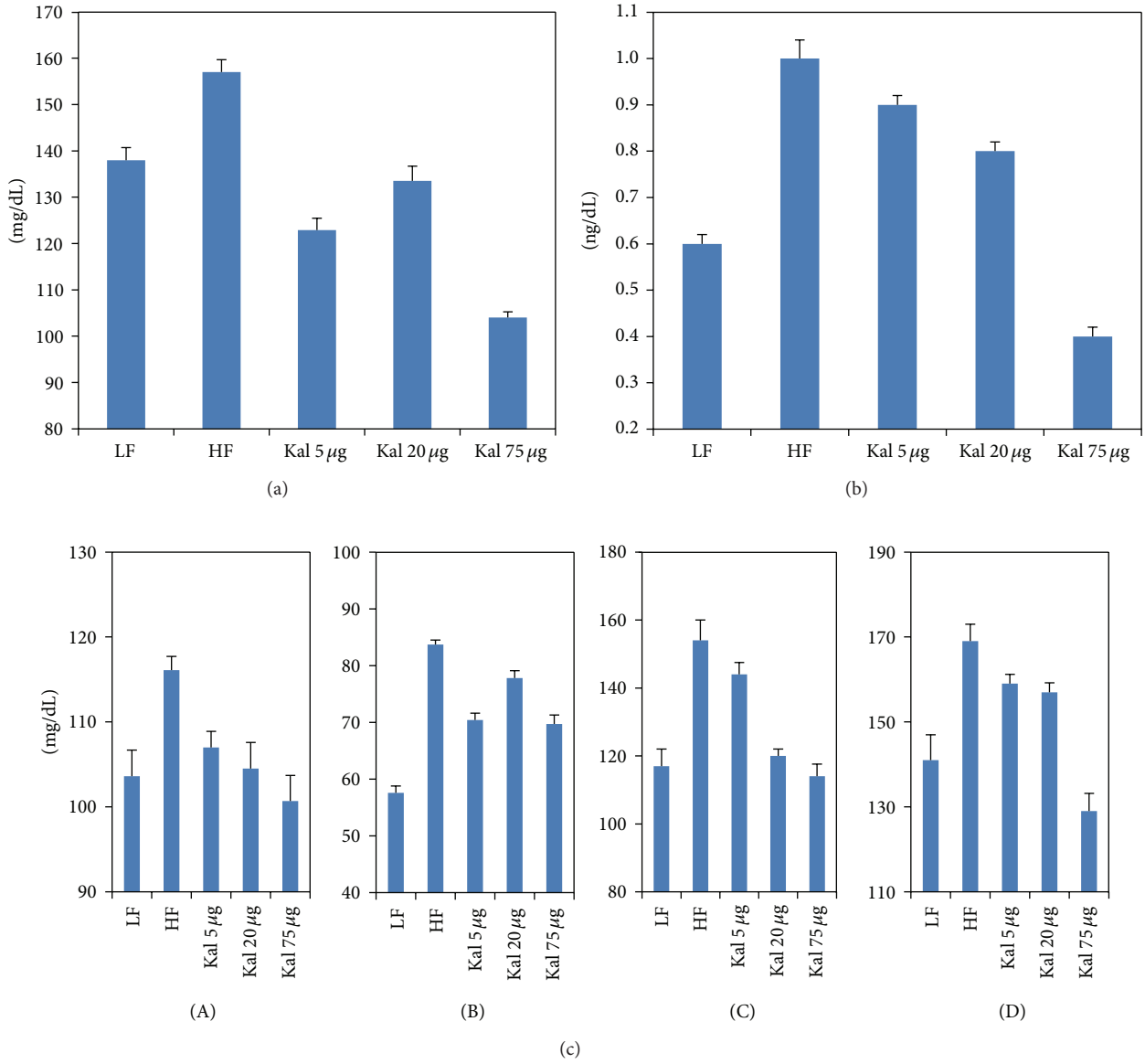

Figure 2: Kal-1 rectifies the metabolic imbalance in mice fed on high-fat high-sugar diets. (a) Effect of Kal-1 on fasting blood glucose levels in high-fat high-sugar fed diet at week 21. (b) Effect of Kal-1 on fasting insulin levels in high-fat high-sugar fed diet at week 21. (c) Effect of Kal-1 on various biochemical parameters (fasting) in high-fat high-sugar fed diet at week 15. (A) HDL. (B) LDL. (C) Cholesterol. (D) Triglycerides. All doses (5, 20, and 75 μL) of Kal-1 were supplemented along with HFHSD. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

nonsignificant for HFHS + Kal-1 75 μL group with IL-10. Comparison between experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control group (LFD) for pro- (IL-1α, IL-1β, IL-6, MCP-1, and TNF-α) inflammatory cytokines was found to be statistically significant ($P < 0.05$) except for IL-1α for which HFHS + Kal-1 20 μL group was nonsignificant on analysis using one-way ANOVA followed by Tukey's multiple comparison test.

Thus, both of the readouts, namely, hormones and cytokines concentrations, showed marked levels of correction in Kal-1 supplemented group.

3.6. Kal-1 Treatment Restores the Inflammatory Balance in Mice Fed on HFHSD. We also monitored the effect of Kal-1 after 21 weeks of obesity induction by feeding the mice on HFHSD, which was the reverse of what we did earlier. Here also we observed similar trends in correction for body weights, fasting blood glucose, blood biochemistry, serum hormones, and cytokines in the same manner as described earlier with the same number of mice per group.

Kal-1 at the three above-mentioned amounts (5, 20, and 75 μL) was then put forth to be administered for next 8 weeks in the HFHSD group. We observed that 8-week

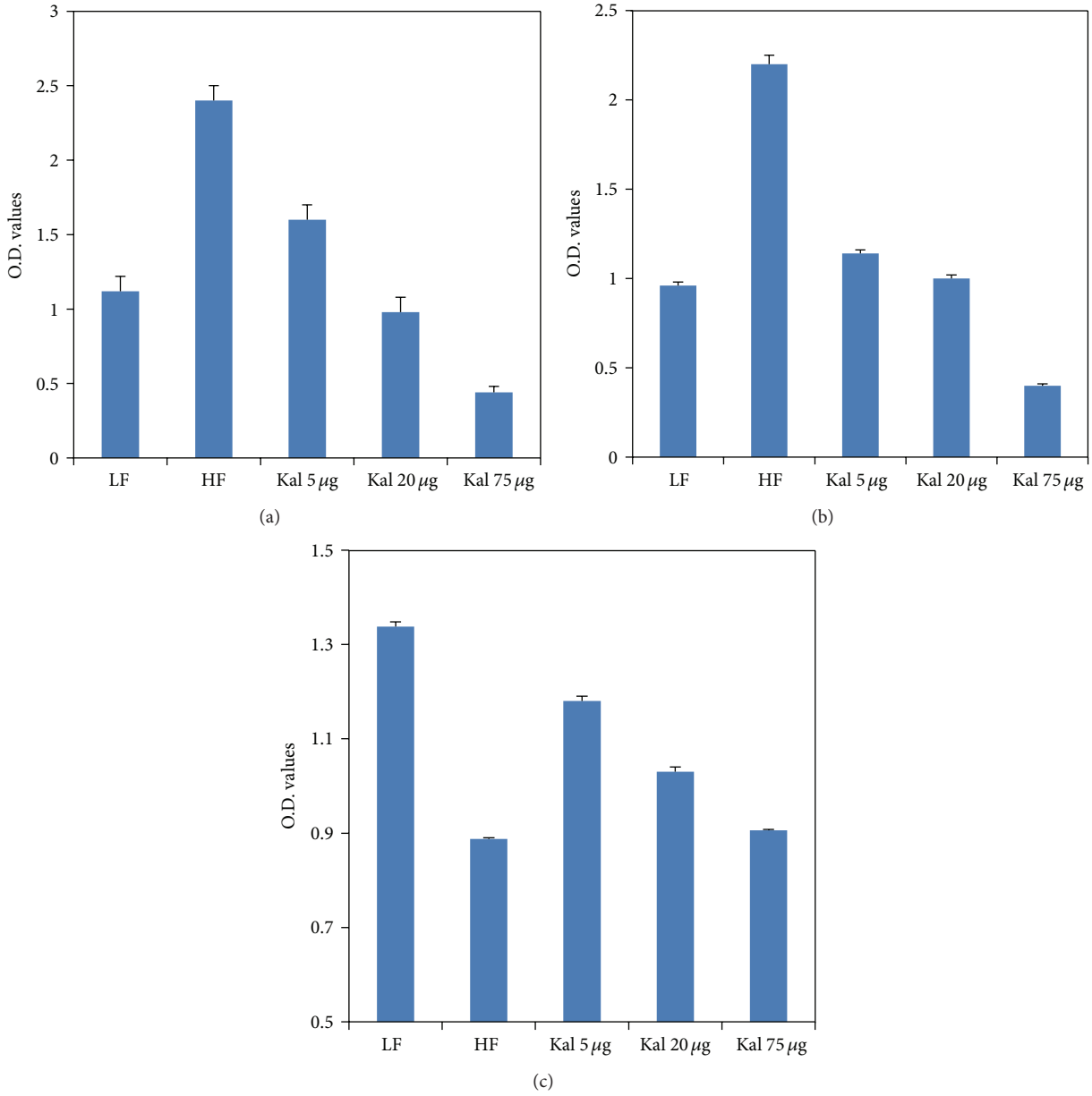

Figure 3: Kal-1 rectifies the hormonal imbalance in mice fed on high-fat high-sugar diets. (a) Effect of Kal-1 on resistin levels in high-fat high-sugar fed mice at week 15. (b) Effect of Kal-1 on leptin levels in high-fat high-sugar fed mice at week 15. (c) Effect of Kal-1 on high-molecular-weight adiponectin levels in high-fat high-sugar fed mice at week 15. All doses (5, 20, and 75 μL) of Kal-1 were supplemented along with HFHSD. LF: low fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

short-term dietary treatment of Kal-1 at 75 μL significantly reduced ($P < 0.05$) the body weight of HFHSD fed animals instead of HFHSD + Kal-1 at 20 μL test animals (as in rescue experiments) on comparison among experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL group) and control groups (HFD) using one-way ANOVA analysis followed by Tukey's multiple comparison test (Figure 5(b)), though the effect of Kal-1 was again dose dependent as seen earlier (Figure 5(a)(A, B)).

3.7. Treatment with Kal-1 Modulates Blood Glucose and Serum Insulin Levels in Obesity-Induced Mice by HFHSD. Fasting blood glucose and serum insulin levels were measured at week 30 in experimental mice to assess the effect of treatment with Kal-1 on these aspects. Fasting blood glucose value of HFHSD fed mice was significantly higher (25 mg/dL or 17%, $P < 0.005$) than LFD fed mice at week 30. The mean concentrations of fasting blood glucose in the Kal-1 75 μL treated mice were significantly less (22 mg/dL, 15% $P < 0.05$) than in mice fed on HFHSD. The rest two amounts of 5 μL and 20 μL of Kal-1 treated animals were found to be nonsignificant on analysis using one-way ANOVA followed by Tukey's multiple comparison test (Figure 5(b)). Moreover, the fasting serum insulin levels were increased >2-fold in HFHS grouped mice

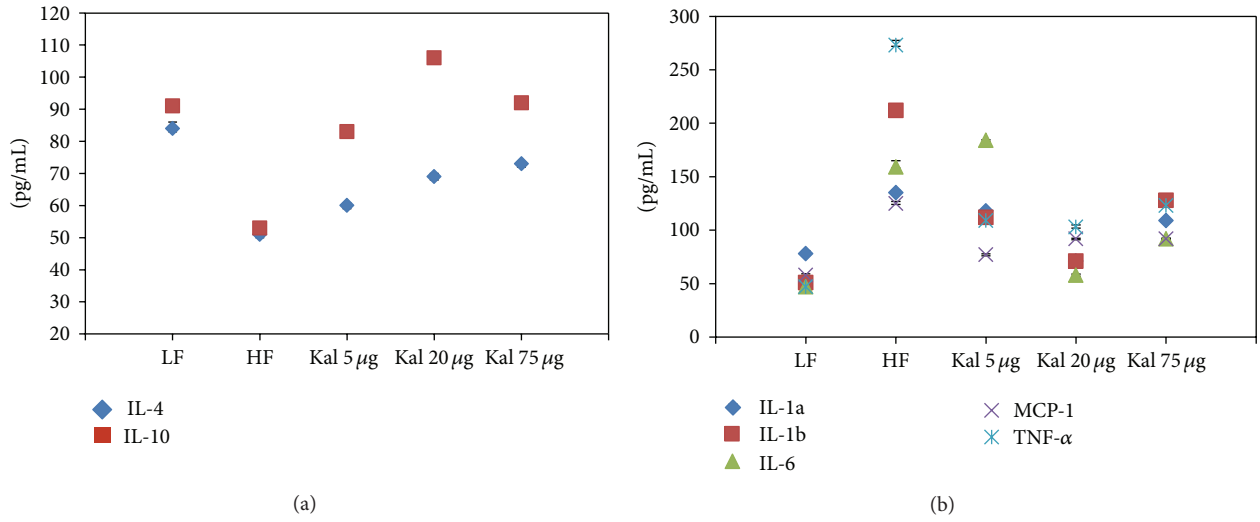

FIGURE 4: Kal-1 rectifies the inflammatory cytokines imbalance in mice fed on high-fat high-sugar diets. (a) Effect of Kal-1 on anti-inflammatory cytokines in high-fat high-sugar fed mice at week 15. (b) Effect of Kal-1 on proinflammatory cytokines in high-fat high-sugar fed mice at week 15. All doses (5, 20, and 75 μL) of Kal-1 were supplemented along with HFHSD. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

than LF grouped mice. Differences among all experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control groups (HFD) on analysis using one-way ANOVA followed by Tukey's multiple comparison test were found to be statistically significant ($P < 0.05$). The levels of HFHS + Kal-1 5 and 20 μL test group were also comparable with LF control group but not as closer as those HFHS + Kal-1 75 μL test group (Figure 5(c)).

3.8. Functional Relevance of Dietary Treatment of Kal-1 on Serum Biochemistry. Following the same approach, serum biochemistry of individual mice in all the five groups (control groups and Kal-1 treated groups) were also examined. All parameters like serum HDL, LDL, cholesterol, and triglycerides were distinguishable between LF and HFHS control groups, although the differences in only HDL and cholesterol levels were statistically significant. The values for HDL and cholesterol of HFHS + Kal-1 75 μL test group were comparable (6 mg/dL or 7.5% and 17 mg/dL or 15.7%, resp.) with LF control group. However, the same was statistically significant ($P < 0.05$) only for cholesterol on analysis using one-way ANOVA followed by Tukey's multiple comparison test. In contrast, values of LDL and triglycerides for HFHS + Kal-1 75 μL test group were found to be little less (1 mg/dL or 2.3% and 3 mg/dL or 2.6%) than LF control group and nonsignificant on analysis using one-way ANOVA followed by Tukey's multiple comparison test (Figures 6(a), 6(b), 6(c), and 6(d)).

3.9. Antiobesity Effect of Kal-1 as Assessed through Measurement of Hormones and Cytokines Secreted during Disease State. To further examine the treatment effect of Kal-1 on pro- and anti-inflammatory parameters, a set of two hormones and seven cytokines was analyzed in the serum of LF and HFHS controls and HFHSD fed mice supplemented with Kal-1. For serum leptin and HMW adiponectin levels, a significant alteration was detected in LF and HFHS control groups ($P < 0.02$ and $P < 0.05$, resp.) at week 30. And differences among experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control groups (HFD) on analysis using one-way ANOVA followed by Tukey's multiple comparison test were found to be statistically significant ($P < 0.05$) with HFHS + KAL-1 20 μL and 75 μL for leptin only and nonsignificant with HFHS + KAL-1 5 μL treated animals for both leptin and adiponectin (Figures 7(a) and 7(b)).

Figure 8(a) revealed that serum levels of LF control mice were 40% ($P < 0.01$) and 47% (0.005) higher than those of HFHS control mice in anti-inflammatory cytokines, IL-4 and IL-10, respectively. On the other hand, 72% and 65% statistically significant decrease ($P < 0.05$) in the levels of both cytokines—IL-4 and IL-10, respectively—were observed for HFHS + Kal-1 75 μL group than LF control group. However, the levels of both cytokines in all test groups (HFHS + Kal-1 5 μL, 20 μL, and 75 μL) were found to be nonsignificantly lower than HFHS control group at week 30. The analysis was done using one-way ANOVA followed by Tukey's multiple comparison test, whereas at week 30, HFHSD fed animals showed significant increase in the pro-inflammatory cytokines levels (Figure 8(b)), when compared with LFD fed animals ($P < 0.005$ for all except MCP-1), while Kal-1 at 75 μL resulted in significant reductions of 259 pg/mL, 198 pg/mL, 123 pg/mL, 88 pg/mL, and 382 pg/mL on the IL-1α, IL-1β, IL-6, MCP-1, and TNF-α levels, respectively, in the serum of HFHSD fed animals ($P < 0.05$ for all except MCP-1 and IL-6) at week 30 on comparison among experimental (HFHS + Kal-1 5 μL, HFHS + Kal-1 20 μL, and HFHS + Kal-1 75 μL groups) and control groups (HFD) on analysis using one-way ANOVA followed by Tukey's multiple comparison test.

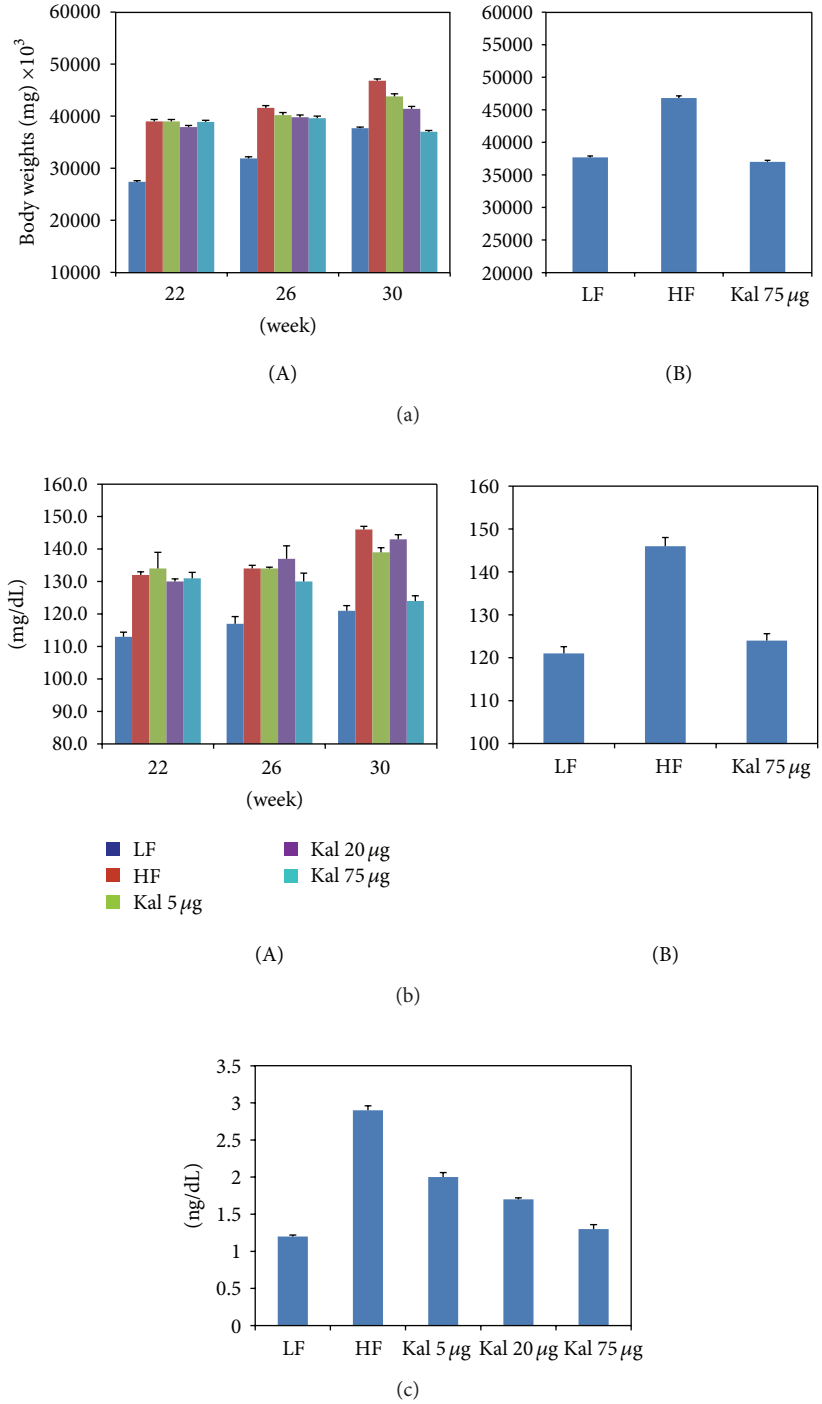

Figure 5: Body weights, fasting blood glucose, and insulin levels in high-fat high-sugar fed control were lower than normal diet fed control mice and Kal-1 treatment restored the body weights and blood glucose levels successfully. After obesity and diabetes induction period up to 21 weeks, Kal-1 treatment was started from week 22 to 30. (a)(A) Effect of Kal-1 treatment on body weights in high-fat high-sugar diet fed mice at weeks 22, 26, and 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. (B) Effect of Kal-1 treatment with optimum dose (75 μL) on body weights in high-fat high-sugar diet fed mice only at week 30. (b)(A) Effect of Kal-1 treatment on fasting blood glucose levels in high-fat high-sugar diet fed mice at weeks 22, 26, and 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. (B) Effect of Kal-1 treatment with optimum dose (75 μL) on fasting blood glucose in high-fat high-sugar diet fed mice only at week 30. (c) Effect of Kal-1 treatment on fasting insulin levels in high-fat high-sugar diet fed mice at week 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

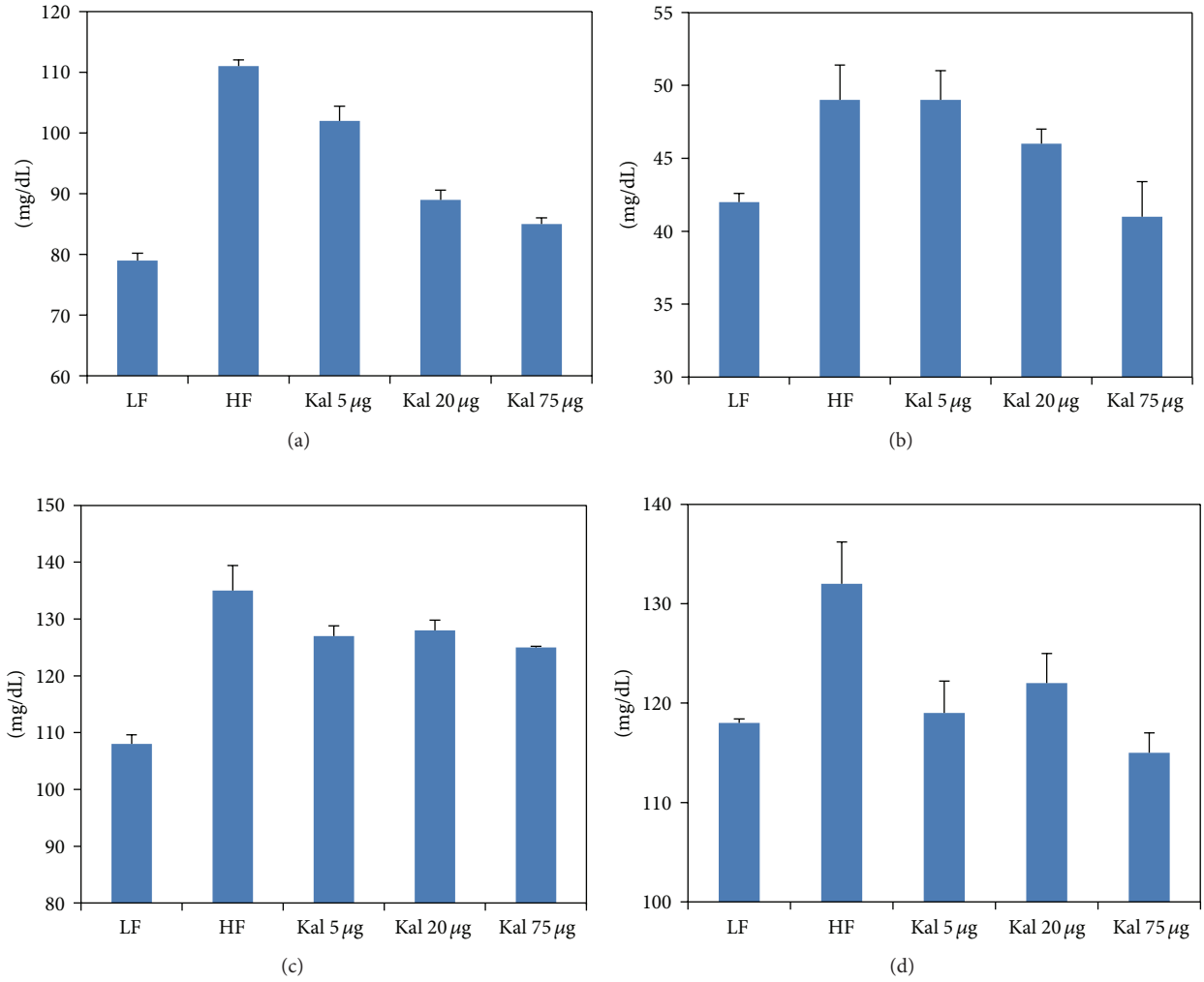

Figure 6: Kal-1 treats the irregularities in blood biochemical parameters in mice due to feeding on high-fat high-sugar diets at week 30. Effect of Kal-1 treatment on different biochemical parameters, namely, (a) HDL, (b) LDL, (c) cholesterol, and (d) triglycerides in high-fat high-sugar diet fed mice at week 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

4. Discussion

The present study confirms immunoregulatory effect of Kal-1, an ayurvedic formulation suggestive of controlling obesity and diabetes. Kal-1 is basically a decoction of seven different ingredients (with synergistic properties) which we suggest (based on information provided by the procuring source) could be useful in regulating heightened or disturbed immune response especially during chronic low-grade inflammatory conditions, namely, obesity and diabetes. We tested the above formulation in well-established diet-induced mice models (C57BL/6J strain of mice) using skewing of immune response from a pro- to anti-inflammatory as one of the key elementary readouts. The effect of Kal-1 on body fat mass, adipose tissues (epididymal and subcutaneous) weights, blood biochemistry including blood glucose level and insulin profile, and adipocytokines were monitored on HFHSD-induced experimental mice.

A number of *in vivo* studies have shown the effect of low- and high-fat diets on body weights, blood glucose level, and inflammatory markers [15–18]. It is noteworthy that diets play an important role in inflammatory modulation; especially high-carbohydrate diet directly contributes to fat mass expansion in adipose tissues and then leads to inflammation and insulin resistance [19]. In the present study, it is observed that HFD with increased sucrose significantly elevates the body weights, blood glucose, and serum insulin levels in mice than LFD fed mice over the observation period. Moreover, preliminary screening study of body weights in mice fed on HFHSD with the different dose amounts of Kal-1 was also performed; consequently, Kal-1 dose-dependent reduction in body weights was also observed (Supplementary Figure 1). On the basis of body weights reduction profile, a single amount of Kal-1, that is, 20 μL, was optimized. This was based on the observation that body weights of animals being fed on HFHSD along with this amount were equal to body weights of

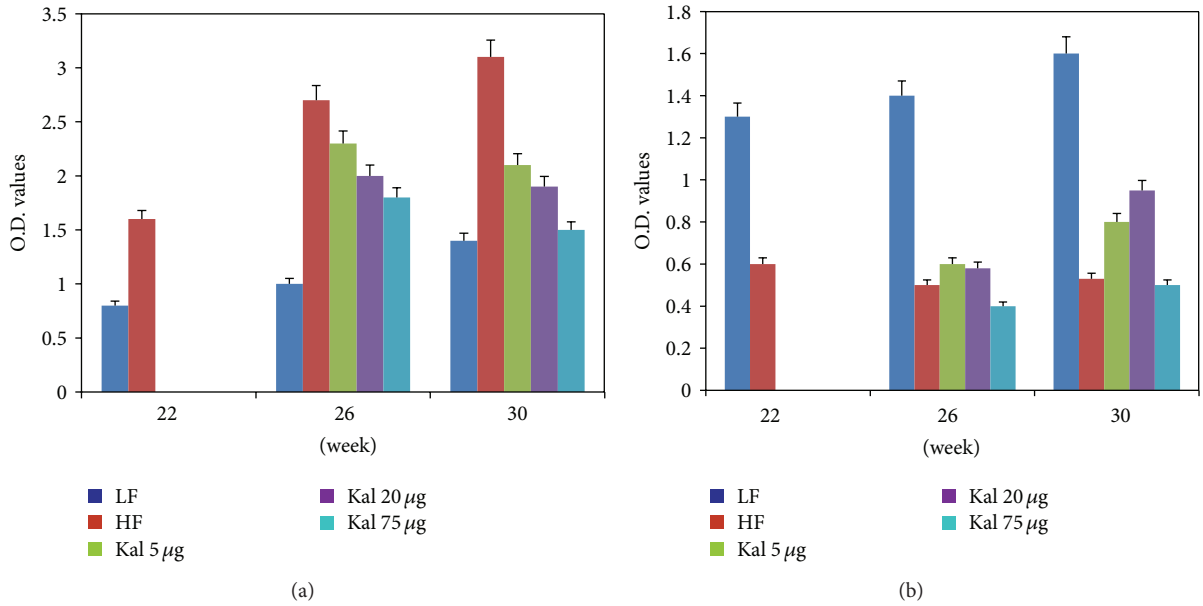

FIGURE 7: Treatment of Kal-1 brings back the levels of pro- and antihormonal levels to normal which were showing metabolic disturbed contour in high-fat high-sugar fed mice at weeks 22, 26, and 30. (a) Effect of Kal-1 treatment on leptin profile in high-fat high-sugar diet fed mice at weeks 22, 26, and 30. (b) Effect of Kal-1 treatment on high-molecular-weight adiponectin profile in high-fat high-sugar diet fed mice at weeks 22, 26, and 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. LF: low-fat control, HF: high-fat high-sugar control. All the values represent mean ± SEM from five animals.

LF fed mice in rescue experiment (Figure 1(a)). Surprisingly, same type of observations were also noticed with Kal-1 in treatment study (Figure 5(a)(A)); however, 75 μL, a higher amount of Kal-1, was the optimal dose (Figure 5(a)(B)). In the same manner, a decrease in the weights of epididymal and subcutaneous fat pads was also exemplified by Kal-1 (Figure 1(b)(A, B)).

With these observational facts, it may be speculated that Kal-1 is effective either at the level of regulating adipocyte hypertrophy, adipogenesis, or both. Second, alteration in the fatty acids present in HFHSD from monounsaturated fatty acid to saturated fatty acid due to Kal-1 is another possibility as circulating saturated fatty acids play a key role in obesity [20]. It must, however, be taken into account that HFHSD comprised almost equal amounts of both saturated and monounsaturated fatty acids (details not given).

Despite the fact that chronic low-grade inflammation is directly linked with the consumption of high-carbohydrate diets [21], sucrose is one of the important elements in HFD which is the leading cause of obesity, high blood sugar, and insulin resistance [22]. In this respect, expected higher levels of blood glucose and serum insulin levels were observed in obese mice control group (fed on HFHSD) than in lean mice control group (fed on LFD). Irrespective of the HFHS constituents in diet, significantly lower levels of blood glucose and serum insulin were observed in both test groups administered with Kal-1 20 and 75 μL (Figures 2(a), 2(b), 5(b)(B), and 5(c)), which could not be explained.

Furthermore, one metabolically active hormone is resistin, which is secreted by adipocytes and may contribute to obesity, insulin resistance, and diabetes in mice. In parallel with the observations of Steppan and Lazar [23], here we also show that serum resistin levels of lean mice were reduced up to more than 2-fold compared to obese mice. In accordance with glucose and insulin profile, decreased levels of resistin were observed in obese mice exposed to Kal-1 and were comparable to those in lean mice (Figure 3(a)). Therefore, it can be speculated that Kal-1 exhibits similar effects on resistin levels as is observed for blood glucose and serum insulin levels [23, 24].

Further, leptin is an adipokine, also secreted by adipocytes, considered a key pro-inflammatory cytokine. In addition to regulating food intake and energy homeostasis, this bioactive molecule also plays a potent role in modulating the immune response and inflammatory processes. Leptin is present in serum in direct proportion to the amount of adipose tissue; therefore, sum of energy in adipose tissue reveals the level of leptin in serum; that is, the more the energy the more the production of leptin. Similar to previous explanation [25], amount of energy stored in adipocytes of obese mice fed with HFHSD was higher than that in mice fed with LFD as leptin levels were found to be significantly more in obese mice than lean mice in current study. In corroboration with previous studies, increased leptin production can be positively correlated with adipocyte hypertrophy and hyperplasia [26, 27]. In both experiments, the serum from mice on HFHSD supplemented with Kal-1 showed that leptin levels came back to the normal levels almost comparable with the levels seen in the LF diet group (Figures 3(a) and 7(a)). One possible explanation could be that Kal-1 stimulates and

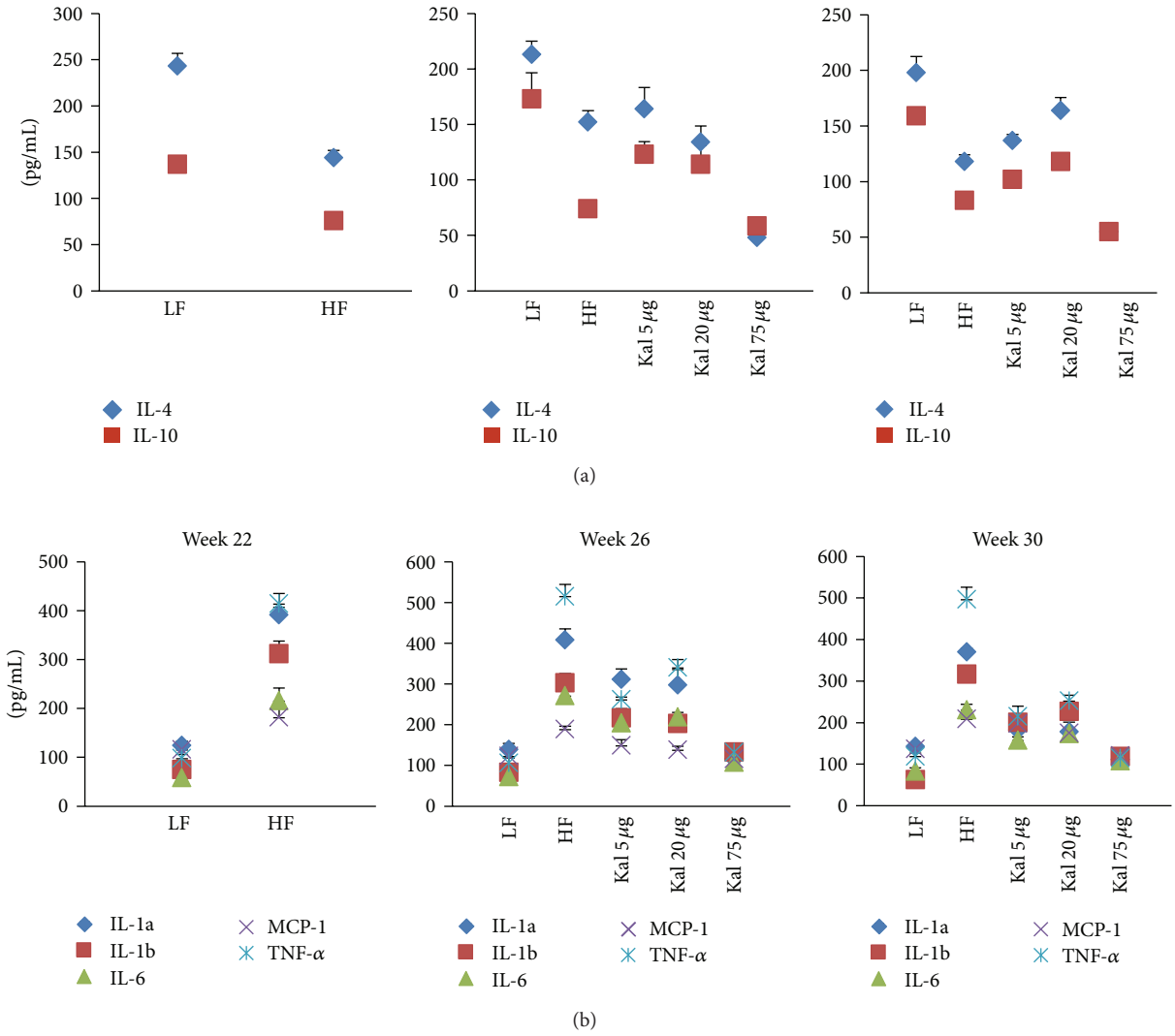

FIGURE 8: Treatment of Kal-1 modulates the anti- (a) and proinfammatory (b) cytokines levels to normal which were showing abnormal pattern in high-fat high-sugar fed mice at weeks 22, 26, and 30. Treatment with all doses (5, 20, and 75 μL) of Kal-1 was started only after 21 weeks (induction period) along with high-fat high-sugar diet. LF: low-fat control, HF: high-fat high sugar control. All the values represent mean ± SEM from five animals.

catalyses lipolysis and at the same time also regulates excess accumulation of fat cells in the body.

Unlike leptin, adiponectin, an adipocyte-specific secretary protein, is well known for its anti-inflammatory action. Shklyaev et al. [28] reported that adiponectin with sustained peripheral expression can improve insulin sensitivity too. The hormone also contributes to the production of anti-inflammatory cytokines and suppresses the pro-inflammatory cytokines [29]. Serum adiponectin levels decrease with obesity or with increased adiposity, though the mechanism behind this reduction is still unclear. Similar to the above-mentioned fact, the adiponectin concentration shrinks with body weight reduction after the administration of Kal-1 in rescue and treatment studies in the present investigation (Figures 3(c) and 7(b)). Consequently, it can be hypothesized that Kal-1 might be a contributing factor for reduction in body weight.

Production and regulation of adipocytokines from adipocytes have been shown to be completely based on dietary conditions as dietary fats are directly associated with obesity and related metabolic disorders like diabetes. LF diet accompanies decreased inflammatory markers whereas HFHS diet improved levels of pro-inflammatory cytokines [30]. In case of obesity and impaired glucose metabolism, chronic low-grade inflammation is considered to be a principal mechanism. The chronic inflammation can be only controlled by equilibrium between pro-inflammatory and anti-inflammatory cytokines.

Out of a number of adipocytokines expressed in and secreted by adipocytes, namely, IL-1α, IL-1β, IL-6, TNF-α, and MCP-1 are considered as classical pro-inflammatory cytokines in chronic inflammatory responses. It has been implicated by earlier studies that these cytokines are involved in the low-grade inflammation, impaired glucose

metabolism, and insulin resistance [31–35]. In accordance with previous studies, it is revealed in the present study that mentioned pro-inflammatory cytokines concentrations are higher in HFHSD fed animals in comparison to LFD fed animals. The serum levels of TNF-α and IL-6, key pro-inflammatory cytokines, are frequently increased in the obese state which is well in concurrence with the present study. TNF-α actively participates in the development of insulin resistance and IL-6 is linked with type II diabetes. IL-1α (cell-associated molecule) and IL-1β (secretary protein) are members of IL family and recognized as immunomodulatory proteins. Both are related with obesity while IL-1β is also linked with obesity-induced diabetes [6, 35]. It has been reported that one of the important pro-inflammatory cytokines is MCP-1 whose circulating levels were high in the obese mice model. Contrary to this, in our study, decreased serum concentrations of two important adipokines IL-4 and IL-10 were found in obese animals compared to lean animals. This finding is in support of the fact that these adipokines have long been considered as anti-inflammatory cytokines [36, 37].

In the rescue experiment, HFHSD supplemented with 20 μL of Kal-1 suppresses obesity and related pro-inflammatory responses like insulin response and blood glucose levels by reducing levels of IL-1α, IL-1β, IL-6, TNF-α, and MCP-1 and simultaneously elevating levels of anti-inflammatory IL-4 and IL-10 at week 15; however, here Kal-1 working amount was lower, that is, 5 μL (Figures 4(a) and 4(b)). On the other hand in treatment experiment, same types of profile were observed for both pro- and anti inflammatory adipokines. The only difference was in the amount of Kal-1; here, it was 75 μL as observed in other biochemical parameters in treatment study. The possible explanation for this higher amount of Kal-1 is that herbal formulation with several ingredients like Kal-1 is required in little amount if this is administered with disease progression. Once disease is old and induced for a certain period of time, more than 2-fold of herbal formulation is required. To the best of our knowledge, this is the first study in which these two aspects of disease are covered simultaneously, and single herbal formulation, Kal-1, controls chronic low-grade inflammation and maintains a balance between pro- and anti-inflammatory cytokines too by its immunoregulatory effect, and then contributes to control weight gain and related metabolic problems.

5. Conclusion

In conclusion, our investigations imply that Kal-1 exhibits substantial antiobesity concomitant with metabolic regulatory effect especially in terms of chronic low-grade inflammation, energy equilibrium, and linked significant disorders. However, as discussed above that Kal-1 is constituted of seven different herbal ingredients, it is very difficult to conclude that a combination or a single ingredient is responsible for these observed responses at this stage. Indeed, clinical trials are needed in order to understand the relevance of formulation. It is also clear that diet rich in carbohydrate with increased sugar may affect body weight, blood biochemistry including blood glucose and serum insulin, the levels of inflammatory markers both pro and anti, and most importantly energy balance. It would also be interesting to investigate Kal-1 mechanism and its influence on metabolic process and pathways at transcriptional level.

Conflict of Interests

The authors have declared that no conflict of interests exists. This does not alter their adherence to all the evidence-based complementary and alternate medicine policies on sharing data and materials.

Authors' Contribution

Kanury V. S. Rao, Parul Tripathi, and Sachin Sharma conceived and designed the experiments. Kamiya Tikoo, Shashank Misra, Parul Tripathi, and Sachin Sharma performed the experiments. Kanury V. S. Rao, Parul Tripathi, and Sachin Sharma analyzed the data. Parul Tripathi and Sachin Sharma wrote the paper.

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Research Article

The Evaluation of the Body Weight Lowering Effects of Herbal Extract THI on Exercising Healthy Overweight Humans: A Randomized Double-Blind, Placebo-Controlled Trial

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We investigated the effects of herbal extracts, a mixture of Scutellariae Radix and Platycodi Radix containing the active ingredients Baicalin and Saponin (target herbal ingredient (THI)), on lowering body weight. The present study was a prospective, randomized, double-blind, and placebo-controlled trial carried out at the outpatient department of a hospital over a period of 2 months. Group 1 patients ($n = 30$) received THI, and group 2 patients ($n = 23$) received placebo three times a day before meals. Weight, waist circumference, BMI, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, and glucose were measured at baseline and again at the 2nd month. For safety evaluation, various hematological and biochemical parameters were assessed. Values of mean change of weight in the THI-treated group were -1.16 ± 1.41 kg and in the placebo-treated group were -0.24 ± 1.70 kg, respectively. The difference in mean change of weight in the THI-treated group compared with that in the placebo-treated group was statistically significant ($P < 0.05$). The incidence of subjective and objective adverse drug reactions was insignificant ($P > 0.05$). THI was statistically significant in its effectiveness on the weight loss.

1. Introduction

Overweight or obesity substantially raises a patient's risk of morbidity from hypertension [1, 2], type 2 diabetes [3, 4], dyslipidemia [5], cardiovascular disease (CVD) [5], stroke [6], gallbladder disease [7], osteoarthritis [8], sleep apnea, respiratory problems [9, 10], and also endometrial, breast, prostate, and colon cancers [11]. Overweight and obesity are a major public health concern not only in western countries but also in Asian countries because of its increasing prevalence and its association to morbidity and mortality [12, 13]. The prevalence of obesity (body mass index (BMI) $\geq 30\,kg/m^2$) in USA was 32.2% in 2004 [14]. While the prevalence of obesity in Asian populations is lower than that of Caucasians, the health risks associated with obesity occur at a lower BMI. Accordingly, the criteria for overweight and obesity in the Asian-Pacific region of WHO has been proposed as BMI $\geq 23\,kg/m^2$ and BMI $\geq 25\,kg/m^2$, respectively [15]. The prevalence of obesity using a BMI $\geq 25\,kg/m^2$ among Koreans over age 19 was 30.8% in 2010 [16] which represented a rapid increase in comparison to rates in 1998 [17].

There is strong evidence that weight loss in overweight and obese individuals reduces risk factors for diabetes and cardiovascular disease. Strong evidence exists that weight loss reduces blood pressure in both overweight hypertensive and nonhypertensive individuals, reduces serum triglycerides, increases high-density lipoprotein (HDL) cholesterol, and generally produces some reduction in total serum cholesterol and low-density lipoprotein (LDL) cholesterol. Weight loss reduces blood glucose levels in overweight and obese persons without diabetes; weight loss also reduces blood glucose levels and HbA1c in some patients with type 2 diabetes. Although there have been no prospective trials to show changes in mortality with weight loss in obese patients, reductions in risk factors would suggest that development of type 2 diabetes and CVD would be reduced with weight loss [18].

Therefore, drugs or supplements to help weight loss need to be developed.

This study is aimed at investigating the body weight lowering effect of extracts from a mixture of herbs with the active ingredients Baicalin and Saponin (target herbal ingredient (THI)). Baicalin is the active ingredient in Scutellariae Radix (the root of *Scutellariae baicalensis*) which has been shown to be efficacious in reducing weight, visceral fat mass, serum cholesterol, free fatty acid, and insulin concentrations on high-fat diet-fed rats in comparison to controls [19]. High-fat diet-fed rats were also shown to suppress expected increases in body weight [20, 21], plasma triacylglycerols, adipose tissues [20, 22], food consumption, expression of leptin, and neuropeptide Y [21] and prevented hepatic steatosis [22] after consuming saponin isolated from ginseng or Platycodi Radix. The present study was a prospective, randomized, double-blind, and placebo-controlled trial carried out at an outpatient department of the hospital over a period of 2-months.

2. Materials and Methods

2.1. Study Subjects. Subjects who participated in the study were all healthy volunteers who were recruited using announcements in leaflets and posters at Chung-Ang University Hospital, Seoul, Korea. The study was approved by the Institutional Review Board (IRB) of the Chung-Ang University Hospital (Ethics Committee reference number C2010114 (410)). In addition, written informed consent was obtained from all subjects before initiation of the trial.

From November 2010 to December 2010, a total of 80 patients visited the Outpatient Department of Family Medicine of Chung-Ang University Hospital where they had a baseline clinical examination (weight, BMI, body and composition) and laboratory investigations. Body composition was assessed by a bioelectrical impedance analysis system (InBody 720, Biospace Co., Seoul, Korea) that was developed for the Korean population.

2.2. Inclusion and Exclusion Criteria. The inclusion criteria were patients with BMI $\geq 23\,kg/m^2$ of either sex between the age of 18 and 70 years. The following categories of patients were excluded: patients with endocrinologic obesity; patients using other medications that can alter body weight or lipid levels; patients with clinically significant cardiovascular, respiratory, or hepatobiliary disorder; chronic diarrhea; and pregnant or lactating women.

2.3. Preparation of Herbal Dietary Supplement. The dietary supplement, THI, was a liquid herbal extract of Scutellariae Radix and Platycodi Radix as its main ingredient. The two herbs were extracted and made into a dietary supplement by Namil Farm & Ginseng Co. (Geumsam, Korea). The two herbs were mixed at a 1 : 1 ratio and extracted with 70% ethanol. The ethanol was then evaporated under low pressure conditions. The final yield of the herbal extract to raw herbs was 25% (w/w). For the standardization of the herbal extract, the contents of Baicalin—an ingredient of Scutellariae Radix—and Saponin—an ingredient of Platycodi Radix—were measured using high performance liquid chromatography resulting in 5.16% of Baicalin and 3.95% of Saponin (w/w). The preclinical study of this herbal extract was reported previously [22]. Each pouch of THI supplement contained 2.28 gm of herbal extract as its main ingredient, 3.18 gm of oligosaccharide as a sweetener, and 0.05 gm of berry flavor and was adjusted to the final volume of 50 mL with water. Placebos, with no herbal extract, contained same sweeter and flavor to have a similar appearance and scent.

2.4. Sample Size. There were no previous studies on the efficacy of THI with respect to body weight lowering effects for healthy overweight humans. We postulated that we would need a sample size of $n \geq 36$ in order to see standard deviation and difference of body weight change between groups with a statistical significance level of 0.05 and power of test of 0.9 based on previous animal study [23]. Therefore it was decided to enroll 60 subjects in the study anticipating a 40% exclusion from the study.

Initially, 69 subjects were enrolled in the study. Fifteen subjects were either disqualified for not complying with the drug regimen or voluntarily dropped from the study. One subject was excluded for excessive exercise. A final sample size of $n = 53$ was used in the final analysis and reported in the results.

2.5. Methods and Statistics. A total of 69 patients were enrolled in the study according to inclusion and exclusion criteria and were randomized into two groups of 39 patients for THI and 30 patients for placebo on the computer-generated list by SAS randomization program at Chung-Ang University, College of Medicine, Department of Microbiology. Group 1 patients received THI three times a day before meals. Group 2 patients received placebo three times a day before meals. After enrollment into the study, tests were carried out for baseline clinical examination (weight, BMI, body composition (muscle (kg), fat (kg), and fat ratio (%)), and blood pressure) and laboratory investigations; hematological tests (red blood cell count, hemoglobin level, (Hb), hematocrit level, white blood cell count, differential white blood cell count, and blood platelet count); blood biochemical tests (total protein, albumin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, blood urea nitrogen, uric acid, serum creatinine, aspartate transaminase (SGOT), alanine transaminase (SGPT), γ-glutamyl transpeptidase (γGTP), and blood glucose); urine analysis (urine protein, urine sugar, and urinary sediment (erythrocytes, leukocytes, and casts)); and Serologic tests (highly sensitive C-reactive protein (hs-CRP), thyroid stimulating hormone (TSH), and free T4)). In the same visit, irrespective of group allotted, all the patients were educated about the importance of lifestyle changes including healthy dietary habits and exercise in weight reduction and maintenance. Patients were given information about the nutritional value of various foods and few simple exercises for decreasing and maintaining near-normal body weight by a clinical dietician. Their compliance for lifestyle change advice was checked through verbal questions at

each visit. Biochemical examinations assessed were above laboratory investigations (hematological tests, lipid profile, liver function test, blood glucose, serum creatinine, urine analysis, etc.) at baseline, the 1st month, and the 2nd month of study period. The efficacy of the drug was assessed by primary efficacy parameters, that is, body weight (kg), BMI (kg/m^2), waist circumference (in cm measured at midpoint between the lower border of rib cage and iliac crest), and body composition. Other parameters assessed were total cholesterol (mg/dL), triglyceride (mg/dL), LDL cholesterol (mg/dL), HDL cholesterol (mg/dL), random blood glucose level (mg/dL), hs-CRP (mg/L), and blood pressure (mmHg). Safety was assessed in terms of both subjective and objective adverse effects with grading scale of Common Terminology Criteria for Adverse Events v3.0 (CTCAE) [24]. Subjective symptoms such as anorexia, nausea, indigestion, loose stools, and constipation were assessed by questioning patients at each visit. Objective signs were assessed by clinical and biochemical examination. Followups were performed at the 1st and 2nd months of the study period, and during each visit, all the efficacy parameters were measured, and safety evaluations were done.

Quantitative data was analyzed by t-test or Mann-Whitney U-test for the differences between two means, while qualitative data was analyzed by Chi-square test or Fisher's exact test for difference between two proportions. P value < 0.05 was taken as significant, while P value > 0.05 was taken as insignificant.

Analyses were performed using the SPSS statistical software Version 12.0KO for windows (SPSS Inc.).

3. Results

Nine patients withdrew from trial in the THI-treated group, and 6 patients withdrew from trial in the placebo-treated group because they were busy and had been taking their supplements irregularly. No patients withdrew due to adverse effects of the supplements. In the placebo-treated group, one male patient was excluded because, unlike the rest of the patients, he exercised over 2 hours a day which was too vigorous for the purpose of this study.

At the time of the final analysis, there were 30 patients in the THI-treated group and 23 patients in the placebo-treated group.

Baseline values of both groups were comparable with respect to age, sex, weight, height, BMI, waist circumference, hip circumference, blood pressure, and laboratory investigations (random blood glucose level, total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, etc.) (Table 1). There were insignificant differences between the two groups except the total bilirubin level ($P < 0.05$).

In the THI-treated group, the mean weight at baseline was 71.84 ± 10.57 kg and reduced to 70.68 ± 10.25 kg 2-months later. In the placebo-treated group, mean weight at baseline was 67.89 ± 7.85 kg and reduced to 67.64 ± 7.96 kg after 2-months.

When the mean value of the change of weight in the THI-treated group was compared with that in the placebo-treated group, it was found that the difference in mean change of weight was statistically significant ($P < 0.05$) (Table 2). When values of the mean change of the other clinical examination variables in the THI-treated group were compared with that in the placebo-treated group, the differences were found to be statistically insignificant ($P > 0.05$).

There were no significant ($P > 0.05$) differences in the mean change of blood glucose level, total cholesterol concentration, triglyceride concentration, HDL cholesterol concentration, LDL cholesterol concentration, hs-CRP level, and systolic and diastolic blood pressure during the 2-month treatment in the THI-treated group compared to the placebo-treated group (Table 2). All changed laboratory investigations were in normal range.

In the present study, the incidence of adverse drug reactions like nausea (16.7%), indigestion (26.7%), abdominal pain (3.3%), loose stools (6.7%), diarrhea (13.3%), constipation (6.7%), insomnia (6.7%), itching (6.7%), satiety (6.7%), suppression of appetite (10.0%), and so forth was insignificant in the THI-treated group compared to the placebo-treated group ($P > 0.05$) (Table 3). Most of these adverse effects were mild and transient. THI had no adverse impact on white blood cell count, hemoglobin, red blood cell count, hematocrit, platelet count, total protein, albumin, total bilirubin, direct bilirubin, SGOT, SGPT, γGTP, blood urea nitrogen, serum creatinine, uric acid, TSH, and free T4 level at the end of study (Table 2).

4. Discussion

A sedentary lifestyle coupled with an increased intake of energy-dense food is contributing to the increased prevalence of obesity worldwide. A range of strategies can be employed for weight loss which includes lifestyle changes (diet, exercise), behavioral therapy, pharmacotherapy, and surgery. Orlistat is an effective and well-tolerated antiobesity drug, which can be employed as an adjunct to therapeutic lifestyle changes to achieve and maintain optimal weight [25]. But most antiobesity drugs act on the central nervous system to suppress appetite, reduce food intake, and have serious adverse effects. Therefore, alternative drugs or supplements to help weight loss need to be developed.

In traditional medicine of East Asian countries including China, Korea, and Japan, Scutellariae Radix extracts are widely used for clinical treatment of hyperlipemia, atherosclerosis, hypertension, dysentery, the common cold, and inflammatory diseases such as atopic dermatitis. Baicalin, 5, 6-dihydroxyflavone-7-glucuronic acid, is a major flavonoid found in Scutellariae Radix and is well known for its anti-inflammatory and antioxidant activities [26, 27], and few reports describe the antiobesity effects of this compound. In vitro study, Baicalin treatment of 3T3-L1 preadipocytes was shown to inhibit triglyceride accumulation and lipid droplet formation during induced adipogenesis. Baicalin inhibits adipogenesis through the downregulation of proadipogenic genes, including PPARγ, C/EBPα, and KLF15, as well as the upregulation of antiadipogenic regulators, including C/EBPγ and KLF2 [28]. Also in animal studies, Baicalin might have

Table 1: General characteristics of study subjects.

Variables	THI ($n = 30$)	Placebo ($n = 23$)	P value
Sex			
Male	3 (10)	5 (21.7)	0.272
Female	27 (90)	18 (78.3)	
Age (years)	42.90 ± 12.67	41.83 ± 14.82	0.778
Height (cm)*	159.22 ± 7.91	160.03 ± 9.46	0.654
Weight (kg)	71.84 ± 10.57	67.89 ± 7.85	0.139
Body mass index (kg/m^2)	28.35 ± 3.95	26.51 ± 2.21	0.050
Waist circumference (cm)	84.02 ± 8.66	82.96 ± 6.61	0.628
Hip circumference (cm)*	102.79 ± 7.09	99.40 ± 3.97	0.337
Waist/hip (%)	81.75 ± 6.51	83.50 ± 6.28	0.329
Body composition			
Muscle (kg)*	42.60 ± 7.91	41.14 ± 8.07	0.355
Fat (kg)	26.68 ± 7.94	24.43 ± 4.24	0.225
Fat ratio (%)	36.90 ± 7.81	36.28 ± 6.48	0.759
Blood pressure (mmHg)			
Systolic	124.80 ± 13.66	125.17 ± 13.58	0.922
Diastolic*	85.47 ± 10.18	84.74 ± 9.79	0.842
Blood glucose (mg/dL)*	94.73 ± 11.43	96.00 ± 6.19	0.570
Total cholesterol (mg/dL)	197.20 ± 38.39	201.39 ± 39.83	0.700
Triglyceride (mg/dL)*	115.03 ± 76.11	117.57 ± 56.93	0.341
HDL cholesterol (mg/dL)*	49.97 ± 9.04	54.04 ± 12.55	0.261
LDL cholesterol (mg/dL)	117.67 ± 31.76	115.91 ± 28.30	0.836
hs-CRP (mg/L)*	0.92 ± 0.88	2.41 ± 6.52	0.495
White blood cell count (10^9/L)*	5.99 ± 1.37	6.72 ± 1.56	0.097
Hemoglobin (g/dL)	13.69 ± 1.23	14.11 ± 1.42	0.262
Hematocrit (%)*	41.03 ± 3.25	42.22 ± 3.65	0.274
Platelet count (10^9/L)*	256.97 ± 43.91	255.87 ± 45.20	0.641
Total protein (g/dL)	7.26 ± 0.25	7.42 ± 0.42	0.091
Albumin (g/dL)*	4.32 ± 0.25	4.36 ± 0.25	0.562
Total bilirubin (mg/dL)*	0.58 ± 0.16	0.74 ± 0.29	0.029‡
Direct bilirubin (mg/dL)*	0.17 ± 0.05	0.21 ± 0.08	0.091
SGOT (IU/L)*	23.10 ± 7.22	21.30 ± 5.49	0.335
SGPT (IU/L)*	25.77 ± 14.86	22.17 ± 13.00	0.243
γGTP (IU/L)*	32.53 ± 29.19	24.65 ± 15.08	0.596
Blood urea nitrogen (mg/dL)*	13.33 ± 3.16	13.70 ± 3.75	0.704
Serum creatinine (mg/dL)*	0.79 ± 0.16	0.83 ± 0.12	0.332
Uric acid (mg/dL)*	4.31 ± 1.03	4.57 ± 1.49	0.713
TSH (mIU/mL)*	2.23 ± 1.58	1.95 ± 1.19	0.634
Free T4 (ng/dL)*	1.08 ± 0.19	1.10 ± 0.14	0.389

Chi-square test (categorical variables); t-test (continuous variables); * P value using Mann-Whitney U-test; ‡ $P < 0.05$.
Values given are number of patients (%) or mean ± SD.
LDL: low-density lipoprotein; HDL: high-density lipoprotein; hs-CRP: highly sensitive C-reactive protein; SGOT: aspartate transaminase; SGPT: alanine transaminase; γGTP: γ-glutamyltranspeptidase; TSH: thyroid stimulating hormone.

beneficial effects on the development of hepatic steatosis and obesity-related disorders by targeting the hepatic AMPK [19].

Platycodi Radix has long been used as an expectorant in traditional oriental medicine. Recently, it was reported that Platycodi Radix has anti-inflammatory, antiallergy, antitumor, apoptosis-inducing, and immune-stimulating activities [29–31]. Recently, it was reported that platycodin D, a major saponin component of Platycodi Radix, inhibits fat accumulation and adipogenesis [22, 32–35]. The antiobesity effect of the crude saponins in mice fed a high-fat diet may be due to the inhibition of intestinal absorption of dietary fat by platycodin D [22]. And the antiadipogenic effect of platycodin D involves the upregulation of KLF2 and subsequent downregulation of PPARγ [36].

Table 2: Changes over time in clinical examination and laboratory investigations between THI and placebo ingestion groups.

Variables	THI ($n = 30$)	Placebo ($n = 23$)	P value
Weight (kg)	−1.16 ± 1.41	−0.24 ± 1.70	0.036‡
Body mass index (kg/m^2)	−0.41 ± 0.55	−0.10 ± 0.70	0.083
Waist circumference (cm)*	−1.77 ± 1.91	−1.04 ± 2.38	0.156
Hip circumference (cm)	−1.23 ± 1.77	−0.71 ± 1.78	0.304
Waist/hip (%)	−0.77 ± 2.23	−0.46 ± 2.32	0.628
Body composition			
Muscle (kg)*	−0.63 ± 1.51	−0.06 ± 0.98	0.151
Fat (kg)	−0.50 ± 1.36	−0.38 ± 1.47	0.761
Fat ratio (%)*	−0.06 ± 1.82	−0.41 ± 1.36	0.957
Blood pressure (mmHg)			
Systolic*	−11.30 ± 13.92	−5.65 ± 16.29	0.368
Diastolic	−8.20 ± 10.60	−6.00 ± 11.07	0.466
Blood glucose (mg/dL)*	−1.93 ± 8.05	1.43 ± 7.26	0.062
Total cholesterol (mg/dL)	−4.63 ± 26.84	−9.73 ± 24.23	0.478
Triglyceride (mg/dL)*	−14.96 ± 59.22	1.43 ± 48.64	0.647
HDL cholesterol (mg/dL)*	3.50 ± 16.99	−2.39 ± 8.47	0.124
LDL cholesterol (mg/dL)*	−3.50 ± 30.39	−3.56 ± 20.53	0.628
hs-CRP (mg/L)*	1.59 ± 6.25	−1.36 ± 6.50	0.251
White blood cell count (10^9/L)	−0.13 ± 1.02	−0.42 ± 1.07	0.328
Hemoglobin (g/dL)*	−0.12 ± 0.51	0.73 ± 5.24	0.208
Hematocrit (%)*	−2.09 ± 7.30	−1.43 ± 1.11	0.258
Platelet count (10^9/L)	−14.50 ± 23.21	−5.65 ± 29.32	0.226
Total protein (g/dL)*	−0.15 ± 1.31	0.00 ± 0.29	0.240
Albumin (g/dL)*	0.04 ± 0.14	0.02 ± 0.17	0.483
Total bilirubin (mg/dL)*	0.04 ± 0.14	0.00 ± 0.33	0.112
Direct bilirubin (mg/dL)*	0.01 ± 0.06	0.01 ± 0.12	0.568
SGOT (IU/L)*	−0.26 ± 6.20	−0.60 ± 3.47	0.971
SGPT (IU/L)*	−0.73 ± 10.41	−1.13 ± 5.40	0.869
γGTP (IU/L)*	−1.26 ± 7.65	1.17 ± 12.64	0.487
Blood urea nitrogen (mg/dL)*	−1.43 ± 2.80	−1.52 ± 3.96	0.474
Serum creatinine (mg/dL)*	0.03 ± 0.10	0.00 ± 0.05	0.223
Uric acid (mg/dL)*	−0.09 ± 0.59	−0.10 ± 0.63	0.621
TSH (mIU/mL)*	0.13 ± 1.45	−0.14 ± 1.15	0.680
Free T4 (ng/dL)*	0.08 ± 0.35	0.07 ± 0.31	0.634

t-test; * P value using Mann-Whitney U-test; ‡ $P < 0.05$.
Values given are number of patients (%) or mean ± SD.
LDL: low-density lipoprotein; HDL: high-density lipoprotein; hs-CRP: highly sensitive C-reactive protein; SGOT: aspartate transaminase; SGPT: alanine transaminase; γGTP: γ-glutamyltranspeptidase; TSH: thyroid stimulating hormone.

In our study, values of mean change of weight during the 2-month treatment in the THI-treated group were −1.16 ± 1.41 kg and in the placebo-treated group were −0.24 ± 1.70 kg, respectively. When the mean change in weight in the THI-treated group was compared with that in the placebo-treated group, it was found that the difference in the mean change of weight was statistically significant ($P < 0.05$), but the mean change of weight during the 2-month treatment in the THI-treated group was −1.16 ± 1.41 kg. Caloric restriction combined with exercise or alone to create a daily energy deficit is the foundation of most weight loss programs. In these programs, many individuals successfully lose at least 4.5 Kg of body weight through behavioral approaches for 6 months [36]. In other studies, the placebo-treated group loses at least −1.49 ± 6.19 kg of body weight during the 2-months [25]. So can we conclude from our study that THI was effective in weight loss?

Our study was conducted in South Korea between November of 2010 to February of 2011, when most Koreans eat and drink more than usual. In addition, the winters in Korea frequently have heavy snowstorms, so our patients did not exercise properly around the time we were completing our

Table 3: Side effects during treatment of THI and placebo.

Side effects	THI ($n = 30$)	Placebo ($n = 23$)	P value
Anorexia grade 1*	0	1 (4.3)	0.434
Nausea grade 1	5 (16.7)	1 (4.3)	0.217
Indigestion grade 1	8 (26.7)	3 (13.0)	0.313
Hunger pain grade 1	1 (3.3)	0	1.0
Abdominal pain grade 1	1 (3.3)	0	1.0
Loose stool grade 1	2 (6.7)	3 (13.0)	0.642
Diarrhea grade 1	4 (13.3)	1 (4.3)	0.374
Constipation grade 1	2 (6.7)	1 (4.3)	1.0
Facial edema grade 1	1 (3.3)	0	1.0
Headache grade 1	1 (3.3)	0	1.0
Insomnia grade 1	2 (6.7)	1 (4.3)	1.0
Itching grade 1	2 (6.7)	0	0.499
Fatigue grade 1	1 (3.3)	1 (4.3)	1.0
Palpitation grade 1	0	1 (4.3)	0.434
Satiety grade 1	2 (6.7)	2 (8.7)	1.0
Relieve constipation grade 1	5 (16.7)	4 (17.4)	1.0
Good appetite grade 1	1 (3.3)	1 (4.3)	1.0
Increased gastric acid secretion grade 1	1 (3.3)	0	1.0
Suppression of appetite grade 1	3 (10.0)	4 (17.4)	0.451
Thirst grade 1	1 (3.3)	0	1.0

Chi-square test; * P value using Fisher's exact test.
Values given are number of patients (%).
Grading scale of Common Terminology Criteria for Adverse Events v3.0 (CTCAE).

study. In our study, even though weight loss was small, THI was statistically significant in its effectiveness on the weight loss.

Prior to this study, THI improved metabolic abnormality in mice that were fed high-fat diets [23]. And the extracts of Platycodi Radix with white balloon flower ameliorated obesity and insulin resistance in obese mice via the activation of AMPK/ACC pathways and reductions of adipocyte differentiation. It also reduced the elevated circulating mediators, including triglyceride, total cholesterol, leptin, resistin, and monocyte chemotactic protein- (MCP-) 1 in obesity [37].

Therefor we expected that THI improved metabolic abnormality with body weight loss in our study. In our study, the values of mean change of triglyceride and HDL cholesterol during the 2-month treatment in the THI-treated group seemed to be improved than those in the placebo-treated group. However, those were not statistically significant ($P > 0.05$), because the remedy was a supplement, not a medication, the group was small, the study period was short, and the body reduction effect was too small. We will, therefore, need further research in order to make conclusions about its long-term effects.

However, in our study, values of mean change of platelet count during the 2-month treatment in the THI-treated group seemed to be decreased than that in the placebo-treated group. But, that was not statistically significant ($P > 0.05$). Scuttellaria has antithrombotic and antiplatelet actions that prevent the pathological changes of atherosclerosis and restenosis because it has several chemical compounds. Baicalin has antiproliferative and lipoxygenase-inhibitory activities [38], and Oroxylin A has antithrombotic activities [39]. Also there was a protective effect from *Scutellaria baicalensis* Georgi on cerebral ischemia injury [40]. So if we studied for a longer time, the result would be statistically significant, and the side effect of bleeding tendency would occur. But in our study the change occurred in normal range.

The adverse drug reactions were indigestion (26.7%), nausea (16.7%), diarrhea (13.3%), and loose stool (6.7%). They were mainly gastrointestinal symptoms. These adverse drug reactions may have been due to the body weight lowering effects of the crude saponins, the inhibition of pancreatic lipase activity and intestinal absorption of dietary fat by platycodin D [28]. However, patients who suffered from constipation before the study began felt that the supplement relieved their constipation (16.7%). In our study, the incidence of subjective and objective adverse drug reactions was insignificant in the THI-treated group compared to the placebo-treated group ($P > 0.05$).

We could not make a capsule or tablet for this study because the THI supplements were 50 mL of liquid extracts. There can, therefore, be problems for long-term storage.

5. Conclusion

In conclusion, the herb extract, a mixture of Scutellariae Radix and Platycodi Radix containing the active ingredients

Baicalin and Saponin (target herbal ingredient (THI)) was statistically significant in its effectiveness on the weight loss without significant side effects, which was demonstrated by our present randomized, double-blind, and placebo-controlled clinical trial.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Oat Protects against Diabetic Nephropathy in Rats via Attenuating Advanced Glycation End Products and Nuclear Factor Kappa B

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Oat, a rich source of soluble fiber, was considered to have a possible preventive effect on the progression of diabetic nephropathy. The present study aimed to assess this preventive activity in a rat model of diabetic nephropathy. Adult Wister rats were injected by streptozotocin (65 mg/kg). Animals were fed with normal diet or with a diet containing 20% oat (W/W) for 21 weeks. At the end of 21 weeks, all the kidney tissues were collected for various examinations. Our results suggested that oat could decrease the Scr and glucose level in blood of diabetic rats significantly ($P < 0.05$), and increase the creatinine clearance ($P < 0.01$). In histopathological examination, oat-fed rats showed a significant decrease in glomerulus segmented sclerosis and incidence of tubule vacuolar degeneration. By ELISA, we reported that oat feeding resulted in decreasing the levels of IL-6 and AGE in serum and kidney homogenate. In addition, the levels of oxidative stress markers were markedly improved as a result of oat feeding. Furthermore, using EMSA, we showed that oat attenuated the activation of NF-κB. Using RT-PCR, we found that oat could downregulate the TGF-β1 and RAGE expression at mRNA levels. This study suggests that oat can suppress diabetic nephropathy in rats effectively and may slow down the renal fibrosis by the disruption of the detrimental AGE-RAGE-NFκB axis.

1. Introduction

Diabetic nephropathy (DN) is the most common cause contributing to end-stage renal disease (ESRD) [1]. The chronic hyperglycemia destroys function and structure the kidney, leading to albuminuria which in turn further damages the renal tubular structure [2]. In diabetes, the kidney is a direct target to the enhanced glucose levels. Advanced glycation end products (AGE) are heterogeneous products formed by the nonenzymatic reactions between reducing sugars and free amino groups of proteins, lips, and nucleic acids [3].

AGE have been implicated as one of the major factors in the pathogenesis of numerous pathologies (aging, atherosclerosis, rheumatoid arthritis, and Alzheimer's disease) notably including diabetic complications [4–6]. AGE can interfere with protein function and promote formation of aggregates. In kidney, AGE can become trapped in glomerular basement membranes and covalently crosslink to collagen resulting in membrane thickening and distortion [7]. In addition to these direct effects, AGE can bind mesangial cell surface receptors stimulating formation of transforming growth factor β1 (TGF-β1) and connective tissue growth factor (CTGF), which in turn mediates mesangial expansion and glomerulosclerosis [8]. Furthermore, AGE interact with their receptor (RAGE) on the cell membrane resulting in activation of the nuclear transcription factor kappa B (NF-κB). NF-κB is found in the cytoplasm of the unactivated cells bound to its inhibitor I-κB. Upon activation, NF-κB migrates to the nucleus where it upregulates the transcription of several inflammatory genes like IL-6, TGFβ, and others [9].

Many AGE inhibitors offer a promising therapeutic approach for diabetes-related complications [10, 11]. AGE-inhibitors include aminoguanidine (AG, Pimagedine) [12], pyridoxamine [13], aspirin [14], OPB9195 [15], and LR compounds [16]. Compounds that break AGE crosslink include phenacylthiazolium bromide (PTB) [17] and ALT-711 (Alagebrium) [18]. Some of the agents targeting AGE have been approved for clinical trials [19].

Cumulative studies demonstrated that dietary fiber can significantly reduce the risk of cardiovascular disease and type 2 diabetes mellitus [20]. This is due in part to the ability of fiber to reduce postprandial glycaemia and improve long-term glycemic control [21, 22]. It was postulated that the rheological properties of soluble dietary fibers are highly related to their effects on control of the glucose concentration [23]. For instance, the ability of oat-derived β-glucan to reduce postprandial glycaemia has been strongly correlated with its viscosity [24], demonstrating an inverse linear relationship between the logarithm of viscosity measures and peak postprandial plasma glucose and insulin responses after consuming various doses of purified oat β-glucan with a 50 g oral glucose load. Despite these findings, the levels of viscosity required to achieve specific glucose-lowering effects are poorly understood. Still, the majority of trials investigating dietary fiber have not accounted for the principles of polysaccharide solubility and viscosity as the main determinants of its physiological outcome. While a small number of studies have shown the effect of oat on diabetes [25, 26], none examined its effect on the development and progression of diabetic nephropathy.

The aim of this study is to evaluate the effect of oat on the hyperglycemia-induced AGE formation and NF-κB activation and if this can attenuate the development of diabetic nephropathy. Because oat is natural dietary supplement and widely used, the results of this study may provide an alternative for enhancing nutrition and diabetic control during diabetic nephropathy.

2. Materials and Methods

2.1. Animals. Adult male Wistar rats (Rattus Rattus) weighing about 190 ± 30 g were used in the present study. All animals were housed in cages and received normal rat diet and tap water *ad libitum* in a constant environment (room temperature 24 ± 3°C, room humidity 55 ± 5%) with a 12 h light, 12 h dark cycle. The animals were kept under observation for two weeks prior to the start of the experiments.

2.2. Induction of Diabetes Model and Study Design. Forty-five Wister rats were used in this experiment. Ten rats were used as normal control group (group 1, $n = 10$), which received a single *ip* injection of 0.1 mol/L citrate buffer. A group of 35 rats were intravenously injected with STZ (65 mg/kg body weight) [27] in a 0.1 mol/L citrate buffer (pH 4.5). Only rats with blood glucose higher than 300 mg/dL after 7 days were considered as being diabetic in the fasting state. Glucose measurement was done by using *OneTouch Select* Analyzer (LifeScan, Inc., UK). Rats with blood glucose lower than 250 mg/dL were excluded from the study (6 rats). All studies were carried out two days after induction of diabetes. Twenty-nine diabetic rats were randomly divided into two groups: diabetic untreated rats ($n = 15$ rats) and diabetic rats that received 20% oat in the diet (W/W) ($n = 14$) [28]. Rats were fed with normal rat meal and oat containing diet 20 g for each rat/day for 21 weeks [29]. Body weight blood glucose and HbA1C levels were measured regularly and at the end of the experiment duration. At the end of the experiment (21 weeks after induction of diabetes), animals were sacrificed. Kidneys were dissected and rinsed with ice cold normal saline and then weighed. An index of renal hypertrophy was estimated by comparing the wet weight of the left kidney to the body weight.

2.3. Kidney Homogenate Preparation. One kidney of each group was washed by cold normal saline solution, then it was homogenized in a homogenization buffer (0.05 M Tris-HCl pH 7.9, 25% glycerol, 0.1 mM EDTA, and 0.32 M $(NH_4)_2SO_4$) containing a protease inhibitor tablet (Roche, Germany). The resulting solution was sonicated in an ice bath for 10 seconds followed by centrifugation at 13000 rpm, 4°C for 5 minutes. The supernatant was aliquoted and stored at −80°C and assayed for protein concentration using BCA kit (Pierce, Rockford, USA) using bovine serum albumin diluted in the lysis buffer as standard. The homogenate was used for the determination of antioxidant biomarkers, concentration of AGE, and level of IL-6 and TGFβ. The other kidney from each group was used for histopathological, isolation of renal DNA, extraction of the nuclear proteins [30].

2.4. Determination of Serum Biomarkers. Blood sample of rats was centrifuged at 8000 rpm for 10 minutes at 4°C, and serum was removed and aliquoted for the respective analytical determinations. The diagnostic kits for determinations of creatinine (Cr) blood urea nitrogen (BUN), sodium, and potassium were purchased from BioSystem (Barcelona, Spain). All analyses were performed according to the instructions of the manufacturer.

2.5. Analysis of Urine Parameters. Before induction of diabetes and the day before the end of the experiment, urine samples were collected by placing the rats in individual metabolic cages for 24 h. The urine albumin concentration was determined using an ELISA kit (Nephrat II, Exocell, Philadelphia, PA, USA) and the concentration of Cr in pooled urine samples was determined by the commercial assay kit. All analyses were performed in accordance with the manuals provided by the manufacturers. The 24 h urinary albumin excretion rate (UAER) was calculated as UAER (μg 24 h − 1) = urinary albumin (μg mL^{-1}) × 24 h urine volume (mL). Cr clearance (Ccr) was calculated using the following equation: Ccr (mL min^{-1} kg^{-1}) = [urinary Cr (mg dL^{-1}) × urinary volume (mL)/serum Cr (mg dL^{-1})] × [1000/body weight (g)] × [1/1440 (min)] [31].

2.6. Determination of the Antioxidant Biomarkers. The activities of total SOD, catalase, and GSH-Px as well as the concentrations of MDA and GSH in the kidney homogenate were determined using commercially available kits from BioVision Research Products (Linda Vista Avenue, USA) according to the instructions of the manufacturer [32–36].

2.7. Determination of IL-6 and TGFβ. The levels of IL-6 and TGFβ in the serum and in the kidney homogenate were assayed by using the commercially available ELISA kits from

R&D (Mannheim, Germany) according to the instructions of the manufacturer.

2.8. Measurement of Urinary and Renal 8-Hydroxy-2′-Deoxyguanosine. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were determined using an ELISA kit from Genox Corporation (Baltimore, MD, USA) according to the method of Vuksan et al. [21] and corrected by using individual urine creatinine concentrations. Extraction of renal DNA was performed using a DNA extraction kit (Promega, Germany) according to the manufacturer's protocol. The genomic DNA samples from kidney tissue were also used for the determination of 8-OHdG using the competitive ELISA kit [37].

2.9. Determination of AGE in the Kidney Homogenate. The renal AGE level was determined according to previous method [38]. The concentration of AGE was determined by a competitive ELISA assay. The ELISA kit for AGE determination was obtained from Roche Diagnostics (Mannheim, Germany). As a standard, the monomeric epitope N-carboxymethyl-aminocaproate was used.

2.10. Electrophoretic Mobility Shift Assay (EMSA). Part of the kidney of each group was dissolved in TOTEX buffer (100 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.35 M sodium, chloride, 1% NP 40, 1 mM magnesium chloride, EGTA 0.5 mM, EDTA 0.5 mM, 10 μg/mL leupeptin, 0.2 mM PMSF, and 0.5 mM DTT) for 30 seconds. The lysate was incubated in ice for 0.5 h, vortexed, and centrifuged at 10000 g for five minutes. The supernatant which contained the total liver extract was transferred to a fresh tube and kept at −80°C for EMSA [25]. The extract was used for determining of the binding activity using the NF-κBp65 consensus sequence: 5′-AG TT GA GG GG AC TT TC CC AG GC-3′. The binding specificity of the mixture was ascertained by competition with a 160-fold molar excess of the unlabeled consensus oligo nucleotides as previously described [39, 40]. Incubation was carried out for 30 min at room temperature in a total volume of 15 mL of buffer that contains 12 mM HEPES/NaOH, pH 7.9, 12% glycerol, 60 mM potassium chloride, EDTA 1 mM, dithiothreitol 1 mM, 1.0 mg of poly (dI-dC), and 10,000 cpm of the labeled probe. The DNA/protein complexes were resolved on nondenaturing 5% polyacrylamide gel electrophoresis, performed with 0.5x Tris/borate/EDTA buffer (4.5 mM boric acid, 0.1 mM EDTA, pH 8.0, and 4.5 mM Tris) [41]. The increase in the expression of NFκBp65 gene was determined by a Phosphor Imager using background substract.

2.11. Reverse Transcription Polymerase Chain Reaction. Total RNA of rats in the three groups was isolated from kidney cortex by using TRIzol reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Gene expressions were determined using real-time quantitative reverse transcription polymerase chain reaction using light cycler and cyber green kit from Roche (Mannheim, Germany). β-actin was used as a reference gene. The transcripts were amplified in a single reaction containing 1 mg cDNA and 0.5 mM each of the sense and antisense primers. PCR was performed at 94°C 1 cycle for five minutes, followed by 33 cycles at 94°C 1 cycle 1 minute, 62°C 1 cycle 1 minute, 72°C 1 cycle 1 minute, and a final extension step at 72°C 1 cycle 20 minutes. An 08% agarose gel electrophoresis in presence of ethidium bromide as a stain was used for separation of the PCR products. A Digital Imaging System (Gel Logic 2200 Pro, Kodak, USA) was used for scanning and analyzing the obtained bands. The band intensities were expressed relative to the intensity of the bands of the control group which was set as 100%. The sequences of the used primers were as follows:

TGFβ1 sense 5′-TGGAGCAACATG TGG AAC TC-3′, antisense 5′-GTC AGC AGC CGG TTA CCA-3′;

RAGE sense 5′-CAC AGC CCG GAT TG-3′, antisense 5′-GCT GTA GCT GGT GGT CAG AAC A-3′;

β-actin sense 5′-GTGCTATGTTGCTCTAGACTT-CG-3′, antisense 5′-ATGCCACAGGATTCCATA-CC-3′.

2.12. Histopathological Examination. Part of the kidneys was fixed in a 10% neutral formaldehyde solution and embedded in paraffin. Sections were cut at 4 mm with a microtome and deparaffined with xylene. They were stained with hematoxylin and eosin (H&E) staining. Stained kidney sections were observed under a light microscope at magnifications of 200x and 400x [42].

2.13. Statistical Analysis. All group values are expressed as the mean ± SD. Data were evaluated using IBM SPSS 20 for windows. An analysis of variance test was performed initially to test for differences in the treatment, and a Tukey post hoc test and Student's t-test were performed to examine whether there were any significant differences between the three groups.

3. Results

3.1. Effects of Oat Feeding on the Blood Glucose and Kidney/Body Weight Ratio. As indicated in Table 1, STZ injection resulted in a nearly 6-fold increase of the fasting blood sugar (FBS) in the rats of group 2. In addition, STZ injection caused a nearly 3-fold increase in the levels of HbA1C. Oat feeding markedly reduced the elevated levels of FBS and HbA1C. The final body weight of the diabetic untreated animals was markedly decreased compared with the normal control rats indicating that these rats suffer from growth retardation as a result of STZ injection. At the end of the experimental period, the kidney/final body weight ratios of the untreated diabetic animals were significantly higher than those of the control rats ($P < 0.01$). As a result of oat feeding, the diabetic animals in the third group showed a significant reduction of this elevated ratio compared to the diabetic animals of group 2 ($P < 0.05$).

3.2. Effects of Oat Feeding on the Renal Function. Data in Table 1 showed that the 24-hour Upro, creatinine, Ccr,

Table 1: Biochemical, physiological, and renal functional parameters of the rats.

	Normal control, $n = 10$	Diabetic untreated, $n = 9$	Diabetic plus 20% oat, $n = 13$
Initial body weight, g	201.3 ± 5.6	202.4 ± 4	199.7 ± 9
Final body weight, g	271.5 ± 8.1	159.5 ± 8.2[a]	224 ± 12.3[a,b]
Glucose, mg/dl	99.98 ± 5.48	530.55 ± 20[a]	237.5 ± 28[a,b]
HbA1c, %	5.11 ± 0.15	13.42 ± 0.34[a]	8.95 ± 0.9[a,b]
Kidney/body weight, g/g, $\times 10^{-3}$	6.45 ± 0.65	11.15 ± 1.76[a]	8.12 ± 0.9[a,b]
BUN, mmol/L	7.23 ± 0.98	14.95 ± 2.91[a]	8.62 ± 2.1[a,b]
Serum creatinine, mmol/L	50.45 ± 8.2	69.52 ± 7.97[a]	53.15 ± 4.5[b]
Ccr $mLmin^{-1}\,kg^{-1}$	3.51 ± 0.3	6.78 ± 0.91[a]	3.43 ± 0.56[b]
U prot, mg/24 h	8.53 ± 0.93	22.56 ± 3.1[a]	14.95 ± 3.12[a,b]
Serum sodium, mmol/L	144.4 ± 3.5	176.1 ± 10.5[a]	150.1 ± 1.2[b]
Serum potassium, mmol/L	4.54 ± 0.83	7.76 ± 0.76[a]	5.34 ± 0.42[b]
Serum IL-6, g/mL	82.2 ± 0.54	695.2 ± 16.46[a]	211.2 ± 13.34[a,b]

Data are expressed as the means ± SD. [a]$P < 0.05$ versus normal control group, [b]$P < 0.05$ versus diabetic untreated group, one way ANOVA.

Table 2: Oxidant/antioxidant parameters as well as concentration of AGE and IL-6 in the rat kidney homogenate.

	Normal control, $n = 10$	Diabetic untreated, $n = 9$	Diabetic plus 20% oat, $n = 13$
MDA, nmol/mg protein	4.04 ± 0.1	9.76 ± 1.3[a]	5.2 ± 0.65[b]
GST, nmol/mg protein	19.76 ± 2.05	11.1 ± 1.23[a]	15.97 ± 2.1[a,b]
GSH-Px, U/mg protein	0.99 ± 0.23	0.29 ± 0.21[a]	0.75 ± 0.21[b]
Catalase, U/mg protein	52.16 ± 3.85	31.76 ± 2.5[a]	42.74 ± 3.95[a,b]
SOD, U/mg protein	25.17 ± 4.2	9.54 ± 1.91[a]	17.65 ± 2.34[b]
GSH, nmol/mg protein	27.76 ± 3.27	14.11 ± 1.95[a]	19.54 ± 3.56[b]
AGE, ng/mg protein	3.91 ± 0.12	9.75 ± 0.54[a]	5.11 ± 0.25[b]
IL-6, ng/mg protein	255 ± 22	1145 ± 69[a]	564 ± 48[a,b]

Data are expressed as the means ± SD. [a]$P < 0.05$ versus normal control group, [b]$P < 0.05$ versus diabetic untreated group, one way ANOVA.

potassium, and sodium levels were markedly higher in the diabetic untreated group when compared with the normal control group ($P < 0.05$). Oat feeding obviously reduced these elevated renal biomarkers in the animals of group 3 compared with rats of group 2 ($P < 0.05$).

3.3. Effects of Oat on Activities of Oxidant/Antioxidant Enzymes. The activity of SOD, catalase, GSH-px, and GST as well as the concentration of GSH was markedly reduced, whereas the concentration of MDA was markedly increased in the kidney homogenate of the diabetic untreated rats (group 2) compared to the control group (group 1) ($P < 0.05$), suggesting that these rats suffered from oxidative stress (Table 2). As a result of oat feeding, these altered parameters were significantly improved in the animals of group 3 ($P < 0.05$). The obtained data showed that oat ameliorated oxidative stress in the diabetic rats.

3.4. Effects of Oat on the Urinary and Renal 8-OHdG. The total amounts of urinary 8-OHdG were significantly greater in diabetic rats (group 2) than in the control rats after 21 weeks after the onset of diabetes ($P < 0.05$). Feeding of oat resulted in the suppression of the increase in urinary excretion of 8-OHdG in the diabetic rats ($P < 0.05$). In parallel with the urine results, the levels of 8-OHdG in the DNA were markedly increased in the kidney cortex of the diabetic rats.

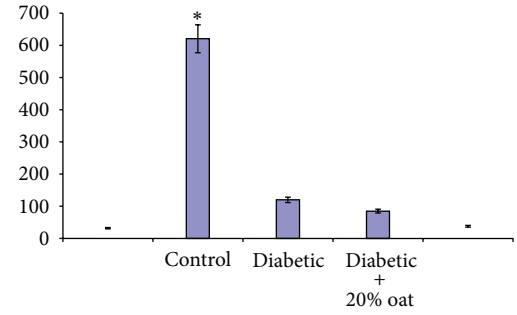


Figure 1: The levels of 8-hydroxy-2′-deoxyguanosine in the urine (a) and renal cortex (b) of rats. The levels of the urinary and renal cortex 8-OHdG in diabetic rats were markedly reduced as a result of oat feeding. Data are expressed as the means ± SD. *$P < 0.05$ versus normal control group, **$P < 0.05$ versus diabetes untreated group.

As shown in Figure 1, all the elevated levels of 8-OHdG were normalized by oat feeding ($P < 0.05$).

3.5. Effect of Oat on the Levels of AGE. As a result of diabetes, the kidneys of diabetic rats are subjected to elevated levels of glucose. The renal levels of AGE and CML were significantly elevated in the STZ-diabetic rats. These elevated levels were effectively lowered by oat feeding for 21 weeks (Table 2).

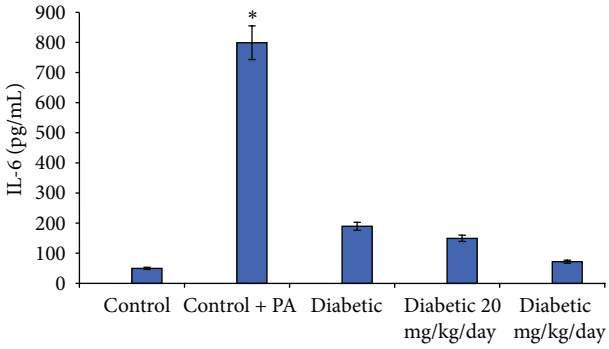


Figure 2: Binding activity of NF-κB. Binding activity of NF-κB to its consensus sequence was assayed by EMSA of total nuclear protein extracts. Quantification of activated NF-κB was performed by densitometric analysis of relative EMSA band intensities. Results are the means ± SE of four individual replicates, value from an unpaired Student's t-test ($^{*}P < 0.05$, compared with normal control group and $^{**}P < 0.001$ comparing with diabetic untreated group).

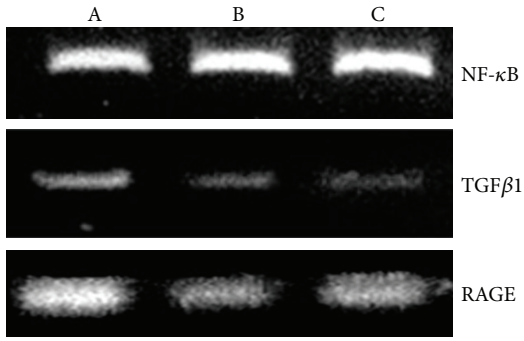


Figure 3: Expression of NF-κB, TGFβ1, and RAGE in the kidney of control, diabetic, and diabetic + oat rats. RT-PCR analysis by using total RNA extracted from the kidney cortex is shown. Data represent the means with SEs of three independent experiments from three different rats. Gene expression is illustrated as a ratio of the integrated density values for the genes in question and the integrated density of the actin values to yield a semiquantitative assessment. A: control, B: diabetic, and C: diabetic + 20% oat.

3.6. Effect of Oat on the Activation of NF-κB. As a result of diabetes, oxidative stress, and high levels of AGE, NF-κB-p65 was markedly activated in the diabatic untreated animals compared with the normal control. Feeding of the diabetic rats with oat in group 3 resulted in a significant reduction of the NF-κB-p65 as indicated in Figures 2(a) and 2(b).

3.7. Effects of Oat Feeding on RAGE and TGF-β in Rats Kidneys. The expression of RAGE and TGF-β1 was significantly higher in the diabetic untreated group compared with the normal control animals. Oat feeding of the animals in group 3 markedly attenuated the expression of both genes compared with the diabetic untreated rats (group 2) as illustrated in Figure 3.

3.8. Effect of Oat Feeding on Serum and Renal IL-6. As a result of diabetes, inflammation increases and the release of cytokines like IL-6 increases. Data in Table 1 showed that the serum level of IL-6 was markedly increased as a result of diabetes in group 2 compared with the normal control group ($P < 0.05$). In parallel, the level of IL-6 in the kidney homogenate of rat in group 2 was significantly increased ($P = 0.012$). Rats of group 3 which fed oat containing diets showed a significantly lower IL-6 levels in both serum and kidney homogenate (Tables 1 and 2) ($P < 0.05$).

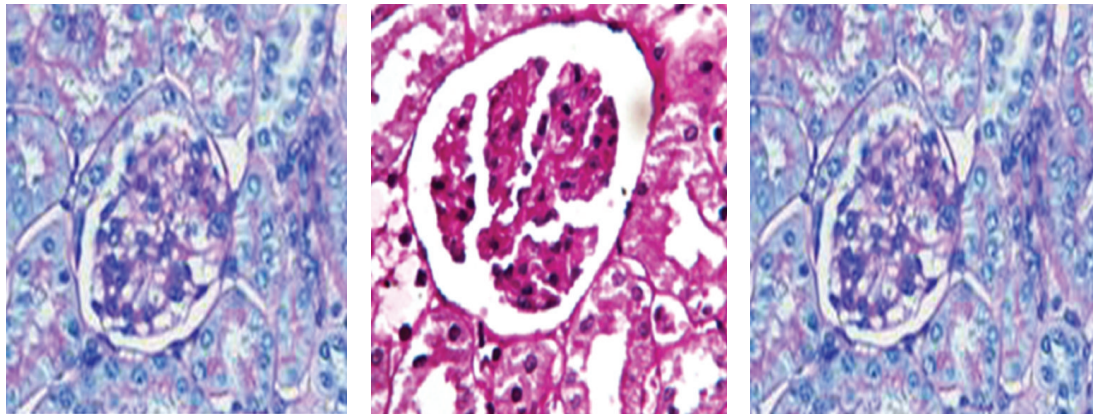
3.9. Histopathological Findings. After feeding with oat for 21 weeks, all the kidneys were collected for histological examination. In diabetes control group, the renal lesions mainly existed in glomerulus and renal tubules. As compared with the diabetes control rats, oat-fed rats showed significantly less severe sclerosis in glomerulus segments. Nevertheless, oat reduced the incidence of tubule vacuolar degeneration and severity level, suggesting some preventive effect on renal tubule lesions. Representative photomicrographs of glomerular changes were shown in Figure 4. The major differences in diabetes controls and oat-fed animals were segmental and diffuse mesangial expansion of the glomeruli. Hypocellular, sclerotic lesions compatible with diabetic glomerulosclerosis were observed in glomeruli of the diabetic animals under a light microscope.

4. Discussion

In the present study, 21 weeks diabetes by STZ produced a diabetic nephropathy which was manifested by increased creatinine, creatinine clearance, serum BUN, and 24 h urinary albumin. Feeding with Oat significantly reversed the alterations of renal function and confers a hypoglycemic effect which contributes at least in part to a reversal of diabetic nephropathy. An increment in the urine albumin excretion in 24 h was significantly aggravated in diabetic nephropathy that is an important sign to indicate progressive damage to glomerular and tubular cells in diabetic kidney. Oat feeding slows down the 24 hour urinary albumin excretion, in association with a significant decline in BUN and creatinine and creatinine clearance in serum, respectively.

Hyperglycemia causes an increased production of free radical which plays a major role in the disruption of the cellular functions of the kidney that correlates to a decline in the endogenous ROS scavengers such as GSH-px, SOD, GST and catalase (Kalia et al., 2004; Cameron et al., 2005; Jandeleit-Dahm et al., 2005). Oxidative stress as indicated by the high levels of 8-OHdG and the overproduction of the superoxide anions is implicated in the pathophysiology of diabetic nephropathy (Vural et al., 2002). Oat feeding confers the benefit to suppress MDA and 8-OHdG production. In addition, the activity of GST, GSH-px catalase, and SOD was elevated which results in the reduction of progression of the renal lesions.

The overproduction of ROS in diabetes results directly in the enhanced formation of AGE [3]. One important receptor for mediating AGE effects is RAGE [24]. RAGE is expressed in the diabetic kidney, both in tubular and in glomerular cells [25]. Binding of AGE such as CML to RAGE augments and propagates oxidative stress in the kidney tissue [25, 26].



(a) (b) (c)

Figure 4: Mesangial expansion and ultra structural changes in diabetic apoE−/− mice after 12 weeks after induction of diabetes. (a) Nondiabetic mice without PA. (b) Diabetic mice without PA. (c) Diabetic mice with PA. PAS staining, original magnification x200.

RAGE signals via the transcription factor NFκB to activate target genes which have deleterious potential for the diabetic kidney [23, 27, 28]. Feeding with oat reduced RAGE expression, NFκB p65 antigen, and NFκB binding activity. The pivotal role of RAGE in the mediation of ROS/AGE-induced diabetic microangiopathy is supported by experiments using transgenic and knockout mice. RAGE-null mice are protected from diabetes-induced kidney damage (H.-P. Hammes, A. Bierhaus et al., unpublished observations). Thus, our data suggest that part of the beneficial effect of oat includes the disruption of the detrimental AGE-RAGE-NFκB axis.

Activation of NFκB results in its migration into the nucleus. In the nucleus, it upregulates the transcription of its controlled genes like IL-6, TGFβ, and so forth. In the diabetic group, the levels of IL-6 in both serum and kidney homogenate as well as the levels of TGFβ were markedly increased. The results are in line with previous data in parallel with the NFκB results in Figure 2. Feeding with oat reduced AGE and NFκB activation and this resulted in the attenuation of the levels of both cytokine.

Diabetic nephropathy is caused by many factors and cannot be sufficiently controlled with a strict control of hyperglycemia. The early stage diabetic nephropathy is induced by ambient hyperglycemia, but secondary effects are not dependent on persistent hyperglycemia (Vestra and Fioretto, 2003) and it is therefore not enough to merely control serum glucose levels in order to retard the development of diabetic nephropathy. The level of oxidative stress, AGE and NF-κB, together with the elevated levels of cytokine llike IL-6 and TGFβ1, play crucial roles in the functional and pathological damage to the kidney. Feeding with oat slows down the progression of the disease progression by normalizing these abnormalities.

In summary, we provide evidence that ROS scavenging is an effective approach for the prevention of diabetic nephropathy. Oat is a paradigm food supplement with a broad spectrum of beneficial effects, based on its ability to reduce the sequelae of hyperglycaemia-induced ROS overproduction. Since oat also has beneficial effects on other target tissues of diabetic angiopathy and shows beneficial effects on mediators of large vessel damage, this concept appears attractive for the prevention or delay of diabetic angiopathy.

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Research Article

Scopoletin Inhibits Rat Aldose Reductase Activity and Cataractogenesis in Galactose-Fed Rats

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Cataracts are a major cause of human blindness. Aldose reductase (AR) is an important rate-limiting enzyme that contributes to cataract induction in diabetic patients. Scopoletin is the main bioactive constituent of flower buds from *Magnolia fargesii* and is known to inhibit AR activity. To assess scopoletin's ability to mitigate sugar cataract formation *in vivo*, we studied its effects in a rat model of dietary galactose-induced sugar cataracts. Galactose-fed rats were orally dosed with scopoletin (10 or 50 mg/kg body weight) once a day for 2 weeks. Administering scopoletin delayed the progression of the cataracts that were induced by dietary galactose. Scopoletin also prevented galactose-induced changes in lens morphology, such as lens fiber swelling and membrane rupture. Scopoletin's protective effect against sugar cataracts was mediated by inhibiting both AR activity and oxidative stress. These results suggest that scopoletin is a useful treatment for sugar cataracts.

1. Introduction

Cataracts are the leading cause of blindness worldwide. Hyperglycemia and diabetes increase the risk of developing cataracts [1]. Cataractogenesis under diabetic or galactosemic conditions is directly linked to the aldose-reductase- (AR-) catalyzed accumulation of sorbitol or galactitol from glucose or galactose, respectively. Accumulating excess sorbitol or galactitol initiates osmotic stress, altering lens cell permeability and redox homeostasis, as well as decreasing ATPase activity and crystallin synthesis [2, 3].

Although cataracts can be successfully treated with surgery, it remains important to find nonsurgical treatments for this condition. Synthetic AR inhibitors (ARIs) have been studied to treat diabetic cataracts. The use of traditional medicines, which are mainly derived from plant sources, has remained critical for managing many chronic diseases [4]. Consuming foods or medicinal plants containing micronutrients with potential anti-AR activities may protect against cataracts [5–9].

Scopoletin (Figure 1) is one of the major coumarin constituents of the flower buds of *Magnolia fargesii*; this plant has been used to treat various inflammatory diseases as a component of traditional Chinese medicines [10]. Several coumarins reportedly block angiogenesis by inhibiting endothelial cell growth [11, 12]. Of the substances found in this plant extract, scopoletin was chosen for study because it possesses a wide range of biological effects, including anti-inflammatory, hypouricemic, and antioxidant activities [13–15]. Recently, scopoletin was reported to regulate hyperglycemia and diabetes [16]. In our previous study, scopoletin from the flower buds of *M. Fargesii* inhibited protein glycation, aldose reductase, and cataractogenesis *ex vivo* [17]. However, *in vivo* anticataract activity and the biochemical mechanism of scopoletin have not been understood yet. In this study, we investigated the effect of scopoletin on galactose-induced cataracts and studied the biochemical mechanism of this protection.

2. Materials and Methods

2.1. Animals and Experimental Design. To elucidate the effect of scopoletin treatment on sugar cataracts *in vivo*, a galactose-fed rat model was used. Scopoletin was purchased from

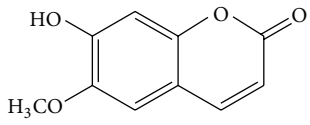

Figure 1: Chemical structure of scopoletin.

Sigma (St. Louis, MO, USA). Male Sprague-Dawley (SD) rats (~200 g) were randomized into four groups of 10 rats: Group 1 rats received a normal diet; Group 2 rats received 50% galactose diet (50% w/w with normal diet); Group 3 and Group 4 rats were fed the galactose diet and treated orally with scopoletin (10 and 50 mg/kg body weight, resp.) once a day for 2 weeks. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee.

2.2. Analysis of Cataract Formation and Its Severity Gradation. After two weeks of treatment, the rats eyes were enucleated under deep anesthesia after an intraperitoneal injection of 10 mg/kg zolazepam (Zoletil, Virbac, Carros, France) mixed with 10 mg/kg xylazine hydrochloride (Rumpun, Bayer, Frankfurt, Germany). The lenses were excised from the eyeball under an optical microscope, and the lenses's wet weights were calculated. The lenses were transferred onto 24-well plates with each well each containing 2 mL saline solution and were photographed under an optical microscope with a CCD camera. The severity of the cataracts was evaluated using the following gradation: grade 0, no opacity; grade I, vacuoles present at a part of the cortical equator; grade II, vacuoles present at all parts of the cortical equator; grade III, vacuoles and their confluents spreading from the cortical equator toward the center of the cortex; grade IV, large, interconnected opacities covering the whole cortex. The opaque areas of the lenses were analyzed using an imaging program (ImageJ, NIH, USA). The data are expressed as the percentage of opaque area relative to the entire lens area.

2.3. Analysis of Lens Fiber Degeneration. The isolated lenses were fixed in 10% neutralized formalin for 24 h and embedded in paraffin. To analysis the lens fiber degeneration, fiber cells were visualized by labeling their membranes with wheat germ agglutinin. The lens sections were deparaffinized in xylene and rehydrated. The sections were reacted with 2.5 mg/mL rhodamine-conjugated wheat germ agglutinin (Vector Laboratories, CA, USA) for 60 minutes. All specimens were examined with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

2.4. Determination of AR Activity. A 10% lens homogenate was prepared from two to three pooled lenses in a 50 mM phosphate buffer (pH 7.4). The incubation mixture contained 135 mmol/L Na, K-phosphate buffer (pH 7.0), 100 mmol/L lithium sulfate, 0.03 mmol/L NADPH, 0.04 mmol/L dL-glyceraldehyde, and 150 μL of lens homogenate, in a total volume of 1.0 mL. The reaction was initiated by adding NADPH at 37°C and stopped by adding 0.3 mL of 0.5 N hydrochloric acid. Subsequently, 1 mL 6 N NaOH containing 10 mmol/L imidazole was added, and the mixture was incubated at 60°C for 10 min to convert NADP into a fluorescent product. The fluorescence was measured at room temperature with a spectrofluorophotometer (Ex/Em = 360 nm/460 nm; Synergy HT, Bio-Tek, VT, USA). All measurements were performed in triplicate.

2.5. Lens Polyol Levels. The lens galactitol was measured as previously reported [18]. Briefly, each lens was homogenized in a ground glass homogenizer, and an aliquot of the homogenate was removed for colorimetric protein quantification using a DC Protein Assay (Bio-Rad Laboratories, CA, USA) with bovine serum albumin (BSA) protein standards. Seventy-five microliters of 10% trichloroacetic acid (TCA) was added to 125 μL centrifuged lens homogenate; the mixture was centrifuged at 12,000 rpm for 5 min. To 15 μL protein-free supernatant, we added 50 μL 1 N HCl and 250 μL 25 nM $NaIO_4$. The mixture was incubated for 30 min at 37°C. Afterward, 50 μL 1.4 N NaOH and 50 μL 10% $ZnSO_4$ were added. The mixture was allowed to stand for a few minutes after vortexing before adding 500 μL 2 M ammonium acetate containing 20 mM pentanedione. The methyltoluidine (absorbance maximum 415 nm) content was measured in the supernatant after incubation for 1 h at 37°C. The polyol standards were treated in the same manner, and the galactitol in the lens homogenate samples was completely recovered.

2.6. Glutathione Levels. Each lens was homogenized in a ground glass homogenizer and the insoluble proteins were removed by centrifugation at 4°C. The remaining cell supernatants were deproteinized with equal volumes of 20% TCA, and the glutathione (GSH) levels in the deproteinized supernatant were measured at 412 nm using the DTNB (5-5′-dithiobis[2-nitrobenzoic acid]) method [19].

2.7. Immunofluorescence Staining. Lens sections were deparaffinized and hydrated by sequential immersions in xylene and graded alcohol solutions. The slides were placed in 10 mM sodium citrate buffer (pH 6.0) and autoclaved at 121°C for 10 min. Sections were then blocked with normal serum obtained from the same species with a secondary antibody developed to block nonspecific staining. The sections were first labeled with mouse anti-AR antibody (1 : 250, Santa Cruz Biotechnology, CA, USA) overnight at 4°C. After washing, the sections were labeled with fluorescein-isothiocyanate- (FITC-) conjugated goat anti-mouse IgG (1 : 1000, Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. Finally, the slides were analyzed using fluorescence microscopy (BX51, Olympus). The negative controls for immunostaining were run by incubating the sections with nonimmune serum instead of the primary antibody.

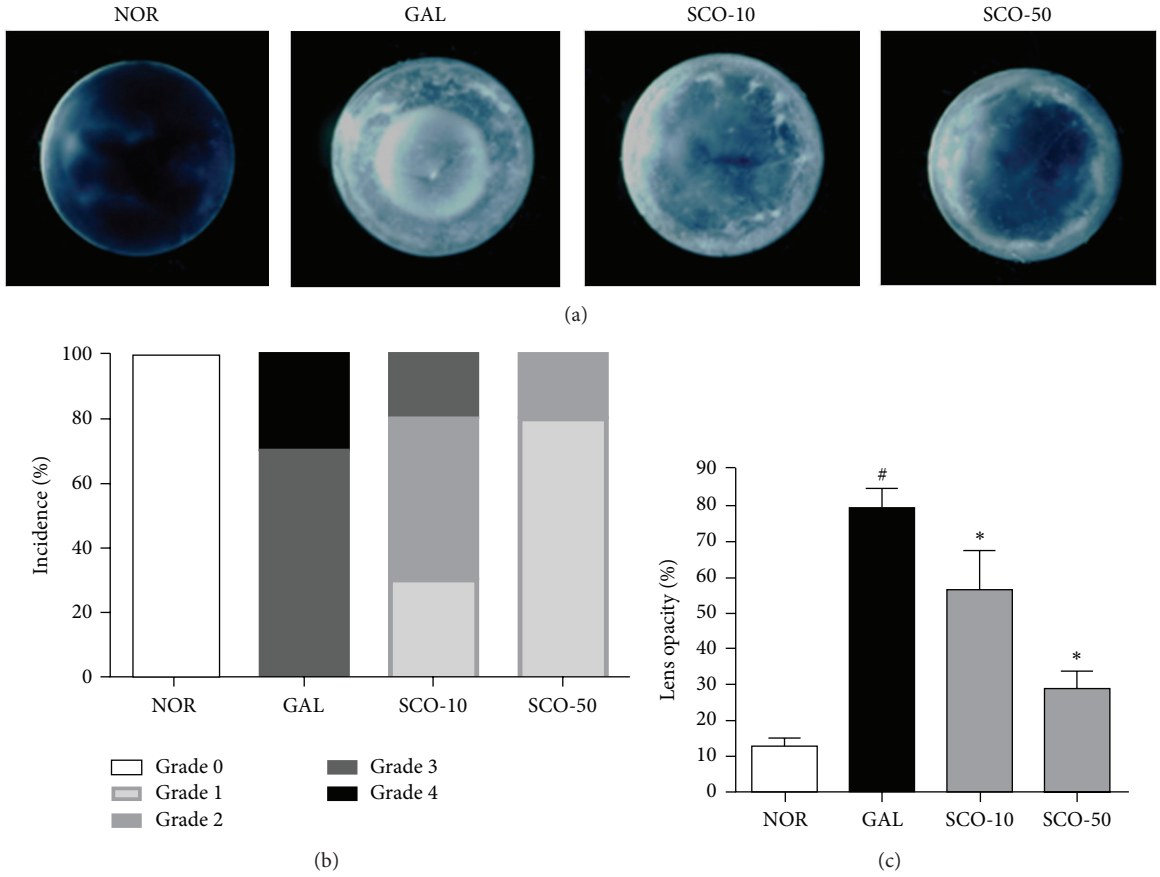

Figure 2: Lens opacity. (a) Representative images of the lenses in each group. (b) Cataract grading. The cataracts were assessed on a scale of 0–4. (c) Analyses of lens opacities. The opacities were analyzed for each lens from the normal rats (NOR), the vehicle-treated galactose-fed rats (GAL), the galactose-fed rats treated with scopoletin at concentration 10 mg/kg (SCO-10), and the galactose-fed rats treated with scopoletin at concentration 50 mg/kg (SCO-50). All data are expressed as the means ± SE, $n = 10$. $^{\#}P < 0.01$ versus normal control rats, $^{*}P < 0.01$ versus vehicle-treated galactose-fed rats.

2.8. Statistical Analysis. The results were statistically evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 5.0 software (GraphPad, CA, USA).

3. Results

3.1. Cataract Formation Analysis. During the cataract analysis, all of the animals fed on the galactose diet developed mature cataracts after two weeks (70% were in grade III and 30% were in grade IV, Figures 2(a) and 2(b)). The scopoletin treatment delayed the onset of galactose-induced cataracts in a dose-dependent manner. The highest dose of scopoletin treatment (50 mg/kg/day) delayed the onset of cataracts (80% were in grade I and 20% were in grade II, Figures 2(a) and 2(b)). When analyzing the lenses' opacification, the mean opaque area of the lenses was increased 8-fold in the galactose-fed rats relative to the normal rats; the opacity was suppressed by the scopoletin treatment in a dose-dependent manner (Figure 2(c), $P < 0.01$). Therefore, scopoletin slowed the development of galactose-induced cataracts.

3.2. Lens Fiber Cell Degeneration. In Figure 3, the membrane-labeled lens section illustrates the histological findings after the two weeks of study. No significant alterations in the cuboidal epithelium or lens fiber were observed in the lenses of the control rats. The lenses of galactose-fed rats were swollen, degenerated, vacuolated, and liquefied with degenerated lens fibers. Although the lenticular damage was severe, no corneal or retinal damage was observed. However, this histological change in the lens fibers of galactose-fed rats was prevented in a dose-dependent manner after scopoletin treatment.

3.3. Polyol Pathway in Lens. The galactose-fed rats were killed after two weeks; some rats were progressing toward grade 3 cataracts, and extensive protection by scopoletin was observed. AR is a key enzyme in the polyol pathway; its activity was significantly elevated in the galactose-fed rats. The AR activity in the lenses from the scopoletin-treated animals was decreased (Figure 4(a)), agreeing with our observations during our *in vitro* studies [17]. In addition, the galactitol levels in the galactose-fed rats increased relative to the control

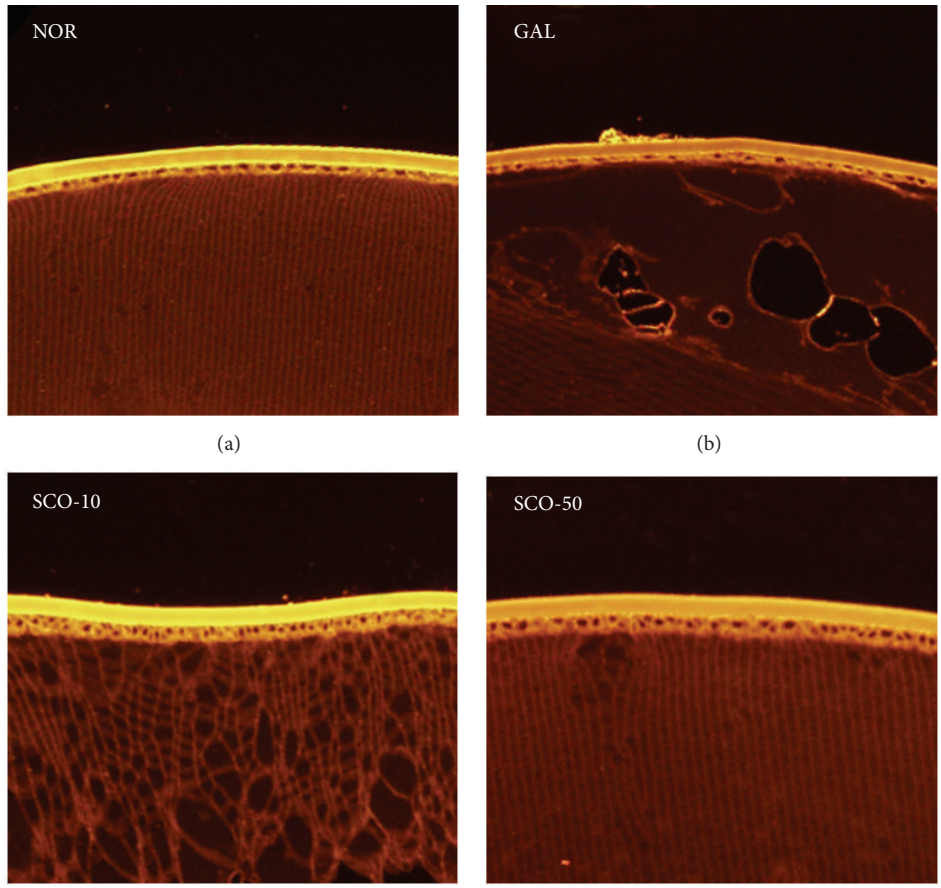

Figure 3: Lens fiber changes. The lens sections from the normal rats (NOR), the vehicle-treated galactose-fed rats (GAL), the galactose-fed rats treated with scopoletin at concentration 10 mg/kg (SCO-10), and the galactose-fed rats treated with scopoletin at concentration 50 mg/kg (SCO-50) were labeled with rhodamine-conjugated wheat germ agglutinin. Fiber cell liquefaction, swelling, and membrane rupture were observed in galactosemic cataractous lens.

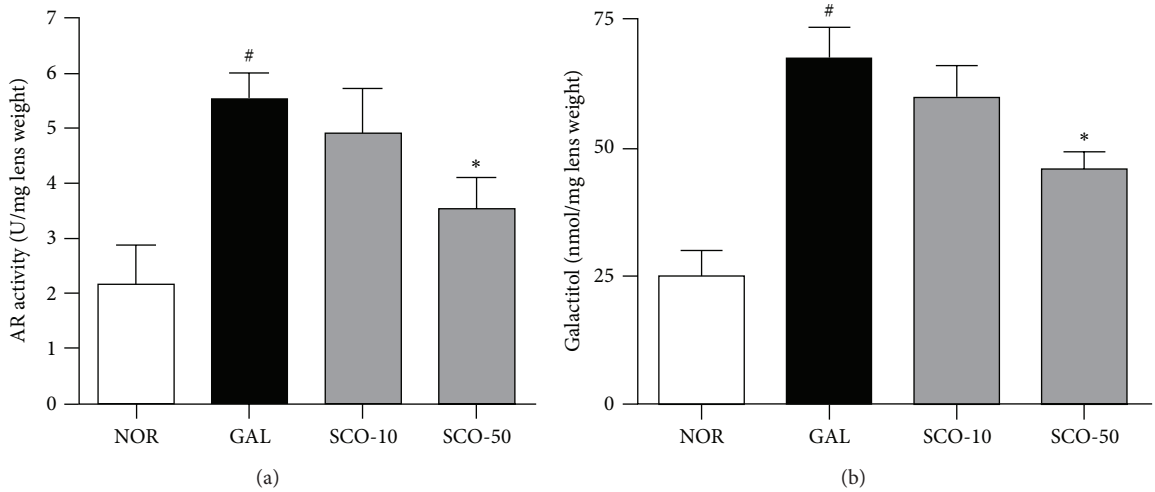

Figure 4: Polyol pathway. (a) Aldose reductase (AR) activity, (b) galactitol levels in lenses from the normal rats (NOR), the vehicle-treated galactose-fed rats (GAL), the galactose-fed rats treated with scopoletin at concentration 10 mg/kg (SCO-10), and the galactose-fed rats treated with scopoletin at concentration 50 mg/kg (SCO-50). All data are expressed as the means ± SE, $n = 10$. $^{\#}P < 0.01$ versus normal control rats, $^{*}P < 0.01$ versus vehicle-treated galactose-fed rats.

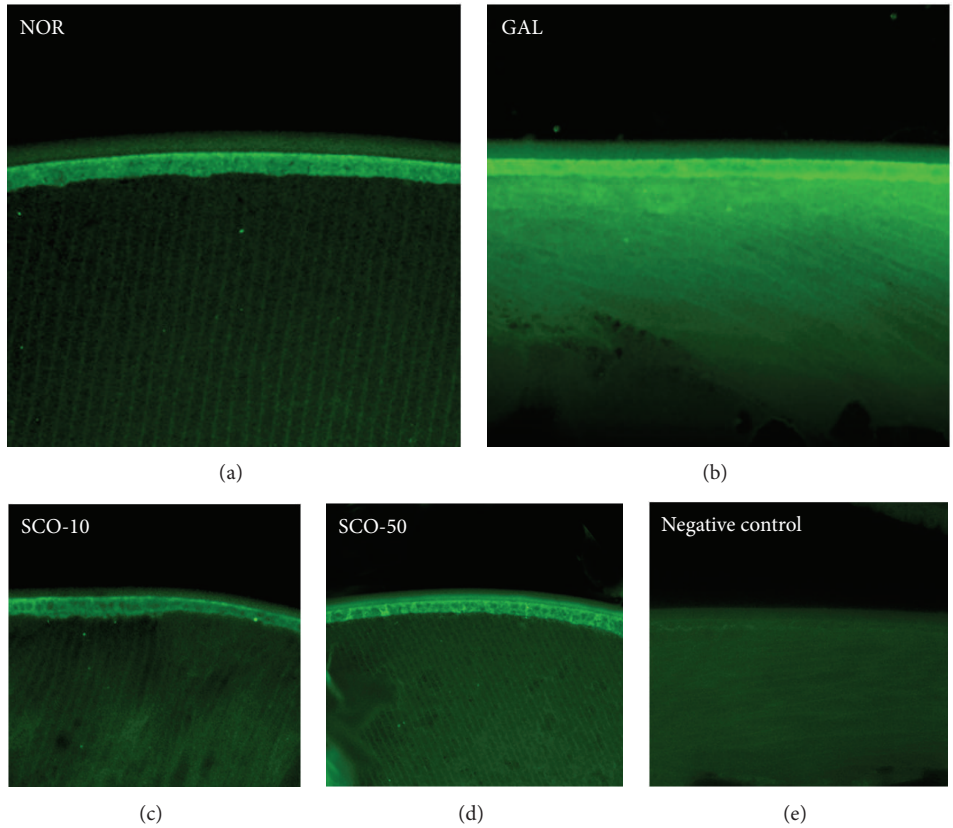

Figure 5: Immunofluorescence stained AR. Representative immunostained AR in lenses from the normal rats (NOR), the vehicle-treated galactose-fed rats (GAL), the galactose-fed rats treated with scopoletin at concentration 10 mg/kg (SCO-10), and the galactose-fed rats treated with scopoletin at concentration 50 mg/kg (SCO-50). AR was strongly immunoreactive in the cytoplasm of the lens epithelial cells and lens cortical fibers. The negative control section was incubated with nonimmune mouse IgG and remained unstained.

rats (Figure 4(b)) as expected because the polyol pathway was activated. However, administering scopoletin resulted in less galactose-induced lenticular galactitol accumulation.

3.4. AR Protein Expression in Lens. In vehicle-treated galactose-fed rats, immunoreactive straining for AR increased in the cytoplasm of lens epithelial cells and extended into the deeper cortical fibers. However, the scopoletin treatment prevented AR expression in the lens epithelial cells and inhibited the extension of AR beneath the epithelial region (Figure 5).

3.5. GSH Levels in Lens. The GSH status after treatment indicated that the rats fed with galactose displayed lower GSH levels in their lenses relative to the control. The scopoletin treatment given with the dietary galactose prevented decreases in GSH levels in the lenses (Figure 6).

4. Discussion

In this study, we investigated the protective effects of scopoletin against cataractogenesis in galactose-fed rats. The scopoletin treatment delayed the progression and reduced the extent of cataract formation. Currently, the only treatment for cataracts is surgery. It has been estimated that a 10-year delay in the onset and progression of a cataract could reduce the need for cataract surgery by 50% [20].

Galactosemic and diabetic cataractogenesis in experimental animals and humans might be primarily due to the increased formation of polyols from the reduced aldose sugars produced by aldose reductase and nicotinamide adenine dinucleotide phosphate (NADPH) [21]. Polyols may accumulate in the lens fiber cells, causing increased cell hydration, membrane stretching, and dysfunction. Galactose-fed rats are a popular model used to examine the role of the AR pathway in diabetic complications. In addition, the galactose-induced cataracts develop within a week of feeding; this model has been used extensively to study the morphological and biochemical changes during cataractogenesis. Galactitol is a metabolite of galactose by AR that can accumulate in the lens. Because the cellular lens membranes are impermeable to galactitol, hyperosmotic cell swelling occurs, causing light scattering and diminished lens transparency [22–24]. In this study, scopoletin inhibited the lenticular AR activity and the accumulation of galactitol in galactose-fed rats. This AR inhibition corresponded to the anticataractogenic activity.

ARIs, such as sorbinil, prevented sugar cataractogenesis in experimental animals [25, 26]. Among the ARI, only sorbinil has reached advanced clinical trials in cataract

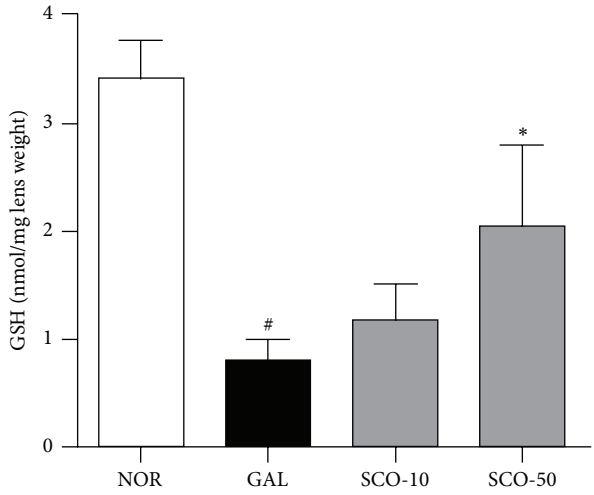

Figure 6: Glutathione (GSH) alteration. GSH was measured in the lenses from the normal rats (NOR), the vehicle-treated galactose-fed rats (GAL), the galactose-fed rats treated with scopoletin at concentration 10 mg/kg (SCO-10), and the galactose-fed rats treated with scopoletin at concentration 50 mg/kg (SCO-50). All data are expressed as the means ± SE, $n = 10$. $^{\#}P < 0.01$ versus normal control rats, $^{*}P < 0.01$ versus vehicle-treated galactose-fed rats.

prevention programs. However, due to the manifestation of skin rashes, the trial was discontinued [27]. Although several previous studies demonstrated that ARIs inhibited sugar cataracts by inhibiting AR, no single agent has been proven clinically effective during the treatment of sugar cataracts. Many naturally occurring compounds have strong AR inhibitory activity *in vitro* [28]. Recently, scopoletin demonstrated effective AR inhibitory activity [17, 29]. Coumarins are bicyclic phenolic compounds that harbor a lactone moiety; this functionality might participate in AR inhibition by hydrogen bonding with the TYR48, HIS110, and TRP111 residues in AR [30]. Based on these results, preventing sugar cataracts with scopoletin is partially related to AR inhibition and galactitol accumulation in the lens.

In our previous study, scopoletin had an excellent inhibitory activity on AR, displaying an IC_{50} value of 4.32 μg/mL [17]. Liu et al. reported that the peak plasma scopoletin concentrations (Cmax) were 0.51, 0.68, and 1.49 μg/mL and reached approximately 40 minutes after administering 50, 100, and 250 mg/kg scopoletin in rabbits, respectively [31]. The peak plasma concentration of scopoletin was 8.2 μg/mL after administering 50 mg/kg scopoletin in rats [32]. Because scopoletin was highly lipophilic, it absorbed effectively after oral administration and spread widely to different tissues [33]. Based on its previously reported pharmacokinetics and our *in vitro* results, we chose 10 and 50 mg/kg doses of scopoletin to evaluate its anti-AR activity in rats. We found that the effective dose of scopoletin was 50 mg/kd, agreeing with the *in vitro* result.

The enzymatic distribution of AR activity was supported by AR's localized immunofluorescence. In human and rat lenses, AR is primarily localized in the epithelial and superficial cortical fiber cells [34]. In galactose-fed rats, the enhanced immunoreactive staining of AR was observed in the epithelial cells and the cortex region. This staining decreased, progressing from the superficial region to the deeper cortex. Scopoletin inhibited the extension of AR beneath the epithelial region. Therefore, the decrease in AR activity observed in the scopoletin-treated rats was caused by the reduced amount of AR protein.

Although the prevention of sugar-induced cataractogenesis by ARIs appears to be caused by AR inhibition, the osmotic hypothesis might not fully explain diabetic cataracts in human subjects because, even during severe hyperglycemia, the examined tissues, including the lens, did not have sorbitol levels >2 mM [35]. Antioxidants effectively slow sugar cataract formation. Butylated hydroxytoluene is a well-known synthetic phenolic antioxidant that slows cataract formation in rat lenses cultured under high-glucose conditions, although the sorbitol and fructose levels in the lenses remains elevated [36]. Consequently, Wolff and Crabbe suggested that the ARIs protected against sugar cataracts due to the antioxidant nature of these inhibitors [37]. Scopoletin has demonstrated benefits for oxidative injury as an antioxidant [38, 39]. In this study, scopoletin preserved the lenticular GSH content. Therefore, one of the possible mechanisms for scopoletin during sugar cataract development may involve the protection of the lens cell membrane from oxidative damage.

In summary, this study reveals that scopoletin may exert beneficial/protective effects during the sugar cataract development. Scopoletin inhibits the AR activity, polyol accumulation, and reduction of the GSH levels. We suggest that the scopoletin may be particularly useful in treating sugar cataracts.

Acknowledgments

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Research Article

The Korean Mistletoe (*Viscum album coloratum*) Extract Has an Antiobesity Effect and Protects against Hepatic Steatosis in Mice with High-Fat Diet-Induced Obesity

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This study investigates the inhibitory effects of Korean mistletoe extract (KME) on adipogenic factors in 3T3-L1 cells and obesity and nonalcoholic fatty liver disease (NAFLD) in mice fed a high-fat diet. Male C57Bl/6 mice fed a high-fat diet were treated with KME (3 g/kg/day) for 15 weeks for the antiobesity and NAFLD experiments. Body weight and daily food intake were measured regularly during the experimental period. The epididymal pad was measured and liver histology was observed. The effects of KME on thermogenesis and endurance capacity were measured. The effects of KME on adipogenic factors were examined in 3T3-L1 cells. Body and epididymal fat pad weights were reduced in KME-treated mice, and histological examination showed an amelioration of fatty liver in KME-treated mice, without an effect on food consumption. KME potently induces mitochondrial activity by activating thermogenesis and improving endurance capacity. KME also inhibited adipogenic factors *in vitro*. These results demonstrate the inhibitory effects of KME on obesity and NAFLD in mice fed a high-fat diet. The effects appear to be mediated through an enhanced mitochondrial activity. Therefore, KME may be an effective therapeutic candidate for treating obesity and fatty liver caused by a high-fat diet.

1. Introduction

Obesity is the most common metabolic disorder and results from the combined effects of excess energy intake and reduced energy expenditure [1]. It is one of the fastest growing major disorders throughout the developed nations [2]. It is associated with a variety of clinical disorders in developed nations [2]. Obesity is associated with a variety of clinical disorders, including hypertension, insulin resistance, glucose intolerance, and dyslipidemia. It is well known that an oversupply of fat is associated with the development of obesity in mice [3]. Long-term feeding with a high-fat diet can induce obesity with hyperlipidemia, insulin resistance, hyperphagia, and hypergluconemia [4, 5].

Nonalcoholic fatty liver disease (NAFLD) is present in up to one-third of the general population and affects all ages and ethnic groups. NAFLD is the second leading cause of death in the general population [6, 7]. At present, there is no pharmacological agent known to reverse NAFLD and effective medical interventions have focused on the modification of risk factors, such as weight reduction and diet [8].

Mistletoe is a hemiparasitic plant growing all over the world on various deciduous trees, such as oak. It has been

used as a traditional herbal medicine, especially in Europe [9]. Korean mistletoe (KM, *Viscum album cololatum*) extract (KME) has been shown to have anticancer [10], antioxidant [11], antidiabetes [12] and antidementia [13] effects, and it enhances immune system function [14]. Recently, we reported that KME significantly increases mitochondrial function [15].

Mitochondria are known as "cellular power plants" because they generate most of the cellular supply of adenosine triphosphate (ATP) which is used as a source of chemical energy. Studies have implicated mitochondria in several human diseases, including metabolic diseases [16], cardiac dysfunction [17], mental disorders [18], and the aging process [19]. Decreased mitochondrial respiration rates [20] and reduced expression of genes involved in mitochondrial oxidative capacity [21] have been reported in diet-induced obese rats. Because KME improves mitochondrial function, we asked whether it has beneficial effects on obesity.

It is well known that adipocyte differentiation and the extent of subsequent fat accumulation are closely related to the occurrence and advancement of various diseases, such as coronary artery disease and obesity [1, 22, 23]. 3T3-L1 cells have served as a useful *in vitro* model for adipocyte differentiation and function [24]. The differentiation of preadipocytes into adipocytes requires a variety of effectors that activate a cascade of transcription factors, such as CCAAT/enhancer-binding protein-α (C/EBP-α), peroxisome proliferator-activated receptorγ (PPAR-γ), and sterol regulatory element binding element protein-1c (SREBP-1c). This cascade begins with the CCAAT enhancer-binding protein- (C/EBP-) β and δ, which induces the expression of C/EBP-α and PPAR-γ [25–27]. These transcription factors coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype, including genes for adipocyte fatty acid-binding protein, glucose transporter 4, lipoprotein lipase (LPL) and leptin [28, 29]. SREBP-1c is strongly associated with SREBP cleavage-activating protein (SCAP). Activated SREBP-1c accelerates adipogenesis through the overexpression of adipogenic enzymes, such as fatty acid synthase(FAS), acyl-CoA synthase (ACC), and acyl-CoA carboxylase (ACS). LPL is the major enzyme that hydrolyzes triglyceride (TG) molecules of chylomicrons and VLDL particles. The released free fatty acids are either oxidized to generate ATP in muscle or stored in adipose tissue [30].

We measured the inhibitory effect of KME on adipogenic factors in 3T3-L1 cells and on the development of obesity and NAFLD in mice fed a high-fat diet. Our data show that KME suppresses adipocyte differentiation through down-regulation of adipogenic factors and could ameliorate obesity and NAFLD in mice fed a high-fat diet. Our results indicate the great potential of KME as a potential metabolic regulator of adipocyte differentiation and a potential therapeutic agent for preventing or treating obesity and NAFLD.

2. Materials and Methods

2.1. Preparation of KME. Mistletoe growing on oak was harvested from Gangwon-do, Republic of Korea, in February. The mistletoe was 1 or 2 years old, and the leaves, stems, and fruits were cut into 2 joints from the end of a branch, washed with distilled water (DW), and dried. The vacuum-packed mistletoe was stored at −80°C until extracted. Then mistletoe leaves, fruits, and stems were freeze-dried, crushed and ground in approximately 10 volumes of DW for 30 seconds. After being washed, they were ground in a mixer for 2 minutes and stirred for 16 hours at 4°C. To obtain fine mistletoe extract, the homogenized mistletoe was centrifuged at 8,000 rpm for 30 minutes at 4°C, and the resulting supernatant was successively filtered through different pore sizes (0.9 and 0.45 μm). The mistletoe extract was freeze-dried and resuspended in DW at an appropriate dilution factor.

2.2. Animals and Diets. Lean, male C57BL/6 mice (7 weeks old) were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). All mice were housed for 1 week under a 12/12-hour light/dark cycle in a temperature—(22 ± 1°C) and humidity—(55 ± 5%) controlled room and fed standard laboratory chow and water ad libitum. KME was mixed with either powdered chow (M07, Feedlab, Republic of Korea) or high-fat (D12327, Research Diets, Inc., New Brunswick, NJ, USA) feed at a concentration of 4 g/kg of food to provide a 3000 mg/kg/day (mpk) dose. The powdered chow was then made into pellets. Control groups received pellets without KME. These dietary amounts represent the maximum amount of chow that these animals were able to consume during a 24-hour period. Seven C57BL/6 mice on the above *ad libitum* diets had their daily average caloric intake and weekly body weight measurement during the course of the study. At the conclusion of the *in vivo* experiment, the mice were sacrificed and the epididymal pads were collected and weighed.

2.3. Cold Test. The adaptive thermogenic response was measured in a cold test, during which the animals were individually housed at 4°C for 6 hours [31].

2.4. Liver Histology. Liver tissues were isolated immediately after sacrifice. For hematoxylin and eosin (HE) staining, tissues were fixed in 10% formalin and processed and embedded in paraffin prior to sectioning (10 μm.) and staining. Liver biopsies for electron microscopy were cut into 1 mm pieces, fixed immediately after collection in Karnovsky fixative (glutaraldehyde in cacodylate buffer), and kept at 4°C. The second step involved postfixation with 1% osmium tetraoxide in 0.1 M cacodylate buffer for 1 hour at 4°C. Tissues were then dehydrated through successive baths of graded alcohol, followed by a propylene oxide bath and a treatment with propylene oxide and resin mix before being embedded in a pure epoxy resin (araldite, Epon 812) that solidified after 48 h at 60°C. Semithin sections were cut at 2 μm, stained with toluidine blue, and then analyzed by light microscopy. Ultrathin sections were cut at 70 nm and examined with an electron microscope (BX 50, Olympus, Japan). Electron micrographs (400x magnification) were digitized and analyzed using ACD SEE, version 4.0. The liver of 1 mouse from each group (control, high-fat diet (HFD), HFD + KME 3000 mg/kg)

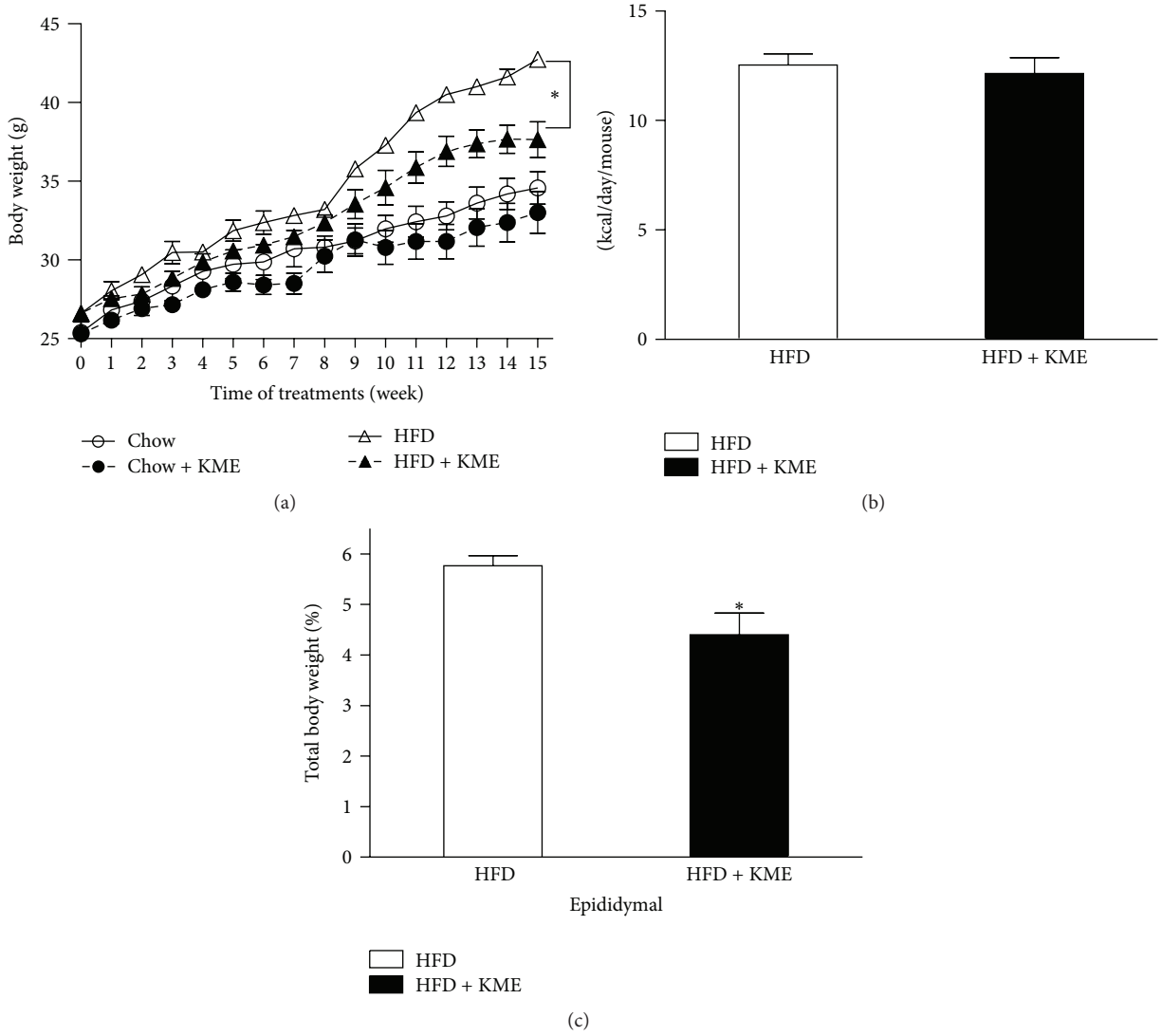

Figure 1: Effect of KME on body weight and food intake in mice fed a high-fat diet for 15 weeks. (a) Changes in body weight gain at each treatment period are shown. (○) C: chow diet; (•) C + KME: chow diet plus KME 3000 mg/kg; (△) HFD: high-fat diet; (▲) HFD + KME: high-fat diet plus KME 3000 mg/kg. (b) Average food intake expressed as kcal/mouse/day. (c) Epididymal fat weight expressed as percentage of total body weight. Values represent mean ± standard error of mean (SEM; $^{*}P < 0.05$, $n = 7$ per group).

was measured. In brief, the following criteria were used for scoring hepatic steatosis: grade 0, grade 1, hepatocytes occupying <33% of the hepatic parenchyma; grade 2, fatty hepatocytes occupying 33–66% of the hepatic parenchyma; and grade 3, fatty hepatocytes occupying >66% of the hepatic parenchyma.

2.5. Endurance Test. Mice were subjected to an endurance test using a variable-speed belt treadmill enclosed in a plexiglass chamber with a stimulus device consisting of a shock grid attached to the rear of the belt (Columbus Instruments Oxymax System, Columbus, OH, USA). The shock grid was set to deliver 0.2 mA, which caused an uncomfortable shock but did not physically harm or injure the animals. Animals were acclimatized to the test using a habituation protocol. Mice were run at 16.2 meter/minutes for 10 minutes with a 5° incline. For high-fat- (HF-) fed animals, the experiment was initiated at 10.8 meter/minutes at a 0° incline. The speed was gradually increased from 10.8 to 24.6 meter/minutes and then maintained until exhaustion [15]. Exhaustion was determined to have been reached if the mice were unable to run for 10 seconds [32], at which point the electric shock was discontinued. Figure 1 shows the running test protocol.

2.6. Cell Culture and Induction of Adipocyte Differentiation. 3T3-L1 preadipocytes purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's-modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) containing 10% bovine calf serum (BCS, Hyclone, Logan, Utah, USA) at 37°C in a humidified atmosphere of 5% CO_2. After 3 or 4 days, the cells had reached 90% confluence and were collected with 0.05% trypsin/0.53 mM

Table 1

Gene name	Accession number		Sequence
ACC	AY451393	Forward	5′-GAG TGA CTG CCG AAA CAT CTC TG-3′
		Reverse	5′-GCC TCT TCC TGA CAA ACG AGT-3′
ACS	NM_007981	Forward	5′-TGA CCT CTC CAT GCA GTC AG-3′
		Reverse	5′-GAG CCT ATG CAC TCA GCC AGT-3′
FAS	NM_007988	Forward	5′-TGG GTT CTA GCC AGC AGA GT-3′
		Reverse	5′-TAC CAC CAG AGA CCG TTA TGC-3′
LPL	BC003305	Forward	5′-GCC CAG CAA CAT TAT CCA GT-3′
		Reverse	5′-GGTCAG ACT TCC TGC TAC GC-3′
SREBP-1c	8C056922	Forward	5′-AAT GGT CCA GGC AAG TTC TGG GT-3′
		Reverse	5′-TCC CTC TCA GCT GTG GTG GTG AA-3′
C/EBP-α	M62362	Forward	5′-TGC TGG AGT TGA CCA GTG AC-3′
		Reverse	5′-AAA CCA TCC TCT GGG TCT CC-3′
PPAR-γ	NM_011146	Forward	5′-TTT TCA AGG GTG CCA GTT TCA ATC C-3′
		Reverse	5′-AAT CCT TGG CCC TCT GAG AT-3′
UCP1	NM_009463	Forward	5′-GGC CCT TGT AAA CAA CAA AAT AC-3′
		Reverse	5′-GGC AAC AAG AGC TGA CAG TAA AT-3′
β-Actin	NM_007393	Forward	5′-GAC TAC CTC ATG AAG ATC-3′
		Reverse	5′-GAT CCA CAT CTG CTG GAA-3′

ACD: acyl-coenzyme A dehydrogenase; ACO: acyl-coenzyme A oxidase; ACS: acetyl-coenzyme A synthetase; COX-2: cyclooxygenase 2; CPT-1: carnitine palmitoyltransferase 1; FAS: fatty acid synthase; HSL: hormone-sensitive lipase; IL-6: interleukin-6; LPL: lipoprotein lipase; MCP1: monocyte chemotactic protein 1; SREBP-1c: sterol regulatory element binding protein-1.

EDTA treatment. After centrifugation (1300 rpm, 5 minutes), the cells were plated in 6-well plates at a concentration of 3×10^4 cell/well. One day after confluence (designated "day 0"), cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 mM), and insulin (5 mg/mL) in DMEM containing 10% fetal bovine serum (FBS). On day 2 and thereafter, DMEM supplemented with 10% FBS and 5 mg/mL insulin was replaced every 2 days. 3T3-L1 adipocytes, 7 to 8 days after differentiation, were treated with KME 6 μg/μL or phosphate-buffered saline (PBS) for 24 hours.

2.7. RNA Preparation and Real-Time RT-PCR. Total RNA was isolated from 3T3-L1 adipocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Cells were homogenized by 4-5 passages through a 23-gauge needle. After a 5-minute reaction, samples were mixed with chloroform by vortex machine for 15 seconds. Samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. The colorless supernatant was transferred into new tubes. Total RNA was precipitated by mixing with isopropyl alcohol and centrifuging at 12,000 rpm. The resulting RNA pellets were washed with 75% ethanol and dissolved in RNase-free water. RNA was quantified by spectrophotometry at A_{260} and then stored at −80°C. Total extracted RNA (1 μg) mixed with annealing oligo dT primer and RNase-free water was incubated in a thermocycler for denaturing RNA. Reactant was mixed with 5X first strand buffer, 20 mM DTT, 10 mM dNTP mix, RNase-free water, and Superscript II reverse transcriptase. The mixtures were incubated in a thermocycler (42°C for 1 hour and 72°C for 7 minutes) to generate cDNA. Real-time RT-PCR analysis was performed with an AB 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Samples containing 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 μmol each of appropriate primers, and cDNA were incubated in the AB 7500 real-time PCR system for an initial denaturation at 94°C for 10 minutes, followed by 40 PCR cycles. Each cycle was run at 95°C for 15 seconds and at 60°C for 1 minute. The oligonucleotide primers for the experiment are listed in Table 1. β-actin was used as an internal housekeeping control. To confirm amplification of specific transcripts, melting curve profiles were produced at the end of each PCR cycle by cooling the sample to 65°C for 15 seconds and heating slowly to 95°C, with continuous fluorescence measurement.

2.8. Statistical Analysis. Data (means ± SE) were analyzed using GraphPad Prism (version 5.04, GraphPad Software, USA). Unpaired two-tailed Student's t-tests were used to evaluate differences between means, as indicated. P values < 0.05 were considered statistically significant.

3. Results

3.1. KME-Induced Body Weight, Dietary Intake, and Adiposity Changes in HFD-Fed Mice. During the 15-week experiment, body weight and food intake were measured weekly. As shown in Figure 1, the KME-treated group had a slight decrease in body weight compared to the control group. However, this effect was significantly pronounced in animals that were fed an HF diet with KME. The difference between the KME-treated and control animals became significant at week 9. At the end of the experiment, the body weight of mice fed KME was 20% lower ($P < 0.05$) than that of the control

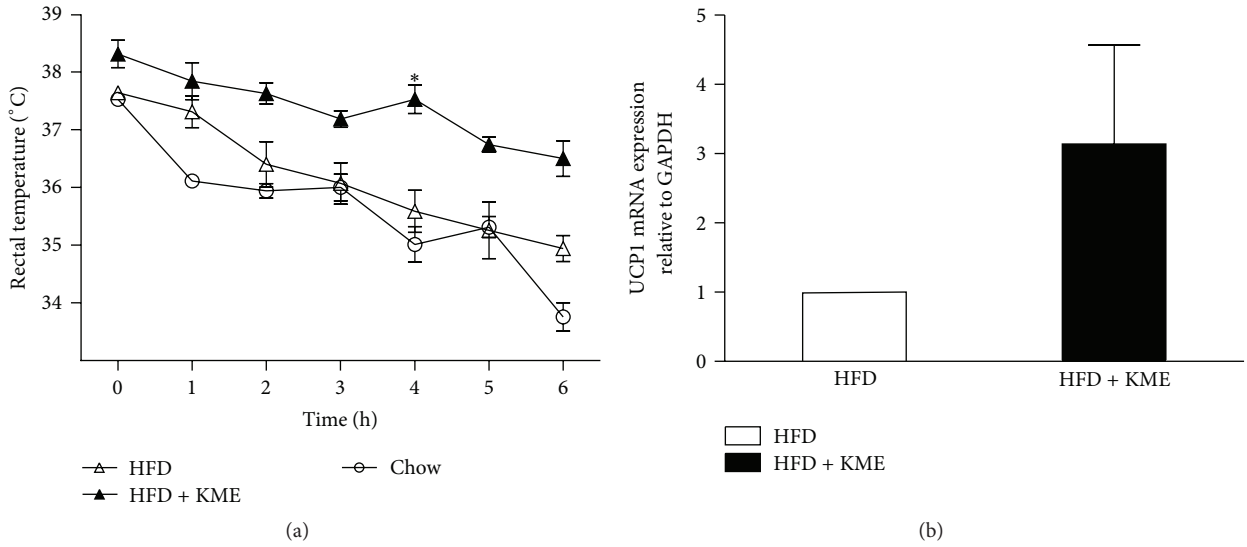

Figure 2: effect of KME on body temperature during the cold test (4°C for 6 h) and on UCP1 mRNA expression in BAT. (a) Hourly changes in body temperature are shown. (△) HFD: high-fat diet; (▲) HFD + KME: high-fat diet plus KME 3000 mg/kg ($^*P < 0.05$, $n = 7$ per group). (b) The relative UCP1 mRNA expression level in BAT of animals treated with HFD or HFD + KME (high-fat diet plus KME 3000 mg/kg) as quantified by real-time PCR. Values represent mean ± SEM ($^*P < 0.05$, $n = 3$).

group, whereas HF diet plus KME mice weighed almost the same as the mice that ate normal chow (Figure 1(a)). These effects of KME on body weight were not due to decreased food intake, as the amount of kcal consumed per mouse over a 24-hour period remained unchanged (Figure 1(b)). The data indicate that KME might have antiobesity effects *in vivo*, without affecting food intake.

To test whether body weight loss was caused by decreased adiposity, animals were sacrificed and epididymal white adipose tissue was dissected and weighed. This analysis revealed that epididymal white adipose tissue was significantly reduced in the HF diet plus KME group (Figure 1(c)).

3.2. Effect of KME on Cold Exposure in HFD-Fed Mice. We performed a cold test to assess the effect of KME on adaptive thermogenesis capacity. KME-treated animals maintained higher body temperatures than nontreated animals (Figure 2(a)), suggesting that it improved this capacity. To test whether thermogenesis is accompanied by changes in the expression of genes involved in thermogenesis, total RNA was prepared from BAT. We examined mRNA levels of UCP1, the major contributor to heat production, by Real-time RT-PCR. Figure 2(b) shows that the mRNA level of UCP1 was remarkably increased in KME-treated mice.

3.3. Effect of KME on Endurance Capacity of HFD-Fed Mice. Because KME reduced body weight (Figure 1(a)) and increased thermogenesis (Figure 3), we evaluated the effect of KME administration on a treadmill endurance test. The mice were trained to run at 16.2 meter/minutes for 10 minutes at a 5° incline the day before the running test, according to the procedure used for our previous report [15]. The experiment was initiated at 10 meter/minutes at a 0° incline with a gradual increase in speed. The mice were run until exhaustion, which was defined as remaining on the shock grid for longer than 10 consecutive seconds. Surprisingly, KME-treated mice ran twice as far as high-fat diet mice (Figure 3).

3.4. Effect of KME on Hepatic Histology of HFD-Fed C57BL/6. One of the most common characteristics among people with obesity is the development of fatty liver [33, 34]. Therefore, we also analyzed the effect of KME on fatty liver development. Histologic evaluation is regarded as the "gold standard" for assessing the presence and severity of NAFLD [35]. We histologically evaluated liver sections to determine the extent to which KME attenuated hepatic steatosis development. As shown in Figure 4, the control group exhibited little histologic evidence of hepatic steatosis. Severe steatosis was observed in mice fed a high-fat diet without KME. However, a marked reduction in the degree of steatosis was seen in livers from high-fat diet mice treated with KME. Furthermore, hepatic steatosis scores were dramatically lower in the high-fat diet mice fed KME (grade 1).

3.5. Effect of KME on mRNA Levels of Adipogenic Factors in 3T3-L1 Adipocytes. To demonstrate the inhibitory mechanism of KME on adipocyte differentiation, we measured the expression levels of the transcription factors PPAR-γ, C/EBP-α, and SREBP-1c in 3T3-L1 cells treated with 6 μg/μL of KME. Our results show that PPAR-γ, C/EBP-α, and SREBP-1c mRNA levels were decreased by 64%, 60%, and 32%, respectively, (Figure 5(a)). We also measured the mRNA expression levels of adipogenic enzymes, such as FAS, ACS, and ACC. We found that they were reduced by 69%, 55%, and 22%, respectively (Figure 5(b)). LPL expression is not directly

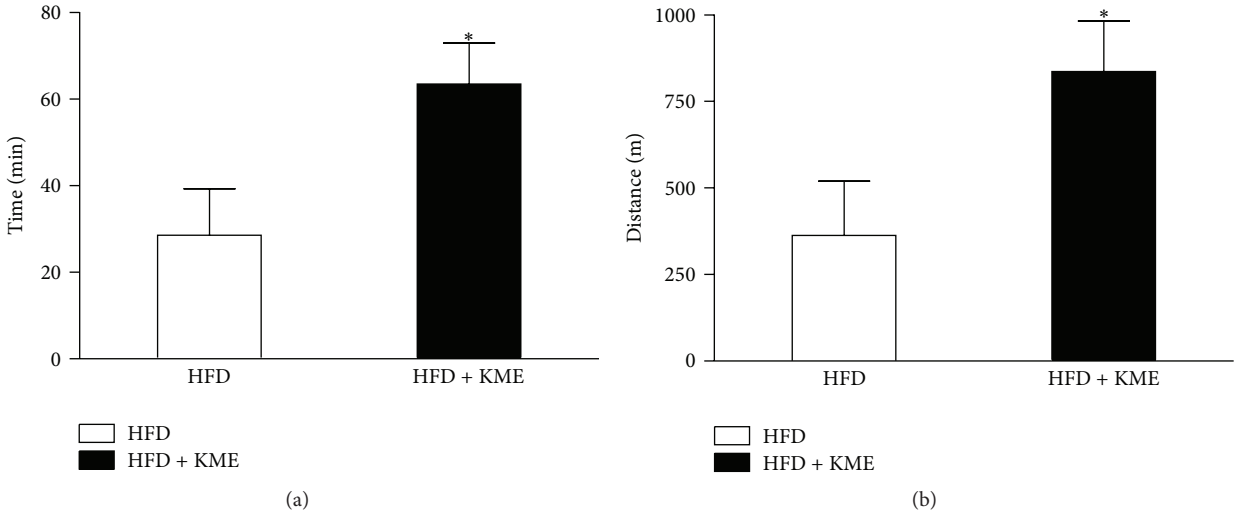

Figure 3: Endurance capacity was enhanced in KME-treated mice over 15 weeks. Average times (a) and distance (b) run until exhaustion are presented for animals treated with HF or HF + KME at 3000 mpk. Values represent the mean ± SEM ($^{*}P < 0.05$, $n = 7$ per group).

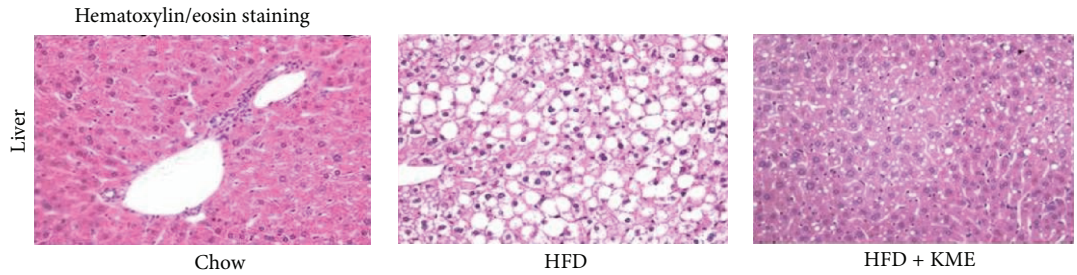

Figure 4: Histologic evaluation of hepatic steatosis. Hematoxylin and eosin staining of liver from mice fed chow diet (c), high-fat diet (HFD), or high-fat diet supplemented with KME at 3000 mpk (HFD + KME) (original magnification 400x). High-fat diet mice had severe hepatic steatosis. High-fat diet-induced mice fed KME at 3000 mpk had significantly reduced evidence of hepatic steatosis.

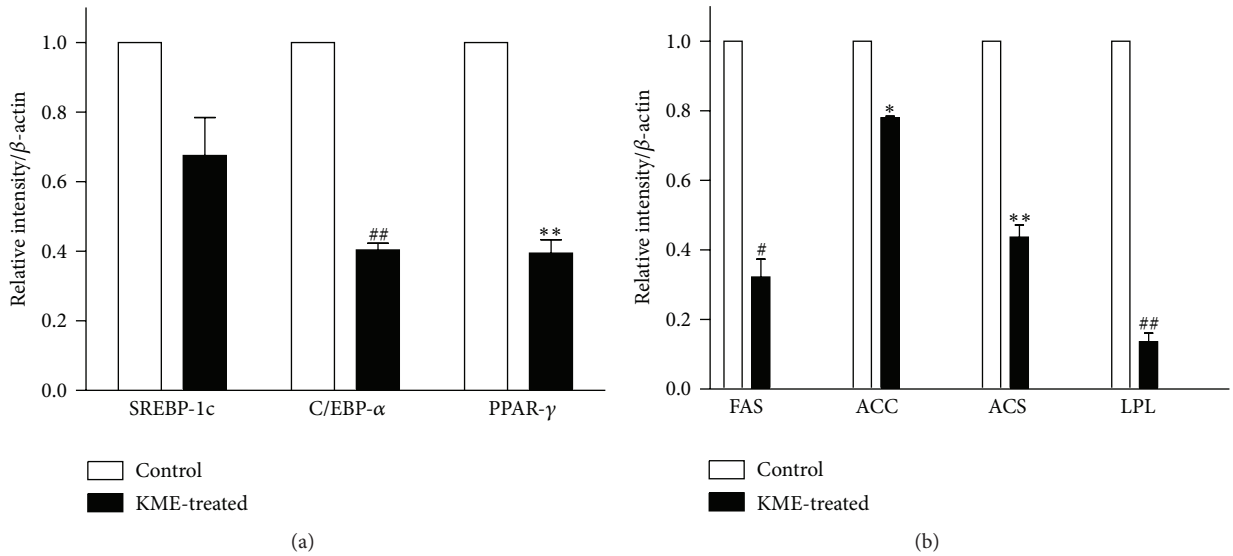

Figure 5: mRNA expressions of adipogenic enzymes and transcription factors in KME-treated 3T3-L1 adipocytes. On day 8 after differentiation, adipocytes were cultured in 6-well plates with or without KME (6 μg/μL) for 18 hours. Graphs represent mRNA expression of (a) adipogenic enzymes ACC, ACS, FAS, and LPL and (b) adipogenic transcription factors SREBP-1c, C/EBP-α, and PPAR-γ. Data are expressed relative to untreated control cells and represent the mean ± SEM ($^{*}P < 0.05$, $^{\#}P < 0.01$, $^{**}P < 0.005$, $^{\#\#}P < 0.001$, $n = 3$).

associated with lipid synthesis but is strongly associated with adipogenesis. Therefore, we measured the expression level of LPL mRNA and found that the level of expression in KME-treated 3T3-L1 cells was decreased by 89% compared to that of untreated cells (Figure 5(a)).

4. Discussion

Our study is the first to demonstrate that KME prevents weight gain in mice. We examined the effects of KME on HFD-induced obesity in C57Bl/6 mice. Our results showed that body weight gain in groups fed a diet supplemented with KME was reduced compared to control HFD mice. This effect was more distinct in animals fed a high-fat diet. Epididymal WAT in C57Bl/6 mice was significantly reduced by KME supplementation. This study also provides evidence that dietary supplementation of KME protects against hepatic steatosis development. We have considered the possibility that the effect of KME may be mediated by food intake because decreased food intake would be expected to significantly affect body weight, which influences hepatic steatosis. In this study, however, food intake did not differ between the groups. This suggests that KME directly protected against obesity and hepatic steatosis independent of food intake.

Brown adipose tissues (BATs) use stored triacylglycerols (TG) to maintain body temperature. These cells produce energy from fatty acid metabolism to generate heat through the action of uncoupling protein 1 (UCP1), a mitochondrial protein found only in brown adipose tissue. Brown adipocytes contain less TG and more mitochondria than white adipocytes, resulting in their unique color. KME treatment increased UCP1 expression levels in BAT, which poised the mitochondria for respiration uncoupling [36]. This effect is consistent with the observed enhancement of cold tolerance and helps explain the increase in energy expenditure and resistance to weight gain.

Mitochondrial function can affect total body metabolism. This is most evident in muscle, a metabolically flexible tissue that switches between lipid and carbohydrate substrates to fulfill energy requirements [37]. One study found that mitochondrial OXPHOS activity within the oxidative *soleus* muscle, which is resistant to fatigue and is dependent on mitochondrial activity for ATP production, was more affected than in the glycolytic muscle *tibialis anterior*. The main changes included a reduction in respiratory chain activity with a concomitant decrease in mitochondrial ATP production [38]. In line with this finding and previously [15], KME significantly improved muscle oxidative capacity, resulting in enhancing the endurance.

To further consolidate our hypothesis that KME inhibits obesity, we evaluated the effects of KME treatment *in vitro* using an adipocyte differentiation development system, which is regulated by transcriptional activators such as PPAR-γ, C/EBP-α, and some transcriptional regulators at the molecular level [39, 40]. PPAR-γ is a member of the nuclear receptor superfamily of transcription factors and is dominantly expressed in adipose tissue. These transcription factors appear to be the main activators of adipocyte differentiation [41].

The C/EBPs belong to a large family of leucine zipper transcription factors [42]. C/EBP-α is a promising candidate transcription factor for directly controlling adipocyte differentiation [43].

SREBP1 is known to critically cross-activate a ligand binding domain of PPAR-γ and activate the production of an endogenous PPAR-γ ligand [27]. SREBP1 also regulates the expression of enzymes involved in fatty acid desaturation and lipogenesis.

Adipocyte differentiation involves a series of programmed changes in the expression of specific genes. Adipogenesis can be induced through the action of some enzymes, such as FAS, ACC, and acyl-CoA synthetase (ACS). The expressions of these genes are regulated by transcription factors, including PPAR-γ, C/EBP-α, and SREBP1, which are known to be crucial activators for adipogenesis and showed early changes in gene expression during adipocyte differentiation [44, 45]. In this study, we showed that KME treatment significantly decreased SREBP-1c, C/EBP-α, and PPAR-γ mRNA expression in cultured 3T3-L1 adipocytes and inhibited expression of adipocyte-specific proteins (FAS, ACC, ACS, and LPL). Collectively, these observations suggest that KME suppresses adipocyte differentiation by regulating the expression levels of adipogenic transcription factors.

Further investigation is necessary to define specific mechanisms by which KME protects against obesity-mediated hepatic steatosis. To elucidate the mechanism associated with the antiobesity effects of KME, additional studies should be conducted with the most abundant component in KME. Sung [46] reported that pentacyclic triterpenoid oleanolic acid has antiobesity activity. We have shown that KME contains oleanolic acid, which has anticancer activity [47]. It is possible that oleanolic acid might be the leading antiobesity component in KME.

5. Conclusion

KME had marked inhibitory effects on the development of obesity and NAFLD in mice fed high-fat diets. Activating endurance capacity and increasing thermogenesis are two possible mechanisms for the antiobesity effect of KME. This finding suggests that KME may be a potential dietary strategy for preventing obesity and NAFLD.

Acknowledgments

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Research Article

Effect of Dietary Cocoa Tea (*Camellia ptilophylla*) Supplementation on High-Fat Diet-Induced Obesity, Hepatic Steatosis, and Hyperlipidemia in Mice

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Recent studies suggested that green tea has the potential to protect against diet-induced obesity. The presence of caffeine within green tea has caused limitations. Cocoa tea (*Camellia ptilophylla*) is a naturally decaffeinated tea plant. To determine whether cocoa tea supplementation results in an improvement in high-fat diet-induced obesity, hyperlipidemia and hepatic steatosis, and whether such effects would be comparable to those of green tea extract, we studied six groups ($n = 10$) of C57BL/6 mice that were fed with (1) normal chow (N); (2) high-fat diet (21% butterfat + 0.15% cholesterol, wt/wt) (HF); (3) a high-fat diet supplemented with 2% green tea extract (HFLG); (4) a high-fat diet supplemented with 4% green tea extract (HFHG); (5) a high-fat diet supplemented with 2% cocoa tea extract (HFLC); and (6) a high-fat diet supplemented with 4% cocoa tea extract (HFHC). From the results, 2% and 4% dietary cocoa tea supplementation caused a dose-dependent decrease in (a) body weight, (b) fat pad mass, (c) liver weight, (d) total liver lipid, (e) liver triglyceride and cholesterol, and (f) plasma lipids (triglyceride and cholesterol). These data indicate that dietary cocoa tea, being naturally decaffeinated, has a beneficial effect on high-fat diet-induced obesity, hepatomegaly, hepatic steatosis, and elevated plasma lipid levels in mice, which are comparable to green tea. The present findings have provided the proof of concept that dietary cocoa tea might be of therapeutic value and could therefore provide a safer and cost effective option for patients with diet-induced metabolic syndrome.

1. Introduction

Metabolic syndrome refers to the clusters of risk factors that would lead to increased episodes of cardiovascular disease (CVD). These risk factors include age, family history of CVD, gender, obesity, insulin resistance, nonalcoholic fatty liver (NAFLD), dyslipidemia, hypertension, and type 2 diabetes [1, 2]. According to the American Association of Clinical Endocrinologists and the International Diabetes Federation (IDF), metabolic syndrome can be defined as a complex of symptoms with central obesity (with waist circumference of >102 cm in men or >88 cm in women), plus two or more of the following factors: elevated serum levels of triglycerides, hyperglycemia, elevated blood pressure, and reduced serum levels of high-density lipoprotein- (HDL-) associated cholesterol [3].

Because of the current epidemic of over-nutrition and sedentary lifestyle worldwide, metabolic syndrome is becoming more common, particularly in developed countries. According to the report from the National Cholesterol Education Program (NCEP), the prevalence of metabolic syndrome is high, with a rate of 35.1% for middle-aged men and 32.6%

for middle-aged women in the United States [3, 4]. However, since the pathogenesis of metabolic syndrome has multiple metabolic origins, pharmacological approaches often consist of separate drugs which target at individual risk factors: lipid-lowering drugs, antihypertensive agents, hypoglycemic drugs, antiplatelet drugs, and weight-loss agents [5, 6]. These drugs nonetheless pose various side effects risks. Functional foods or nutraceuticals which have potentially important antiobesity properties have thus attracted great attention.

Tea (*Camellia sinensis*) is one of the most consumed beverages in the world, second only to water [7, 8]. Depending on the fermentation process, tea can be classified into three main types: the "nonfermented" green tea, the "semifermented" oolong tea, and the "fully fermented" black/red tea [9]. Due to the differences in processing, the chemical compositions of tea also differ, leading to the differences in the biological properties of different types of tea extracts [8]. Whereas the fully fermented black tea contains theaflavins and thearubigins as products from catechins oxidation, green tea has only undergone minimal fermentation and therefore contains predominately catechins [10]. It is estimated that a typical brewed green tea beverage (2.5 g green tea in 250 mL hot water) usually contains approximately 240–320 mg catechins including (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), (+)-catechin (C), and (+)-gallocatechin (GC) [10–12]. EGCG is the most abundant catechin present in green tea comprising approximately 30–50% of the total catechins content [13]. In addition to the catechin constituents, green tea also contains considerably high amounts of caffeine (3–6%) [11, 12, 14]. Although it has been well documented that green tea consumption could exhibit antiobesity, hypolipidemic, and hypoglycaemic properties and thereby improving CVD health, effects of caffeine contents within green tea on CVD have been contradictive [15, 16]. The various negative effects that caffeine poses on human behaviours [17] and sleep deprivation [18] have also caused great dilemma to obese or diabetic individuals. There is thus an urge for the market to search for decaffeinated green tea which could pose similar health benefits [19].

Cocoa tea (*Camellia ptilophylla*), which belongs to the genus *Camellia*, is a naturally decaffeinated tea plant. For many years, it has been widely consumed by local inhabitants in the Longmen area of Guangdong Province of China but has only started attracting scientific interest since 1988 [20, 21]. Early work suggested that cocoa tea exhibits profound cytotoxic effect on various cancer cell lines including HeLa, CNE2, and MGC-803 [22]. Peng et al. also demonstrated the potential of cocoa tea extract to inhibit prostate cancer cell proliferation and tumour growth *in vivo* and *in vitro* [23]. More recently, our laboratory showed that administration of cocoa tea to HepG2 xenograft nude mice resulted in dose-dependent reduction in tumour growth via caspase-3-regulated pathway [24]. Despite having high catechins content, the beneficial effects of cocoa tea on cardiovascular health have not been studied in details. Kurihara et al. demonstrated that a single oral administration of 500 mg/kg of cocoa tea extract significantly suppressed plasma triglyceride (TG) induction in mice given 5 mL/kg of lard oil [25]. Although this experiment suggested the potential of cocoa tea extract to reduce plasma TG, this effect is acute and the chronic effect of cocoa tea remains to be determined. Furthermore, there exists no experimental data on the effects of cocoa tea on diet-induced obesity and NAFLD. In order to determine whether cocoa tea supplementation to a high-fat diet results in an improvement in obesity and plasma and liver lipid levels, we carried out the present study in C57BL/6 mice to compare the effects of different doses of cocoa tea and green tea supplementations on high-fat diet-induced metabolic syndrome.

2. Materials and Methods

2.1. Herbal Materials Preparation. Green tea leaves were purchased from renowned supplier in China. Cocoa tea leaves were obtained from the Tea Research Institute, Guangdong Academy of Agricultural Sciences, China. Herbarium voucher specimens of the green tea and cocoa tea were deposited at the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher specimen numbers 3336 and 3401, respectively. For the extracts preparation, green tea or cocoa tea leaves (100 g) were brewed 3 times, each with 1 L of hot distilled water (80°C) for 15 min. The infusion was then cooled to room temperature and filtered with cellulose filter paper (0.45 μm, Millipore, Billerica, MA, USA). The filtrate was then concentrated using a vacuum rotary evaporator. The resulting extracts were freeze-dried overnight to produce tea powder.

2.2. HPLC Analysis of Tea Aqueous Extract. High-performance liquid chromatography (HPLC) analysis was performed using Hewlett Packard Agilent 1100 series HPLC System, equipped with G1329A ALS Autosampler and G1315A Diode Array Detector (Agilent Technologies, USA). Sample solution was injected onto a Supelco Discovery RP Amide C16 guard column (15 cm × 4.6 mm, 5 μm) (Sigma-Aldrich, Inc., USA). All solvents were prefiltered with 0.45 μm Millipore filter disk (Millipore, MA, USA) and degassed. A gradient elution was carried out using the following solvent systems: mobile phase A: double distilled water/phosphoric acid (99.95/0.05; v/v); mobile phase B: acetonitrile. The elution was performed with a gradient procedure as follows: 0-1 min, 2% B; 2–60 min, from 2% B to 98% B. The column heater was kept at 35°C. The flow rate used was 0.8 mL/min, and detection was performed at 210 nm. Each sample (10 μL) was injected into the column after filtration through a 0.45 μm filter disk. Identification of the tea polyphenols was carried out by comparing the retention times and the UV absorbance of the unknown peaks to those of the standards. A standard mixture containing theanine (Thea), theobromine (TB), caffeine (CAF), epigallocatechin (EGC), catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), gallocatechin (GC), gallocatechin gallate (GCG), and epicatechin gallate (ECG) in methanol was prepared and analyzed. Calibration curves for standard mixture was carried out using standard solutions injecting with volumes: 5, 10, and 20 μL. The system

was monitored by a computer equipped with the 32 Karat Software (Beckman Instrument Inc., Fullerton, CA, USA) for data collection, integration, and analysis.

2.3. Animals and Diets. Eight-week-old male C57BL/6 mice were obtained from the Laboratory Animal Services Centre (LASEC) of the Chinese University of Hong Kong. The care and use of the animals were in compliance with the institutional guidelines, and the experimental procedures were approved by the Animal Experimentation Ethics Committee of the CUHK. Animals were housed in standard cages (5 animals per cage) at a constant temperature of 20°C with a 12 h light/dark cycle. They were allowed *ad libitum* access to normal-chow diet and water. After 1 week of acclimatization, they were divided into six groups ($n = 10$ mice per group): (1) normal chow-fed group (N) that received normal chow diet containing 6% by weight of fat (SF04-057, Specialty Feeds, Glen Forest, Western Australia); (2) high-fat fed group (HF) that received a high-fat semipurified diet containing 21% wt/wt butterfat and 0.15% cholesterol (SF00-219, Specialty Feeds, Glen Forest, Western Australia); (3) high-fat fed group supplemented with 2% wt/wt green tea aqueous extract (HFLG); (4) high-fat fed group supplemented with 4% wt/wt green tea aqueous extract (HFHG); (5) high-fat fed group supplemented with 2% wt/wt cocoa tea aqueous extract (HFLC); and (6) high-fat fed group supplemented with 4% wt/wt cocoa tea aqueous extract (HFHC). The high-fat semipurified diet SF00-219 was formulated to be equivalent to western type diet Harlan Teklad TD88137. The normal chow diet SF04-057 was designed to be the control diet for the high-fat semipurified diet SF00-219. A detailed composition of SF00-219 and SF04-057 are summarised in Table 1. Food intake was recorded twice weekly, and body weights were measured twice a week.

2.4. Tissue Processing. Mice were fed diets for 8 weeks. At the end of the study, all mice were sacrificed after a 16-hour overnight fast. Animals were anaesthetised using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p., and whole blood was withdrawn by cardiac puncture. Blood was collected using heparin containing tubes, and plasma was separated by centrifugation (3,000 rpm, 10 min). Plasma were used immediately for plasma lipid measurement or frozen (−80°C) for future use. Livers were immediately excised, weighed, and divided into smaller pieces for storage at −80°C (for lipid analysis) or in 4% paraformaldehyde for histological analysis. Epididymal, inguinal, and perirenal fat pads were excised and weighed.

2.5. Biochemical Analyses. Plasma triglyceride (TG) and cholesterol (Chol) concentrations were measured by enzymatic methods, using GPO-PAP and CHOD-PAP kits (Roche Diagnostics, Switzerland), respectively. Total liver lipids were determined gravimetrically after extraction by the method of Bligh and Dyer [26]. Individual hepatic lipids were quantitated enzymatically (as described above) after solubilization in isopropanol.

Table 1: Ingredient composition of normal control diet, SF04-057, and high-fat semipurified diet, SF00-219.

Ingredients	SF04-057 (g/kg)	SF00-219 (g/kg)
Casein (acid)	195	195
Sucrose	341	341
Canola oil	60	0
Clarified Butter (ghee)	0	210
Cellulose	50	50
Wheat starch	306	154
DL methionine	3.0	3.0
Calcium carbonate	17.1	17.1
Sodium chloride	2.6	2.6
AIN93 trace minerals	1.4	1.4
Potassium citrate	2.5	2.6
Potassium dihydrogen phosphate	6.9	6.9
Potassium sulphate	1.6	1.6
Choline chloride (60%)	2.5	2.5
SF00-219 vitamins	10	10
Cholesterol	0	1.5
Oxicap E2	0.04	0.04

2.6. Histology. 5 μm thick sections of paraffin embedded tissue were cut with a microtome and placed on SuperFrost Plus microscope slides (Thermo Scientific, USA). Sections were dried at 37°C overnight and dewaxed by immersion with two exchanges in histolene (2 min) followed by rehydration to water through a series of graded ethanol washes comprising two washes in absolute ethanol, two washes in 95% ethanol, and two final washes in 75% ethanol. Sections were subjected to haematoxylin and eosin (H&E) staining, according to the method of Harris [27]. They were then dehydrated using graded ethanols comprising two washes in 75% ethanol, two washes in 95% ethanol, and two washes in absolute ethanol, after which they were subjected to two changes of xylene, and mounted with dibutyl phthalate in histolene (DPX) (Sigma-Aldrich, Inc., USA). Liver samples were examined histologically after embedding in paraffin, sectioning, and staining with H&E.

2.7. Gene Expression Analysis. Hepatic mRNA levels were measured by real-time PCR. Total RNA was isolated by selective binding to a silica gel-based membrane following the lysis and homogenisation of liver samples in a denaturing guanidine thiocyanate buffer (RNeasy kit, Qiagen, USA). RNA (100 ng) was reversely transcribed into cDNA using random primers provided with the iScript cDNA Synthesis kit (Bio-Rad, USA). Selected genes were amplified using iQ SYBR Green Supermix (Bio-Rad, USA) in an iCycler system (Bio-Rad, USA) with 12 pmol of both forward and reverse primers. PCR conditions were as follows: 1 cycle of 95°C for 3 min, 50 cycles of 95°C for 30 s, 55–60°C for 30 s and 72°C for 30 s, followed by 1 cycle of 95°C for 1 min. Purity of PCR products was assessed by melt curve analysis.

Relative gene expression was calculated by normalizing cycle threshold (Ct) values for genes of interest with Ct values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or cyclophilin using the delta-delta Ct method. Primer sequences were as follows: CYCLOPHILIN (forward: 5′-CAAATGCTGGACCAAACACAA-3′; reverse: 5′-CCATCCAGCCATTCAGTCTTG-3′); GAPDH (forward: 5′-GGCATCACTGCAACTCAGAA-3′; reverse: 5′-TTCAGCTCTGGGATGACCTT-3′); HMGCR (forward: 5′-CTTGTGGAATGCCTTGTGATTG-3′; reverse: 5′-AGCCGAAGCAGCACATGAT-3′); LDL-R (forward: 5′-CTGTGGGCTCCATAGGCTATCT-3′; reverse: 5′-GCGGTCCAGGGTCATCTTC-3′); PPAR-γ (forward: 5′-CCAGAGTCTGCTGATCTGCG-3′; reverse: 5′-GCCACCTCTTTGCTCTGCTC-3′); CD36 (forward: 5′-GAACCTATTGAAGGCTTACATCC-3′; reverse: 5′-CCCAGTCACTTGTGTTTTGAAC-3′).

2.8. Statistical Analysis. Values given in the text are means ± SEM, and Prism 5 for Window (version 5.0c, GraphPad Software, Inc., USA) was used for statistical analysis. Significant differences among all groups were assessed by one-way ANOVA, followed by Bonferroni's multiple comparison test. A probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Chemical Profiles of Green Tea and Cocoa Tea Extracts. Figure 1 shows the HPLC profiles of (a) green tea and (b) cocoa tea aqueous extracts, respectively. The retention time point of the standards were compared to the HPLC profiles of both tea extracts, with the quantities for each of the chemical markers within the tea extracts calculated and summarized in Table 2. Cocoa tea possessed a chemical profile different from green tea, with a relative concentration of the six major tea catechins ranked GCG > C > EGCG > EGC > ECG > EC, whereas green tea extract had a relatively higher concentration of EGCG, followed by EGC, ECG, EC, C, and GCG. Cocoa tea extract also contained theanine and theobromine, with the content of 1.05 ± 0.09 and 10.32 ± 0.18%, respectively. Green tea extract contained a theanine composition of 1.59 ± 0.09% and a much lower contents of theobromine (0.35 ± 0.02%) compared to that of cocoa tea. The caffeine contents of green tea and cocoa tea extracts were 6.12 ± 0.03% and 0%, respectively.

3.2. Effect of Green Tea and Cocoa Tea Supplementations on Daily Food Intake, Body Weight Gain, and Final Body Weight in Mice Given High-Fat Diet. Among all the high-fat-fed animals, addition of green tea or cocoa tea extract at 2% did not affect the daily food consumption of the animals. However, 4% green tea supplementation significantly reduced daily food consumption of C57BL/6 mice (2.6 ± 0.1 versus 3.0 ± 0.1 g, $P < 0.01$), whereby there was no significant difference on food intake for 4% cocoa tea supplemented group compared to HF group (Table 3). HF-fed animals gained significantly more weight than chow-fed animals

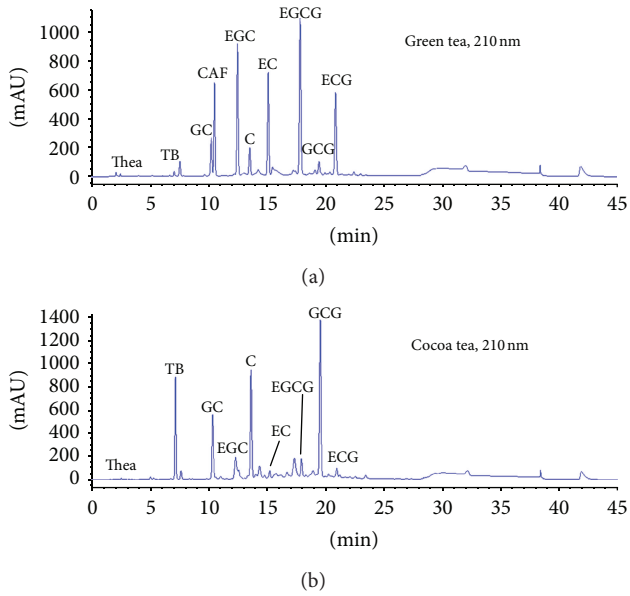

Figure 1: HPLC profiles of (a) green tea and (b) cocoa tea aqueous extracts. Detection was performed at UV 210 nm.

(33.9 ± 1.3 versus 26.7 ± 0.8 g, $P < 0.001$). Both green tea and cocoa tea supplementation significantly reduced the final body weight and total body weight gain compared to mice given high-fat diet alone in a dose-dependent manner, as shown in Table 3. There was however no significant difference on final body weight or total weight gain between 2% green tea and cocoa tea supplemented groups or between 4% green tea and cocoa tea supplemented groups.

3.3. Effect of Green Tea and Cocoa Tea Supplementations on Liver Weight and Liver to Body Weight Ratio in Mice Given High-Fat Diet. High-fat diet alone significantly increased liver weights of mice compared to mice given normal-chow diet. Mean liver weight was 35 ± 8% higher in the HF compared to the N group ($P < 0.001$). Both 2% and 4% green tea and cocoa tea supplementations had significant effect on liver weight in HF-fed mice (Table 4). When liver weights were expressed as a ratio relative to body wt, HF had a mean of 4.41 ± 0.16 g/100 g body wt, compared to the 4.34 ± 0.05 g/100 g for normal-chow-fed mice, which did not reach statistical significance. Both green tea and cocoa tea supplemented groups significantly reduced the liver to body weight ratio dose dependently compared to HF group. The % of reduction in liver/body weight ratio were 7.3 ± 3.4% and 11.9 ± 3.6% for 2% and 4% green tea supplementation and 12.3 ± 3.0% and 13.6 ± 2.6% for 2% and 4% cocoa tea supplementation, respectively. No significant difference was observed among all tea supplemented groups.

3.4. Effect of Green Tea and Cocoa Tea Supplementations on Epididymal, Perirenal, and Inguinal Fat Pad Weight in Mice Given High-Fat Diet. High-fat diet induced obesity in C57BL/6 male mice compared to normal-chow-fed mice after 8 weeks of diet, as evidenced by the significant increase in

Table 2: Amount of each chemical markers within cocoa tea and green tea extracts.

Chemical markers	Quantity (mg/100 mg)	
	Green tea	Cocoa tea
Thea	1.59 ± 0.19	1.05 ± 0.09
TB	0.35 ± 0.02	10.32 ± 0.18
CAF	6.12 ± 0.03	Not detected
EGC	5.57 ± 0.04	1.03 ± 0.14
C	1.43 ± 0.07	7.44 ± 0.11
EC	4.95 ± 0.48	0.44 ± 0.01
EGCG	8.54 ± 0.09	1.17 ± 0.08
GCG	0.58 ± 0.01	11.07 ± 0.18
ECG	5.17 ± 0.04	0.68 ± 0.02

Values represent means ± SEM ($n = 3$).

all three types of fat pad mass: epididymal fat mass (1467 ± 165 versus 397 ± 52 mg, $P < 0.001$), perirenal fat mass (517 ± 55 versus 139 ± 25 mg, $P < 0.001$), and inguinal fat mass (752 ± 135 versus 257 ± 52 mg, $P < 0.001$) (Table 5). Both green tea and cocoa tea supplementations significantly reduced all fat pad mass in a dose-dependent manner. There was no significant difference on epididymal fat mass or epididymal fat/body wt ratio and inguinal fat mass or inguinal fat/body weight ratio between 2% green tea and cocoa tea supplemented groups and likewise between 4% green tea and cocoa tea supplemented groups. However, perirenal fat mass tended to be lower for 2% green tea supplemented group compared to 2% cocoa tea supplemented group and was significantly lower when expressed as perirenal fat wt/body wt ratio. There was however no significant difference on perirenal fat mass and perirenal fat wt/body wt ratio between 4% green tea and cocoa tea supplemented groups (Table 5).

3.5. Effect of Green Tea and Cocoa Tea Supplementations on Liver Histology and Liver Lipid Contents in Mice Given High-Fat Diet. Livers from mice given different diets were analyzed histologically, and representative stained sections are shown in Figure 2. N group animals demonstrated the histological sections of normal livers. In contrast, H&E sections from HF animals revealed the presence of a large number of circular lipid droplets in between hepatocytes. These lipid inclusions were clearly reduced in both size and number in livers of both doses of green tea- and cocoa tea-treated animals.

The beneficial effect of both tea supplementations on HF-induced hepatomegaly was associated with a significant reduction in total hepatic lipid content, expressed as mg of lipid per grams of liver (Figure 3(a)) or expressed as mg of lipid per whole liver (Figure 3(b)). Total liver lipid content (in mg/g tissue) was significantly higher in HF mice compared to N mice (32 ± 11% higher, $P < 0.05$). HFLG-, HFHG-, HFLC-, and HFHC-fed mice had liver lipid levels that were significantly lower than HF-fed mice (i.e., 47 ± 4%, 56 ± 6%, 26 ± 6%, and 47 ± 4%, resp., for mg/g tissue). No significant difference was observed among green tea and cocoa tea supplemented groups at either dose. As shown in Figure 4, measurement of liver TG (a) and Chol (b) revealed that HF mice had elevated levels of both lipids compared to N mice (i.e., 1.2-fold (±0.1) increase in TG and 1.9-fold (±0.1) increase in Chol), of which only the latter had reached statistical significance. 2% and 4% green tea supplementation significantly reduced liver TG and Chol levels in HF-fed mice. Cocoa tea supplementation, on the other hand significantly reduced liver Chol at both 2% and 4% (55.9 ± 3.2%, $P < 0.001$, and 72.1 ± 1.6%, $P < 0.001$). Although both 2% and 4% cocoa tea additions resulted in reduced levels of liver TG (11.5 ± 5.5% and 20.8 ± 4.9%, $P < 0.01$), only 4% supplementation reached statistical significance.

3.6. Effect of Green Tea and Cocoa Tea Supplementations on Plasma Lipid in Mice Given High-Fat Diet. Plasma lipid levels of mice are shown in Table 6. Compared to N-fed mice, HF mice had significantly elevated levels of plasma TG and Chol. Both green tea and cocoa tea additions resulted in significant reduced levels of plasma TG and Chol at either dose. However, there was no significant difference on TG or Chol between 2% green tea and cocoa tea supplemented groups or between 4% green tea and cocoa tea supplemented groups.

3.7. Effect of Green Tea and Cocoa Tea Supplementations on Hepatic Gene Expression in Mice Given High-Fat Diet. In order to shed light on the mechanism by which cocoa tea extract contributes to its beneficial effects, mRNA levels of proteins controlling lipid metabolism in the liver were determined (Figure 5). Relative mRNA levels were compared by expressing levels relative to N mice. High-fat diet tended to increase level of expression of genes controlling cholesterol uptake—LDL-R, and cholesterol synthesis—HMG Co-A reductase, which were tended to be lowered by green tea and cocoa tea supplementations. mRNA levels for genes involved in fatty acid uptake, CD36 was also higher in HF group. Likewise, green tea and cocoa tea supplementations dose-dependently reduced the level of expression. mRNA levels for the transcription factor controlling lipid metabolism, PPAR-γ was markedly increased by high-fat diet. Both green tea and cocoa tea supplementations dose-dependently downregulated the expression at 2% and 4%, however only 2% and 4% green tea and 4% cocoa tea supplementation had reached statistical significance.

4. Discussion

The present study demonstrates that the addition of cocoa tea to mice on a high-fat diet (containing 21% butterfat and 0.15% cholesterol) resulted in a dose-dependent reduction in: (a) body weight, (b) fat pad mass (epididymal, perirenal and inguinal fat), (c) liver weight, (d) total liver lipid, (e) liver triglyceride and cholesterol, and (f) plasma lipid levels. These results provided evidence for the first time indicating that cocoa tea has a potential beneficial effect on weight control and lipid metabolism in C57BL/6 mice with diet-induced obesity. We are also the first time comparing the effects of cocoa tea and green tea using a diet-induced mice model which mimics human with metabolic syndrome.

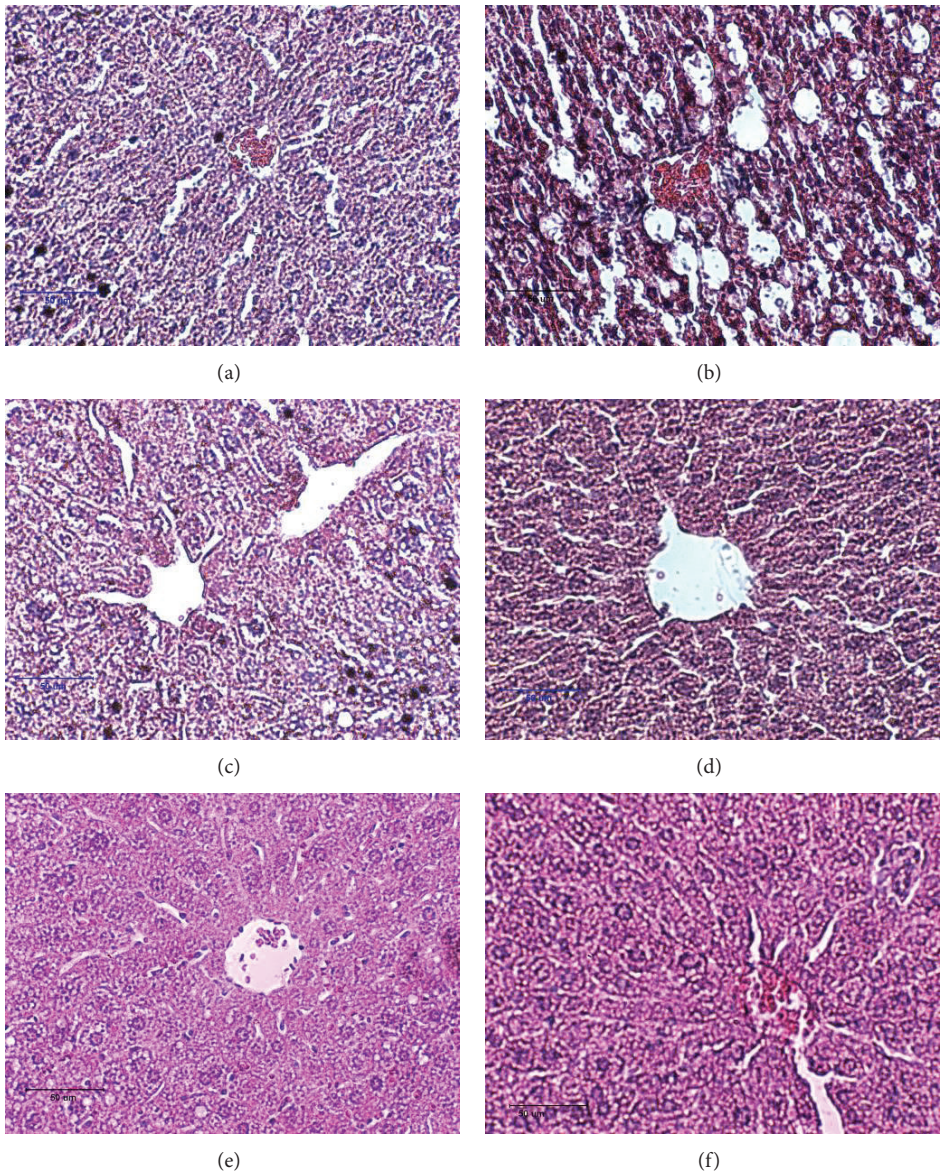

Figure 2: Histological appearance of liver sections of (a) a normal chow-fed mice; (b) a high-fat fed mice; (c) a high-fat fed mice supplemented with 2% green tea extract; (d) a high-fat fed mice supplemented with 4% green tea extract; (e) a high-fat fed mice supplemented with 2% cocoa tea extract; and (f) a high-fat fed mice supplemented with 4% cocoa tea extract. Sections were stained with haematoxylin and eosin. Lipid accumulation in the liver of HF-fed mice was very evident due to the presence of circular lipid droplets. Circular lipid droplets were significantly reduced in sections from both green tea and cocoa tea supplemented animals.

Table 3: Body weights and daily food intake of mice fed normal chow or a high-fat diet with or without green tea or cocoa tea supplementation at different concentrations.

	N	HF	HFLG	HFHG	HFLC	HFHC
Initial body wt. (g)	22.7 ± 0.4	23.1 ± 0.3	22.8 ± 0.6	22.5 ± 0.6	22.7 ± 0.6	22.5 ± 0.5
Final body wt. (g)	26.7 ± 0.8	$33.9 \pm 1.3^{***}$	$26.4 \pm 0.7^{\#\#\#}$	$23.8 \pm 0.7^{\#\#\#}$	$28.5 \pm 0.5^{\#\#\#}$	$25.6 \pm 0.4^{\#\#\#}$
Wt. gain (g)	4.0 ± 0.5	$10.8 \pm 1.1^{***}$	$3.6 \pm 0.4^{\#\#\#}$	$1.3 \pm 0.3^{\#\#\#}$	$5.9 \pm 0.4^{\#\#\#}$	$3.1 \pm 0.3^{\#\#\#}$
Daily food intake (g)	2.6 ± 0.1	3.0 ± 0.1	2.8 ± 0.1	$2.6 \pm 0.1^{\#\#}$	2.8 ± 0.1	2.7 ± 0.1

Values represent means ± SEM ($n = 10$).
Significant difference between N and HF mice using one-way ANOVA: $^{***}P < 0.001$.
Significant difference between HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

Table 4: Liver weights and liver to body weight ratio of mice fed normal chow or a high-fat diet with or without green tea or cocoa tea supplementation at different concentrations.

	N	HF	HFLG	HFHG	HFLC	HFHC
Liver wt. (g)	1.08 ± 0.04	1.43 ± 0.10***	0.99 ± 0.03$^{\#\#\#}$	0.84 ± 0.03$^{\#\#\#}$	1.02 ± 0.02$^{\#\#\#}$	0.87 ± 0.02$^{\#\#\#}$
Liver wt./body wt. (g/100 g)	4.34 ± 0.05	4.41 ± 0.16	4.05 ± 0.05$^{\#}$	3.83 ± 0.04$^{\#\#\#}$	3.83 ± 0.04$^{\#\#\#}$	3.77 ± 0.03$^{\#\#\#}$

Values represent means ± SEM ($n = 10$).
Significant difference between N and HF mice using one-way ANOVA: $^{***}P < 0.001$.
Significant difference between HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

Table 5: Fat pad weights of mice fed normal chow or a high-fat diet with or without green tea or cocoa tea supplementation at different concentrations.

	N	HF	HFLG	HFHG	HFLC	HFHC
Epididymal fat pad wt. (mg)	397 ± 52	1467 ± 165***	442 ± 64$^{\#\#\#}$	233 ± 19$^{\#\#\#}$	632 ± 50$^{\#\#\#}$	329 ± 31$^{\#\#\#}$
Epididymal fat pad wt./body wt. (g/100 g)	1.55 ± 0.17	4.46 ± 0.37***	1.77 ± 0.21$^{\#\#\#}$	1.07 ± 0.08$^{\#\#\#}$	2.35 ± 0.16$^{\#\#\#}$	1.42 ± 12$^{\#\#\#}$
Perirenal fat pad wt. (g)	139 ± 25	517 ± 55***	135 ± 19$^{\#\#\#}$	67 ± 6$^{\#\#\#}$	232 ± 20$^{\#\#\#}$	88 ± 12$^{\#\#\#}$
Perirenal fat pad wt./body wt. (g/100 g)	0.54 ± 0.08	1.58 ± 0.13***	0.54 ± 0.06$^{\#\#\#}$	0.31 ± 0.03$^{\#\#\#}$	0.86 ± 0.07$^{\#\#\#+}$	0.38 ± 0.05$^{\#\#\#}$
Inguinal fat pad wt. (g)	257 ± 52	752 ± 135***	258 ± 41$^{\#\#\#}$	205 ± 10$^{\#\#\#}$	394 ± 32$^{\#\#}$	214 ± 20$^{\#\#\#}$
Inguinal fat pad wt./body wt. (g/100 g)	0.99 ± 0.17	2.25 ± 0.34***	1.03 ± 0.14$^{\#\#\#}$	0.95 ± 0.06$^{\#\#\#}$	1.47 ± 0.10$^{\#}$	0.92 ± 0.08$^{\#\#\#}$

Values represent means ± SEM ($n = 10$).
Significant difference between N and HF mice using one-way ANOVA: $^{***}P < 0.001$.
Significant difference between HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.
Significant difference between HFLG and HFLC mice using one-way ANOVA: $^{+}P < 0.05$.

Over the years, the beneficial effects of dietary green tea on plasma and hepatic lipid levels have been well documented in various animal studies [8, 9, 28, 29]. The positive findings from these pre-clinical studies have also been confirmed in a number of human clinical trials suggesting the antiobesity effect of green tea extract [7, 30, 31]. The beneficial effects of green tea have been attributed to the presence of tea catechins, in particularly EGCG [32–34]. Our data pertaining to the catechins contents of both tea extracts (Table 2) indicate that the major type of catechins in green tea extract was EGCG. This was however not the case for cocoa tea extract, which contained only approximately one-seventh the amount of EGCG detected in green tea extract, but with higher quantities of other catechins such as GCG and C. Previous work using GCG produced by heat epimerization of EGCG suggested GCG are more effective in lowering plasma cholesterol concentration compared to EGCG, possibly because GCG are more effective in inhibiting lymphatic absorption of endogenous cholesterol [35–37], but not triglyceride [36]. In consonance with these studies, when comparing the individual Chol and TG lowering effects of green tea and cocoa tea in our animal experiment, it appeared that green tea extract are more potent in lowering both plasma and hepatic TG, whereby cocoa tea extract are comparatively more potent in lowering plasma and hepatic Chol. The above studies therefore support the notion that GCG has played a role in contributing the beneficial effects of cocoa tea.

The preceding discussion assumes that the lipid-lowering and hepatoprotective properties of cocoa tea extract are dependent on its catechins content and/or its distinct catechin composition. It cannot be ruled out however, that the noncatechins containing portion of cocoa tea extract may also be responsible for the observed effects. HPLC analysis suggested that cocoa tea extract also contained high levels of theobromine. Although researches focusing on the effect of theobromine on lipid metabolism are limited, Eteng and Ettarh showed that acute theobromine administration (700 mg/kg) caused significant reduction in total serum cholesterol, and triglycerides [38]. Only additional studies with fractionated cocoa tea will provide the necessary evidence as to determine the bioactive component of cocoa tea extract.

A number of different mechanisms could be responsible for the beneficial effects of cocoa tea on obesity and plasma and liver lipid metabolism. It had been suggested that green tea catechins could exert modifications in appetite control, leading to decreased nutrient absorption and thereby contributing to a downregulation of enzymes involved in lipid metabolism within the liver and adipose tissues [39, 40]. In the present study, 4% green tea supplemented animals had significantly reduced appetite, as evidenced by the reduced food intake compared to high-fat-fed animals. This was however not the case for cocoa tea supplemented animals. Cocoa tea supplementation dose-dependently reduced the body weight and plasma and hepatic lipid without affecting the food intake of the animals. Furthermore, this reduction in hepatic cholesterol levels induced by cocoa tea extract was associated with a trend in the reduction in the expression of enzymes affecting cholesterol uptake (LDL-R) and cholesterol synthesis (HMG Co-A reductase). Cocoa tea could also downregulate the expression of enzyme affecting fatty acid uptake (CD36), thereby leading to its TG-lowering effect. Interestingly, cocoa tea could reduce high-fat diet-induced increase in PPAR-γ expression. PPAR-γ is a ligand-activated transcription factor that played an important role in the regulation of lipid and glucose metabolisms. It regulates a serial of

Table 6: Plasma lipid in mice fed normal chow or a high-fat diet with or without green tea or cocoa tea supplementation at different concentrations.

	N	HF	HFLG	HFHG	HFLC	HFHC
Triglyceride (mmol/L)	0.92 ± 0.02	1.15 ± 0.05*	0.57 ± 0.02$^{\#\#\#}$	0.49 ± 0.04$^{\#\#\#}$	0.92 ± 0.02$^{\#}$	0.65 ± 0.02$^{\#\#\#}$
Cholesterol (mmol/L)	2.72 ± 0.13	5.89 ± 0.20***	3.68 ± 0.09$^{\#\#\#}$	3.54 ± 0.13$^{\#\#\#}$	3.67 ± 0.07$^{\#\#\#}$	3.33 ± 0.10$^{\#\#\#}$

Values represent means ± SEM (n = 10).
Significant difference between N and HF mice using one-way ANOVA: $^{*}P < 0.05$, $^{***}P < 0.001$.
Significant difference among HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

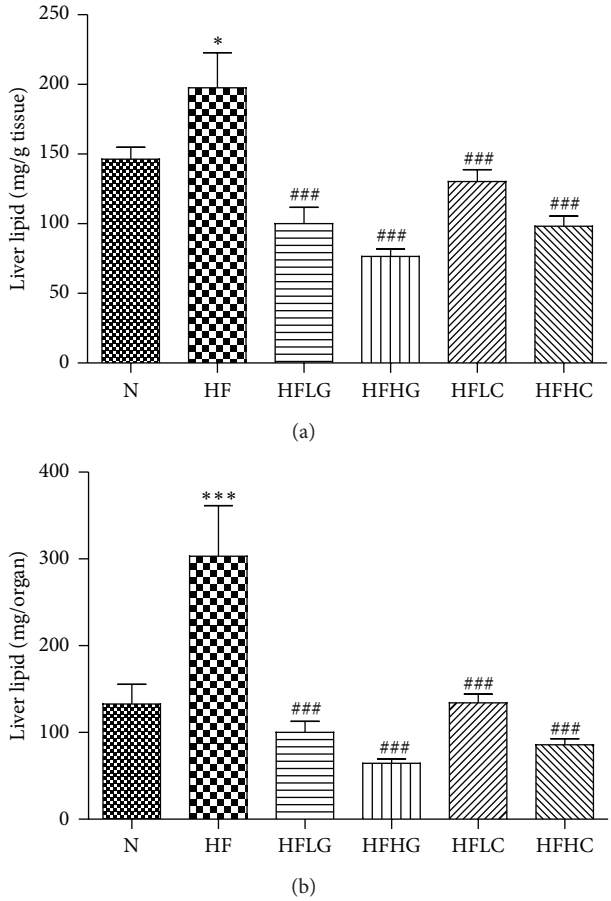

Figure 3: Total lipid in the liver of mice fed a normal chow or high-fat diet, with or without the addition of green tea or cocoa tea supplementation at different concentrations. (a) shows results obtained gravimetrically expressed as mg of lipid per grams of liver; (b) shows results expressed as mg of lipid per whole liver. Mice were fed diets for 8 weeks. Values represent means ± SEM (n = 10). Significant difference between N and HF mice using one-way ANOVA: $^{***}P < 0.001$. Significant difference among HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#\#\#}P < 0.001$.

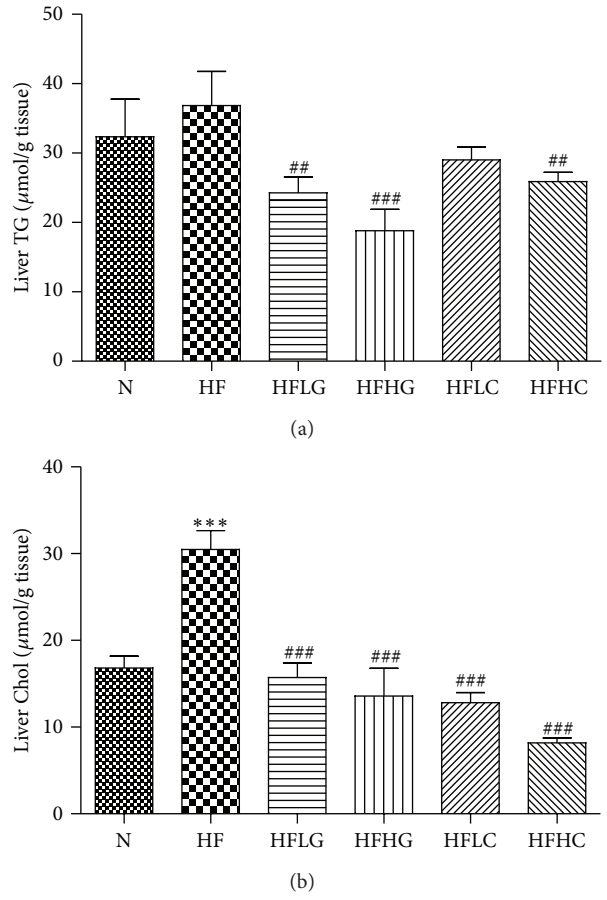

Figure 4: Liver TG (a) and Chol (b) of mice fed a normal chow or high-fat diet, with or without the addition of green tea or cocoa tea supplementation at different concentrations. Results expressed as μmol/grams of liver. Mice were fed diets for 8 weeks. Values represent means ± SEM (n = 10). Significant difference between N and HF mice using one-way ANOVA: $^{***}P < 0.001$. Significant difference among HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

genes involved in lipid metabolism including SCD-1, CD36, FAS, LDL-R, and SREBP-1. In rodent models of metabolic syndrome demonstrating diet-induced hepatic steatosis and obesity, hepatic expression of PPARγ is markedly upregulated [41, 42]. The fact that the elevated transcription levels of PPARγ and its targeted genes including LDL-R, HMG Co-A reductase and CD36 in the livers of high-fat-fed mice are dose dependently downregulated in cocoa tea supplemented animals suggested cocoa tea could possibly regulate hepatic lipid metabolism via PPAR-γ regulated pathway. Kurihara et al. suggested that cocoa tea reduced blood TG via suppression of lymphatic TG absorption [25]. Whether the effect of cocoa tea supplementation on body weight and downregulation of plasma and liver lipid observed in our study was due to the ability of cocoa tea to primarily regulate hepatic

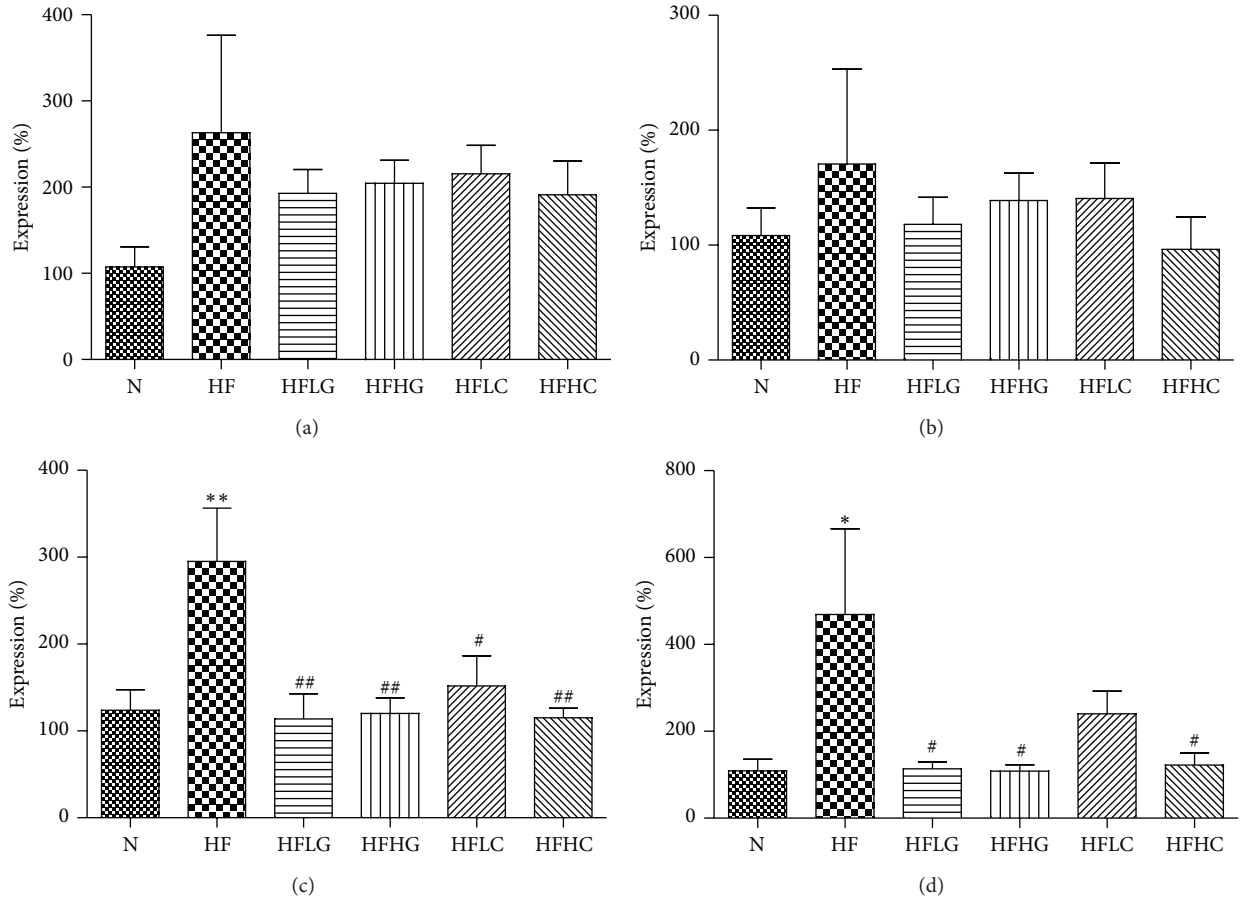

Figure 5: Effect of green tea and cocoa tea supplementations on (a) LDL-R; (b) HMG Co-A reductase; (c) CD36; and (d) PPAR-γ mRNA levels in the liver of chow-fed and high-fat-fed mice. Mice were fed diets for 8 weeks. Values represent means ± SEM (n = 3-4). Significant difference between N and HF mice using one-way ANOVA: $^{*}P < 0.05$, $^{**}P < 0.01$. Significant difference among HF and HFLG, HFHG, HFLC, and HFHC mice using one-way ANOVA: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

PPAR-γ expression and its targeted genes or secondary as a result of reduced lipid absorption within the intestine, the present study has the limitation in providing direct evidence of reduced lipid absorption and/or reduced bile acid production. Additional experiments including gene and protein expression analysis within the liver, adipose tissues, and intestine will be required to define which of these effects of cocoa tea are primary and secondary.

The potent ability of dietary cocoa tea to reduce both plasma and liver lipid content in high-fat-fed mice, to an extent similar to green tea of the same dosage, suggests it might be of therapeutic benefit in humans with diet-induced metabolic syndrome, in particularly for obese individuals with hyperlipidemia and nonalcoholic fatty liver disease (NAFLD). NAFLD affects 10–20% of the general population and is commonly found in obese or diabetic patients [43]. At present, there is no established treatment for it, and current suggested management strategy relies on diet regimen, weight loss, and exercise. Dietary supplements/nutraceuticals that might help to delay the development or alleviate this condition are therefore of great importance [44, 45]. Although green tea promotes beneficial effect on health, the fact that green tea contains caffeine has caused various concerns. Caffeine can cause insomnia, anxiety, irritability, upset stomach, nausea, diarrhea, or frequent urination [46] and has been reported to cause interact with various drugs [47]. It is generally recommended that green tea should not be taken by patients suffering from heart conditions or major cardiovascular problems [9, 48]. Pregnant and breastfeeding women and children are suggested not to drink more than one or two cups per day due to their slow detoxification rate of caffeine [8, 19]. Current green tea decaffeination technique predominately relies on the use of supercritical carbon dioxide fluid extraction (SC-CO_2) method. Although this method has great advantages over conventional methods which employ the use of solvents that could be toxic [19], SC-CO_2 are relatively cost ineffective and require high capital costs for batch extraction [49]. During the decaffeination process, much of the volatile and aroma-active compounds in the green tea will also be removed, thereby resulting in weaker aroma and stronger bitter taste [19, 50]. The fact that cocoa tea, being a naturally occurring tea plant, contains no caffeine therefore poses major advantage. This unique feature of cocoa tea also provides an opportunity for the more feasible

through-the-day consumption, so that it could be integrated with the daily dietary regimen of obese individuals and subjects with cardiovascular problems, without the excitatory effects of caffeine. Further studies will help to determine whether cocoa tea is of therapeutic benefit in patients with NAFLD or in overweight and insulin-resistant individuals at increased risk of coronary artery disease.

In conclusion, the present study indicates that cocoa tea has a beneficial effect on obesity, hepatomegaly, hepatic steatosis, and elevated plasma lipid levels in mice fed with a high-fat diet. These results provide the proof of concept that cocoa tea, which contains no caffeine, might be of therapeutic value as a nutraceutical in patients with increased risk of fatty liver disease and/or cardiovascular disease. Furthermore, the unique feature of the naturally decaffeinated cocoa tea has posed major advantage over green tea or decaffeinated green tea in providing individuals whom are prohibited from caffeine intake with the feasibility and flexibility to enjoy the benefits of tea, without the concern of the side effects induced by caffeine or the high cost incurred during the processing of decaffeinated green tea.

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Review Article

Update on Berberine in Nonalcoholic Fatty Liver Disease

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Berberine (BBR), an active ingredient from nature plants, has demonstrated multiple biological activities and pharmacological effects in a series of metabolic diseases including nonalcoholic fatty liver disease (NAFLD). The recent literature points out that BBR may be a potential drug for NAFLD in both experimental models and clinical trials. This review highlights important discoveries of BBR in this increasing disease and addresses the relevant targets of BBR on NAFLD which links to insulin pathway, adenosine monophosphate-activated protein kinase (AMPK) signaling, gut environment, hepatic lipid transportation, among others. Developing nuanced understanding of the mechanisms will help to optimize more targeted and effective clinical application of BBR for NAFLD.

1. Introduction

As the global waistline continues to expand, metabolic abnormalities including obesity, type 2 diabetes, hypertension, and dyslipidemia, collectively termed the "metabolic syndrome," are reaching epidemic proportions. The pathogenesis of these disease state is hypothesized to begin with abnormal accumulation of lipids in nonadipose tissues (steatosis), known as nonalcoholic fatty liver disease (NAFLD), a chronic condition that is currently the leading cause of referrals to hepatology clinics. Due to serious adverse effects and the limited effectiveness of currently available pharmacological therapies for "metabolic syndrome," many research efforts have focused on the development of drugs from natural products.

Berberine (BBR, $C_{20}H_{18}NO_4$) is an isoquinoline alkaloid of the protoberberine type, which presents in an array of plants, including Hydrastis canadensis (goldenseal), Coptis chinensis (Coptis or goldenthread), Berberis aquifolium (the Oregon grape), Berberis vulgaris (barberry), and Berberis aristata (tree turmeric) among others. The isoquinoline alkaloid drug belongs to the structural class of protoberberines which includes a quaternary base (Figure 1). There are many derivates and analogues available, such as berberine hydrochloride, berberine sulfate, and berberine citrate or phosphate, contributing to its multiple pharmacological and biochemical effects.

BBR is traditionally used as an antimicrobial and antiprotozoal drug, the antimicrobial activity against a variety of organisms, including bacteria, viruses, fungi, protozoans, helminths, and chlamydia, which have been applied in Chinese medicine for many decades. Recent researches have revealed novel pharmacological properties and multiple therapeutic applications, mainly concerning metabolic diseases, such as obesity and type 2 diabetes [1]. Kinetic study shows that BBR metabolites are widely distributed into various tissues, including liver, heart, kidney, spleen, lung, and even brain, with the liver being the most predominant organ, and average concentration of BBR in liver is approximately 70-fold greater than that in plasma [2]. Other dosing routes, such as femoral vein administration also identified the disposition of BBR in blood, liver, and bile fluid [3]. Additionally, BBR has longer half-life in liver than other tissues [4], suggesting liver as the main target organ of BBR.

In the past several decades, BBR's action on glucose and lipid metabolic disorders has been widely studied; although the effects or mechanisms of BBR on NAFLD could not separate completely from other metabolic diseases, the role of BBR

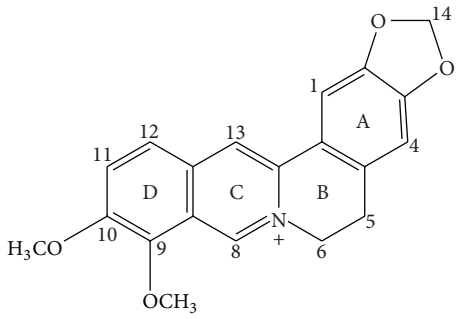

Figure 1: Chemical structure of BBR.

on NAFLD might be different due to the uniqueness of the organ. This review will mainly focus on BBR on the effect NAFLD and its potential mechanisms.

2. Effect of BBR on Fatty Liver

BBR is reported to inhibit cholesterol and triglyceride synthesis in human hepatoma cell line (HepG2) cells and primary hepatocytes [5, 6], and treating rat hepatoma H4IIE cells with BBR shows increased glucose consumption in dose-dependent manner [7]. In vivo data from various animal models also confirm BBR's beneficial role in preventing or treating NAFLD. Intraperitoneal injection of BBR for three weeks has been shown to alleviate hyperlipidemia and fatty liver in obese db/db and ob/ob mice [8]. In Zucker diabetic fatty rats, two-week treatment with a BBR-containing formula could attenuate fatty degeneration [9]. Treatment of hyperlipidemic hamsters with BBR strongly reduces fat storage in the liver [5]. As for mice with high-fat diet (HFD) induced fatty liver, sixteen weeks BBR supplement could alleviate hepatic steatosis and decrease liver lipid content by 14% [10]. This antisteatosis effect of BBR is also reported in diabetic hyperlipidemic rats, which demonstrates that BBR prevents the pathological progression of liver and reverted the increased hepatic triglyceride to near the control levels [11]. In addition, BBR further prevents the development of obesity and insulin resistance in HFD-fed rats [12], and BBR may also prevent liver fibrosis experimental models [13]. Clinical investigations showed that BBR supplement may reduce alanine and aspartate transaminase levels in patients with type 2 diabetes, indicating the restoration of liver function [14, 15]. Furthermore, BBR has been shown to reduce liver necrosis both in nonalcoholic steatosis and in steatosis due to hepatitis C infection [13]. In elderly hypercholesterolemic patients who were previously statin-intolerant, BBR demonstrates reduced cholesterolemia and plasma low density lipoprotein-cholesterol (LDL-c) levels [16].

3. Mechanisms of BBR in NAFLD

The precise mechanisms of the development of NAFLD or BBR improving fatty liver remain largely unclear. Defects in lipid metabolism pathways, insulin resistance, and inflammation are crucial players in the process of NAFLD. The recent literature points out that BBR may be integrated into lipid and glucose regulation, combating fatty liver and related syndromes, and the beneficial role of BBR on NAFLD might be achieved through multiple mechanisms.

3.1. BBR in Mediating Insulin Resistance. Insulin resistance plays a critical role in the pathogenesis of NAFLD [17]; hence, improving insulin sensitivity is of great importance in dealing with NAFLD. Evidence of BBR on insulin resistance has been elucidated in clinical trials as well as experimental animals and cells lines [14, 18, 19]. Although there is no definitive explanation of how BBR in regulating sensitivity, there are several encouraging observations revealing the possible mechanisms.

After meal, pancreatic islets secrete insulin, and insulin's presence at the cell surface is transduced to cytoplasmic and nuclear responses by tyrosine phosphorylation of insulin receptor substrates (IRSs). Nutrient-induced serine phosphorylation of IRS proteins is proved to be the counter-regulation of the signaling pathway, which blunts insulin action in stressed target tissues and stems the influx of nutrients into already overwhelmed cells [20]. Recently, low grade inflammation and endoplasmic reticulum (ER) stress have been proposed to be in close association with insulin resistance, activation of inflammatory pathways, such as Jun N-terminal kinases (JNKs), inhibitor of nuclear factor B(IkB) kinaseβ (IKKβ), inositol requiring enzyme 1 (IRE-1), could coverage [21–23].

BBR was reported to stimulate insulin secretion in HIT-T15 cells and pancreatic islets, which may have certain influence on its antidiabetic activity [24]. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) production in HepG2 cells represent a state of inflammation and consequently impair insulin pathway, and treatment with BBR effectively inhibits IL-6 and TNFα production in a concentration-dependent manner, and improves insulin signaling cascade by modification of IRS-1 and Protein kinase B (PKB, Akt) Ser/Thr phosphorylation, indicating the insulin enhacing potential of BBR is through the anti-inflammatory activity [25]. Moreover, pretreating with BBR could block tunicamycin induced ER stress, coexisting with the inhibition of PKR-like eukaryotic initiation factor 2α kinase (PERK) and eukaryotic translational initiation factor 2α (eIF2α) phosphorylation, two protein markers of ER stress, and this results in increasing IRS-1Tyr phosphorylation whereas decreasing its Ser (307) phosphorylation, thus improving insulin resistance under the condition [26]. Other mechanisms, including BBR-caused insulin receptor (InsR) promoter activation and InsR messenger RNA (mRNA) transcription, might also contribute to effect of BBR in regulating insulin sensitivity. In vitro studies further suggest that BBR-induced InsR gene exoression depends on protein kinase C (PKC) activity [27], possibly depends on protein kinase C (PKC) activity in the liver, and BBR mediated PPARs restoration is observed to be in parallel with hepatic glycogen and triglyceride attenuation actions [11]. Our previous work also identified the direct action of BBR on IRS [28]. Taken together, these studies indicate that BBR might be of great insulin sensitive potential and an active player in the liver.

3.2. BBR in Regulating APMK Pathway. Adenosine monophosphate-activated protein kinase (AMPK) is an attractive drug target that plays a key role in regulation of whole-body energy homeostasis. Increasing empirical evidence points towards AMPK activation as the target of BBR. In different animal models and various cell lines, phosphorylation of AMPK is identified, attributing to the hypoglycemic and hypolipidemic effect of BBR [5, 7, 29, 30]. To further verify this target, exposure cultured cells with Compound C, an AMPK inhibitor, BBR mediated effect is then abolished [8, 31], implying AMPK as a crucial player of BBR to dissipate stored fat and elevated glucose levels.

The mechanism of BBR on AMPK activation is still elusive, and studies suggest mitochondrial function as the key of this issue. BBR was used as cationic fluorescent probe for investigating energized state of liver mitochondria several decades ago, penetration of BBR into mitochondria could inhibit NAD-linked respiration, and the inhibition is totally dependent on the energization of the membrane [32]. Later, in vitro experiments provided detailed insight into the mechanisms of BBR in regulating this pathway. BBR inhibits glucose oxidation in mitochondria, which leads to an increase in the AMP/ATP (adenosine monophosphate/adenosine triphosphate) ratio in cells, thus accounting for AMPK activation [7, 19, 33, 34]. Other studies further identified complex I inhibition in the mitochondrial respiratory chain as the target of BBR, which blocks AMP conversion into ATP, allowing AMP accumulation in mitochondrial [19, 33, 34]. When elevated AMP binds to the subunit γ, the inhibitory domain of the α1 subunit is released from the kinase domain, results in an active conformation of AMPK [35].

The liver is a vital organ present in vertebrates and has a wide range of functions. Activation of hepatic AMPK leads to increased fatty acid oxidation and simultaneously inhibition of hepatic glucose production as well as lipogenesis and cholesterol synthesis. In vivo data from obese db/db and ob/ob mice indicate that BBR stimulates the expression of fatty acid oxidative genes, while suppresses that of lipogenic genes [8], and BBR also improves insulin resistance in nutrient stressed mice through activation of AMPK [36]. Hepatic sterol regulatory element-binding proteins (SREBPs), liver X receptor α (LXRα) and peroxisome proliferator-activated receptor α (PPARα) transcriptional programs are also observed to be involved in the therapeutic mechanisms of BBR in type 2 diabetic hamsters [37], supporting the concept that BBR prevents dyslipidemia and fatty liver by directly promoting the activation of hepatic AMPK. As an alternative to animal studies, BBR hydrochloride regulates the transcription of hepatic genes that involve in glucose and fatty acid metabolism in vitro experiment using rat primary hepatocytes [6]. More importantly, AMPK specifically binds to and directly phosphorylates SREBP-1c and SREBP-2, and the Ser372 phosphorylation of SREBP-1c by AMPK could inhibit the proteolytic cleavage and nuclear translocation of SREBP-1c in hepatocytes, thereby preventing its autoregulation and transcription of target lipogenic genes [acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1)] [38]. Indeed, BBR was reported to inhibit both SREBP1c and SREBP2 expression in hepatocytes [34, 39], quite consistent with its AMPK regulation mechanism, explaining the triglyceride and cholesterol lowering effect of the compound.

3.3. BBR in Modifying Gut Microenvironment. Roughly 75% of the blood entering the liver is venous blood from the portal vein, which is all from the digest system. Therefore, the liver gets "first pickings" of everything absorbed in the small intestine. While simple steatosis seems to be well tolerated and to have only mild consequences, a significant proportion of patients with NAFLD develop nonalcoholic steatohepatitis (NASH), a condition that may result in hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [40, 41]. Although the circumstances that may lead to fatty liver progress remain largely unknown, components from the intestinal microflora may contribute to the regulation of proinflammatory processes in the liver that has been investigated in recent years [42].

Due to the low bioavailability and poor absorption through the gut wall, BBR might exert its effect in the intestinal tract before absorption. BBR has been shown to have significant antimicrobial activity against bacteria, fungi, parasites, worms, and viruses. In terms of bacteria, BBR has demonstrated highly significant activity against Staphylococcus, Streptococcus, Salmonella, Klebsiella, Clostridium, Pseudomonas, Proteus, Shigella, Vibrio, and Cryptococcus species [43]. BBR also exhibited effectiveness in combating enterotoxigenic *Escherichia coli* diarrhea [44]. Moreover, BBR inhibited the overgrowth organisms such as staphylococci and coliforms, while having no effect on indigenous lactobacilli and bifidobacteria.

Alternatively, BBR inhibits a wide range of intestinal microbes, modulates shift of the gut microbiota structure, and enriches some short-chain fatty acid (SCFA) producers in HFD-fed rats [12]. It has been shown that the antimicrobial activity of BBR can be mediated by inhibiting FtsZ (an essential cytoskeleton protein for bacteria cytokinesis) assembly and halting cell division of the bacteria [45]. Additionally, BBR-regulated reversion of inducible cyclooxygenase-2 (COX-2) might also contribute to this process [46]. Dysfunction of gutmicrobiota has more effective capacity to harvest energy from the diet, and when germ-free mice were transplanted with caecal microbiota from ob/ob mice, they developed obesity and insulin resistant within 2 weeks [47], thus the antimicrobial role of BBR might putatively affect energy absorption, which could partially explain its lipid-lowering action under nutrient oversupplying conditions.

BBR could regulate integrity of tight junction in cultured human Caco-2 cells [48], and similar effect was reflected in a mice model of endotoxemia; pretreatment with BBR attenuates disruption of tight junctions in intestinal epithelium in the animals [49], suggesting BBR's action in reducing epithelial gut permeability under pathogen stressed conditions, with the possible involvement of nuclear factor-κB (NF-κB) and myosin light chain kinase pathways [49, 50]. Therefore, BBR mediated intestinal barrier improvement could block the endotoxemia into circulation, thus reducing hepatic inflammation and preventing NAFLD progression.

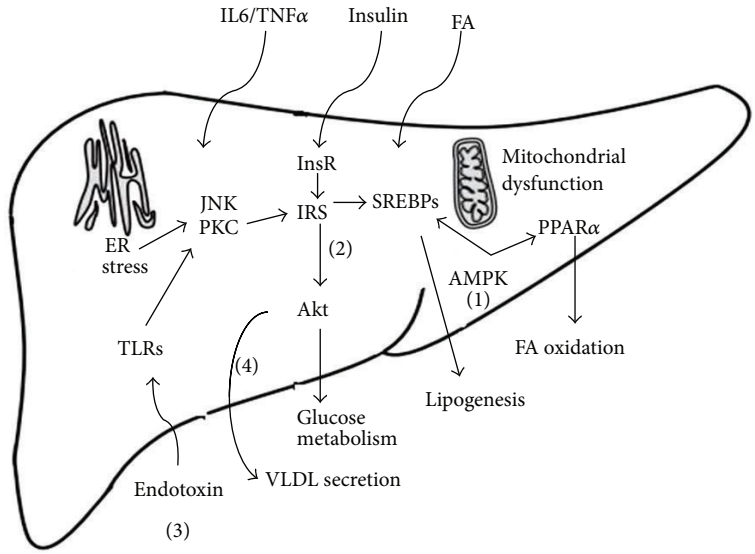

Figure 2: Cellular mechanisms of BBR in reverting dysfunction in NAFLD. Nutrient stress induced a series of alterations in the liver, including mitochondrial dysfunction, ER stress, proinflammatory cytokines and endotoxin elevation, and decreased VLDL secretion. BBR is partitioned toward several pathways in protecting fatty liver. (1) BBR phosphorylates α subunit of AMPK through regulating AMP/ATP ratio, and activation of AMPK can inhibit SREBPs to suppress de novo lipogenesis, increasing PPARα expression to enhance fatty acid oxidation in the liver. (2) BBR improves insulin sensitivity by normalize insulin signaling pathway, and BBR reduces pro-inflammatory cytokines production, counteracting ER stress, thus leading to the reviving of insulin signaling transduction. (3) BBR blocks intestinal endotoxin into liver, endotoxin is a major risk factor for NAFLD progression, BBR may mediate gut environment and reduce epithelial gut permeability, which are subsequently avoid the endotoxemia into circulation; (4) BBR promote VLDL secretion by increase ApoB assembly. Additionally, the extrahepatic role of BBR that mediates fatty acid, hormones, and cytokines entering liver also contributes to the lipid-lowering effects of BBR.

3.4. BBR on Hepatic Lipid Secretion. Secretion of triglycerides as very low-density lipoprotein (VLDL) is an important aspect in maintaining hepatic lipid homeostasis. Hepatocytes have a unique ability to present triglycerides to the organelles where VLDL assembly takes place. Efficient assembly of apolipoprotein B (apoB) 100 with triglyceride and cholesterol into VLDL requires the activity of an ER-resident microsomal triglyceride transfer protein (MTTP), and loss-of-function mutations within the MTTP gene are the cause of human abetalipoproteinemia, an autosomal recessive disease characterized by the total absence of triglyceride-rich lipoproteins in the plasma [51]. In an HFD-induced NAFLD rat model, Chang et al. [10] observed three increased DNA methylation sites in the MTTP promoter, which account for the reduced MTTP expression in the liver. BBR treatment could cause demethylation of the abnormal regions in MTTP promoter, counteracting HFD-induced MTTP dysregulation. BBR regulated restoration of MTTP expression and VLDL assembly further increase triglyceride secretion and alleviate fatty liver [10].

3.5. BBR on Cholesterol Metabolism. BBR has shown considerable impact on cholesterol metabolism, and preclinical and clinical studies both suggest lipid-lowering acting of the drug [52, 53]. The molecular mechanism has been proposed to be through stabilization of the low-density lipoprotein receptor (LDLR) messenger RNA, which led to upregulation of LDLR protein [54, 55]. Other studies illustrate that BBR-induced stabilization of LDLR mRNA is mediated by the extracellular signal-regulated kinase (ERK) signaling pathway through interactions of Cis-regulatory sequences of 3′ untranslated region (UTR) and mRNA binding proteins that are downstream effectors of this signaling cascade [56]. Additionally, SREBPs also act as regulators of hepatic cholesterol levels and activate genes involved in the synthesis of cholesterol and free fatty acids [57]. SREBP cleavage-activating protein has a cholesterol-sensing domain that senses intracellular cholesterol levels and directs the activity of SREBPs. Therefore, the ability of BBR in suppressing SREBPs could be another mechanism for its role in cholesterol metabolism, further explain the beneficial effects on NAFLD.

3.6. Other Possible Mechanisms. BBR can downregulate hepatic expression of uncoupling protein-2 (UCP2) mRNA protein in NAFLD rats, promote the recovery of hepatocyte steatosis, and improve lipid metabolism disorder [58]. BBR also demonstrates to have antioxidative activities in cultured cells and animals, and this effect may help reducing reactive oxygen species (ROS) production in the liver [59, 60], thus preventing liver damage. Extrahepatic factors might also affect NAFLD pathology, and in adipocytes, studies illustrate that BBR inhibits adipogenesis in murine-derived 3T3-L1 preadipocytes and human white preadipocytes [61], while enhancing glucose and fatty acid uptake by muscle cells has been proved [62], BBR is proved to play a role in pancreatic islets as well [24]. Both intrahepatic and extrahepatic

mechanisms integrate into the BBR regulating action in combating metabolic diseases.

4. Prospects of BBR on NAFLD

There is a long history of safe usage of BBR in clinic, sporadic single case reports on the adverse effect of BBR include gastrointestinal side effects, allergic skin reaction and arrhyth-mia [63]. Even though some reports indicate that BBR could induce hepatoma cells apoptosis, the cytotoxic effects were absent in healthy hepatocytes [64, 65], and BBR actually shows antihepatotoxic action in a series of studies. BBR is investigated in rats with acetaminophen-induced hepatic damage, which shows decreased serum levels of alkaline phosphatase (ALP) and aminotransaminases, suggestive of hepatoprotection of the alkaloid [66]. In cultured rat hepatocytes, Hwang et al. [67] demonstrated bioactivity of BBR in protecting the cells against oxidative damage.

However, despite the promising effects of BBR on animal models and cells, large clinical investigations are not available. Due to low bioavailability and poor absorption of BBR via oral administration [2], high dose oral administration usually causes gastrointestinal side effects, which greatly limit its clinical application. There are some reports, however, trying to explore new dosage forms of BBR to increase its bioavailability. Preclinical studies that use sodium caprate, one of the intestinal absorption enhancers, showed 1.5- to 2.3-fold increase of BBR bioavailability in different models [68, 69]. P-glycoprotein (P-gp) inhibitors, such as cyclosporine and verapamil, also illustrate marked increase of BBR absorption [70]. Though the beneficial action of BBR is obvious, further studies are still in need to optimize its clinical application in NAFLD.

5. Summary

NAFLD is the liver manifestation of obesity and the metabolic syndrome and is marked by lipid deposition and/or inflammation. In this review, we introduced the beneficial potential of a nature compound, BBR, in NAFLD and the possible mechanisms under the therapeutic actions (Figure 2). Though most evidence based on experimental studies, and clinical trials need to be further confirmed, we still hold the belief that BBR is a promising candidate in preventing and treating NAFLD in the future. However, more studies should be cautiously performed to clarify the mechanisms and optimize clinical application of the drug.

Abbreviations

ACC1: Acetyl-CoA carboxylase 1
ALP: Alkaline phosphatase
AMP: Adenosine monophosphate
AMPK: Adenosine monophosphate-activated protein kinase
apoB: Apolipoprotein B
BBR: Berberine
COX-2: Cyclooxygenase-2
eIF2α: Eukaryotic translational initiation factor 2α
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase
FAS: Fatty acid synthase
HFD: High-fat diet
IKKβ: Inhibitor of kappaB kinase β
IL-6: Interleukin-6
IRE-1: ER-to-nucleus signaling 1
IRS: Insulin receptor substrate
IκB: Inhibitor of nuclear factor B
JNK: Jun N-terminal kinases
LDL-c: Low-density lipoprotein cholesterol
LDLR: Low-density lipoprotein receptor
LXRα: Liver X receptor α
mRNA: Messenger RNA
MTTP: Microsomal triglyceride transfer protein
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NF-κB: Nuclear factor-κB
PERK: PKR-like eukaryotic initiation factor 2α kinase
P-gp: P-glycoprotein
PKB, Akt: Protein kinase B
PKC: Protein kinase C
PPARs: Peroxisome proliferator-activated receptors
ROS: Reactive oxygen species
SCD1: Stearoyl CoA desaturase 1
SCFA: Short-chain fatty acid
Ser/Thr: Serine/threonine
SREBPs: Sterol regulatory element-binding proteins
t-BHP: Tert-butyl hydroperoxide
TNF-α: Tumor necrosis factor-α
UCP2: Uncoupling protein-2
UTR: Untranslated region
VLDL: Very low-density lipoprotein.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

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Research Article

Swertiamarin: An Active Lead from *Enicostemma littorale* Regulates Hepatic and Adipose Tissue Gene Expression by Targeting PPAR-γ and Improves Insulin Sensitivity in Experimental NIDDM Rat Model

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Enicostemma littorale (EL) Blume is one of the herbs widely used for treating and alleviating the effects of both type I and type II diabetes. However, lack of understanding of mechanism precludes the use of the herb and its molecules. In this study, we attempt to unravel the molecular mechanism of action of swertiamarin, a compound isolated form EL, by comparing its molecular effects with those of aqueous EL extract in alleviating the insulin resistance in type II diabetes. We further investigated hypolipidemic and insulin sensitizing effect of swertiamarin in experimentally induced noninsulin dependent diabetes mellitus (NIDDM) in rats. Swertiamarin (50 mg/kg) and aqueous extract (15 grams dried plant equivalent extract/kg) were administered to rats orally for 40 days and tight regulation of serum glucose, insulin, and lipid profile was found in both groups. Their mode of action was by restoring G6Pase and HMG-CoA reductase activities to normal levels and restoring normal transcriptional levels of PEPCK, GK, Glut 2, PPAR-γ, leptin, adiponectin, LPL, SREBP-1c, and Glut 4 genes. This suggests that both treatments increased insulin sensitivity and regulated carbohydrate and fat metabolism. This is the first report on the role of SM in regulating the PPARγ-mediated regulation of candidate genes involved in metabolism in peripheral tissues *in vivo*.

1. Introduction

Diabetes mellitus is the third most prevalent fatal disease in the world. Epidemiology shows that it is one of the major global health problems in the current scenario, targeting 6.4% of total world population. Around fifty million people are diabetic in Indian subcontinent; hence, India leads globally in this disease [1]. Indian population is more prone to diabetes than western population as their metabolism is quite different with lots of epigenetic modifications. Constant migration of people from rural to urban areas has contributed significantly in availability of food, calorie intake, and physical activities, which has major impact on the metabolic programming of an individual [2]. To understand the physiological and metabolic alterations of this disorder, many animal models are used. NA-STZ rat is a nonobese type 2 diabetes model that reflects the majority of diabetic patients among Asian races [3].

Type II diabetes mellitus is a heterogeneous metabolic disorder. Liver, skeletal muscles, and adipose tissues being insulin sensitive tissues show significant metabolic changes [4]. Insulin, apart from governing glucose uptake and metabolism, also influences the expression level of many genes related to energy metabolism [5].

Glut 2 plays a major role in glucose uptake and its metabolism in hepatic tissue, whose gene expression is influenced by glucose and insulin concentrations; hence, remarkable alterations in glucose would lead to significant change in the expression profile of this transporter. In diabetic condition, the expression of glucokinase (GK) enzyme is downregulated, which eventually leads to insulin resistance and hyperglycemia [6, 7]. PEPCK is a rate-limiting enzyme in gluconeogenesis; hence, its elevated expression leads to increased hepatic glucose production (HGP). The processes of glycolysis, gluconeogenesis, glycogenesis, and glycogenolysis are governed

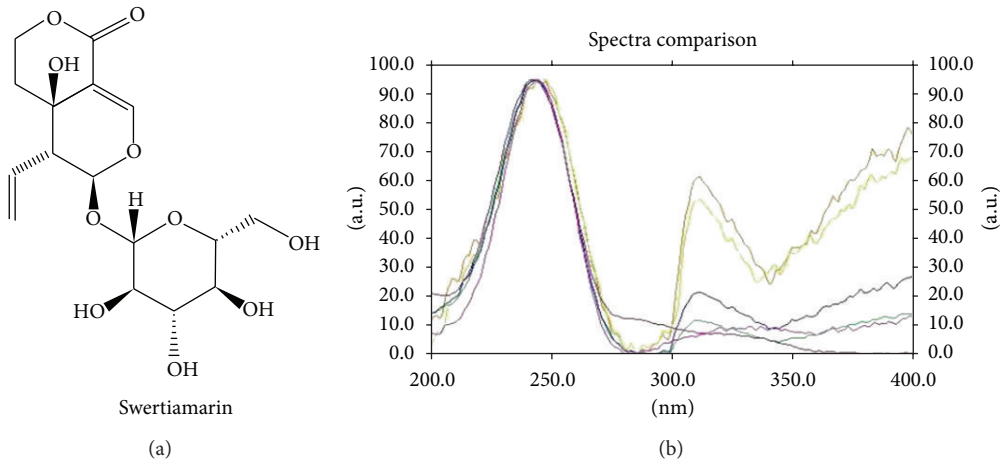

Figure 1: (a) Chemical structure of swertiamarin. (b) Overlay of ultraviolet absorption spectrum of swertiamarin isolated in lab and reference standard (λmax: 240–245 nm).

by insulin action, which are hampered due to altered action of insulin in TIIDM [8].

Obesity and dyslipidemia lead to the development of type II diabetes. Secretion of adipokines play a key role in controlling glucose and fat homeostasis of the entire body. PPAR-γ, playing a major role in regulation of transcription, is responsible for adipogenesis, mature adipocyte function, insulin signaling, carbohydrate metabolism, fat metabolism, and secretion of various adipokines like adiponectin, leptin, and so forth. Adiponectin positively and leptin negatively regulate insulin signaling in liver and muscle tissues [9]. Looking at the importance of this regulator, most studies have been directed toward developing synthetic PPARs ligands for insulin resistance and dyslipidemia in amelioration of TIIDM complications [10].

There are many treatments available ranging from synthetic drugs, like metformin, thiazolidinediones, GLP-1, DPP-4 inhibitor, and so forth, to herbal formulations like *Momordica charantia*, *Artemisia dracunculus*, *Gymnema sylvestre*, and so forth in amelioration of obesity and TIIDM [11]. In recent years, there has been renewed interest in the treatment of diabetes using herbal drugs, as World Health Organization (WHO) has recommended evaluation of the effectiveness of plants due to side effects of modern drugs [12]. Our lab has well documented *Enicostemma littorale* Blume owing antioxidant, hypolipidemic, and antidiabetic activities both in animals and human diabetic patients [13, 14]. Apart from antidiabetic activity, islet neogenic property of swertisin and normoglycemia in a diabetic rat are also reported [15]. Hence, it can be presumed that the EL extract potentially owes varied beneficiary activities due to the presence of many compounds within it. Swertiamarin (SM) is the major compound found in EL Blume and its pharmacokinetic study suggest that it is rapidly distributed in most of the tissues. Among all the tissues, the highest concentrations were found to be absorbed in the liver and its elimination was through kidney [16].

Many studies have been done to explore the mechanistic action of SM on TIIDM but are restricted to in vitro only. Also, other groups have focused on physiological and biochemical studies in neonatal-STZ models to understand role of SM for the treatment of obesity and dyslipidemia. However, the mechanism of action of SM has not been explored at a systemic level [17, 18].

Therefore, for the first time in this study we aim to assess the antidiabetic efficiency of SM in treating nicotinamide-streptozotocin (NA-STZ) diabetic rats and to elucidate its probable mechanisms of action. The current study was designed to answer the key question, "what is the mechanism of SM in regulating the expression levels of the candidate genes involved in carbohydrate, fat metabolism and insulin signaling in the liver and adipose tissue in TIIDM?"

2. Materials and Methods

2.1. Plant Material, Preparation of Aqueous EL Extract, and Isolation of Swertiamarin. The plant material of dry *E. littorale* was procured from Saurashtra region, Gujarat, India, during the month of August. Specimen was authenticated at Botany Department, M. S. University, Baroda with Voucher Specimen number [Oza 51,51(a)] deposited at the Herbarium of Botany Department, M. S. University, Baroda. Whole plant material was cleaned and dried. The fine powder of 40–60 mesh particle size was prepared in an electric grinder. The powder was soaked in thrice the amount of water for 2 hours and then boiled for 30 minutes. Three such extractions were done from each batch. Residue was removed by filtration, and water-soluble filtrate was pooled and evaporated to obtain extract concentration of 1 g dry plant weight equivalent per mL as per the method described above [19]. The yield of dry EL extract was found to be 28% (w/w). Isolation and characterization of swertiamarin from *E. littorale* were carried out by recording melting point and UV spectrometry with the standard sample of swertiamarin (Figure 1(a)). Purity of the sample was checked by HPTLC on ethyl acetate: methanol : water (0.7 : 0.2 : 0.1) as a solvent system [20].

2.2. Animals and Housing. Male *Charles Foster* rats housed at animal house facility of Department of Biochemistry were used for the study with *ad libitum* access to water

Table 1: List of primer sequences of RT-PCR with its amplicon size.

Gene	Accession number	Sequence forward primer $5'\rightarrow3'$	Sequence reverse primer $5'\rightarrow3'$	Product size
Glucokinase	NM_012565.1	AGTATGACCGGATGGTGGAT	CCGTGGAACAGAAGGTTCTC	139
Glut 2	NM_012879.1	CATTGCTGGAAGAAGCGTATCAG	GAGACCTTCTGCTCAGTCGACG	408
PEPCK	NM_198780	GTCACCATCACTTCCTGGAAGA	GGTGCAGAATCGCGAGTTG	84
Adiponectin	NM_144744.2	AATCCTGCCCAGTCATGAAG	CATCTCCTGGGTCACCCTTA	215
LPL	NM_012598.1	GAGATTTCTCTGTATGGCACA	CTGCAGATGAGAAACTTTCTC	276
Leptin	NM_013076.2	ACACCAAAACCCTCATCAAGA	GAAGGCAAGCTGGTGAGGA	184
SREBP-1c	XM_213329.4	GGCCTGCTTGGCTCTTCTC	GCCAGCCACAGCTGTTGAG	150
PPARγ	NM_013124	GGATTCATGACCAGGGAGTTCCTC	GCGGTCTCCACTGAGAATAATGAC	156
Glut 4	NM_012751.1	GCCTTCTTTGAGATTGGTCC	CTGCTGTTTCCTTCATCCTG	457
β-ACTIN	NM_031144	CCTGCTTGCTGATCCACA	CTGACCGAGCGTGGCTAG	505

and commercial chow (Pranav Agro Industries Ltd, Pune, India) in a well-ventilated animal unit (26–28°C, humidity 60%, 12 h light—12 h dark cycle). Care and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee (938/a/06/CPCSEA, BC/14/2009-10). NIDDM rat model was developed by intraperitoneal injection of nicotinamide dissolved in normal saline at a concentration of 230 mg/kg body weight 15 minutes before giving an intraperitoneal injection of streptozotocin (Sigma, Aldrich) which was dissolved in 0.1 M citrate buffer (pH 4.5) at a concentration of 65 mg/kg body weight [21]. Hyperglycemia was confirmed by the elevated glucose level in fasting and postprandial blood sugar (PP_2BS) at 15–20 days of streptozotocin-nicotinamide injection.

2.3. Dosing of Swertiamarin and Aqueous Extract in NIDDM Rats. Swertiamarin and aqueous extract were orally administered for 40 days, and fasting serum glucose levels, OGTT profiles, and serum triglyceride levels were monitored. Rats were divided into five groups having six rats in each group; group I: normal control (NC), group II: DM, group III: DM + Aqueous extract (15 grams dried plant equivalent extract/kg b.w/day, p.o.), group IV: DM + swertiamarin (50 mg/kg/day, p.o.), and group V: DM + metformin (500 mg/kg b.w/day, p.o.).

2.4. Biochemical Parameters

2.4.1. Oral Glucose Tolerance Test (OGTT), Serum Insulin, and Lipid Profile. Rats were kept for overnight (10–12 hrs) fasting, and blood was collected from retro orbital sinus for estimation of fasting blood sugar. To measure OGTT of the rats, 2 gms/kg body weight of glucose was given orally and blood was collected at regular interval of every 30 min. till 2 hours. Serum was separated and glucose level was estimated using GOD-POD method by commercially available kit (Enzopak, India). Fasting serum insulin was estimated by rat insulin ELISA kit (Mercodia, Sweden). Total cholesterol, HDL-cholesterol, and TG was estimated using commercially available kits (Enzopak, India), and then the values of LDL-cholesterol and VLDL-cholesterol were derived from Friedewald's formula.

2.4.2. Determination of Liver Enzymes. Glucose-6-phosphatase was assayed according to the method of Koida and Oda, 1959, and the inorganic phosphorus (Pi) liberated was estimated by Fiske and Subbarow method, 1925. The ratio of absorbance of HMG CoA/absorbance of mevalonate was taken as an index of the activity of HMG CoA reductase activity required to convert HMG CoA to mevalonate, in the presence of NADPH [14].

2.4.3. RNA Isolation and Semiquantitative PCR. Animals from each group were sacrificed, and tissues (liver and adipocytes) were pooled. RNA was isolated from the homogenized liver and adipose tissue using the TRIzol reagent (Sigma Aldrich) as per manufacturer's instructions. A reverse-transcription reaction was performed using 2 μg RNA with MuLV reverse transcriptase in a 20 μL reaction volume containing DEPC treated water (Fermentas Kit). PCR product was amplified using gene-specific primers (Table 1). β-Actin was used as an internal control. The PCR products were analyzed by electrophoresis on 2.0% agarose gels or 15% DNA-PAGE, the gels were photographed after staining with ethidium bromide, and intensities of the band were calculated by densitometric analysis using the Image J software.

2.4.4. Immunoprecipitation and Immunoblotting for Insulin Signaling Proteins. Tissues were collected, suspended in lysis buffer containing 1X protease inhibitor cocktail, and homogenized. After centrifugation at 16000 g for 15 min. at 4°C, the supernatant was collected. Total protein content was quantified using Bradford assay (Biorad Bradford Solution, USA). Immunoprecipitation with insulin receptor (anti-IRβ 1 : 50) was performed using dynabeads G-protein IP kit (Invitrogen). Protein was loaded on a 10% SDS-polyacrylamide gel and then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare). The membrane was then incubated for 1 h at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further incubated overnight with the primary antibodies for insulin receptor (1 : 1000), p-Tyr (1 : 1000), and PI(3)K (1 : 1000) at 4°C. Membrane was then washed four times with TBS-T and incubated with HRP-conjugated secondary antibodies (1 : 2500) for 1 h. Finally, membrane was developed and visualized with enhanced chemiluminescence western blotting detection system (Millipore Inc. USA).

2.5. Statistical Analysis. The results were analyzed using one-way analysis of variance (ANOVA) and student's t-test to determine the level of significance. $P < 0.05$ was considered to be significant. Results were expressed as mean ± SEM. The statistical analysis was carried out by using the Graph Pad Prism 3.0 software.

3. Results

3.1. Isolation and Confirmation of Swertiamarin from Enicostemma Littorale Blume. The n-butanol fraction yielded 7.31% w/w of swertiamarin. HPTLC densitogram confirmed identity of compound with standard swertiamarin as well as established purity of the same (Figure 1(b)). Ultraviolet absorption spectrum showed λmax in the range of 240–245 nm. Melting point of the compound was 190–192°C. The mass fragmentation pattern of compound represented base peak m/z of 374 representing molecular weight and m/z of 212 (M-162) a characteristic peak after removal of sugar moiety from the compound.

3.2. Swertiamarin Positively Regulates Various Physical and Biochemical Parameters in TIIDM. Decrease in body weight is a characteristic hallmark of TIIDM that happens due to the loss of the stored energy reserves. A classical way of determining the efficacy of drug treatment is the ability to restore body weight. As expected, we found a drastic decrease in the body weight in NA-STZ-induced diabetic rats as compared to the controls. The animals treated with standard drug metformin (MFO), aqueous extract, and SM showed significant increase in the body weight, which indicated reversal of DM condition (Figure 2).

Further, in an attempt to confirm the NIDDM condition in the animal model, oral glucose tolerance test was performed to ascertain severity of the diabetic condition. Our observation is in agreement with the known facts: the diabetic rats have a high PP_2BS and showed glucose intolerance as compared to the control rats (Figures 3(a) and 3(b)). MFO effect, as expected in Type II diabetes, reduced the PP_2BS in our rats (Figures 3(a) and 3(b)). The aqueous extract and SM-treated diabetic rats were observed to be normoglycemic.

Hyperinsulinemia is a characteristic feature of type II DM. However, in our DM group of rats, the serum insulin levels were lower than those in the control rats which matched with the reported model where insulin content was reduced up to 40% [21] (Figure 3(c)) and which mimic the later stage TIIDM. The EL extract and SM treatments were not capable of significantly ameliorating the hypoinsulinemic condition by increasing the serum insulin levels.

3.3. Swertiamarin Reduces Glucose 6 Phosphatase Activity. Changes due to diabetes are not only seen at the mRNA level but also at the protein levels. Many of the enzyme activities are altered in the peripheral tissues of diabetes. G-6-Pase is the key enzyme of gluconeogenesis in hepatic tissue. Its activity increases under diabetic condition due to deficiency of insulin or insulin action. The EL extract and SM restored elevated specific activity of G-6-Pase to normal levels (Figure 4).

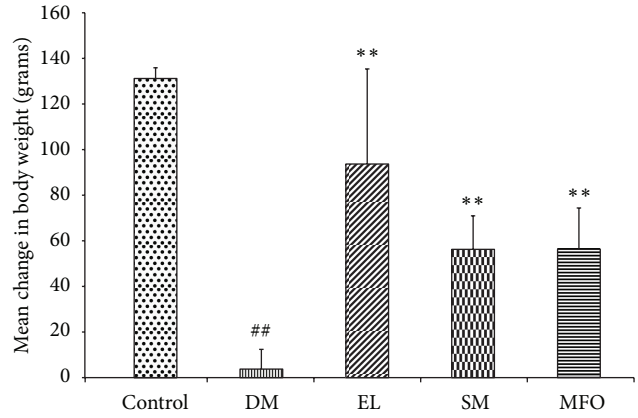

Figure 2: Change in body weight upon induction of diabetes and treatment of diabetic rats with EL, SM, and standard drug MFO. Data presented as mean ± SEM of 6 independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus Diabetic rats.

3.4. Swertiamarin Regulates the Expression Levels of Candidate Genes of Carbohydrate Metabolism in TIIDM. Diabetes affects the expression of many candidate genes in the insulin dependent peripheral tissues like liver, adipose, and skeletal muscles. Glut transporters are the main glucose transporters in different peripheral tissues. Glut 2 is present in liver and is insulin independent. In diabetic rats, Glut 2 expression decreased significantly as compared to the control rats. We observed that the two treatments, EL extract and SM, rescue this decrease in expression, and the extract was more efficacious than the compound. PEPCK and glucokinase are the main enzymes of gluconeogenesis and glycolysis, respectively. They are regulated by insulin at the transcriptional level. In the diabetic condition, PEPCK has increased expression, while GK has decreased expression in liver. Treatments for diabetes should thus decrease the expression of PEPCK and increase the expression of GK. It was observed that EL extract and SM showed this (Figures 5(a) and 5(b)).

3.5. Swertiamarin Regulates the Altered Expression of Insulin Signaling Proteins in Liver. The liver homogenate was subjected to immunoprecipitation and immunoblotting with antiphosphotyrosine and anti-insulin receptor antibody. There was a decrease in the protein expression of insulin receptor in the diabetic group as compared to control. However, treatment with SM and EL extract restored not only the level of insulin receptor protein but also increased its phosphorylation. PI(3)K is a molecule downstream to insulin receptor, which gets recruited via IRS signaling pathway (Figures 6(a) and 6(b)).

3.6. Lipid Profile. We observed a significant increase in the serum triglyceride levels in the diabetic rats as compared to the control rats. This observation assertively showed that the serum triglyceride levels increase due to peripheral insulin resistance. Metformin (MFO) did not bring down the serum triglycerides levels significantly. But EL extract and its compound, SM, had a higher efficacy and reduced the serum triglyceride level near to control levels, thus making them out

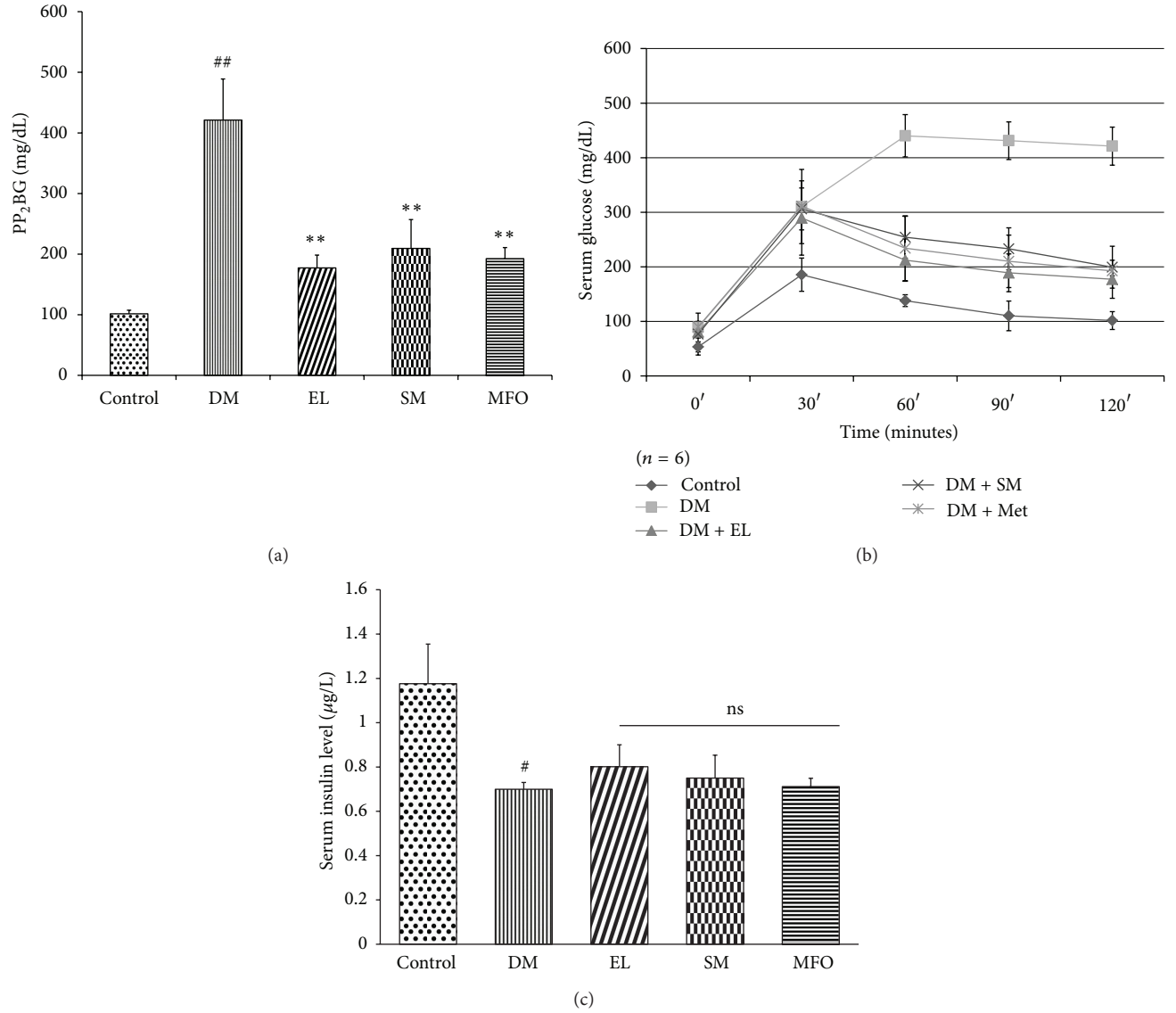

Figure 3: (a) Effect of EL extract, SM, and MFO treatments for 40 days on the postprandial serum glucose levels in diabetic conditions. Serum glucose levels were measured using GOD-POD. Data presented as mean ± SEM of $n = 6$ independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus diabetic rats. (b) Effect of EL extract, SM, and MFO treatments for 40 days on the OGTT profile in diabetic conditions. Serum glucose levels were measured using GOD-POD. Data presented as mean ± SEM of $n = 6$ independent observations. (c) Effect of EL extract, SM, and MFO treatments for 40 days on the serum insulin levels in diabetic conditions. Serum insulin levels were measured using ELISA kit. Data presented as mean ± SEM of 4 independent observations. $^{\#}P < 0.05$ versus control rats. P value ns versus diabetic rats.

to be a safer alternative than the available anti-diabetic drugs. Aqueous extract and SM both were able to decrease serum cholesterol, serum LDL, and VLDL levels and increase HDL-cholesterol (Table 2).

3.7. Swertiamarin Regulates Activity of HMG-CoA Reductase Enzyme of Cholesterol Biosynthesis. HMG-CoA reductase is the major regulatory enzyme of cholesterol biosynthesis in the liver. An estimate of the enzyme activity can be used as a measure of the severity of the diabetic condition. The results of the present study showed inhibition of the HMG-CoA reductase activity in the diabetic rats treated with SM and EL extract as observed by higher HMG-Co/mevalonate (substrate/product) ratio compared to that of the diabetic control rats (Figure 7), thus supporting earlier reported hypolipidemic activity of SM.

3.8. Swertiamarin Regulates the Expression Levels of Various Key Enzymes of Lipid Metabolism and Glucose Transporter. Glut 4, an insulin dependent glucose transporter present in adipocytes and skeletal muscles, has decreased expression in diabetic rats due to increased insulin resistance. The key regulators of fat metabolism like adiponectin, SREBP-1c, PPAR-γ, and lipoprotein lipase 1 (LPL 1) are also found to be downregulated, while leptin is upregulated. The treatments with EL extract and SM helped in overcoming the insulin resistance by restoring the above gene expressions to normal levels (Figures 8(a), 8(b), and 8(c)).

Table 2: Lipid profile of control and treated diabetic rats.

Groups	Control	DM	DM + EL	DM + SM	DM + metformin
Triglyceride[#]	55.86 ± 4.69	110.94 ± 14.32[*]	49.63 ± 6.37[a]	63.34 ± 7.86[b]	94.54 ± 19.31
Total cholesterol[#]	88.55 ± 7.28	123.26 ± 15.80[*]	95.15 ± 10.88[a]	86.75 ± 13.64[b]	106.78 ± 12.54
HDL-C[#]	55.86 ± 4.66	27.44 ± 8.27[*]	44.63 ± 5.49[a]	40.94 ± 6.77[b]	41.89 ± 4.69
LDL-C[#]	21.52 ± 1.93	73.63 ± 3.2[*]	40.59 ± 4.37[a]	33.14 ± 5.86[b]	45.99 ± 5.64[c]
VLDL-C[#]	11.17 ± 0.69	22.19 ± 4.76[*]	9.93 ± 1.3[a]	12.67 ± 1.01[b]	18.90 ± 2.31

[#]Units: mg/dL. Values are given as mean ± SEM from 6 rats in each group, [*]$P < 0.005$ compared to control and a, b, c compared to diabetic group.

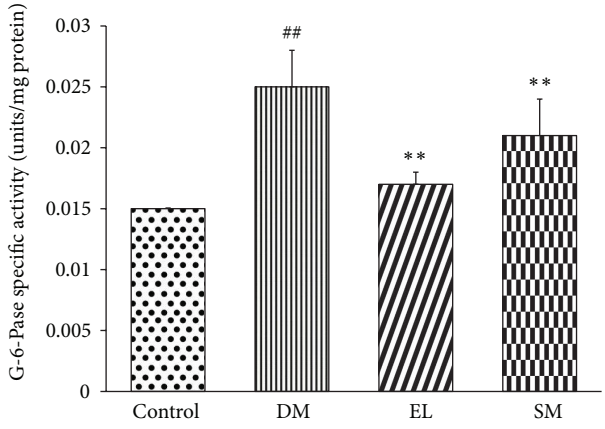

Figure 4: Effect of EL extract, SM, and MFO treatments for 40 days on the specific activity of G-6-Pase enzyme from hepatic tissue in diabetic conditions. It was assayed according to Koida and Oda method, and released Pi was estimated using Fiske-Subbarao method. Data presented as mean ± SEM of 5 independent observations. [##]$P < 0.05$ versus control rats; [**]$P < 0.05$ versus diabetic rats.

3.9. Expression of Insulin Signaling Proteins in Adipose Tissue. The adipose tissue homogenate was subjected to immunoprecipitation and immunoblotting with antiphosphotyrosine and anti-insulin receptor antibodies. There was a decrease in the protein expression of insulin receptor and PI(3)K in the diabetic group as compared to control (Figure 9(a)). Treatment with SM and aqueous extract restored the level of insulin receptor, IR phosphorylation, and PI(3)K protein level (Figure 9(b)).

4. Discussion

Obesity and insulin resistance are major causes of TIIDM. Multiple problems in diabetes lead to a cascade of complications in peripheral tissues. For controlling hyperglycemia, dyslipidemia, and insulin resistance, many synthetic drugs have been used. Also, the beneficiary effects of herbal extracts and compounds have been exploited [12].

Enicostemma litorrale Blume belonging to Gentianaceae family has been evaluated for its hypoglycemic, antioxidant, and hypolipidemic activities [13, 14]. Further, the author's research group is continuously involved in exploring the wide spectrum hidden potentials of this plant for islet neogenesis and various diabetic complications [15, 22, 23]. Qualitative analyses of EL have demonstrated the presence of flavonoids and secoiridoid glycosides. Swertiamarin, a secoiridoid glycoside, is one of the most valuable compounds that is present in abundance and possesses various therapeutic activities: antidiabetic, antinociceptive, antilipidemic, and anti-inflammatory [17, 24]. Hence, it is interesting to unravel the mechanism of this compound's action against the development and progression of TIIDM.

Various animal models are available for studying TIIDM. NA-STZ nonobese NIDDM rat model was selected for this study that best mimics the non-insulin dependent diabetes condition prevalent in humans [21]. Glucose intolerance, altered insulin content and skewed lipid profile of the experimental animals, resemble the hallmarks of this model that actually persist in a later stage of human TIIDM patients. Effect of SM in restoration of body weight, OGTT profile, and hypolipidemic activity on the experimental animals potentially proves the reported characteristics of this compound in the present study. Many herbal compounds are reported in regulating the expressions of the candidate genes involved in metabolic pathways and thus ameliorating TIIDM. This led to our interest in unraveling the molecular mechanism of aqueous extract and SM in restoring the altered expressions of the candidate genes involved in TIIDM.

Carbohydrate and fat metabolism regulation is governed mainly in insulin sensitive peripheral tissues like liver, muscle, and adipose tissue. Liver is the master organ in metabolism where glucokinase, PEPCK, and glycogen phosphorylase are rate limiting enzymes in glucose flux, gluconeogenesis, and glycogenolysis, respectively [8].

Decreased activity of G-6-Pase was observed in SM-treated diabetic rats, which correlate to the results reported by us previously in aqueous extract-treated diabetic rats. Reduction in the PEPCK gene expression was observed when diabetic rats were treated with SM in the current study. PEPCK expression restoration reflects increased insulin sensitivity [25–27]. Increased activity of this limiting enzyme leads to more hepatic glucose production (HGP), which worsens the diabetic condition. Glucose concentration increases the binding of SREBP-1c on promoter of Glut 2, increasing its transcription which is regulated by glucose and insulin. Expression levels of glucokinase and Glut 2 have been shown to be decreased in the hepatocytes of the diabetic rats [28]. In agreement with earlier reports, our results show that diabetic rats have decreased glucokinase and Glut 2 expressions which are reversed upon treatment with EL aqueous extract and SM.

It is well documented that diabetic patients exhibit dyslipidemia. Our lab previously reported a decrease in serum triglycerides, cholesterol, LDL, and VLDL with increased

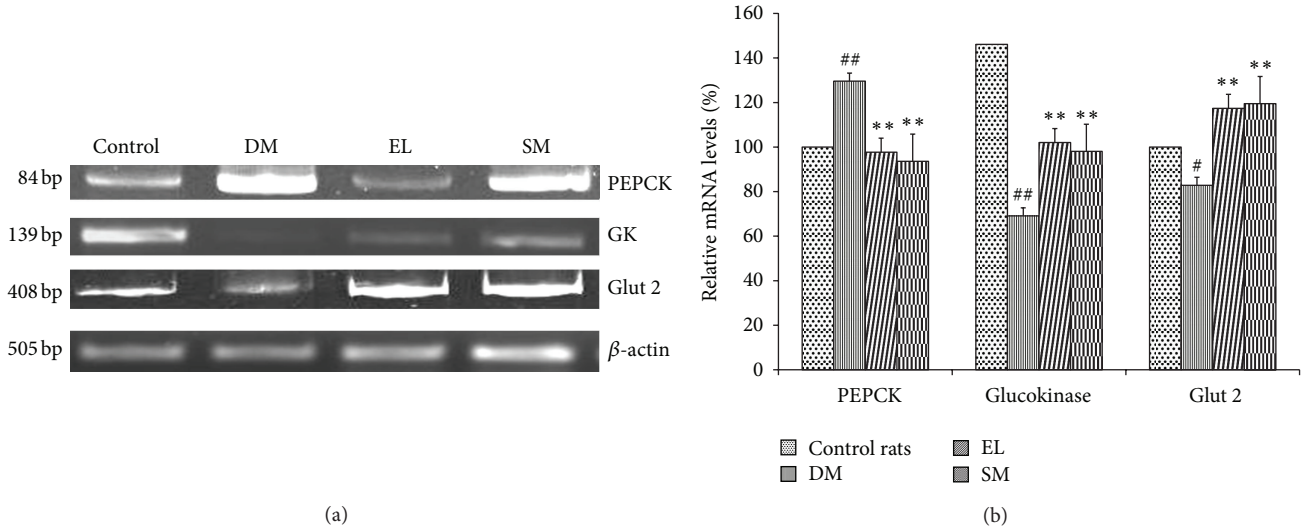

Figure 5: (a) Effect of EL extract and SM treatments on the mRNA expression of PEPCK, GK, Glut 2, and β-actin in the hepatic tissue as compared to diabetic rats (Gel image). (b) Effect of EL extract and SM treatments on the expression of PEPCK, GK, and Glut 2 in the hepatic tissue as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as mean ± SEM of 4 independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus diabetic rats.

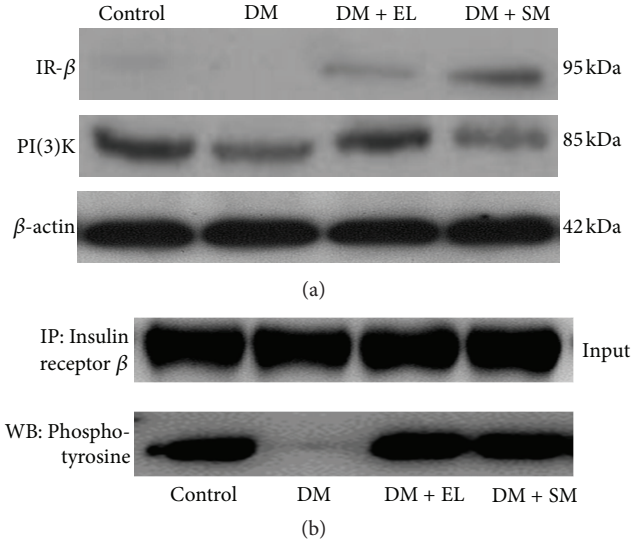

Figure 6: (a) Western blot study showing the effect of EL extract and SM treatments on the expression of insulin signaling proteins: IR and PI(3)K in the hepatic tissue as compared to diabetic rats. β-actin was taken as an internal control. (b) Immunoprecipitation study showing the effect of EL extract and SM treatments on the tyrosine phosphorylation of insulin signaling proteins: IR in the hepatic tissue as compared to diabetic rats (200 ug protein).

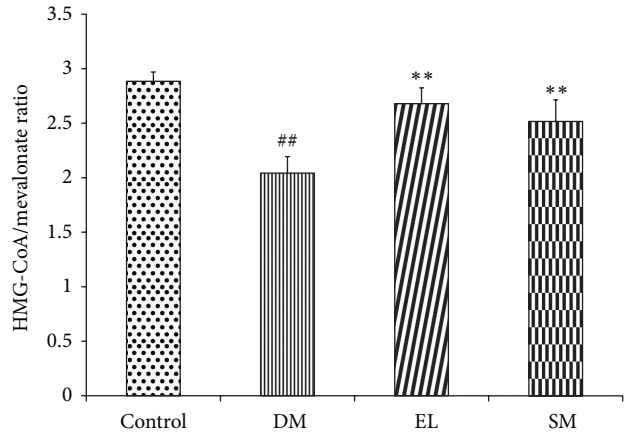

Figure 7: Effect of EL extract and SM treatments for 40 days on the ratio of absorbance of HMG CoA/absorbance of mevalonate was taken as an index of the of HMG CoA reductase activity from hepatic tissue in diabetic conditions. Data presented as mean ± SEM of 5 independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus diabetic rats.

HDL level in aqueous extract-treated cholesterol fed rats [14]. SM is beneficial in bringing the lipid profile in neonatal-STZ rats to normal. [18]. HMG-CoA reductase is a key enzyme involved in the cholesterol biosynthesis in the hepatic tissue, which increases the free fatty acid level that leads to insulin resistance. SM and aqueous extract correct lipid profile and HMG-CoA reductase activity [29].

Adipose tissue plays an important role in fat metabolism. In TIIDM, increased lipolysis and decreased lipogenesis occur in liver and adipose tissues. Obesity decreases expression of lipogenic genes like SREBP-1c, PPAR-γ, and aP2, which causes increase in hepatic lipogenesis hence leading to a fatty liver [30]. PPAR-γ is a key transcriptional factor regulating the expression of SREBP-1c, leptin, adiponectin, and LPL. Low adiponectin and high leptin levels can cause insulin resistance in adipocytes thus leading to diabetes [31]. In the present study, aqueous extract and SM both regulate PPAR-γ mRNA levels in NA-STZ-induced diabetic rat model along with induced expression of adiponectin, LPL, and SREBP-1c suggesting it as a potent modulator of diabetes-related modification in adipocytes and thus corrects overall lipid

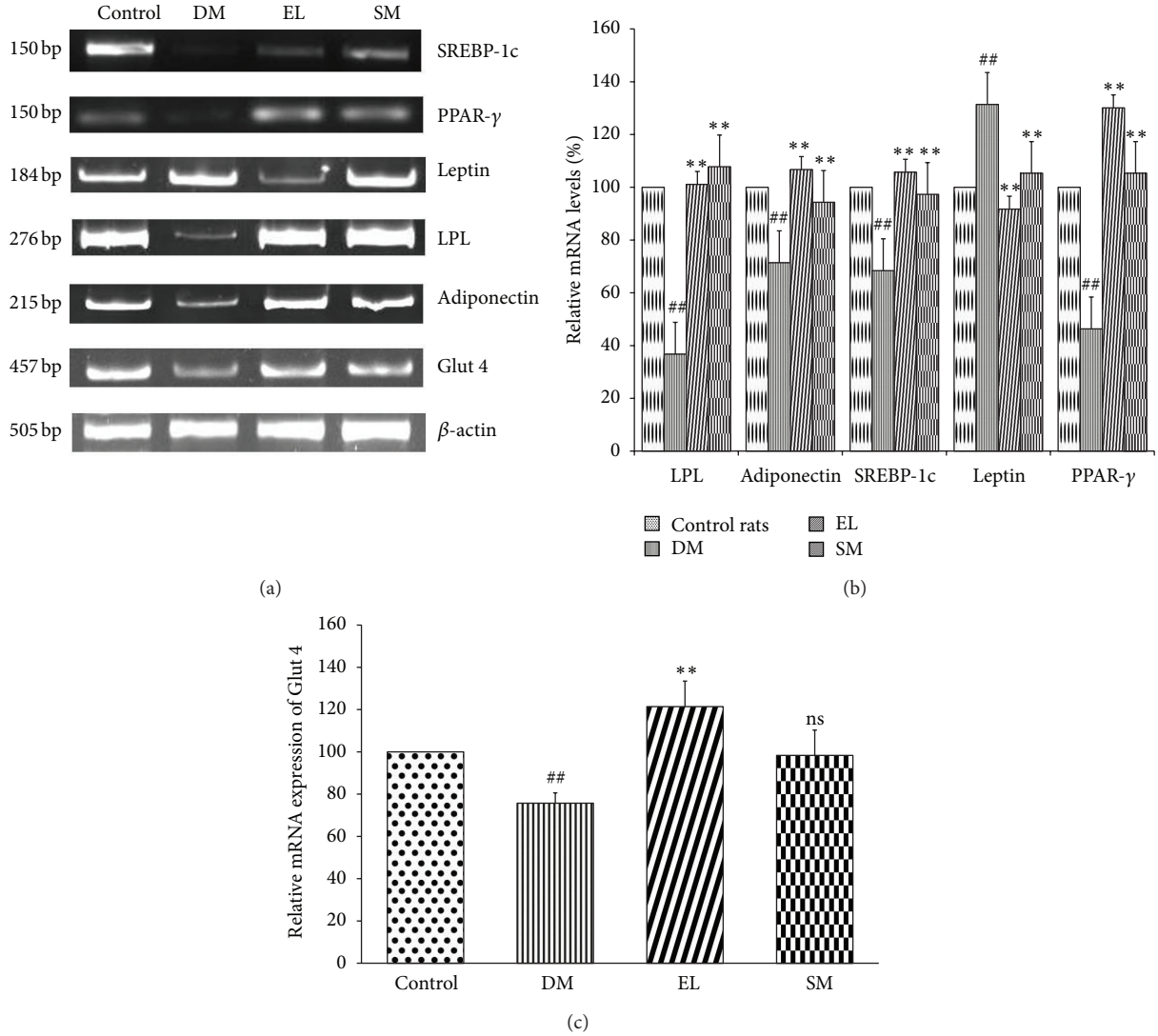

FIGURE 8: (a) Effect of EL extract and SM treatments on the mRNA expression of SREBP-1c, PPAR-γ, leptin, LPL, adiponectin, Glut 4, and β-actin in the adipose tissue as compared to diabetic rats. (Gel image). (b) Effect of EL extract and SM treatments on the expression of the major genes regulating the fat metabolism in the adipocytes as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as Mean ± SEM of 4 independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus diabetic rates. (c) Effect of EL extract and SM treatments on the expression of Glut 4 in the adipocytes as compared to diabetic rats. The expression levels were checked using semi-quantitative RT-PCR and densitometric analysis. Data presented as mean ± SEM of 4 independent observations. $^{\#\#}P < 0.05$ versus control rats; $^{**}P < 0.05$ versus diabetic rates; P value ns versus diabetic rats.

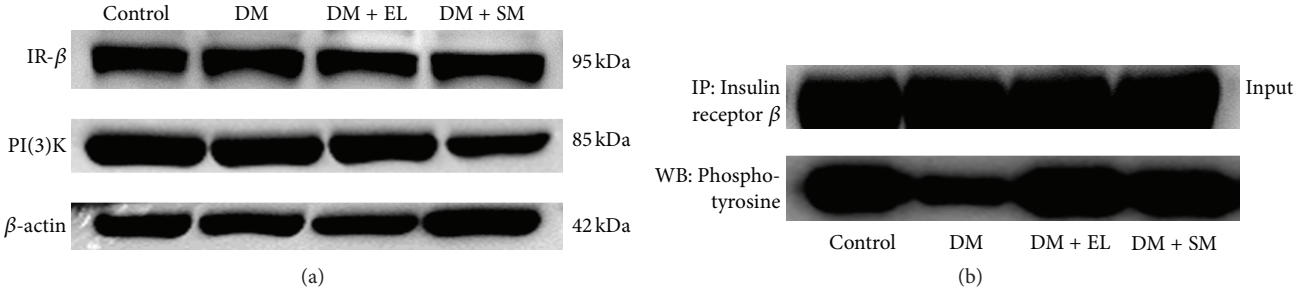

FIGURE 9: (a) Western blot study showing the effect of EL extract and SM treatments on the expression of insulin signaling proteins: IR and PI(3)K in the adipose tissue as compared to diabetic rats. β-actin was taken as an internal control. (b) Immunoprecipitation study showing the effect of EL extract and SM treatments on the phosphorylation of insulin signaling proteins: IR in the adipose tissue as compared to diabetic rats (100 ug protein).

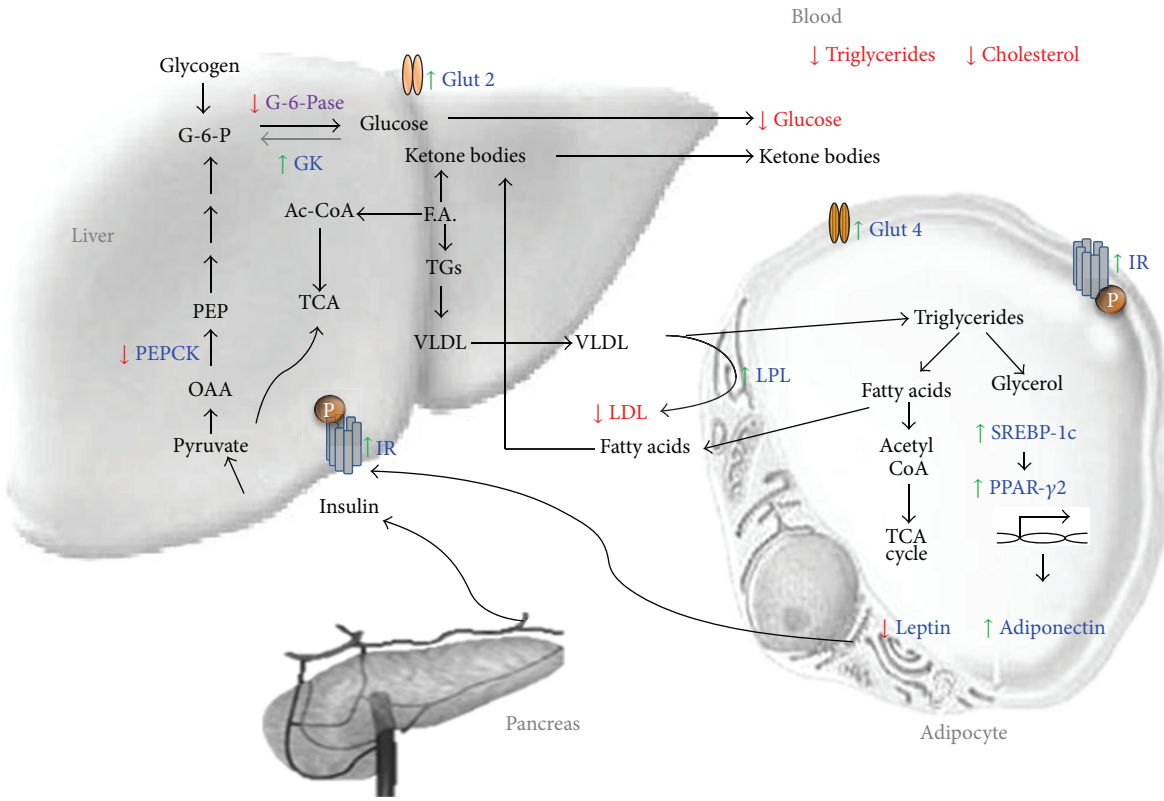

Figure 10: Schematic representation of swertiamarin in amelioration of insulin resistance and TIIDM. Figure shows carbohydrate and fat metabolic pathways and candidate genes which are altered during diabetes. Swertiamarin treatment modulates not only the expression of these target genes which is marked in blue but also the metabolite levels in blood marked in red.

metabolism, which can correct dyslipidemia by increasing insulin sensitivity [32].

Insulin sensitivity depends on the binding of insulin to its receptor, which autophosphorylates and further leads to downstream signaling cascade. Treatment of diabetic animals with aqueous extract and SM showed increased insulin receptor protein synthesis and its autophosphorylation in liver and adipose tissues, which improves insulin sensitivity in TIIDM. Phosphorylation of PI(3)K is mainly responsible for insulin stimulated glucose uptake by Glut 4, which is responsible for peripheral glucose disposition in muscle and adipose tissue. It has been reported that cinnamon extract improves insulin action and glucose uptake by enhancing the insulin signaling pathway in skeletal muscle [33].

The results of the current study proves that SM activates PPAR-γ and its regulatory genes, which improves fat metabolism in adipose tissue. By controlling PPAR-γ, SM can maintain the status of small adipocytes that reduces expression of leptin and TNF-α and increases expression of adiponectin. Increased Adiponectin secretion acts in an autocrine and paracrine manner, which improves expression of insulin receptor, its autophosphorylation, and downstream insulin signaling in liver as well as in adipose tissue [34]. This is the need of the hour, a drug which is able to maintain a balance between all the players involved in the carbohydrate and fat metabolism in the peripheral tissues (Figure 10).

5. Conclusion

The NA + STZ-treated rats show glucose intolerance, increased serum TG, and decreased serum insulin levels, indicating NIDDM-like condition. Treatment with aqueous EL extract and swertiamarin has been found to reduce the glycemic burden as monitored by OGTT profile. In diabetic rats, swertiamarin enhances insulin sensitivity resulting in restoration of altered gene expression of glucose metabolism in liver. In dyslipidemic condition, swertiamarin plays a crucial role in lowering surplus cholesterol by inhibiting HMG-CoA reductase activity. This is the first report *in vivo* that highlights a significant role of SM as a regulator of gene expression under the control of transcriptional factors like PPAR-γ, hence suggesting that SM improves insulin sensitivity and modulates carbohydrate and fat metabolism by regulating PPAR-γ. Present results thus strongly suggest that SM can be a potent therapeutic agent against TIIDM.

Abbreviations

DPP-4:	Dipeptidyl peptidase-IV
EL:	*Enicostemma littorale*
G-6-Pase:	Glucose 6 phosphatase
GK:	Glucokinase
GLP-1:	Glucagon-like peptide-1

Glut 2:	Glucose transporter 2
Glut 4:	Glucose transporter 4
HGP:	Hepatic glucose production
HMG CoA Reductase:	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPTLC:	High performance thin layer chromatography
IR:	Insulin receptor
LPL:	Lipoprotein lipase 1
MFO:	Metformin
NA:	Nicotinamide
NIDDM:	Noninsulin dependent diabetes mellitus
OGTT:	Oral glucose tolerance test
PEPCK:	Phosphoenolpyruvate carboxykinase
PI(3)K:	Phosphatidylinositide 3-kinases
PP_2BS:	Postprandial (2 hours) blood sugar
PPAR-γ:	Peroxisome proliferator-activated receptor gamma
SM:	Swertiamarin
SREBP-1c:	Sterol regulatory element-binding protein-1c
STZ:	Streptozotocin
TG:	Triglycerides
TIIDM:	Type II diabetes mellitus
WHO:	World Health Organization.

Conflict of Interests

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

Authors' Contribution

Sarita Gupta and Tushar P. Patel conceived and designed the experiments; Sanket Soni and Sarita Gupta were responsible for isolation and characterization of compound; Tushar P. Patel, Pankti Parikh, Jeetendra Gosai, and Ragitha Chruvattil performed other experiments; Tushar P. Patel and Sarita Gupta analyzed the data; Tushar P. Patel and Sarita Gupta wrote the paper.

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Research Article

Antiobesity Effect of *Codonopsis lanceolata* in High-Calorie/High-Fat-Diet-Induced Obese Rats

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The antiobesity effects of *Codonopsis lanceolata* (CL) were evaluated in a high-calorie/high-fat-diet (HFD-) induced obesity rat model and 3T3-L1 cells. The Sprague-Dawley male rats were fed a normal diet (ND) or a HFD for a period of 12 weeks. The rats were subdivided into groups: ND, ND + wild *Codonopsis lanceolata* (wCL) (900 mg/kg/day, p.o.), ND + cultivated *Codonopsis lanceolata* (cCL) (900 mg/kg/day, p.o.), HFD, HFD + wCL (100, 300, or 900 mg/kg/day, p.o.), HFD + cCL (100, 300, or 900 mg/kg/day, p.o.), and HFD + sibutramine. The body weight gains of the administered HFD + CL (wCL or CCL) were lower than those of the rats fed with only the HFD group. Moreover, the weight of adipose pads and the serum levels of triglycerides, total cholesterol, and low density lipoprotein cholesterol in the group administered HDL + CL were significantly lower than in the HFD group. The inhibitory effect of lipid accumulation in 3T3-L1 cells was measured by Oil Red O staining and reverse transcription-polymerase chain reaction (RT-PCR). Treatment of 3T3-L1 cells with wCL inhibited lipid accumulation and expression of C/EBPα and PPARγ. These results suggest that CL has a great potential as a functional food with anti-obesity effects and as a therapeutic alternative in the treatment of obesity.

1. Introduction

As obesity has increased in the population, multiple methods of dieting to achieve weight loss have been introduced. Among these diets, controlling calorie intake and increasing calorie consumption expenditure through a proper exercise have been considered as the ideal. However, a large number of people depend on fasting and the consumption of diet food and medicine to control their weight. This tendency has affected food consumption trends and has led to an increase in the consumption of functional foods for antiobesity, leading to a strong demand for the development of new sources of functional foods and related studies on their efficacy.

In recent years, there have been many efforts to find promising functional foods that can be used on a daily basis to improve physical constitution. A good example of this is *Clerodendron glandulosum* Coleb. This herb, found in north eastern states of India [1], is a folklore medicine consumed by urban and tribal populace of Manipur against obesity, diabetes, and hypertension [2, 3]. Recently, it has been reported that aqueous extracts from *Clerodendron glandulosum* Coleb. can regulate high-fat-diet induced hyperlipidemia in rats [3], improve fructose induced insulin resistance and hypertension [4], and prevent high-fat-diet-induced hepatic steatosis [5].

As part of these recent endeavors, this study was performed to investigate the influence of the water extracts of wild and cultivated *Codonopsis lanceolata* on the serum and body fat levels of rats fed a high-fat-diet. *Codonopsis lanceolata* (CL) is a perennial plant in the family of Campanulaceae, often called *Baishen* or *Shashen* in China. CL can be classified as *Bei Sha Shen*, which shows a red surface and has a thick root and many rootlets, or *Nam Sha Shen*, which has a light brown surface with a long and fine root. Wild CL (wCL) collected from an alpine district of Gangwondo and cultivated CL (cCL) from Yeongcheon of Gyeongbuk in Korea were used for this study. CL has long been used as traditional fork medicine to treat asthma, phthisis, tuberculosis, bronchitis, dyspepsia and psychoneurosis in Korea, Japan, and China [6–8]. The root of CL, which is composed of various active

components including tannins, saponins, polyphenolics, alkaloidss, essential oils, and steroids [6–9], has pharmacological properties including antiobesty, antioxidant, antimicrobial, anti-inflammatory, and immunomodulatory activites [10–13]. Recent studies have documented the biological activities of CL in which water extracts effectively suppressed the high-fat-diet-induced accumulation of neutral fat and total cholesterol in serum and the liver in the experimental model [13]. The ethanol extracts of CL demonstrated very strong antioxidant effects comparable to those of ginseng extract [14]. In addition, Lee et al. (1995) and Lee (2002) reported that the aqueous extracts of CL acted on the immune system in direct and indirect manners and increased the cellular immune reaction [15, 16]. Another report showed that 70% MeOH extracts of CL increased the number of helper T cells [17]. Our study was performed to evaluate the antiobesity activity of CL from different sources: the wild type grown naturally on the mountain and the cultivated type. This study attempted to analyze and compare the potential of wCL and cCL as promising resources for the development of a functional food to control obesity.

2. Materials and Methods

2.1. Plant Material and Reagents. wCL (voucher specimen number SNBA200505262013, the National Biospecies Knowledge Information System, Korea) produced from the Youngmun Mt. Corporation of the Agricultural Association and cCL (voucher specimen number KBNA200408101015, the National Biospecies Knowledge Information System, Korea) obtained from Yeongcheon of Gyeongbuk were used for this study. These two products were ground separately, and 1 liter of distilled water was added to 100 g of each of the ground wCL and cCL samples, followed by three extraction at 100°C over 4 hours using a shaking extraction method. The extracts were filtered, vacuum-dried, and then freeze-dried. The final yields of the extract from the dried samples of wCL and cCL were 7.2%, respectively.

Test kits for measuring the total cholesterol, triglyceride, and HDL-cholesterol in the serum were purchased from Asan Pharmaceutical, Korea. Glucose and all other reagents were purchased from Sigma, USA.

2.2. Preparation of Samples for LC-MS Analysis. The powder of water extracts from roots of the wCL (200 mg) was dissolved in 100 mL of distilled water, which was then partitioned three times with the same volume of ethyl acetate [18]. The water fraction was evaporated to dryness under a vacuum. The dried samples were dissolved in distilled water (1 mg/mL) and then subjected to a reverse phase HPLC column.

2.3. HPLC-ESI-MS Analysis. The HPLC column used was an Atlantis T3 RP18 (4.6 × 150 mm, 3 μm; waters) equipped with an Atlantis T3 RP18 guard column (4.6 × 20 mm, 3.5 μm, waters). The mobile phases were constituted with solvent A (distilled water) and solvent B (acetonitrile). The linear gradient program was as follows: 0–60 min 5%–50% B; 60–90 min 50%–70% B; 90–120 min 70%–100% B, running at a constant flow rate of 0.2 mL/min (P680 Pump, DIONEX). The injection volume of the sample was 10 μL for every injection. A UVD340U diode array detector performing the wavelength scanning from 210 to 365 nm and an ASI-100 automatic injector were used. The operating conditions of the ESI ion source (Ion Sense, Tokyo, Japan) coupled with a JMS-T100TD (AccuTOF-TLC) in the positive ion modes were a desolating chamber at 250°C and an orifice at 180°C. The first orifice lens was set to 85 V, and the ring lens voltage was 10 V. The TOF-MS was set with a peak voltage of 1500 V with a detector voltage of 2200 V. The nebulizing and desolvating gases were set to 1.0 L/min and 1.5 L/min, respectively.

Table 1: Composition of the normal diet and the high-calorie/high-fat diet (g/100 g).

Groups ingredient	Normal diet	High-calorie/high-fat diet*
Casein	20.0	29.0
Corn starch	60.0	10.0
Sucrose	—	10.0
Corn oil	9.0	5.0
Cellulose	5.0	5.0
Lard	—	35.0
AIN-76 mineral mix	3.5	3.5
AIN-76 vitamin mix	1.0	1.0
DL-Methionine	0.3	0.3
Choline bitartrate	0.2	0.2
kcal/100 g diet	390.2	458.0
Calorie from fat (%)	11.5	35.0

*High-calorie/high-fat diet was modified from the AIN-76 dietary composition [21].

2.4. Animals and Treatment. Six-week-old male Sprague-Dawley rats, which is a good animal model for studying the obesity in humans [19, 20], were obtained from Samtako (Osan, Korea) and raised under constant conditions (temperature: 20 ± 2°C; humidity: 40%–60%; lights: 12 hours of light/dark cycle) for 7 days. Body weights ranged between 200 and 210 g. The rats were provided commercially available normal diet (Jongang Lab Animal, Seoul, Korea), prior to the dietary manipulation. All of the samples were administered at a specific time.

During the study period, two diets were used: the normal diet or the high-calorie/high-fat-diet which modified the AIN-76 dietary composition [21], as shown in Table 1. A high-calorie/high-fat-diet, which is commonly used in nutritional experiments as a strategy to induce fat deposition and overweight conditions in SD rat [22, 23], was applied for six weeks to induce obesity. The body weight of these rats increased more than 125% compared to that of the rats with normal diet. The rats of the normal test group were fed with a normal diet (ND) for six weeks, while the rats of the control group were fed with a high-calorie/high-fat-diet (HFD) for six weeks to elicit diet-induced obesity.

Then the animals were subdivided into 11 groups (n = 10)—ND, ND + wCL (900 mg/kg), ND + cCL (900 mg/kg), HFD, HFD + wCL (100, 300, or 900 mg/kg), HFD + cCL

Table 2: Classification of experimental groups.

Group	Treatment
ND	Normal diet
ND + wCL 900 mg/kg	ND + wild *Codonopsis lanceolata* 900 mg/kg/day
ND + cCL 900 mg/kg	ND + cultivated *Codonopsis lanceolata* 900 mg/kg/day
HFD	High-calorie/high-fat diet
HFD + sibutramine	HFD + sibutramine 7.5, 11 mg/kg/day
HFD + wCL 100 mg/kg	HFD + wild *Codonopsis lanceolata* 100 mg/kg/day
HFD + wCL 300 mg/kg	HFD + wild *Codonopsis lanceolata* 300 mg/kg/day
HFD + wCL 900 mg/kg	HFD + wild *Codonopsis lanceolata* 900 mg/kg/day
HFD + cCL 100 mg/kg	HFD + cultivated *Codonopsis lanceolata* 100 mg/kg/day
HFD + cCL 300 mg/kg	HFD + cultivated *Codonopsis lanceolata* 300 mg/kg/day
HFD + cCL 900 mg/kg	HFD + cultivated *Codonopsis lanceolata* 900 mg/kg/day

(100, 300, or 900 mg/kg) group and HFD + sibutramine—and orally administered with CL or sibutramine once per day for another 6 weeks (Table 2) [23]. All experimental procedures were carried out in accordance with the protocol that was approved by the Institutional Animal Care and Use Committee guideline of Kyung Hee University.

2.5. Specimen Collection. Blood was collected from the abdominal aorta of the rat anesthetized using CO_2 gas after fasting for 12 hours on the last day of the 6-week feeding period. The collected blood was processed using a microcentrifuge method, and the serum was stored in a freezer at −70°C. The retroperitoneal, epididymal, and brown fat pads were removed, rinsed with phosphate-buffered saline, and then weighed.

2.6. Blood Sampling and Plasma Assay. The total cholesterol (TC), triglyceride (TG), and high density lipoprotein (HDL)-cholesterol of the serum were measured using a reagent kit (Asan Pharm., Hwaseong, Korea) based on the enzymatic colorimetric method. The level of low density lipoprotein-(LDL-) cholesterol was determined using the Friedewald formula, where LDL-cholesterol = TC − HDL-cholesterol − (TG/5).

2.7. Cell Culture and Differentiation. 3T3-L1 cells were cultured to confluence in 6 plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Bovine Calf Serum (BCS). On the second day of postconfluence (designated as day 0), the cells were induced to differentiate DMEM supplemented with 10% (v/v) FBS (Fetal Bovine Serum), 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 5 μg/mL insulin. After 48 hours, the media were replaced with DMEM supplemented with 10% FBS and 1 μg/mL insulin. The cells were subsequently refed every 48 hours with DMEM supplemented with 10% FBS.

2.8. Oil Red O Staining. 3T3-L1 cells were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 10% formalin and then stained with 0.5% Oil Red O.

2.9. Reverse Transcriptase-PCR Analysis of C/EBPα and PPARγ. Total RNA was isolated from cells using the Trizol reagent. One microgram of total RNA was used for single strand cDNA synthesis. Reverse transcription was performed at 30°C for 10 min, 42°C for 30 min, and 99°C for 5 min. The following primers were used: CCAAT/enhancer binding protein-alpha (C/EBPα), forward 5-CGCAAGAGCCGAGATAAAGC-3, and reverse 5-CACGGCTCAGCTGTTCCA-3; peroxisome proliferator-activated receptor-gamma (PPARγ), forward 5-CGCTGATGCACTGCCTATGA-3, and reverse 5-AGAGGTCCACAGAGCTGATTCC-3. The C/EBPα and PPARγ amplifications were performed by denaturing at 94°C for 1 min, annealing at 54°C for 1 min, extending at 72°C for 30 sec for 30 cycles, administering a final extension of ethidium bromide, and then visualizing under a UV light.

2.10. Statistical Analysis. The results of the tests are presented as the mean ± S.D. values for each test group using the Statistical Package for the Social Sciences (SPSS). The statistical significance between test groups was analyzed using a one-way ANOVA method. Differences were analyzed using Duncan's Multiple Range Test at the $P < 0.05$ level; their significances were also analyzed.

3. Results

3.1. Analysis of the Wild Codonopsis lanceolata Extract. Figure 1 shows the HPLC chromatogram and the total ion current chromatogram of the total extract of wCL from the HPLC-UV/ESI-MS analysis. The protonated molecular ion of wCL (m/z 1191 ([M + H]+)) was detected at 11 min as a codonoposide, a compound known to be one of the active components of CL [24]. In addition, spinasterol, cycloartenol, and taraxerone were identified at 77 min, 98 min, and 106 min, respectively [25]. These compounds have already been reported as chemical constituents of this plant. The extracts used in this study were standardized to a codonoposide (yield: 0.3%).

3.2. Body Weight and Weight Gain. Table 3 presents the influences of wCL and cCL on weight, the ingested diet amount, and the food efficiency of the rats fed with the diet. There were no particular differences between the test groups fed with the normal diet (ND) and with the normal diet supplemented with CL (ND + wCL or cCL 900 mg/kg).

Table 3: Effects of water extracts of wild and cultivated *Codonopsis lanceolata* on body weight, food efficiency ratio, and food intake in SD rats fed with normal or high-fat diets.

Group	Body weight (g)		Food efficiency ratio[1]	Food intake (g/day)
	Initial	Final		
ND	213.7 ± 10.11	481.60 ± 35.68$^{\#\#}$	0.21 ± 0.03$^{\#}$	57.24 ± 8.17$^{\#}$
ND + wCL 900 mg/kg	213.6 ± 8.97	473.40 ± 26.38$^{\#\#}$	0.17 ± 0.05$^{*\#\#}$	55.14 ± 10.06$^{\#}$
ND + cCL 900 mg/kg	213.6 ± 8.11	484.20 ± 26.15$^{\#\#}$	0.22 ± 0.04	57.47 ± 12.33$^{\#}$
HFD	213.6 ± 7.89	546.80 ± 36.84**	0.24 ± 0.05*	71.46 ± 6.80*
HFD + Sibutramine	213.6 ± 7.87	474.00 ± 30.20$^{\#\#}$	0.14 ± 0.03$^{**\#\#}$	42.72 ± 6.56$^{*\#\#}$
HFD + wCL 100 mg/kg	213.6 ± 7.66	516.22 ± 32.73*	0.21 ± 0.05$^{\#}$	52.60 ± 5.42$^{\#}$
HFD + wCL 300 mg/kg	213.6 ± 7.50	524.22 ± 58.10*	0.22 ± 0.07	49.92 ± 11.57$^{\#\#}$
HFD + wCL 900 mg/kg	213.6 ± 7.70	499.75 ± 30.32$^{\#}$	0.17 ± 0.08$^{*\#\#}$	46.35 ± 10.68$^{*\#\#}$
HFD + cCL 100 mg/kg	213.6 ± 7.55	504.20 ± 31.20$^{\#}$	0.26 ± 0.06*	48.85 ± 4.38$^{\#\#}$
HFD + cCL 300 mg/kg	213.6 ± 7.55	509.33 ± 36.86$^{\#}$	0.22 ± 0.04	47.37 ± 6.50$^{\#\#}$
HFD + cCL 900 mg/kg	213.6 ± 7.05	520.75 ± 22.52*	0.17 ± 0.08$^{*\#\#}$	50.28 ± 9.09$^{\#\#}$

[1]Food efficiency ratio (FER) = increased body weight (g)/Food intake (g).
Values are the means ± S.D. ($n = 10$), $^{*}P < 0.05$, $^{**}P < 0.01$ versus ND group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HFD group.

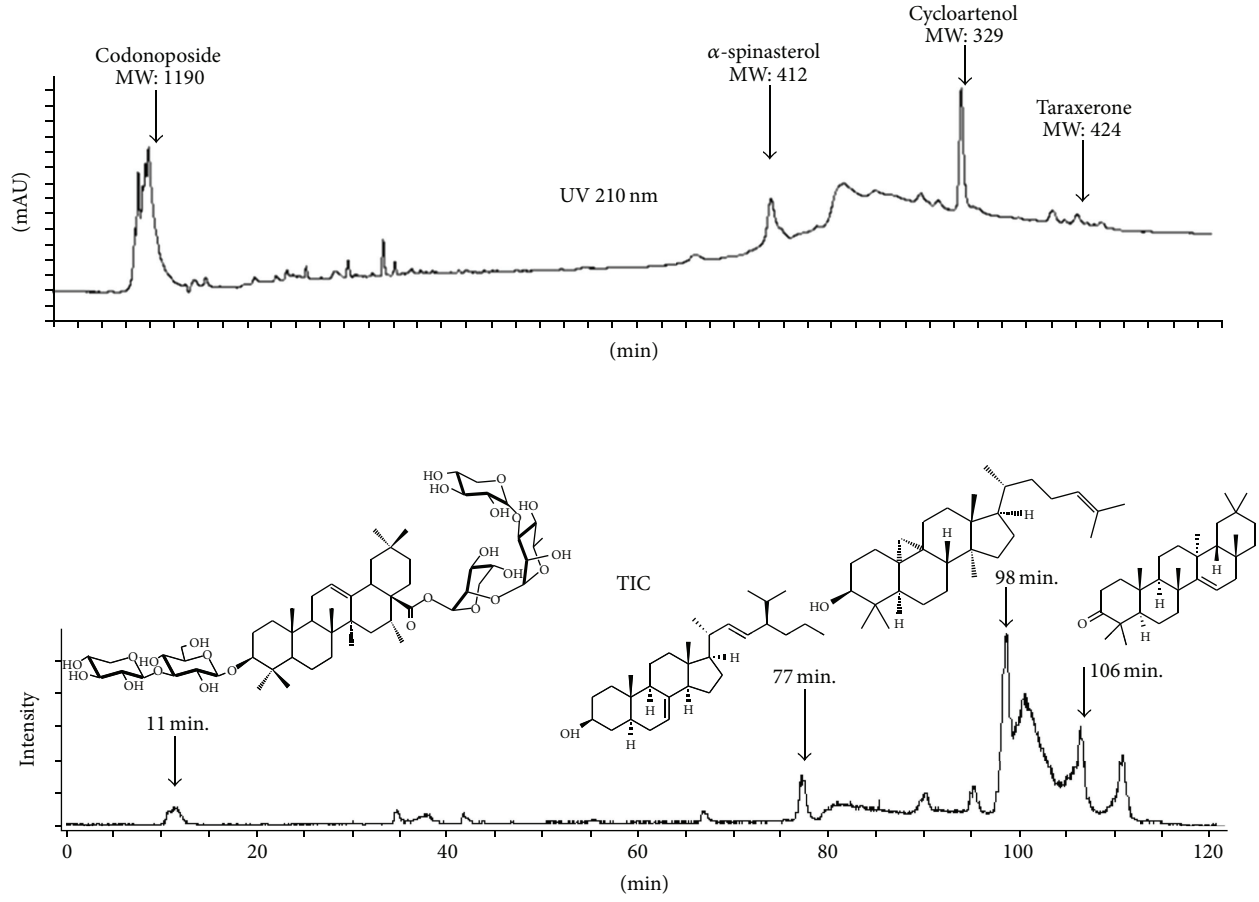

Figure 1: HPLC chromatogram and total ion current chromatogram of the crude extract of wCL by LC-UV-ESI-MS.

However, the rats in the test group fed with the high-calorie/high-fat-diet (HFD) showed a significant increase in their weight compared to the test group fed with the normal diet (ND) ($P < 0.01$). In addition, the amount of daily ingested calories in the control group fed with the high-calorie/high-fat-diet (HFD) represented a significantly higher level compared to that of the normal diet (ND).

Interestingly, the amount of ingested diet in the groups fed with the high-fat-diet supplemented with wCL (HFD + wCL 900 mg/kg) showed a slight decrease over the high-calorie/high-fat-diet (HFD) ($P < 0.01$). There was also a significant decrease ($P < 0.01$) in weight at the high concentration (900 mg/kg) of wCL extracts, while the low (100 mg/kg) and medium (300 mg/kg) concentrations of wCL

Table 4: Effects of water extracts of wild and cultivated *Codonopsis lanceolata* on retroperitoneal, epididymal, brown fat, and total abdominal fat in SD rats fed with normal or high-fat diets.

Group	Retroperitoneal (g)	Epididymal (g)	Brown fat (g)	Total abdominal (g)
ND	$6.21 \pm 3.30^{\#\#}$	$9.40 \pm 4.00^{\#\#}$	0.43 ± 0.08	$13.80 \pm 4.05^{\#\#}$
ND + wCL 900 mg/kg	$5.89 \pm 1.69^{\#\#}$	$9.76 \pm 2.63^{\#\#}$	0.54 ± 0.16	$14.50 \pm 2.92^{\#\#}$
ND + cCL 900 mg/kg	$6.24 \pm 1.20^{\#\#}$	$9.95 \pm 3.36^{\#\#}$	0.52 ± 0.11	$14.58 \pm 3.09^{\#\#}$
HFD	$11.01 \pm 1.79^{**}$	$15.19 \pm 2.57^{**}$	0.44 ± 0.10	$25.78 \pm 3.67^{**}$
HFD + Sibutramine	$6.81 \pm 1.54^{\#\#}$	$11.16 \pm 3.89^{\#}$	0.33 ± 0.07	$18.60 \pm 2.86^{*\#}$
HFD + wCL 100 mg/kg	$8.88 \pm 1.20^{*\#}$	$15.77 \pm 4.34^{**}$	0.40 ± 0.07	$23.26 \pm 3.12^{**}$
HFD + wCL 300 mg/kg	$8.69 \pm 0.83^{*\#}$	$13.42 \pm 1.36^{*}$	0.53 ± 0.12	$21.94 \pm 4.84^{**}$
HFD + wCL 900 mg/kg	$8.25 \pm 1.69^{*\#}$	$12.67 \pm 3.79^{*\#}$	$0.64 \pm 0.10^{**\#\#}$	$19.91 \pm 4.46^{*\#}$
HFD + cCL 100 mg/kg	$9.61 \pm 2.28^{*}$	$13.12 \pm 2.01^{*}$	0.54 ± 0.07	$20.73 \pm 5.21^{*\#}$
HFD + cCL 300 mg/kg	$10.60 \pm 2.23^{*}$	$14.58 \pm 3.10^{**}$	$0.67 \pm 0.11^{**\#\#}$	$24.23 \pm 5.71^{**}$
HFD + cCL 900 mg/kg	$9.99 \pm 3.10^{*}$	$14.10 \pm 3.81^{**}$	$0.56 \pm 0.20^{**}$	$22.82 \pm 3.23^{**}$

Values are means ± S.D. ($n = 10$), $^{*}P < 0.05$, $^{**}P < 0.01$ versus ND group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HFD group.

extracts and the high concentration of cCL extracts showed no significant changes in weight compared to the high-calorie/high-fat-diet (HFD) group. For the HFD + cCL groups, there were some changes in the final weight at the low and medium concentration groups, but there were increases in the food efficiency and no decreases in the ingested amount. Therefore, it can be assumed that the results are related to dehydration due to the increase in fat and the decrease in muscle rather than the decrease in body weight.

3.3. Influences on the Food Efficiency. To investigate the effect of test samples (sibutramine as positive control and CL) on obesity, changes in body weights and ingested food amounts were observed, and the food efficiencies were calculated from the equation outlined below (Table 3). The body weight was measured every day, and the ingested food was calculated by measuring the amount of remaining food, where food efficiency ratio (FER) = increased body weight (g)/food intake (g).

According to this food efficiency ratio equation, a change in body weight is the most important factor affecting the food efficiency ratio, as there is no large change in the amount of ingested food. Thus, it is possible to apply the food efficiency ratio as a scale of obesity and to consider that a small value for the food efficiency ratio is also an effective parameter to predict the avoidance of obesity. When verification of diet-induced obesity using this food efficiency ratio was applied in this study, it was possible to observe that upon administration of CL (i.e., wCL or cCL) for six weeks, obesity was reduced in a concentration-dependent manner to the levels recorded in the normal diet (ND) group. The high-capacity wCL 900 mg/kg group showed the results from the ND group and the HFD group significantly lower FER. This, as showing the proper diet inhibition from all of the normal diet or the high calories diet, is regarded to be a necessary result in diet control for keeping the standard weight. In addition, the groups treated with the highest concentrations (900 mg/kg) of wCL and cCL demonstrated a decrease in the level of obesity. It can be suggested that the high concentration of CL is effective in reducing obesity.

3.4. Plasma Lipid Levels and Fat Pad. The obese rats with no CL treatment showed an increase in the weight of the total fat in the abdominal cavity and in the weight of the retro peritoneum and around the epididymis in the abdominal cavity. However, the obese rats treated with the CL (i.e., wCL or cCL) showed a significant latency in the increase in weight of these fat tissues. In particular, the group treated with wCL (HFD + wCL) had a more efficient control of body weight increase in a concentration-dependent manner compared to that of the group treated with cCL (HFD + cCL). The group treated with high concentrations (900 mg/kg) of wCL showed the most significant effects on the weight of fat tissues, with the retroperitoneal fat tissues decreasing approximately 75% and the weight of epididymal fat tissues decreasing approximately 83%, compared to the high-calorie/high-fat-diet (HFD) control group. The group treated with high concentrations (900 mg/kg) of wCL also demonstrated a decrease of approximately 77% in abdominal fat levels and an increase of approximately 145% in brown adipose tissue levels, which facilitates thermal production (Table 4). Among the test groups treated with CL (HFD + CL), the group treated with the medium concentration (600 mg/kg) showed the smallest effects. To investigate the effects of wCL on the rats fed with the high-calorie/high-fat-diet (HFD) in more detail, the effects on serum triglycerides (TG), total cholesterol (TC) and high density lipoprotein-(HDL-) cholesterol levels in the rats were measured (Figure 2). Additionally, the serum fat concentrations of the rats with diet-induced obesity were measured. From the results of the measurements, the HFD control group (i.e., not treated with CL) showed high values in serum TG and TC compared to those of the normal diet (ND) group. It was evident that the HFD resulted in obesity and high cholesterol levels in rats subjected to this diet. The administration of CL significantly suppressed the increase of these levels. In the case of the TG levels, these effects were observed to be concentration dependent.

3.5. T3-L1 Cells Differentiation. Adipocytes adjust their own growth and development, not only for lipid metabolism but also for the performance of functions to maintain a consistent supply of energy in the body [26].

In this study, we observed the effects of wCL on adipocyte differentiation by a cell-based in vitro experiment with Oil

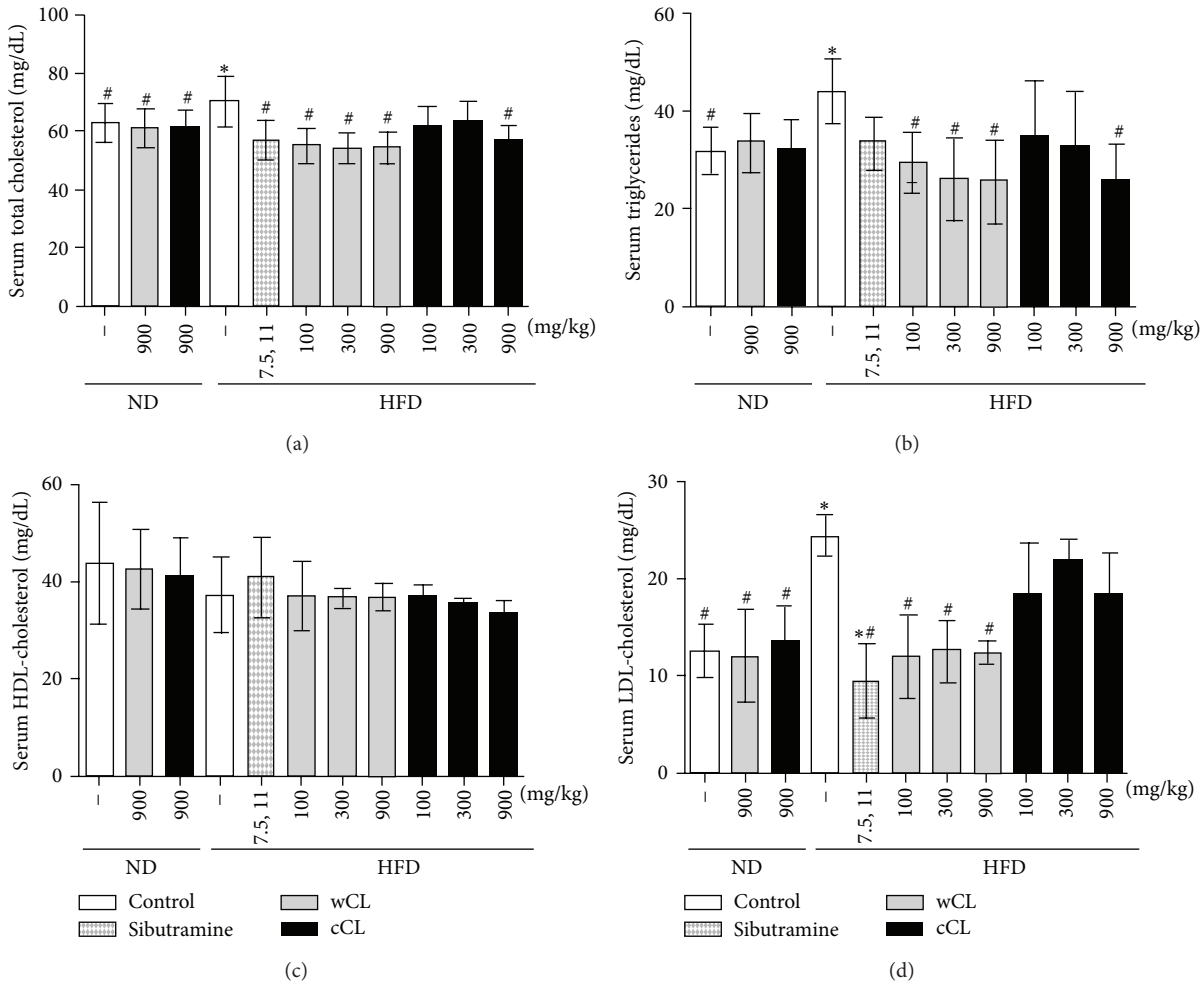

Figure 2: Effects of the wild and cultivated *Codonopsis lanceolata* extracts on (a) serum total cholesterol, (b) serum triglycerides, (c) serum high density lipoprotein (HDL)-cholesterol, (d) serum low density lipoprotein-(LDL-) cholesterol levels (mg/dL) in rats. Values are means ± S.D. ($n = 10$), $^{*}P < 0.05$, $^{**}P < 0.01$ versus ND group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus HFD group.

Red O dye. The results shown in Figure 3 confirmed that 3T3-L1 fat generation is suppressed with treatment of wCL in a concentration-dependent manner. A similar concentration-dependent effect of wCL on the triglyceride levels in the blood samples confirmed the strong relationship between adipocyte differentiation and triglyceride levels in the blood (Figure 2). Because the level of triglycerides in serum increases with increases in the obesity index and plays an important role in lipid metabolism, it may be considered to be a major determinating parameter for obesity. To investigate the possible molecular mechanism of wCL on the suppression of obesity, the levels of CCAAT/enhancer binding protein α (CEBPα) mRNA and peroxisome proliferator-activated receptor γ (PPARγ) mRNA were monitored in 3T3-L1 cells treated with increasing concentrations of wCL. It was observed that the expression of CEBPα mRNA, one of the most important transcription factors in cell differentiation, decreased with increasing concentrations of wCL and the expression of PPARγ mRNA, which plays an important role in promoting storage of fatty acids in adipose tissue, also decreased with wCL treatments in a concentration-dependent manner.

4. Discussion

It is widely accepted that the levels of saturated fat and cholesterol in the diet increase the level of serum cholesterol, while a diet with a low fat content decreases the level of serum cholesterol. There have been tremendous efforts to find efficient food sources from traditional herbal medicines that can be used to optimize the level of serum cholesterol, thereby controlling obesity. Ginseng is one of the most promising candidates for a functional food of this type. It has been reported that the saponin in ginseng increased the absorption of LDL in the liver and facilitated the removal rate of abnormally increased VLDL by the intake of high levels of cholesterol from the diet [27]. It has also been reported that the decrease in the level of blood cholesterol by the administration of saponin derived from ginseng was mainly dependent on the decrease in the concentrations of VLDL and LDL in serum. In this present study, it was also assumed that the significant decreases in the total blood cholesterol of all groups fed with HFD and treated with wCL were due to the action of the saponins in the wCL, as saponins have

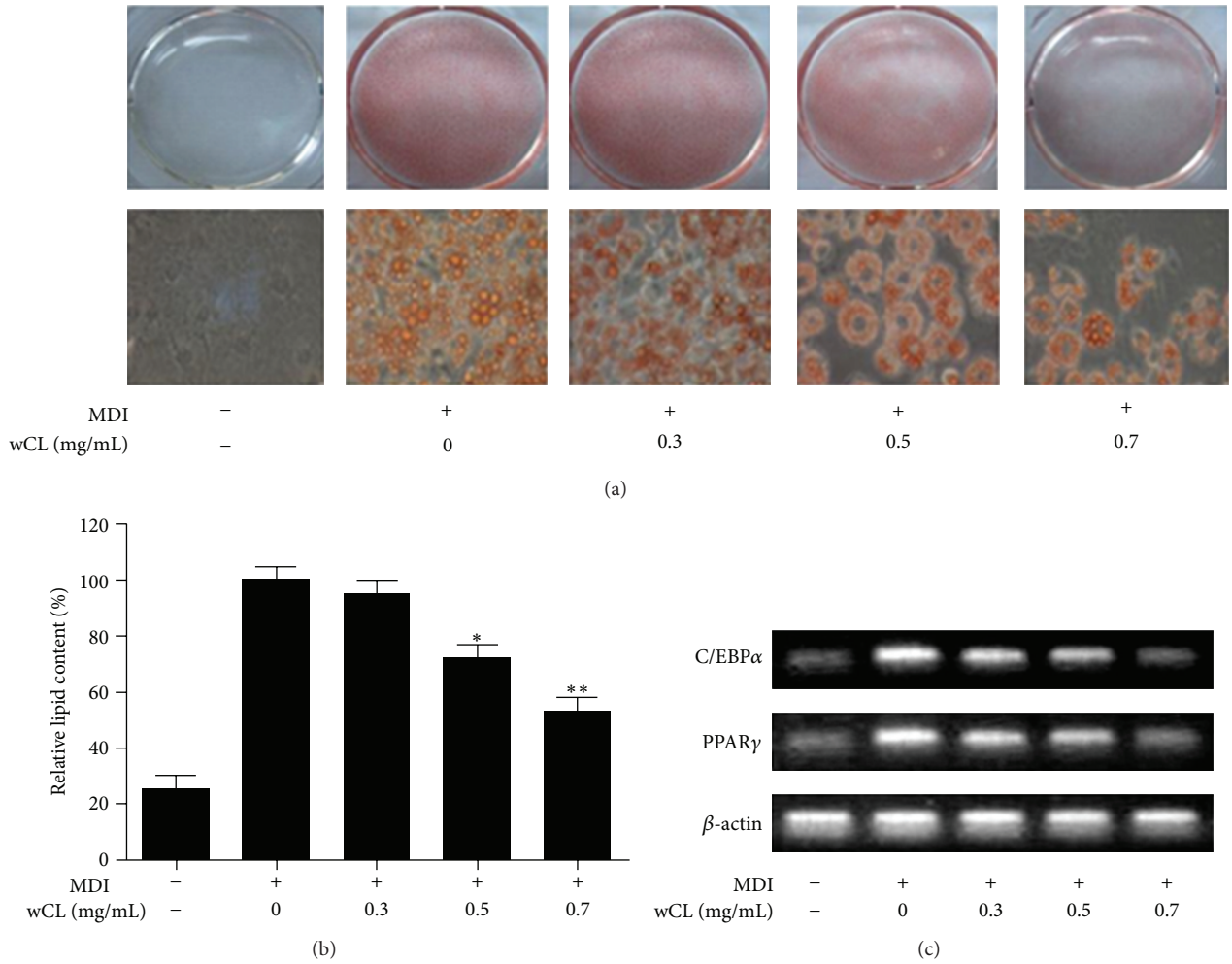

Figure 3: The effect of wCL on 3T3-L1 adipocyte differentiation. (a) 3T3-L1 cells were differentiated with MDI in the absence or presence of wCL (0, 0.3, 0.5, 0.7 mg/mL) for eight days, followed by measurement of lipid contents by Oil Red O staining. (b) Stained oil droplets were dissolved with isopropanol and quantified by spectrophotometric analysis at 510 nm. The results were represented as relative lipid contents. $^{*}P < 0.05$, $^{**}P < 0.01$ by SPSS compared to MDI-treated cells. (c) Expression of C/EBPα and PPARγ mRNA from 3T3-L1 cells as described in (a) was measured by RT-PCR.

been reported as active components of CL [28]. Although the uniform effects on the levels of serum cholesterol and lipids were obtained in the group treated with the highest concentration level of wCL (HFD + wCL 900 mg/kg), there were small improvements in the fat and blood lipid levels in the group treated with cCL (HFD + cCL). The reason that wCL water extracts showed more effective results than cCL extracts is likely due to differences in the content and types of active ingredients between wCL and cCL products.

Mitochondrial β-oxidation of long-chain fatty acids is the major source of energy. Prior to undergoing β-oxidation in mitochondrial matrix, the long-chain fatty acyl-CoA must be transferred from cytosol into matrix. The lipid metabolism related many enzymes in this process. The present study has investigated the effects of wCL on lipid and cholesterol metabolisms in normolipidemic and hyperlipidemic rat. We were able to get the results, wCL inhibiting activity of fat oxidation.

The cholesterol lowering of property wCL could be due to increased excretion of cholesterol and bile acids. These observed effects can be attributed to the presence of phytosterol in wCL as phytosterols possesse greater affinity for micelles than cholesterol and reduce incorporation of cholesterol in micelles [29]. We can see from the configuration that the phytochemical components of this wCL are composed of the enzyme controlling largely physiological vitality of adipose metabolism and joining matters.

The effect of CL extract on obesity control does not seem to be related to a direct inhibition of the neurotransmitter cascade, the main mechanism of sibutramine and a serotonin-norepinephrine reuptake inhibitor. The treatment with wCL resulted in the control of serum lipid levels, a decrease in the content of the total body fat, and a significant increase in the level of brown adipose tissue. However, sibutramine-treated models (HFD + sibutramine) have shown that the obesity control effect does not accompany an increase in the

brown adipose tissue that is known to have high capability in fat burning and fatty acid oxidation. Direct action of CL on the lipid levels in serum, rather than a central control of appetite, could exclude various side effects in the central nervous system (CNS) found in antiobesity agents such as sibutramine.

Growth of adipose tissues is broadly divided into two stages: the increase in fat cell size and the differentiation of the adipocyte from the preadipocyte [30]. During differentiation from precursor adipocytes into fully mature adipocytes, either ecologically or biochemically, differentiating factors are activated, which are important for regulating the adipocyte genes [30, 31].

Various transcription factors like PPARγ and C/EBP family are involved during adipocyte differentiation. Among them, the expression of PPARγ and C/EBPα in 3T3-L1, which is a wellknown and frequently used preadipocyte cell line for in vitro adipocyte differentiation [32], is upregulated [33]. PPARγ and C/EBPα, the key adipogenic and lipogenic transcriptional regulators, are crucial to the regulation of obesity and adipocyte differentiation [34]. Recently, studies have shown that PPARγ is necessary and sufficient to promote adipogenesis and that C/EBPα is influential in maintaining the expression of PPARγ [35–37]. The results of our study demonstrate that the treatment with wCL extract suppresses the accumulation of triglycerides within the cells and the differentiation of 3T3-L1 preadipocytes into adipocytes. Total CL group significantly reduced fat accumulation by inhibiting adipogenic signal transcriptional factors, such as PPARγ and C/EBPα mRNA, which functions via AMPK (5′ AMP-activated protein kinase or 5′adenosine monophosphate-activated protein kinase) signaling, in vitro. In other words, the concentration-dependent suppression of C/EBPα and PPARγ mRNA expression in CL-treated cells represented one of the possible mechanisms of the suppression of 3T3-L1 cells differentiation.

AMPK is an enzyme that plays a role in cellular energy homeostasis. The net effect of AMPK activation is stimulation of hepatic fatty acid oxidation and ketogenesis, and inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis [38]. AMPK and PPARγ appear to be involved in adipocyte differentiation and maturation and thus can be potential drug targets for the treatment of obesity. wCL significantly inhibited the expression levels of C/EBPα and PPARγ, two master regulators of adipogenesis (Figure 3), indicating that wCL might inhibit 3T3-L1 differentiation via suppressing the expression of adipogenesis-related transcription factors and markers. Meanwhile, PPARγ transcriptional activity was reduced supporting the wCL downregulated PPARγ expression as well as its transcriptional activity. AMPK phosphorylates the transcriptional coactivator p300 and induces its interaction with PPARγ [33]. It was supposed that wCL might suppress PPARγ transcriptional activity via activating AMPK and phosphorylating the transcriptional coactivators and hence leading to the inhibition of their abilities to interact with PPARγ.

In conclusion, we can observe that wCL improved the restraint of excessive adipose formation and accumulation by these matters. The potential of CL as a promising source for the development of a functional food, which can be efficiently used to control obesity, should be appreciated and evaluated with more detailed molecular studies.

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Research Article

Populus balsamifera Extract and Its Active Component Salicortin Reduce Obesity and Attenuate Insulin Resistance in a Diet-Induced Obese Mouse Model

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Populus balsamifera L. (BP) is a medicinal plant stemming from the traditional pharmacopoeia of the Cree of Eeyou Istchee (CEI—Northern Quebec). *In vitro* screening studies revealed that it strongly inhibited adipogenesis in 3T3-L1 adipocytes, suggesting potential antiobesity activity. Salicortin was identified, through bioassay-guided fractionation, as the active component responsible for BP's activity. The present study aimed to assess the potential of BP and salicortin at reducing obesity and features of the metabolic syndrome, in diet-induced obese C57Bl/6 mice. Mice were subjected to high fat diet (HFD) for sixteen weeks, with BP (125 or 250 mg/kg) or salicortin (12.5 mg/kg) introduced in the HFD for the last eight of the sixteen weeks. BP and salicortin effectively reduced whole body and retroperitoneal fat pad weights, as well as hepatic triglyceride accumulation. Glycemia, insulinemia, leptin, and adiponectin levels were also improved. This was accompanied by a small yet significant reduction in food intake in animals treated with BP. BP and salicortin (slightly) also modulated key components in signaling pathways involved with glucose regulation and lipid oxidation in the liver, muscle, and adipose tissue. These results confirm the validity of the CEI pharmacopoeia as alternative and complementary antiobesity and antidiabetic therapies.

1. Introduction

Obesity results from a variety of risk factors, including unhealthy dietary habits and a sedentary lifestyle, resulting in higher energy input than output [1]. It also increases the risk for other chronic illnesses such as type 2 diabetes (T2D) and insulin resistance (IR) [1]. Insulin resistance is characterized by a decreased ability of insulin sensitive tissues to respond to insulin action. Skeletal muscle is the principal tissue involved in glucose metabolism through insulin-dependent or exercise-sensitive glucose transport (Glut4) [2] implicating the Akt [3] and AMPK [4] pathways, respectively. These pathways are also implicated in glucose metabolism in the liver [5–7] and adipose tissue [3, 8–12]. The liver is considered to be the principal tissue involved in glucose storage and production [13]. Adipose tissue synthesizes and stores fatty acids and is recognized as an endocrine organ; releasing adipokines (leptin, adiponectin) that are implicated in glucose and lipid metabolism [14–20]. Obesity not only leads to

excessive fat storage in adipose tissue, but also to ectopic fat storage in other insulin sensitive tissues such as the muscle and liver (nonalcoholic fatty liver disease; NAFLD). This, in part, contributes to the development of insulin resistance [21, 22].

Several metabolic and signaling pathways are involved in perpetrating the disturbances of obesity and insulin resistance in the three main insulin-sensitive tissues. PPARγ is involved in the differentiation of adipose tissue; inducing lipid accumulation [23]. Other pathways are involved in lipid entry (FAT/CD36, FABP4) [24–27], lipid metabolism (SREBP-1c and FAS) [28, 29], and oxidation (ACC, CPT-1, PPARα, UCP pathways) [30]. The ERK pathway, involved in cell proliferation and differentiation, also seems to play an important role in both the liver (leads to NAFLD) [31] and adipose tissue [32, 33]. The IKK$\alpha\beta$ pathway is involved in the inflammatory response characteristic of obesity and indirectly mediates insulin resistance [27].

In Canada, the Cree of Eeyou Istchee (CEI) of Eastern James Bay have a prevalence of obesity and T2D that is, respectively, at least 1.5 [34, 35] and 4 times higher [36] than the general Canadian population. This may be the consequence of major lifestyle changes (decreased physical activity and gradual adoption of nontraditional diets), as well as cultural difficulty to comply with modern T2D treatments. Our team has been working with the CEI to identify plants stemming from their traditional pharmacopoeia that could offer culturally adapted complementary and alternative treatments for obesity and T2D. As part of an ethnobotanical survey, *Populus balsamifera* L. (Salicaceae) (balsam poplar) was identified as a plant used by the CEI to treat a variety of symptoms associated with T2D. As part of an *in vitro* bioassay platform used to screen for the antidiabetic potential of CEI plants, the 3T3-L1 cell line was selected to assess glitazone-like activity and stimulation of adipogenesis. *Populus balsamifera* L., also known as balsam poplar, unexpectedly and potently inhibited the accumulation of intracellular triglycerides [37–39], suggesting potential antiobesity activity. This plant extract contains a number of active components, namely, salicin, salicortin, salireposide, and populoside [37]. In subsequent studies conducted in the same cell line, a bioassay-guided fractionation approach identified salicortin, a salicylate glycoside, as the principal active component of *P. balsamifera* responsible for the observed inhibition of adipogenesis [39]. Salicortin is abundant in poplar, willow bark, and throughout the Salicaceae family [39]. Although salicylates are well known for having anti-inflammatory properties, improving insulin sensitivity [40–42], and even having antiproliferative effects [43], antiadipogenic activity had never been ascribed prior to the studies conducted by our team. We thus introduced *P. balsamifera* extract, alongside a high-fat diet (HFD), to study the plant's ability to mitigate the development of obesity using the *in vivo* diet-induced obese (DIO) C57BL/6 mouse model. The results clearly demonstrated that the plant extract substantially attenuated weight gain and the development of insulin resistance [44].

In the present studies, we sought to evaluate the effectiveness of *P. balsamifera* as well as its active principle salicortin at treating obesity and insulin resistance once they have been established in the same model [45, 46]. As previously described by other researchers [47] and discussed further below, DIO mice respond in a stratified manner to the HFD; some animals being resistant to the HFD (low responders—LR) while others show the clearcut profile of metabolic disease (high responders—HR). *P. balsamifera* and salicortin were thus administered to the latter DIO mice in order to determine their potential effectiveness in countering obesity and insulin resistance.

2. Materials and Methods

2.1. Plant Extracts. Specimens of *Populus balsamifera* L. (Salicaceae) were collected on CEI territory (Eastern James Bay, Quebec, Canada). Dr. Alain Cuerrier, taxonomist at the Montreal Botanical Garden, confirmed that the botanical identity and voucher specimens were deposited in the Marie-Victorin herbarium of the Montreal Botanical Garden in Montreal, Canada (Mis03-49). A crude 80% ethanolic extract of *P. balsamifera* was prepared as previously described [37]. Salicortin, the active principle of *P. balsamifera*, was produced through fractionation, isolation, and purification of the crude plant extract as previously described [39]. The structure of the purified compound was identified and confirmed by ^{1}H and ^{13}C NMR and by comparison with previously reported data. 1D- and 2D-NMR spectra were generated using an Avance 400 spectrometer (Bruker Biospin Corporation) [39].

2.2. Animals and Diets. Four-week-old male nondiabetic C57BL/6 mice (Charles River Laboratories, Saint-Constant, QC, Canada) were housed in individual cages, maintained on a 12 h light-dark cycle in a temperature and humidity-controlled animal room, and given free access to food and water. Following acclimatization, the mice were divided into groups of approximately 12 mice each. Chow controls received a standard diet (SD; 18% protein content, 4.5% crude fat; Charles River Animal rodent diet) for 16 weeks. Other groups were fed a high fat diet (HFD; Bio-Serv Diet #F3282; 60% energy from fat) for eight weeks. *P. balsamifera* at 125 or 250 mg/kg, and salicortin at 12.5 mg/kg were incorporated in the HFD and treatments continued for an additional 8 weeks (DIO controls receiving only HFD). Balsam poplar extract studies were initiated first while the active compound was being identified, isolated, or purified. Since, salicortin composes 10% of the whole plant extract, and that the most efficient dose of balsam poplar was 125 mg/kg, salicortin was administered at 12.5 mg/kg. Hence, experimental groups of plant extract and salicortin are compared to distinct CHOW nonobese and DIO controls. However, both experimental protocols were conducted in an identical manner, and DIO controls reacted in a fully comparable fashion relative to nonobese Chow congeners in both studies. Body weight, food and water intake, as well as glycemia were measured 3 times/week during the entire study. Glycemia was measured by pricking the tail vein and by using a commercial glucometer (Accu-Check Roche, Montreal, QC, Canada). Measurements were always performed at the same time/day, in the same order and by the same person. All experimental

protocols were approved by the animal experimentation ethics committee of the Université de Montréal and were carried out in full respect of the guidelines from the Canadian Council for the Care and Protection of Animals.

2.3. Data and Animal Segregation. The area under the curve (AUC) was calculated for parameters measured in a continuous manner throughout the study. The total AUC was then separated into two parts: fraction 1 (F1), representing AUC between week 0 and 4 (first month of treatment), and fraction 2 (F2) corresponding to the AUC between week 4 and 8 of plant extract administration (second month of treatment). This segregation served to determine the plant extract's temporal course of action, that is, whether early in onset (first 4 weeks), later (last 4 weeks), or present throughout the study. Once the experimental feeding protocol had been carried out, we became aware of the studies of Peyot and collaborators [47] discriminating low responders (LR) and high responders (HR) in the DIO mouse model. As discussed by these authors and observed in our own studies, pooling animals with different characteristics, such as low weight gain, weak IR and near-normal glycemia, with animals with high weight gain, frank IR, and hyperglycemia, can yield misleading results [47]. Therefore, we segregated the DIO animals based on these published criteria and analyzed our data accordingly. As expected, low responder animals exhibited a near normal metabolic profile and treatment with the plant extract or its active component essentially had little if any effect. This is positive in the sense that *P. balsamifera* extract and salicortin may have a desirable safety margin by being active only in metabolically compromised animals. This also confirms the validity of the segregation. Hence, data are presented for the effects of plant extract and active principle in HR animals only. This segregation did however reduce our sample size, hence contributing to data variability.

2.4. Surgical Procedure. At the end of the experimental protocol, mice were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital and sacrificed by exsanguination. Livers were flushed with physiological saline, dissected, immediately placed in liquid nitrogen, and stored until further use at −80°C. Soleus skeletal muscle, white adipose tissue (WAT; epididymal and retroperitoneal fat pads), subscapular brown adipose tissue (BAT), and kidneys were also collected, placed in liquid nitrogen, and stored at −80°C until further use.

2.5. Blood Parameters. Plasma insulin, adiponectin, and leptin were assessed by radioimmunoassay (RIA: Linco Research, St.Charles, MO, USA). To avoid interrupting dietary plant treatment and disturbing the HFD feeding pattern (hence affecting the DIO model), mice were not fasting when blood parameters were measured. Plasma levels of AST, ALT, LDH, creatinine, alkaline phosphatase, and circulating lipids (triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol) were measured by standard clinical biochemistry assays at the Department of Biochemistry of Sainte-Justine's Children Hospital (Montreal, QC, Canada).

2.6. Tissue Triglyceride Measurement. Part of the frozen liver and muscle sections (around 100 mg of each sample) were ground into powder under liquid nitrogen and extracted using Folch's chloroform/methanol (2 : 1) method [48]. Triglyceride content was quantified using a commercial kit (Randox Laboratories Ltd., UK).

2.7. Western Blot Analysis. Western blot analysis was performed on frozen liver, muscle, and WAT using the following antibodies: p-Akt (Ser 473), Akt, p-AMPK (Thr 172), AMPK, Glut4, p-ACC, ACC, FAS, FABP4, phospho p44/42 MAPK, p44/42 MAPK, p-IKK$\alpha\beta$, and β-actin (each at 1 : 1000 in blocking buffer incubated overnight at 4°C; Cell Signaling Tech Inc., Danvers, MA, USA). PPARα, PPARγ, CPT-1, CD36, UCP-2, and SREBP1-c were measured using a 1 : 200 dilution in blocking buffer and incubated either at 1 h room temperature (RT) or overnight (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The following HRP-conjugated secondary antibodies were used: anti-rabbit (1 : 10000; Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA), anti-mouse (1 : 4000; Cell Signaling Tech Inc., Danvers, MA, USA), or anti-goat (1 : 5000; Santa Cruz Biotechnology inc., Santa Cruz, CA, USA). Immunoreactive proteins were detected by enhanced chemiluminescence method (GE Healthcare, Baie d'Urfé, QC, Canada). Densitometric analysis was performed using NIH Image J software (version 1.42q, NIH, USA).

2.8. Statistical Analysis. Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni post hoc analysis, or by unpaired Student's t test (Sigma Stat software, Jandel Scientific, San Rafael, CA, USA), as appropriate. Areas under the curve (AUC) were calculated with PRISM software (GraphPad, San Diego, CA, USA). Data are expressed as the mean ± SEM of the indicated number of determinations. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Metabolic Profile of Responders to the High Fat Diet (DIO Controls). As anticipated in relation to recent data by Peyot et al. [47], roughly half of the mice consuming the HFD became obese and insulin resistant. Compared to Chow controls, such DIO control animals gained body weight, increased liver, BAT and WAT weights (Table 1, $P < 0.05$), and displayed hyperlipidemia (total cholesterol, LDL, HDL). These mice also exhibited increased plasma glucose and insulin levels, an enhanced leptin/adiponectin ratio (Table 2, $P < 0.05$) as well as elevated hepatic and muscle triglyceride (TG) levels (Table 3; $P < 0.05$ compared to Chow), thus confirming the presence of an insulin resistant state. Only mice displaying an altered metabolic profile at 8 weeks of HFD feeding were selected for the present study and randomized to receive *P. balsamifera* or its active component, salicortin, for an additional 8 weeks.

3.2. P. balsamifera and Salicortin Decrease Body Weight, Liver Weight, and Steatosis in DIO Mice. Treatment with

Table 1: Effects of obesity, *P. balsamifera*, and salicortin treatments on body and organ weights at sacrifice.

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Body Weight	138 ± 1†	120 ± 7*	131 ± 3	142 ± 2†	130 ± 3§
Retroperitoneal Fat Pad	229 ± 12†	190 ± 28	209 ± 20	242 ± 13†	218 ± 10
Epididymal Fat Pad	77 ± 3†	98 ± 17	103 ± 5*	97 ± 2	149 ± 11§
Brown Adipose tissue	189 ± 14†	136 ± 22 $P = 0.057$	168 ± 14	200 ± 6†	170 ± 11§
Liver Weight	167 ± 6†	108 ± 12*	122 ± 10*	166 ± 9†	118 ± 7§
Total Kidney	111 ± 4†	93 ± 3*	104 ± 4	104 ± 2	101 ± 3

Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with the active salicortin at 12.5 mg/kg. All values are expressed as a percentage of respective Chow controls (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol was: CHOW ($n = 12$); DIO ($n = 8$); *P. balsamifera* 125 ($n = 5$); *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$); DIO ($n = 7$); salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferroni *post hoc* test; $P < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

Table 2: Effects of obesity, *P. balsamifera*, and salicortin treatments on systemic parameters at sacrifice.

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Glucose (mmol/L)	135 ± 14†	105 ± 8	121 ± 10	121 ± 6†	114 ± 6
Insulin (ng/mL)	3056 ± 1074†	450 ± 238*	832 ± 423 ($P = 0.052$)	1035 ± 150†	272 ± 62§
Leptin (ng/mL)	211 ± 28†	108 ± 18*	145 ± 9 ($P = 0.051$)	246 ± 19†	197 ± 13§
Adiponectin (μg/mL)	70 ± 3†	78 ± 10	82 ± 5 ($P = 0.054$)	97 ± 5	101 ± 6
Leptin/adiponectin ratio	304 ± 37†	138 ± 16*	181 ± 16*	248 ± 12†	196 ± 18§
TG (mmol/L)	99 ± 8	80 ± 10	82 ± 8	118 ± 10	106 ± 14
LDL (mmol/L)	391 ± 31†	355 ± 37	307 ± 42	344 ± 20†	207 ± 22§
HDL (mmol/L)	141 ± 8†	112 ± 17	125 ± 8	157 ± 10†	137 ± 7
Total cholesterol (mmol/L)	180 ± 9†	152 ± 14	151 ± 12	203 ± 11†	151 ± 7§
ALT (U/L)	281 ± 39†	229 ± 47	271 ± 77	341 ± 123†	157 ± 27
AST (U/L)	172 ± 30†	153 ± 22	137 ± 22	163 ± 27†	86 ± 5§
Creatinine (U/L)	184 ± 50	491 ± 160	557 ± 138	276 ± 113	136 ± 30
Alkaline phosphatase (U/L)	115 ± 20	85 ± 15	100 ± 9	106 ± 9	88 ± 17
LDH (U/L)	341 ± 83†	137 ± 16§	193 ± 61	376 ± 156†	154 ± 38

Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with the active salicortin at 12.5 mg/kg. All values are expressed as a percentage of their respective Chow controls (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol was: CHOW ($n = 12$); DIO ($n = 8$); *P. balsamifera* 125 ($n = 5$); *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$); DIO ($n = 7$); salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferroni *post hoc* test; $P < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

Table 3: Effects of obesity, *P. balsamifera*, and salicortin treatments on hepatic and muscular triglyceride accumulation.

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Liver TG Levels (mg/g total liver)	930 ± 65†	436 ± 146*	521 ± 116*	1084 ± 180†	559 ± 93§
Muscle TG levels (μg/mg)	223 ± 54†	342 ± 81	267 ± 38	230 ± 32†	219 ± 24

The colorimetric dosage of TG levels in both the liver and muscle was determined using a commercial kit (Randox Laboratories ltd). Measurements were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with the active salicortin at 12.5 mg/kg. All values are expressed as percentage of respective Chow (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol was: CHOW ($n = 12$); DIO ($n = 8$); *P. balsamifera* 125 ($n = 5$); *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$); DIO ($n = 7$); salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferroni *post hoc* test; $P < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

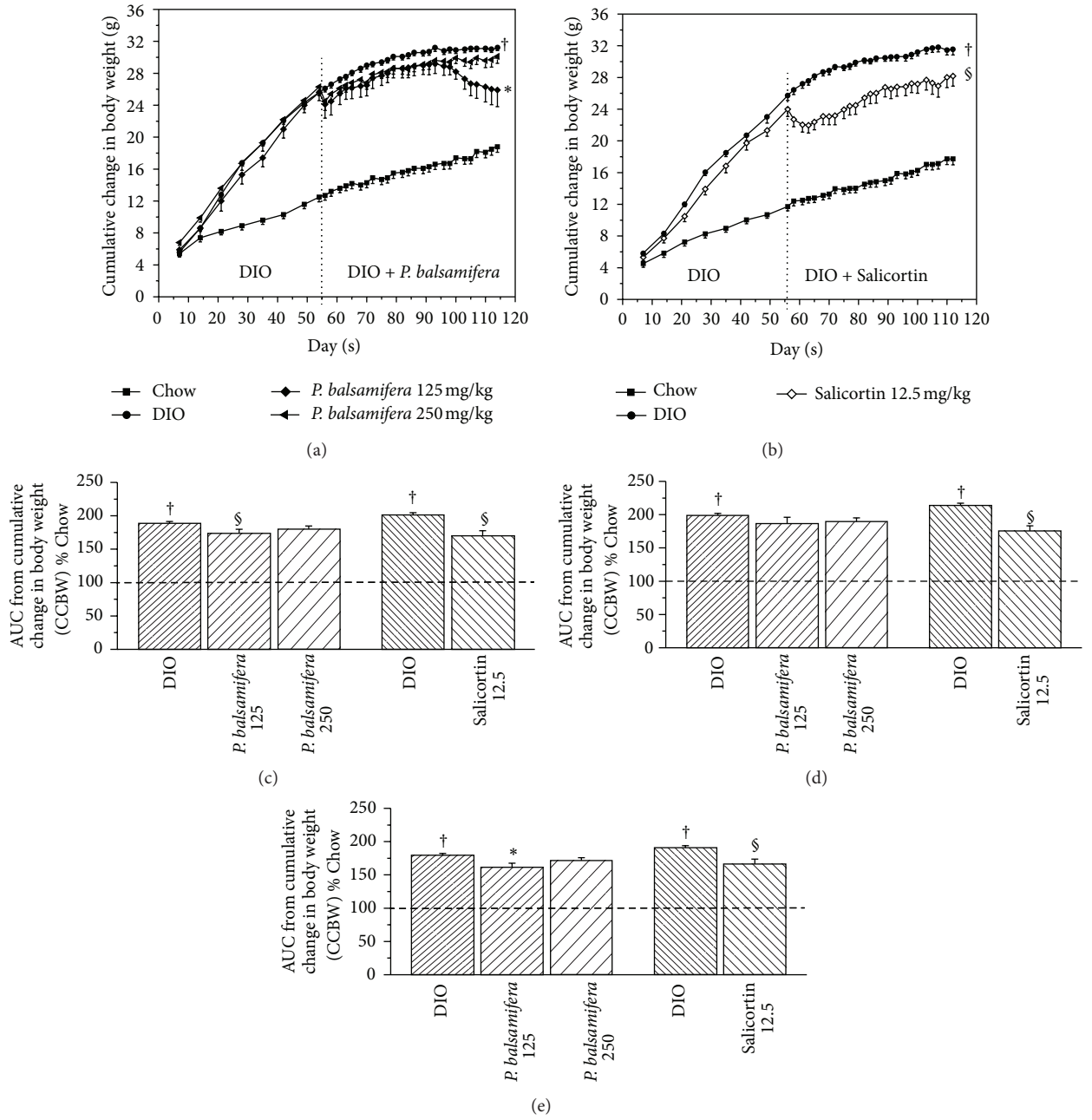

Figure 1: Cumulative changes in body weight (CCBW) in C57BL/6 mice treated with either standard diet (Chow), HFD (DIO), and (a) HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or (b) HFD in combination with salicortin. Area under the curve (AUC) of CCBW for (c) total 8-week treatment period, (d) first 4 weeks of treatment (F1), and (e) second 4 weeks of treatment (F2). As mentioned, C57BL/6 mice were administered either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks a HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with Salicortin 12.5 mg/kg. All values are mean ± SEM. Fraction 1 (F1) consists in the AUC between week 0 and 4, and fraction 2 (F2) corresponds to the AUC between week 4 and 8 of administration of the plant extract. The number of animals for the crude plant extract protocol was CHOW ($n = 12$), DIO ($n = 8$), *P. balsamifera* 125 ($n = 5$), and *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$), DIO ($n = 7$), salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA; $P < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

P. balsamifera (at 125 mg/kg) significantly decreased body weight. This decrease reached 13% at sacrifice when compared to DIO controls ($P < 0.05$; Table 1). When taking into account continuous measurements of cumulative changes in body weight (CCBW; Figures 1(a) and 1(b)), the area under the curve (AUC) was lowered by 8% ($P < 0.05$; Figure 1(c)) with 125 mg/kg of *P. balsamifera*. This effect was gradual, beginning within the first month ($F1$ = 6% reduction; N.S.; Figure 1(d)), but becoming more pronounced in the second month of the treatment ($F2$ = 10% decrease; $P < 0.05$; Figure 1(e)).

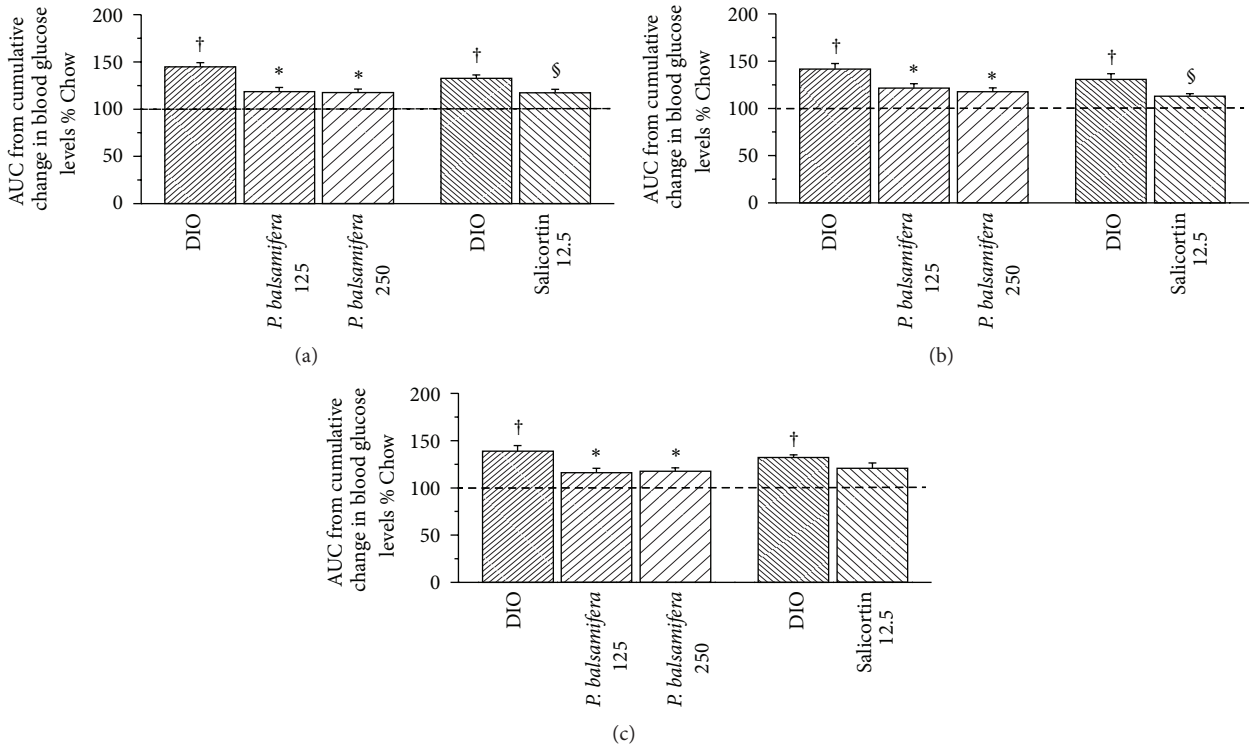

Figure 2: Area under the curve (AUC) of cumulative changes in blood glucose levels for (a) total 8-week treatment period, (b) first 4 weeks of treatment (F1), and (c) second 4 weeks of treatment (F2). C57BL/6 mice were administered either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks a HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with salicortin 12.5 mg/kg. All values are mean ± SEM. Fraction 1 (F1) consists in the AUC between week 0 and 4, and fraction 2 (F2) corresponds to the AUC between week 4 and 8 of administration of the plant extract. The number of animals for the crude plant extract protocol was CHOW ($n = 12$), DIO ($n = 8$), *P. balsamifera* 125 ($n = 5$), and *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$), DIO ($n = 7$), salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA; $P < 0.05$). §denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

Animals receiving 250 mg/kg of *P. balsamifera* exhibited a similar pattern of effects, albeit without reaching statistical significance. Mice fed the active compound, salicortin, also displayed a significant decrease in body weight amounting to an 8% drop at sacrifice ($P < 0.05$; Table 1) and a 15% decrease in total AUC of CCBW ($P < 0.05$; Figure 1(c)). Interestingly, its effect was immediate with 18% decrease in the F1 AUC ($P < 0.05$; Figure 1(d)) that continued into the second month, albeit slightly less prominently ($F2 = 13\%$; $P < 0.05$; Figure 1(e)).

Concomitantly, epididymal fat pad weight increased, whereas retroperitoneal fat pad weight was smaller when animals were treated with either dose of balsam poplar (9%–17% reduction) or with salicortin (10% decrease) than in those receiving HFD alone, although the latter changes failed to reach statistical significance (Table 1; N.S). In contrast, the drop in liver weight was significant in the three aforementioned treated groups (decrease by 27%–35%; $P < 0.05$ versus DIO controls; Table 1). Consistent with these results, hepatic triglyceride (TG) content was also reduced by 44% to 53% in the treated animals in comparison to DIO controls ($P < 0.05$; Table 3). Muscle triglycerides, however, were not significantly altered by any of the treatments (N.S.; Table 3).

It must be noted that a weak anorexic effect was observed in animals receiving *P. balsamifera* at 125 mg/Kg dose. Indeed, the AUC of cumulative food intake of this group was significantly reduced by 6% as compared to DIO congeners ($P < 0.05$, data not shown). No such effect was observed with the higher dose of *P. balsamifera* or with salicortin.

3.3. P. balsamifera and Salicortin Improve Insulin Sensitivity, While only the Active Principle Modulates Lipidemia in DIO Mice. Along with body weight changes, *P. balsamifera* (at 125 and 250 mg/kg) and salicortin improved insulin sensitivity, albeit with slightly different profiles. Firstly, continuous measurement of glycemia showed that both doses of whole plant extract as well as the active principle significantly reduced total AUC by 17%-18% and by 11%, respectively. Although *P. balsamifera* and salicortin effects were rather rapid in onset, the effect of the whole plant was constant throughout the treatment period ($F1 = F2$), whereas that of the active principle decreased with time ($F1 = 14\%$, $P < 0.05$ versus DIO controls; $F2 = 9\%$, N.S.; Figures 2(b) and 2(c)). At sacrifice, glycemia of the three treatment groups was reduced as compared to their respective controls, albeit not in a statistically significant manner (Table 2).

Secondly, insulinemia diminished by 85% with balsam poplar at 125 mg/Kg ($P < 0.05$) and by 73% with 250 mg/Kg ($P = 0.052$) as well as with salicortin ($P < 0.05$) in comparison to DIO controls (Table 2). Thirdly, the two doses of *P. balsamifera* decreased leptin/adiponectin ratio by 41%–54% as compared to congeners receiving HFD alone ($P < 0.05$; Table 2). Salicortin also significantly decreased this ratio, although to a lesser extent (by 21%; $P < 0.05$; Table 2). In terms of the circulating lipid profile, only the salicortin treated group exhibited significantly lowered total plasma cholesterol and LDL levels, which were reduced by 25% and 40%, respectively (Table 2; $P < 0.05$) as compared to the DIO controls. Altogether, these findings illustrate an improvement in insulin sensitivity when balsam poplar or its active principle are added to the HFD.

Finally, *P. balsamifera* and salicortin tended to normalize several systemic parameters of toxicity, although this did not reach statistical significance, except in the case of AST levels for salicortin ($P < 0.05$; Table 2) and LDH levels for *P. balsamifera* at 125 mg/Kg ($P < 0.05$; Table 2).

3.4. P. balsamifera Tends to Increase Skeletal Muscle Glut4 and Improves Components Related to Muscle Lipid Oxidation without Affecting the Akt and AMPK Pathways, Whereas Salicortin Tends to Increase Akt Phosphorylation and Activates p44/42 MAPK. Despite the significant reduction of overall glycemia exerted by the plant extract and its active principle, analysis of protein components involved in muscle glucose homeostasis did not exhibit any statistically significant changes. There was a tendency for Glut4 expression to increase in animals treated with *P. balsamifera* at 125 mg/kg, (Table 4; N.S. balsam poplar versus corresponding DIO controls). Similarly, insulin-dependent Akt phosphorylation tended to increase in animals fed with salicortin, although data variability precluded any definitive interpretation of these results. The insulin-independent AMPK pathway remained more clearly unchanged.

In contrast, components involved in muscle lipid homeostasis showed evidence of improvement with balsam poplar treatment. Indeed, *P. balsamifera* at 125 mg/kg more than doubled muscle PPARα expression levels (Table 4; 137% increase compared to DIO $P < 0.05$). When looking at components involved in muscle fatty acid oxidation and synthesis, again only the plant extract seemed to act on such pathways, by tending to increase phosphorylated ACC levels and to normalize FAS levels back down to Chow levels (Table 4; N.S. compared to DIO controls). The p44/42 MAPK pathway linked to exercise and insulin stimulation was significantly activated with salicortin (Table 4, $P < 0.05$), and showed a tendency to do so with the plant extract at 250 mg/kg (Table 4, N.S.).

3.5. The Effects of P. balsamifera and Salicortin on Liver Components of Glucose and Lipid Homeostasis. Both doses of *P. balsamifera* significantly increased hepatic phosphorylated Akt in HFD-fed animals (Table 4; increases by 111% and 87% for 125 and 250 mg/kg groups, respectively; $P < 0.05$ compared to DIO controls), while the active principle showed only a slight tendency to do so (22% increase). A number of parameters related to hepatic lipid homeostasis or inflammation showed interesting tendencies, but none of these effects reached statistical significance. In all cases, tendencies were more pronounced with the 125 than the 250 mg/kg dose of *P. balsamifera*. Notably, PPARα appeared to be increased by both balsam poplar and the active principle, while CPT-1 seemed to be increased only by the plant extract (Table 4, N.S.). As for IKK$\alpha\beta$ it appeared to be affected only by the plant extract, exhibiting a decrease of 43% and 30% with 125 and 250 mg/kg doses, respectively (Table 4; N.S.).

3.6. The Effect of P. balsamifera and Salicortin on Adipose Tissue Components of Glucose and Lipid Homeostasis. *P. balsamifera* at 125 mg/kg showed a strong tendency to increase phosphorylated Akt levels in adipose tissue (Table 4; increase by 65%, $P = 0.068$ compared to DIO controls). Likewise, CPT-1 expression in animals treated with the plant extract at 250 mg/kg exhibited a strong tendency to be enhanced (Table 4; increase by 47%, $P = 0.079$ compared to DIO), whereas the active principle had a similar albeit much weaker effect on this parameter (Table 4; 11% increase; N.S.). In contrast, FABP4 was clearly and significantly increased by *P. balsamifera* at both doses (54% and 60% at 125 and 250 mg/kg, respectively, Table 4, $P < 0.05$ compared to DIO controls), while salicortin showed only a slight tendency to do so (16%, N.S., Table 4;). Salicortin and balsam poplar showed a tendency to normalize PPARγ and phosphorylated p44/42 MAPK to levels similar to those observed in Chow animals (Table 4). Other components failed to show any significant changes in plant or active-principle treated treated animals compared to their respective DIO controls.

4. Discussion

According to the World Health Organization (WHO), 75% of the world population still relies on traditional medicine for primary health care needs and this often involves crude preparations of medicinal plants [49]. In the Canadian province of Quebec, regional health authorities assigned to the CEI are currently considering the usefulness of Cree traditional medicine, notably its associated pharmacopoeia, to deal with several health concerns such as type 2 diabetes; a condition that has reached epidemic proportions in the region [36]. Our group has been working since 2003 with communities and health authorities in CEI to build the scientific evidence base in support of this initiative. An ethnobotanical study was conducted in collaboration with CEI Elders and healers that identified several plants used to treat diabetes symptoms [37, 50, 51]. One of these was *Populus balsamifera* L. (Salicaceae) or balsam poplar. The plant did not demonstrate much antidiabetic potential in *in vitro* bioassays; for instance, it had little effect on muscle glucose uptake [37]. However, the plant caught our attention by its complete inhibition of triglyceride accumulation and adipogenesis in the 3T3-L1 adipocyte cell line [37], suggesting potential therapeutic usefulness against obesity. Salicortin, a salicylate glycoside, abundant in poplar, willow bark, as well as throughout the Salicaceae family, was identified through bioassay-guided fractionation as the constituent of

Table 4: Effects of obesity, *P. balsamifera,* and salicortin treatments on tissue components involved in glucose and lipid homeostasis.

	DIO	*P. balsamifera* 125 mg/kg	*P. balsamifera* 250 mg/kg	DIO	Salicortin 12.5 mg/kg
Muscle					
Glut4	150 ± 63	321 ± 174	151 ± 52	70 ± 33	59 ± 7
pAkt/Akt	214 ± 59†	195 ± 47	267 ± 82	96 ± 23	120 ± 29
phospho p44/42 Mapk/44/42Mapk	178 ± 83	99 ± 15	273 ± 84	47 ± 11	146 ± 34§
pAMPk/AMPk	138 ± 43	79 ± 10	97 ± 22	108 ± 16	108 ± 22
pACC/ACC	122 ± 28	171 ± 56	157 ± 38	106 ± 22	89 ± 26
PPARα/β-actine	97 ± 23	229 ± 50§	143 ± 44	195 ± 90	162 ± 69
FAS/β-actine	118 ± 30	100 ± 39	77 ± 18	114 ± 16	143 ± 29
Liver					
pAkt/Akt	66 ± 14	139 ± 18*	124 ± 17*	106 ± 28	129 ± 37
phospho p44/42 Mapk/44/42Mapk	68 ± 21	48 ± 21	66 ± 14	109 ± 35	96 ± 44
pACC/ACC	105 ± 41	117 ± 74	114 ± 62	71 ± 26	77 ± 31
PPARα/β-actine	63 ± 6†	88 ± 20	75 ± 13	107 ± 26	147 ± 26
UCP-2/β-actine	94 ± 19	107 ± 29	88 ± 26	151 ± 32	149 ± 27
CPT-l/β-actine	84 ± 12	100 ± 8	86 ± 9	86 ± 6	83 ± 9
FAS/β-actine	69 ± 17	69 ± 22	59 ± 25	86 ± 27	74 ± 15
SREBPl-c/β-actine	88 ± 16	112 ± 27	121 ± 37	109 ± 14	109 ± 17
CD36/β-actine	63 ± 7†	93 ± 19	64 ± 3	69 ± 8†	67 ± 6
pIKK$\alpha\beta$/β-actine	108 ± 23	62 ± 31	75 ± 12	114 ± 21	129 ± 21
Adipose tissue					
pAkt/Akt	112 ± 13	186 ± 37 ($P = 0.068$)	110 ± 15	138 ± 18	109 ± 14
phospho p44/42 Mapk/44/42Mapk	133 ± 31	103 ± 12	79 ± 10	156 ± 23†	128 ± 21
PPARγ/β-actine	73 ± 17	74 ± 15	102 ± 30	85 ± 12	116 ± 15
pACC/ACC	139 ± 46	119 ± 66	157 ± 70	95 ± 30	86 ± 20
CPT-l/β-actine	81 ± 9	86 ± 10	119 ± 19 ($P = 0.079$)	89 ± 11	99 ± 14
FABP4/β-actine	85 ± 11	131 ± 11§	136 ± 19§	71 ± 6	83 ± 10
FAS/β-actine	40 ± 5†	33 ± 8	56 ± 11	49 ± 7†	45 ± 7
SREBP-1 c/β-actine	77 ± 6	88 ± 9	96 ± 19	86 ± 7	87 ± 11

Samples of muscle, liver, and WAT were obtained after 16 weeks of treatment with either standard diet (Chow), HFD (DIO), and for the last 8 of the 16 weeks with HFD in combination with *P. balsamifera* at 125 or 250 mg/kg, or with the active salicortin at 12.5 mg/kg. The samples were homogenized and analyzed by immunoblotting. Blots were quantified by densitometry. All values are expressed as percentage of respective Chow (reference set at 100%) and represent the mean ± SEM. The number of animals for each group for the *P. balsamifera* protocol was: CHOW ($n = 12$); DIO ($n = 8$); *P. balsamifera* 125 ($n = 5$); *P. balsamifera* 250 ($n = 7$); and for the salicortin protocol: CHOW ($n = 12$); DIO ($n = 7$); salicortin ($n = 9$). †denotes DIO significantly different as compared to Chow (unpaired Student's t test; $P < 0.05$). *denotes significantly different as compared to respective DIO (one way ANOVA, Bonferroni *post hoc* test; $P < 0.05$).§denotes significantly different as compared to respective DIO (unpaired Student's t test; $P < 0.05$).

P. balsamifera having the most potential to inhibit adipogenesis in the 3T3-L1 cell line [39]. Prior to our studies, antiadipogenic activity had never been ascribed to balsam poplar, to members of its botanical family, or to its known phytochemical constituents, such as salicortin [39]. The goal of the present study was to evaluate the effectiveness of balsam poplar and salicortin as antiobesity, antiadipogenic, and consequently antidiabetic agents in an *in vivo* mouse model.

The DIO mouse model was used in this study. It closely mimics human metabolic syndrome (notably obesity and insulin resistance) and requires lesser quantities of plant extracts (also, more importantly, of active principles) than larger animals for long-term studies. Indeed, in this model, a period of 8 weeks is necessary to establish obesity and insulin resistance, as confirmed in the present studies. The plant was then incorporated into the high fat diet for a further 8 weeks to fully assess its potential to treat obesity and the associated metabolic disturbances. After 16 weeks on a high fat diet, control DIO animals develop obesity, mild hyperglycemia, hyperinsulinemia, hyperleptinemia, and increased ectopic fat storage (notably hepatic steatosis), all reflecting the establishment of the metabolic syndrome and an insulin resistant state. In previous studies, a less severe

model was used whereby animals were subjected to a HFD for only 8 weeks; *P. balsamifera* being administered from the onset of the HFD feeding in order to evaluate its potential to prevent obesity and its associated insulin resistant state [44]. The plant extract effectively reduced body weight gain, retroperitoneal fat pad weight, liver lipid content, as well as circulating glucose, insulin and leptin levels. It also activated pathways that were involved with glucose and lipid oxidation, as well as thermoregulation. The onset of action of the plant extract was immediate and sustained throughout its course of administration.

The results of the current study clearly demonstrate that in mice subjected to a continuous hypercaloric fat-laden diet, *P. balsamifera* significantly reduced body weight, whereas its active salicortin prevented further weight gain. The plant's effect was more potent and statistically significant at 125 mg/kg than 250 mg/kg. Several anthropomorphic, systemic and tissue parameters were thus examined to circumscribe the possible mechanisms of action of the plant extract and its active principle, salicortin.

A first potentially important lead came from data on cumulative food intake. Indeed, the plant extract at 125 mg/kg slightly but significantly reduced energy intake, and this was visible in the second month of treatment (F2; data not shown). This correlated well with the plant's temporal action on body weight. An initial reduction in body weight was observed upon introduction of the plant extract in the diet and may have resulted from a behavioral response to the food change. However, body weight rapidly resumed its course such that cumulative weight gain in the first month period (F1) was not significantly different among treatment groups. This contrasts with the reduced energy intake in the second month period (F2) that coincided with a significant decrease in weight gain. Such results suggested that the plant may exhibit slight appetite-modifying effects. Interestingly, these putative anorexic effects were seen only with the 125 mg/kg dose and indicate an unconventional dose-response relationship, as discussed further below. Nevertheless, such anorexic effects warrant further investigation. Notably, appetite-related hormones, such as leptin, as well as gut-brain appetite control mechanisms will need to be examined.

However, the reduction in caloric intake was weaker than the weight loss measured, roughly half to two-thirds as important when considering total or F2 AUC measurements of cumulative weight changes, respectively. In contrast, the active salicortin decreased the overall AUC of CCBW without affecting cumulative energy intake. This not only suggests different profiles of biological activity between the active principle and the plant extract, but also that other phytochemical components present within balsam poplar are contributing to its appetite-modifying effect.

On the other hand, although obesity was only partly countered by *P. balsamifera* and salicortin, systemic glucose homeostasis was more significantly improved. Indeed, continuous glycemia measurements showed that the plant and its active principle had an overall effect to reduce blood glucose variations toward normal values observed in Chow-fed controls. Even more telling was the dramatic decrease of insulinemia seen with *P. balsamifera* at 125 mg/kg and with salicortin. Likewise, the leptin-to-adiponectin ratio, also reflective of insulin resistance, was essentially halved with the plant extract and decreased by 1.5-fold with the active principle. Interestingly, salicortin also significantly improved the blood lipid profile by decreasing LDL and total cholesterol levels, whereas the plant extract had no significant impact on systemic parameters of lipid homeostasis. This again points to variations in biological activity between the crude extract and the purified active principle.

Further analysis of the major insulin responsive tissues, notably skeletal muscle, liver and adipose tissue, yielded data that highlights potential mechanisms at several levels of metabolic control. Firstly, excessive skeletal muscle TG accumulation was not corrected by *P. balsamifera* or salicortin treatment. In fact, if anything, balsam poplar extract at 125 mg/kg tended to increase this parameter, albeit large variations in the data preclude any definite interpretations. One possibility is that the two-month treatment was not sufficient to significantly affect muscle TG accumulation, yet improvements in muscle lipid and glucose metabolism could have been initiated. Indeed, the crude plant extract did more than double the expression of PPARα, which could lead to increased fatty acid oxidation [30] and improved muscle insulin sensitivity [52]. In animals receiving 125 mg/kg of the plant, muscle Glut4 expression tended to increase and this is consistent with enhanced insulin sensitivity. In contrast, salicortin treatment only significantly affected muscle p44/42 MAPK activation, again hinting at different actions of the plant extract and its active principle.

In contrast, in the liver, *P. balsamifera* and salicortin treatment more than halved the elevated levels of accumulated TGs. Since hepatic steatosis is increasingly recognized as a major contributor to systemic insulin resistance [53, 54], this action may have played a significant role in improving systemic glucose homeostasis and insulin sensitivity. Indeed, analysis of key tissue proteins indicated that *P. balsamifera* treatment induced a doubling of liver Akt phosphorylation. Since Akt is a major component of the insulin-signaling cascade, part of the effect of balsam poplar could involve improved hepatic insulin sensitivity. Indeed, Akt inhibits glucose production and promotes glycogen deposition in the liver [5, 6, 13]. In hepatic cell lines, our group recently found that *P. balsamifera* inhibits glucose-6-phosphatase [55]. Other components also tended to be modulated by *P. balsamifera* in the liver and suggested that the plant may favor salvaging lipid metabolism. Indeed, PPARα levels were increased by treatment with the plant and its active principle, this transcription factor being known to enhance fatty acid oxidation [30]. The tendency for a reduction of IKK$\alpha\beta$ by the plant treatment, on the other hand, points to a potential improvement of inflammatory components known to be involved in nonalcoholic fatty liver disease and ensuing metabolic disturbances [27, 56]. Such effects of *P. balsamifera* and salicortin on liver lipid homeostasis and inflammation will require confirmation in future studies.

Despite large reductions in retroperitoneal fat pad weight at sacrifice, consistent with the significant reduction in body weight, such changes induced by *P. balsamifera* and salicortin

failed to reach statistical significance due to data variability. In contrast, epididymal fat pad weight was reduced in DIO mice relative to Chow controls and this was normalized by balsam poplar extract treatment. The paradoxical decrease in epididymal fat pad weight in DIO mice may reflect the redistribution of fat towards more visceral sites in response to the high fat diet as observed by other investigators [57, 58].

On the other hand, obesity, especially visceral, leads to low-grade inflammation, releasing into circulation proinflammatory cytokines that contribute to the development of insulin resistance and diabetes. In addition, since both balsam poplar and salicortin belong to the salicylates family, well known for their anti-inflammatory properties, it would be of interest to evaluate the effect of these treatments on circulating proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, resistin, C-reactive protein, and so on). Our group recently assessed the effects of *P. balsamifera* on TNF-α production in THP-1 monocytes (ATCC TIB-202). Preliminary results indicate that *P. balsamifera* displays moderate anti-inflammatory properties in LPS-stimulated THP-1 monocytes [59].

Nonetheless, analysis of adipose tissue components yielded a number of insightful results. Firstly, the tendency for *P. balsamifera* and salicortin to reduce the p44/42 ERK MAP kinase is consistent with the parallel tendency for WAT weight reductions. Indeed, the ERK pathway is involved in adipogenesis and insulin resistance [32, 60] and our group observed that *P. balsamifera* inhibits clonal expansion in 3T3-L1 adipocytes [38]. On the other hand, FABP-4, a lipid chaperone carrying fatty acids to cellular pathways of oxidation, was significantly increased by plant treatment. As in liver, adipose tissue Akt and CPT-1 expression also showed a strong tendency to be increased, supporting the notion that *P. balsamifera* can enhance insulin-dependent lipid oxidative pathways. The active principle showed much weaker actions on adipose tissue components, notably mild tendencies to increase FABP-4 and CPT-1 expression.

The effects of both the crude plant extract and of the active principle salicortin occurred without any overt sign of toxicity, albeit balsam poplar extract tended to increase blood creatinine whereas salicortin tended to reduce the same parameter. Future studies should assess kidney function in a more detailed manner. However, unaltered liver function parameters support the interpretation that the plant and its active salicortin are fairly innocuous. Indeed, products of this tree have been used safely for generations by several Aboriginal peoples of the Northern hemisphere [61, 62]. The inner bark of *P. balsamifera* (from which the plant extract used in the current studies was derived) is even documented as a survival food [62, 63].

Interestingly, the majority of metabolically and statistically significant changes were obtained with the lower dose of 125 mg/kg of *P. balsamifera*, whereas the larger 250 mg/kg dose exerted lesser or no effects. Such counterintuitive dose-response relationships are not uncommon with polymolecular drug mixtures. Synergistic and antagonistic interactions may occur between the phytochemical components, yielding unconventional dose-response profiles [64]; for instance, observing an anorexic effect at the 125 mg/kg dose but not at that of 250 mg/kg. Such interactions are also supported by the aforementioned differences in the biological activity profile between the crude plant extract and salicortin. It is conceivable that other components in the crude extract may complement salicortin's activity.

Indeed, the action of the active principle alone on continuously measured parameters (body weight and blood glucose) appeared to wane with time, since effects were more pronounced in the first month of administration (F1) than in the second (F2). This may limit the use of the active principle at this dose and may have contributed to mask effects on protein components in insulin-sensitive tissues. Further studies need to be conducted in order to determine if this apparent time-dependent decline in activity develops at any dosage, and if so, with what time course. Nevertheless, salicortin has a sufficiently promising biological profile in DIO mice to warrant further studies potentially leading to clinical assessments.

In summary, *P. balsamifera* and salicortin exerted significant weight-reducing properties in obese, insulin resistant mice in the face of continued HFD feeding. Part of the plant extract's effect appears to emanate from a putative weak anorexic effect that will need to be defined, taking into consideration the loss of effect with higher doses. The plant and the active principle had even more profound beneficial effects on systemic glucose homeostasis and indirect indices of insulin sensitivity. Analysis of tissue components involved in glucose and lipid homeostasis uncovered several potential lead mechanisms in key insulin responsive organs such as skeletal muscle, liver, and adipose tissue. Generally, components involved in insulin-dependent lipid oxidative pathways were most prominently and coordinately modulated in animals treated with the plant extract. Such actions would favor the "wastage" of energy derived from excess lipids consumed through the HFD, thereby reducing the negative metabolic impact of obesity. This is highly relevant for Aboriginal populations like the CEI whose rapid changes in dietary habits over the last decades also involve a higher intake of lipid-enriched calorie-dense foods. It is noteworthy that salicortin did not always activate the same pathways and to the same degree as the plant extract, suggesting that other plant constituents in the crude extract may also participate in beneficial biological activity toward metabolic disease.

In conclusion, the present studies confirm the high potential of *P. balsamifera* as a complementary treatment derived from CEI traditional medicine, which can help combat the devastating effects of obesity, often leading to type 2 diabetes. Having identified salicortin as an important active principle *in vitro*—its anti-obesity and mild antidiabetic effects having also been validated by the present *in vivo* treatment study—it can now be considered as a valuable tool to ensure the quality and efficacy of *P. balsamifera* preparations. Salicortin can also serve as a template to develop novel therapeutic agents for the treatment of obesity and type 2 diabetes. Additional studies should further clarify the mode of action of the plant and its active principle. This will pave the way toward clinical studies designed to determine if *P. balsamifera* and salicortin can be used in a safe and efficacious manner, alongside conventional medical treatments, for the treatment of metabolic diseases.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Beta-Glucan-Rich Extract from *Pleurotus sajor-caju* (Fr.) Singer Prevents Obesity and Oxidative Stress in C57BL/6J Mice Fed on a High-Fat Diet

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Mushrooms have been used in folk medicine for thousands of years. In this study, the effect of β-glucan-rich extract of *P. sajor-caju* (GE) on lipid lowering and antioxidant potential was assessed in C57BL/6J mice fed on a high-fat diet. Obesity was induced in C57BL/6J mice by feeding a high-fat diet. The control groups in this study were ND (for normal diet) and HFD (for high-fat diet). The treated groups were ND240 (for normal diet) (240 mg/kg b.w) and HFD60, HFD120, and HFD240 (for high-fat diet), where the mice were administrated with three dosages of GE (60, 120, and 240 mg GE/kg b.w). Metformin (2 mg/kg b.w) served as positive control. GE-treated groups showed significantly reduced body weight, serum lipid, and liver enzymes levels. GE also attenuated protein carbonyl and lipid hydroperoxide levels by increasing the enzymic antioxidants (SOD, CAT, and GPx) activities in the mice. GE-treated groups induced the expression of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) while downregulated the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), sterol regulatory binding protein-1c (SREBP-1c), and lipoprotein lipase (LPL). Hence, GE prevented weight gain in the mice by inducing lipolysis and may be valuable in the formulation of adjuvant therapy for obesity.

1. Introduction

Obesity has reached epidemic proportions and is a major contributor to the global burden of chronic disease and disability because of its increasing prevalence in all age groups, sex, and race with the changes of lifestyles and dietary intake. A recent statistical report by the World Health Organization showed that one out of ten adults were overweight; hence, there are more than one billion overweight adults [1, 2]. Besides that, according to the National Health and Morbidity Surveys (2011), 15.1% of Malaysians aged 18 and above were obese thus Malaysia has the highest rate of obesity in south east Asia and the 6th in Asia. Obesity is a chronic metabolic disorder that results from the disequilibrium between energy intake and energy expenditure. It is characterized by enlarging fat mass and elevated lipid concentration in blood. The amount of fat mass is increased when the number and size of adipocytes are increased by proliferation and differentiation [3]. The obvious alternatives for the treatment of obesity are diet, exercise, and surgical intervention such as bariatric surgery, Roux-en-Y gastric bypass, gastric banding, and sleeve gastrectomy. However, it is proven to be successful only in a small minority of the population [4, 5]. Drugs that are currently available for the management of obesity, include orlistat (Xenical) which reduces intestinal fat absorption through inhibition of pancreatic lipase and sibutramine (Reductil), and appetite suppressant [2] which was found to cause numerous side-effects which include valvular heart disease, high blood pressure, dry mouth, constipation, and headache [6]. Multiple risk factor

syndrome or metabolic syndrome such as insulin resistance [7], diabetes mellitus [8], cardiovascular disease, stroke, hypertension [9], and dyslipidemia [10] is a growing medical problem in industrialized countries. Obesity is the central and causal component in this syndrome [11]. Furukawa et al. [11] reported that in obese individuals, elevated reactive oxygen species (ROS) upregulates the expression of NADPH oxidase, establishing a vicious cycle that augments oxidative stress in adipocytes and blood circulation. The ROS will increase the expression of chemoattractants such as monochemoattractant proteins-1 (MCP-1), by-products of lipid oxidation (lipid hydroperoxides and malondialdehyde (MDA)), and protein oxidation (protein carbonyl) [12, 13] which are linked with systemic inflammation which then lead to the development of metabolic syndrome. However, it is also well reported that antioxidants can inactivate these ROS and thus prevent metabolic deregulation including metabolic syndrome [14].

Mushrooms are well known for their medicinal properties and have been widely used in traditional medicine. The medicinal effects of mushrooms include antioxidant, antiviral, antibacterial, antifungal, antiparasitic, detoxification, immunomodulatory, antitumor, radical scavengers, antiinflammatory, antihyperlipidemic, or antihypercholesterolemic, hepatoprotective, and antidiabetic [14]. In Malaysia, the genus *Pleurotus* (oyster mushroom) which has been shown to have definite nutritive (high quality proteins, vitamins, and very little lipids or starch) and medicinal values is widely cultivated. This mushroom is mostly popular in countries such as India, China, and Japan and is reported to be able to reduce the cholesterol level in blood [15] and prevent hyperglycemia, insulin resistance, and inflammation in adipose tissue [16]. *Pleurotus* mushroom is rich in fiber yet low in calories and fat, and it has been cited as a potential weight-loss aid. The dietary fibers in the mushroom consist of chitin, hemicelluloses, mannans, and β-glucans. Beta-glucans are polysaccharides with glucose residue linked by beta glycosidic bonds. The fermentability of β-glucans and their ability to form highly viscous solutions in the human gut may constitute the basis of their antiobesity benefits [17]. Natural products containing β-glucans have been used for thousands of years, but β-glucans were only identified as active components recently. Therefore, this study was undertaken to investigate the effects of β-glucan-rich extract (GE) from *P. sajor-caju* on prevention of obesity and oxidative stress in C57BL/6J mice fed on a high-fat diet.

2. Materials and Methods

2.1. Mushroom Samples. All necessary permits and permission for the collection of materials for the described field study were obtained, and the party involved is duly acknowledged. Fresh fruiting bodies of *Pleurotus sajor-caju* (10 kg) were grown and collected from Mr. Kuan Kek How mushroom farm in Semenyih, Selangor Darul Ehsan, Malaysia. Authentication of *P. sajor-caju* was carried out by the Mushroom Research Centre (MRC), University of Malaya, and a voucher material (KUM 50082) for this study was deposited at the MRC culture collection.

2.2. Isolation and Purification of GE. The isolation and purification of GE were carried out based on the method described by Roy et al. [18]. The β-glucan level in GE was estimated using the β-glucan kit (specific for mushroom and yeast) purchased from Megazyme International (Ireland). The enzyme kit contains exo-1,3-β-glucanase, β-glucosidase, amyloglucosidase and invertase, glucose determination reagent (GOPOD-glucose oxidase, peroxidase, and 4-aminoantipyrine), and glucose standard solution. The estimation of total glucan content was done by hydrolysing GE with 37% hydrochloric acid (v/v) for 45 minutes at 30°C and continued for 2 hours at 100°C. After neutralization with 2 M potassium hydroxide, glucose hydrolysis was carried out using a mixture of exo-β-(1-3)-D-glucanase and β-glucosidase in sodium acetate buffer (pH 5.0) for 1 hour at 40°C. To measure the total glucan content, glucose oxidase-peroxidase mixture was added to GE and incubated for 20 minutes at 40°C. The absorbance of the resulting colour complex was measured using a spectrophotometer (Bio-Tek Instruments Inc, USA) at 510 nm. The α-glucan content was estimated according to the same method as described above after enzymatic hydrolysis with amyloglucosidase and invertase. The β-glucan content was calculated by subtracting the α-glucan from the total glucan content. Glucan content was expressed as percentage (w/w) of dry weight (DW).

2.3. Animals and Ethics Statement. This study was conducted in conformity with the policies and procedures of the Animal Care and Use Guidelines of Faculty of Medicine, University of Malaya, with reference to the 8th edition of Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Research, National Academy of Science, USA. The animal ethics approval was obtained from Animal Care and Use Committee of Faculty of Medicine, University of Malaya (IACUC, UM) (approval number: ISB/14/07/2010/GK [R]). Female C57BL/6j mice (7 weeks old) were purchased from BioLasco Laboratory, Taiwan. The animals were maintained in stainless steel wire mesh cages in a room kept at 21°C with a standard condition of 12-hour light/dark cycle (light period: 8:00–20:00 hour) with free access to food and water which were provided fresh every day.

2.4. Experimental Design. After one week of acclimatisation, the mice were randomly assigned (based on weight) into seven groups ($n = 6$). Table 1 shows the type of diet and concentration of GE administered to each group. On caloric basis, the normal diet contained 5% fat, 69.2% carbohydrate, and 25.8% protein whereas the high-fat diets (TestDiet, USA) comprised 45% of fat (46.1% fat from lard, 35.8% carbohydrate, and 18.1% protein) and 60% of fat (61.6% fat from lard, 20.3% carbohydrate, and 18.1% protein). GE was administered thrice a week via epigastric route using a feeding needle (size 20) to groups ND240, HFD60, HFD120, and HFD240 for 16 weeks. In this study, metformin (2 mg/kg b.w) was used as the positive control (HFDMET) since metformin has been reported to have comparable effects with orlistat (antiobesity drug) [19], and it is also widely used to treat type 2 diabetes which is closely associated with obesity [11]. After 7 weeks

Table 1: Type of diet and concentration of GE/metformin administrated to each group.

Type of diet	Groups	Treatment
Normal diet	ND	Normal diet only + saline
	ND240	Normal diet + 240 mg/kg of body weight GE
High-fat diet	HFD	High-fat diet only + saline
	HFD60	High-fat diet + 60 mg/kg of body weight of GE
	HFD120	High-fat diet + 120 mg/kg of body weight of GE
	HFD240	High-fat diet + 240 mg/kg of body weight of GE
	HFDMET	High-fat diet + 2 mg/kg of body weight of metformin

of feeding with 45% of fat, the animal diet was substituted with 60% of fat for groups HFD, HFD60, HFD120, HFD240 and HFDMET. The diet for groups ND and ND240 was not altered throughout the experiment. For the normal diet group, only 240 mg/kg of body weight of GE (highest dose) was administrated to the mice in order to reduce the usage of mice.

2.4.1. Sample Collection and Analytical Methods. Body weight and food consumption were monitored daily. During the experimental period, urine was collected from each group weekly (every Monday morning at 10:00 hour). At the end of the 16 weeks, the mice were anesthetized with ether after withholding food for 12 hours and were sacrificed by aortic exsanguination. Blood samples were collected in a SST glass serum tube with gold BD Hemogard closure (BD Vacutainer, USA). Serum samples were separated after centrifugation at 2400 ×g for 15 minutes. The serum samples from each mouse (within a group) were pooled together in order to have sufficient serum for further analysis. The pooled serum samples were sent to the Clinical Diagnostic Laboratory Unit, University Malaya Medical Centre, for the serum lipid and liver analysis. Immediately after blood collection, the liver and kidney were perfused *in-situ* with ice-cold saline. The weight of the liver and kidney of mice from each group were recorded. Eight mL of ice-cold phosphate buffer saline (PBS) was added to one gram of liver or kidney. The samples were then homogenized using a homogenizer (WiseMix HG-15A, Germany). Adipose tissues were removed and stored in RNAlater solution (Applied Biosystems, USA) and refrigerated at 4°C overnight. All samples were then stored at −80°C until further analysis was carried out.

2.5. Urinary Oxidative Indices Measurement. The protein carbonyl content (AOPP) was determined as previously described [20]. Chloramine-T solution of known concentrations (0 to 500 μM) was used as a standard for the estimation of AOPP concentration, and the result was expressed as μM of chloramine-T. Lipid hydroperoxide level was determined based on the method described by Esterbauer and Cheeseman [21] with modifications. 1,1,3,3-Tetraethoxypropane (TEP) solution of known concentration (2.5 to 20 μM) was used as a standard for quantification, and the result was expressed as μM of TEP. The DNA damage level was quantified using 8-hydroxy-2-deoxy-Guanosine (8-OHdG) EIA kit (Cayman Chemical, USA). 8-Hydroxy-2-deoxy-Guanosine hydroxyl EIA standard (10.3 pg/mL to 30 ng/mL) was used for quantification, and the result was expressed as pg/mL.

2.6. Enzymic Antioxidant Activity Measurement. The kidney and liver tissue homogenates were used to measure the activities of superoxide dismutase (SOD [EC-1.15.1.1]), glutathione peroxidase (GPx [EC-1.11.1.9]), and catalase (CAT [EC-1.11.1.6]). Commercially available kits were used for SOD, CAT, and GPx assays (Calbiochem, Germany). The protein content of the homogenates was determined using the Bio-Rad Protein Assay (Barcelona, Spain) [22] with bovine serum albumin as a standard. Enzyme activities were expressed in units per milligram of protein. One unit of SOD activity was defined as the amount of enzyme that exhibited 50% dismutation of the superoxide radical. One unit of CAT activity was defined as the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min. The unit of GPx activity was expressed as nanomoles of NADPH per min (calculated using an extinction coefficient of 0.00373 μM^{-1}).

2.7. Lipid Peroxidation Assay (LPO). The LPO assay was determined according to the modified method of Kuppusamy et al. [23] based on thiobarbituric acid reaction in which MDA was used as an index of lipid peroxidation. Trichloroacetic acid (15%) and thiobarbituric acid (1%) were added to the tissue homogenates in triplicates. The mixtures were incubated in boiling water bath for 10 minutes and were centrifuged at 6000 ×g for 10 minutes to remove the sediments. The absorbance was read at 532 nm using a spectrophotometer (Bio-Tek Instrument Inc., USA). 1,1,3,3-Tetraethoxypropane (TEP) solution of known concentration (2.5 to 20 μM) was used as a standard for quantification, and the result was expressed as mmol/L of TEP.

2.8. Gene Expression Using Real Time: RT-PCR. The total RNA was isolated from the adipose tissue using Ambion RNAqueous-Micro Kit (Applied Biosystems, USA). The purity of recovered total RNA was estimated by calculating the ratio of absorbance reading of 260 nm and 280 nm. The integrity of RNA was estimated using Agilent 2100 Bioanalyzer (Applied Biosystems, USA). Purified RNA with an A_{260}/A_{280} ratio between 1.8–2.0 and RIN values 8–10 was further used to synthesize complementary DNA (cDNA) by polymerase chain reaction (PCR) approach. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) which contained all reagents needed (RT buffer, dNTP mix, random primers, Multiscribe reverse transcriptase enzyme, and nuclease free water) for reverse transcription (RT) of total RNA to single-stranded cDNA was used in this study. The mixture was then loaded into a thermal cycler (Eppendorf, USA), and PCR was carried out according to optimized

Table 2: Genes investigated.

Number	Gene name and abbreviation	Assay ID	Accession number
1	Adipose triglycerides lipase (ATGL/Pnpla2)	Mm 00503040_m1	NM_025802
2	Hormone sensitive lipase (HSL/Lipe)	Mm 00495359_m1	NM_001039507
3	Lipoprotein lipase (LPL)	Mm 00434770_m1	NM_008509.2
4	Peroxisome proliferator-activated receptor γ (PPAR-γ)	Mm 01184322_m1	NM_011146
5	Sterol regulatory binding protein (SREBP-1c)	Mm 00550338_m1	NM_011480.3

General abbreviation of genes selected for this study and corresponding assay ID and accession number was obtained from the Applied Biosystems website and NCBI database. Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan probe mix. Assay ID with "Mm" is referred to as "*Mus musculus*." All Gene Expression Assay kits indicated are FAM/MGB probed.

thermal cycling conditions provided by the manufacturer. Table 2 shows the list of genes investigated in this study and the corresponding accession numbers. Endogenous control used in this study was eukaryotic 18S rRNA with FAM/MGB probe. All TaqMan (Applied Biosystems, USA) probes used in this investigation were labeled with FAM reporter dye at the 5′ end and a MGB quencher at the 3′ end. The quantification approach used was the comparative CT method, also known as $2^{-\Delta\Delta Ct}$ method [24].

2.9. Statistical Analysis. Data are shown as mean ± SD of triplicate assays. One-way analysis of variance was used to estimate the significant differences between groups. Statistical significance was accepted at $P < 0.05$. Duncan's multiple range tests (DMRT) was used to determine the significant differences between groups. Statgraphics Plus software (version 3.0, Statistical Graphics Corp., Princeton, NJ, USA) was used for all statistical analyses. All figures were drawn using GraphPad Prism 5 (GraphPad Software Inc., California, USA).

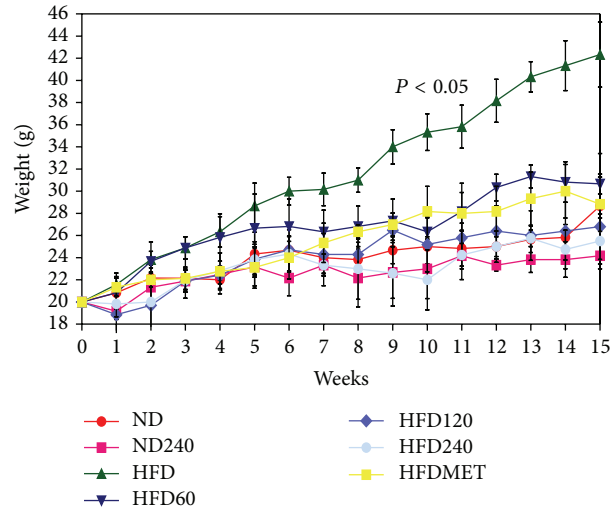

Figure 1: Effects of GE and metformin on body weight changes in C57BL/6J mice fed on a high-fat diet or normal diet. The concentrations of GE were 60, 120, and 240 mg/kg/day. Metformin (2 mg/kg/day) was used as positive control. Values expressed are means ± S.D of ($n = 6$ per group) measurements.

3. Results and Discussion

3.1. Weight and Estimation of β-Glucan Concentration in GE. Fresh *P. sajor-caju* (5.5 kg) was boiled for 8 hours to obtain 12.31 g of GE. The concentration of total glucan in GE was 85.95% (w/w) meanwhile the concentrations of α-glucan and β-glucan were 5.4% (w/w) and 80.55% (w/w) which corresponded to 0.01% and 1.5% in fresh mushroom, respectively [17].

3.2. Effects of GE on the Changes in Body Weight and Serum Lipid Levels. The test compounds (GE/metformin/vehicle) were only administered thrice a week to the mice in order to avoid physical stress. The mean food consumption was not significantly different between high-fat diet-treated mice and high-fat diet plus GE-treated mice. Figure 1 shows the effects of GE and metformin on body weight changes in the mice. The body weight in the ND group gradually increased during the 16-week period. In contrast, the body weight of mice in the HFD group showed a rapid increase of body weight. The descending order of the percentages of weight gain in each group was HFD > HFD60 > HFD120 > HFDMET > ND > HFD240 > ND240. The mice in HFD60, HFD120, and HFD240 groups had 27.55%, 36.69%, and 39.76% lower body weight, respectively, compared to HFD group. HFDMET group showed 31.90% lower body weight compared to HFD group; hence, the potential weight lowering effect of GE-treated groups were comparable to HFDMET group. Obesity has been associated with increased triglycerides (TG), very low-density lipoprotein (VLDL), total cholesterol (TC), and decreased high-density lipoprotein cholesterol (HDL-c) and thus is also a risk factor of cardiovascular disease [25]. Table 3 shows the serum lipid profile which includes the levels of TG, TC, HDL-c, low-density lipoprotein cholesterol (LDL-c), and atherogenic index (AI). In HFD control group, the TG level was increased by 33.3%, TC increased by 40%, HDL-c increased by 34.6%, and LDL-c increased by 171.4% compared to those in the ND group, thus the mice in HFD were considered to be hyperlipidemic. Meanwhile, mice in HFD60, HFD120, and HFD240 groups showed considerably reduced levels of TG, TC, and LDL-c compared to the HFD group, and this effect was dose dependent. The percentages of reduction for TG, TC, and LDL-c levels in HFD60 were 12.5%, 7.1%, and 60.5%, respectively. The percentages of reduction for TG, TC, and LDL-c levels in HFD120 were 25%, 10.7%, and 81.6%, respectively. The percentages of reduction for TG,

Table 3: Effects of GE and metformin on lipid profile and AI in C57BL/6J mice fed on a high-fat diet or normal diet.

Groups	Serum concentration (mmol/L)				
	TG	TC	HDL-c	LDL-c	AI
ND	0.60 ± 0.02^{ab}	2.03 ± 0.5^{a}	1.87 ± 0.05^{a}	0.14 ± 0.0^{ab}	0.07
ND240	0.70 ± 0.02^{b}	1.80 ± 0.4^{a}	1.79 ± 0.01^{a}	0.01 ± 0.0^{a}	0.01
HFD	0.8 ± 0.2^{bc}	2.80 ± 0.3^{b}	2.52 ± 0.3^{b}	0.38 ± 0.1^{c}	0.11
HFD60	0.70 ± 0.1^{b}	2.60 ± 0.2^{b}	2.35 ± 0.2^{b}	0.15 ± 0.0^{ab}	0.11
HFD120	0.60 ± 0.1^{ab}	2.50 ± 0.2^{ab}	2.41 ± 0.3^{b}	0.07 ± 0.0^{a}	0.07
HFD240	0.60 ± 0.4^{ab}	2.10 ± 0.1^{a}	2.35 ± 0.2^{b}	0.02 ± 0.0^{a}	0.02
HFDMET	0.50 ± 0.0^{a}	2.60 ± 0.3^{b}	2.55 ± 0.3^{b}	0.18 ± 0.0^{ab}	0.02

Values expressed are means ± S.D of ($n = 6$ per group) measurements. For same assay with various treatment groups, superscripts in the different bar with different alphabets (a–c) were significantly different ($P < 0.05$). Superscripts with same alphabets were not significantly different between the treated groups ($P > 0.05$). TG is triglycerides; TC is total cholesterol; HDL-c is high-density lipoprotein cholesterol; LDL-c is low-density lipoprotein cholesterol; AI is atherogenic index.

TC, and LDL-c levels in HFD240 were 25%, 25%, and 94.7%, respectively. However, there were no significant differences ($P > 0.05$) in the HDL-c level between the treated groups and control group. The HFDMET group showed decreased levels of TG (37.5%), TC (7.1%), and LDL-c (52.65%) levels and increased level of HDL-c (1.2%) compared to the HFD group. The AI and cardiac risk factor were calculated based on the measurement obtained from the lipid analysis. The AI was defined by TC minus HDL-c divided by HDL-c, whilst the cardiac risk factor was calculated as TC divided by HDL-c [26]. In this study, the AI risk predictor indices for the HFD group were increased compared to those in ND and GE or metformin-treated groups. In accordance to the high AI risk factor, the cardiac risk factor was also elevated in the HFD group compared to those in ND and GE or metformin-treated groups. The reductions in the atherogenic and cardiac risk indexes in GE-treated groups indicate a decreased risk of cardiovascular disease [27]. Beta-glucan has been shown to decrease LDL-c and increase HDL-c to alleviate possibly dyslipidemia and reduce cardiovascular disease [28]. Oats were first found to have a cholesterol-lowering effect, and the active component was identified as beta-glucans [29]. Similar serum cholesterol-lowering activity was also observed in Maitake, Shiitake, and Enokitake mushrooms [30]. The mechanism for LDL-c lowering by β-glucans is speculated to involve bile acid binding. The increased exclusion of bile acids activates cholesterol 7α-hydroxylase and upregulates low-density lipoprotein receptor (LDLR) and thus increases the transport of LDL-c into hepatocytes and the conversion of cholesterol into bile acids [31].

3.3. Effects of GE on Liver Enzymes. Table 4 shows the effects of GE and metformin-treated groups on liver enzymes. Increased liver enzyme concentrations and activity in the serum are conventionally interpreted as a marker of liver damage. In this study, the alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphate (ALP) levels of mice in the HFD group were significantly elevated compared to the other groups. However, there were no changes in the glutamyl transferase (GGT) level between these groups. A recent study demonstrated that obese patients with increased serum TG level showed raised levels of each of the four liver enzymes [32]. Weight reductions have been shown to reduce the liver enzyme levels [33]. The present study shows that GE confers protection against high-fat diet-mediated liver damage.

3.4. Effects of GE on the Urinary Oxidative Indices. Oxidation products can be found in the urine and are considered to reflect local and systemic oxidative stress [34]. Figures 2(a)–2(c) show the AOPP, lipid hydroperoxide, and 8-OHdG levels in each group during the 16 weeks of experiment. The AOPP, lipid hydroperoxides, and 8-OHdG levels in the ND group gradually increased every week, however, these oxidative stress indices were significantly elevated in the HFD group compared with all other groups. The mice in HFD group were obese, and this may have contributed to the increased level of oxidative stress indices in the animals [35]. The AOPP and lipid hydroperoxide levels in GE-treated groups were lower compared to the HFD group, and this effect was dose dependent. Similarly, HFDMET also showed a decrease in AOPP and lipid hydroperoxide levels compared to the HFD group. The 8-OHdG level was elevated in HFD group, however, no significant differences were observed between all the groups tested ($P > 0.05$). Studies have shown that elevated levels of MDA [36], AOPP [37], and 8-OHdG [38] in obese animals or humans are associated with several disease conditions including hypertension, diabetes, cardiovascular diseases, and renal diseases [39].

3.5. Effects of GE on Enzymatic Antioxidant Levels in Liver and Kidney Homogenates. Fruits, vegetables, spices, herbs, and mushrooms have been studied for their antioxidant properties *in-vitro* extensively [40, 41]. However, the demonstration of the antioxidant properties of these components *in-vivo* is scarce but is gaining importance nowadays. Previously, antioxidant capacity has been mainly assessed in serum or plasma after an oral intake of a food infusion. Nevertheless, numerous studies have also suggested that oxidative processes occurring in various tissues and organs in the human body may be crucial in the onset of metabolic diseases [42]. It is reported that, after absorption, the antioxidant

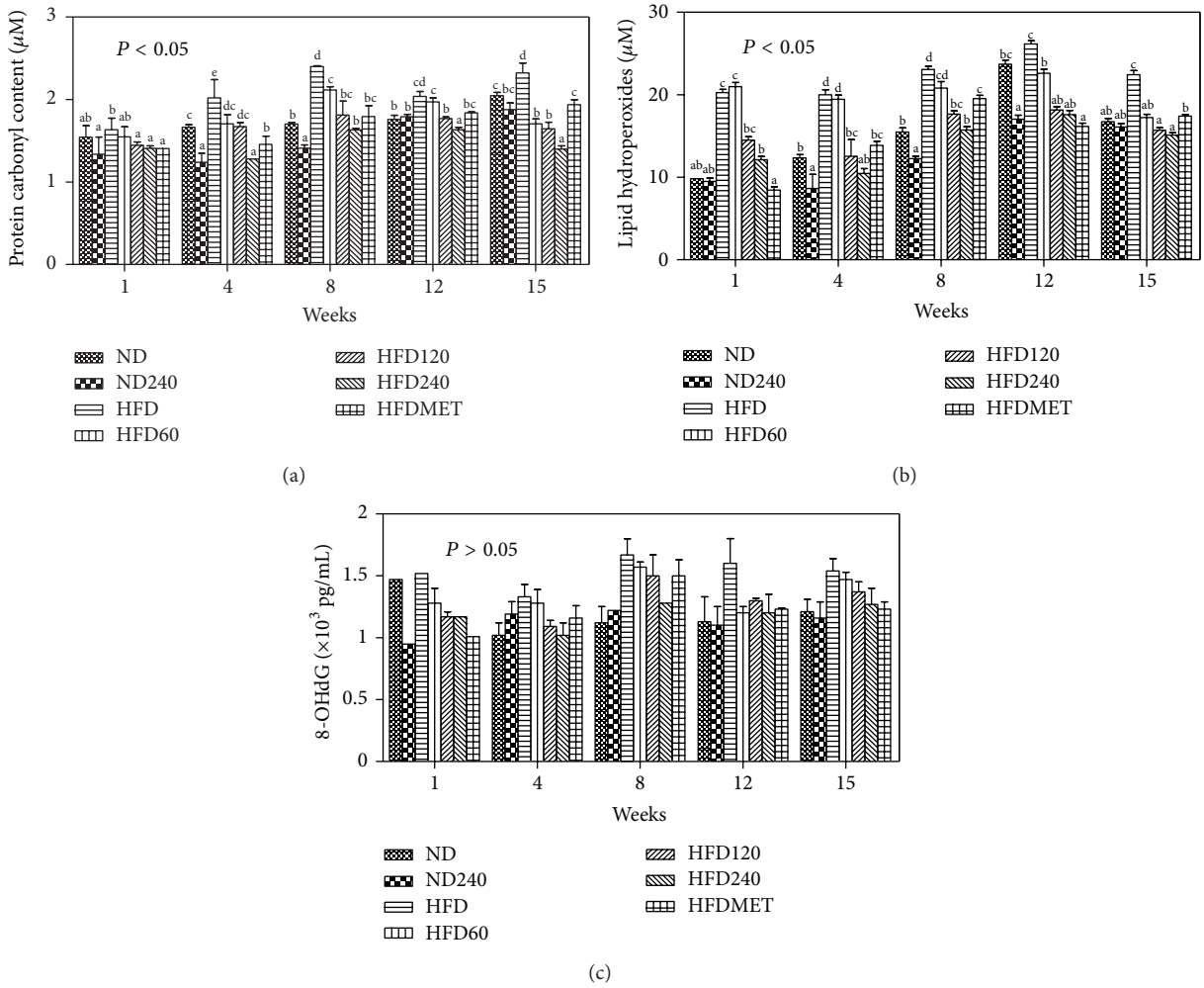

Figure 2: Effects of GE and metformin on (a) AOPP (b) lipid hydroperoxide, and (c) 8-OHdG levels in urine samples of C57BL/6J mice fed on a high-fat diet or normal diet. Values expressed are means ± S.D of triplicate measurements ($n = 6$ per group). For same assay with various treatment groups, superscripts in the different bar with different alphabets (a)–(e) were significantly different ($P < 0.05$). Superscripts with same alphabets were not significantly different between the treated groups ($P > 0.05$). There was no significant difference observed in the 8-OHdG levels between the groups tested ($P > 0.05$).

Table 4: Effects of GE and metformin on liver enzymes in C57BL/6J mice fed on a high-fat diet or normal diet.

Groups	Liver enzymes (mmol/L)			
	Alanine transaminase (ALT)	Aspartate transaminase (AST)	Alkaline phosphate (ALP)	G-glutamyl transferase (GGT)
ND	45 ± 1.2^{c}	182 ± 11.2^{b}	39 ± 1.2^{a}	<3
ND240	29 ± 1.1^{a}	148 ± 10.2^{a}	30 ± 1.1^{a}	<3
HFD	48 ± 1.4^{c}	210 ± 8.2^{c}	54 ± 0.9^{c}	<3
HFD60	46 ± 2.3^{c}	194 ± 12.1^{b}	44 ± 1.4^{b}	<3
HFD120	32 ± 2.4^{b}	181 ± 10.1^{b}	38 ± 1.3^{a}	<3
HFD240	27 ± 1.4^{a}	176 ± 9.7^{ab}	35 ± 1.3^{a}	<3
HFDMET	39 ± 1.33^{b}	189 ± 9.5^{b}	39 ± 1.5^{a}	<3

GE concentrations were 60, 120, and 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements. For same enzyme level with various treatment groups, superscripts in the different bar with different alphabets (a–c) were significantly different ($P < 0.05$). Superscripts with same alphabets were not significantly different between the treated groups ($P > 0.05$).

Table 5: Effects of GE on enzymic antioxidants and MDA levels in the kidney and liver homogenates of C5BL/6J mice fed on a high-fat diet.

Groups		Antioxidant activity (nmol/min/mg protein)			
		GPx	CAT	SOD (U/mg protein)	LPO (mmol/L)
ND	Kidney	71.08 ± 6.3^{cd}	103.93 ± 4.5^{d}	0.34 ± 0.0^{d}	0.89 ± 0.01^{e}
	Liver	83.95 ± 10.8^{b}	29.96 ± 3.9^{c}	0.17 ± 0.0^{b}	0.76 ± 0.03^{b}
ND240	Kidney	82.72 ± 6.9^{d}	112.96 ± 4.6^{e}	0.37 ± 0.0^{d}	0.83 ± 0.04^{d}
	Liver	90.42 ± 11.7^{b}	32.57 ± 6.7^{d}	0.32 ± 0.0^{d}	0.7 ± 0.1^{ab}
HFD	Kidney	32.31 ± 3.2^{a}	52.64 ± 1.2^{a}	0.017 ± 0.0^{a}	0.9 ± 0.04^{e}
	Liver	45.22 ± 5.5^{a}	15.18 ± 8.7^{a}	0.01 ± 0.0^{a}	0.92 ± 0.1^{c}
HFD60	Kidney	32.46 ± 4.9^{a}	79.72 ± 1.3^{b}	0.23 ± 0.0^{b}	0.86 ± 0.02^{de}
	Liver	68.20 ± 8.3^{b}	22.98 ± 5.7^{b}	0.19 ± 0.0^{b}	0.62 ± 0.1^{a}
HFD120	Kidney	36.16 ± 5.1^{ab}	86.40 ± 11.6^{bc}	0.27 ± 0.0^{c}	0.64 ± 0.03^{b}
	Liver	74.86 ± 8.7^{b}	24.91 ± 9.7^{bc}	0.23 ± 0.0^{bc}	0.6 ± 0.04^{a}
HFD240	Kidney	44.58 ± 5.3^{b}	84.29 ± 1.5^{bc}	0.22 ± 0.0^{b}	0.57 ± 0.04^{a}
	Liver	61.39 ± 8.9^{b}	24.30 ± 1.1^{bc}	0.26 ± 0.0^{c}	0.61 ± 0.06^{a}
HFDMET	Kidney	66.83 ± 5.5^{c}	89.49 ± 2.7^{c}	0.20 ± 0.0^{b}	0.74 ± 0.03^{c}
	Liver	119.06 ± 9.3^{c}	25.80 ± 8.5^{bc}	0.19 ± 0.0^{b}	0.64 ± 0.05^{ab}

GE concentrations were 60, 120, and 240 mg/kg/day. Metformin (MET) is used as positive control. Values expressed are means ± S.D of triplicate measurements. For same antioxidant activity with various treatment groups, superscripts in the different bar with different alphabets (a–e) were significantly different ($P < 0.05$). Superscripts with same alphabets were not significantly different between the treated groups ($P > 0.05$). GPx is glutathione peroxidase; CAT is catalase; SOD is superoxide dismutase; LPO is lipid peroxidation.

compounds are transferred through the blood circulation to various organs [43]. In the present study, the enzymic antioxidant activities and LPO level were assessed in the liver and kidney homogenates (Table 5), since these are the key organs in the mammalian oxidative metabolism. The natural antioxidant system consists of a series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds that react with and inactivate ROS. The primary antioxidant enzymes include SOD, CAT, and GPx. Meanwhile, the nonenzymatic antioxidants include vitamin C, vitamin E, β-carotene, reduced glutathione (GSH), and numerous phytochemicals. Cells must maintain their levels of antioxidants, often defined as their antioxidant potential, through dietary intake and/or de novo synthesis [44, 45]. Increased levels of ROS in cells and tissues may act as a signal to enhance the activity and expression of antioxidant enzymes. A high-fat diet is known to increase the superoxide anion ($O_2^{\bullet-}$) radicals in the body. Superoxide dismutase converts the $O_2^{\bullet-}$ radicals to hydrogen peroxide (H_2O_2) which in turn is converted to water by CAT and GPx. In this study, the HFD group showed reduced levels of SOD, CAT, and GPx activities in the kidney and liver homogenates compared to the ND group. Whereas, GE- and metformin-treated groups showed increased levels of SOD, GPx, and CAT activities compared to the control groups (ND and HFD) (Table 4). Overall, the increased level of antioxidant enzyme activities in GE- and metformin-treated groups conferred protection against oxidative damages in the mice, and this speculation is supported by the attenuated levels of oxidative stress indices such as AOPP and lipid hydroperoxide levels in the urine as well as MDA level in the kidney and liver homogenates.

3.6. Effects of GE on the Expression of Differentiation and Lipolysis Genes in Adipose Tissue. Adipose tissue is a complex and active secretory organ that both sends and receives signals that modulate energy expenditure, appetite, insulin sensitivity, endocrine function, inflammation, and immunity [46]. Table 6 shows the expression of the selected genes involved in the differentiation and lipolysis processes in adipose tissue. The mice fed on a high-fat diet (HFD group) weighed more and developed substantially more adipose tissue than the mice on a normal diet (ND group) (Figure 1). The mice became hyperlipidemic, and this is typically associated with obesity [47] (Table 3). PPAR-γ and SREBP-1c genes are the key adipose transcription factors that play important roles in lipogenesis [48]. These genes act cooperatively and sequentially to trigger terminal adipocyte differentiation. The PPAR-γ is expressed selectively in the adipose tissues, and it promotes the differentiation and proliferation of the preadipocytes thereby causing an increase in fat mass [49], while SREBP-1c controls the production of endogenous ligands for PPAR-γ as a mechanism for coordinating the actions of these adipogenic factors [48] and has been implicated as being a key regulator for fatty acid and triglyceride synthesis [50]. Meanwhile, LPL is the key enzyme that regulates the disposal of lipid in the body, and its role is to hydrolyse triglyceride circulating in the lipoprotein particles in order to facilitate the uptake fatty acids into the cells [51]. GE-treated groups had lower expression of PPAR-γ, SREBP-1c, and LPL compared to HFD group. PPAR-γ protein binds to the promoter regions of adipocyte-expressed LPL gene [52], and the attenuation of PPAR-γ gene expression in GE-treated groups could have attributed to the reduced expression of LPL as well. HSL and ATGL genes are reported to play an important role in the mobilization of stored triacylglycerol (TAG) [53]. The activation of these genes leads to mobilization of TAG to form glycerol and fatty acids where HSL mainly breaks down TAG to form diacylglycerol (DAG) whilst ATGL breaksdown DAG to form monoacylglycerol (MAG). Subsequently, MAG is converted to free fatty acids

Table 6: Effects of GE on the expression of genes in adipose tissue.

Genes investigated	ND240	HFD60	HFD120	HFD240	HFDMET
Lipolysis					
ATGL	1.34 ± 0.34	1.78 ± 0.67[a]	6.05 ± 0.42[c]	5.69 ± 0.34[c]	3.84 ± 0.98[b]
HSL	1.98 ± 0.07	2.99 ± 0.17[a]	6.73 ± 0.42[c]	6.54 ± 0.32[c]	4.63 ± 1.16[b]
Differentiation					
LPL	−1.05 ± 0.09	−1.93 ± 0.18[a]	−1.12 ± 0.42[b]	−1.17 ± 0.47[b]	−2.22 ± 0.99[a]
PPAR-γ	−1.69 ± 0.19	−2.08 ± 0.69[a]	−1.69 ± 0.48[ab]	−1.02 ± 0.36[c]	−1.07 ± 0.16[c]
SREBP-1c	−1.27 ± 0.65	−1.01 ± 0.16[c]	−3.10 ± 0.44[a]	−2.25 ± 0.30[ab]	−2.30 ± 1.13[ab]

Results are expressed as fold variation over the appropriate control groups; ND240 indicates fold increase over ND (normal diet control group), and HFD60, HFD120, HFD240, and HFDMET indicate fold increase over HFD (high-fat diet control group). Fold variations less than one were expressed as negative numbers (e.g., a fold variation of 0.50 is expressed as −2.00). Values expressed are means ± S.D of triplicate measurements. Statistical significance was calculated based on the mean ΔCT values by DMRT for only mice fed with high-fat diet with or without GE. For same gene with various treatment groups, superscripts in the different bar with different alphabets (a–c) were significantly different ($P < 0.05$). Superscripts with same alphabets were not significantly different between the treated groups ($P > 0.05$).

and glycerol by monoacylglycerol lipase (MGL) [54]. The GE-treated groups had significantly upregulated expressions of HSL and ATGL genes, and the effect was dose dependent. Therefore, it is feasible to suggest that the reduced weight gain in the high-fat diet fed mice treated with GE was due to the reduced adipose differentiation and increased lipolysis in adipocytes.

4. Conclusion

Previous studies have demonstrated that the lipid lowering potential of β-glucans was mainly mediated by either bile acid binding, delay in the digestion/absorption of fat, or suppressed appetite. However, in this study, GE prevented weight gain and hyperlipidemia in C57BL/6J mice fed on a high-fat diet by inducing lipolysis and inhibiting the differentiation of adipocytes. GE also prevented oxidative stress caused by obesity by increasing the enzymic antioxidant activities, hence, GE could serve as a potential candidate for the management of obesity.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Combined Ethanol Extract of Grape Pomace and Omija Fruit Ameliorates Adipogenesis, Hepatic Steatosis, and Inflammation in Diet-Induced Obese Mice

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The aim of this study was to evaluate the long-term effects of grape pomace ethanol extract (GPE) with or without omija fruit ethanol extract (OFE) on adiposity, hepatic steatosis, and inflammation in diet-induced obese mice. Male C57BL/6J mice were fed a high-fat diet (HFD) as the control diet and HFD plus GPE (0.5%, w/w) with or without OFE (0.05%, w/w) as the experimental diet for 12 weeks. GPE alone did not significantly affect adipogenesis and hepatic steatosis. However, the supplementation of GPE + OFE significantly lowered body weight gain, white adipose tissue weight, adipocyte size, and plasma free fatty acid and adipokines (leptin, PAI-1, IL-6, and MCP-1) levels in HFD-fed mice compared to those of the control group. These beneficial effects of GPE + OFE were partly related to the decreased expression of lipogenic and inflammatory genes in white adipose tissue. GPE + OFE supplementation also significantly lowered liver weight and ameliorated fatty liver by inhibiting expression of hepatic genes involved in fatty acid and cholesterol syntheses as well as inflammation and by activating hepatic fatty acid oxidation. These findings suggest that the combined ethanol extract of grape pomace and omija fruit has the potential to improve adiposity and fatty liver in diet-induced obese mice.

1. Introduction

Obesity, a metabolic disease characterized by an excessive accumulation of fat in white adipose tissue (WAT), is associated with chronic inflammation and is considered a risk factor of nonalcoholic fatty liver disease (NAFLD). WAT secretes various inflammation-related adipocytokines, and the dysregulated production of adipocytokines promotes NAFLD. In particular, visceral WAT is directly linked to the severity of hepatic inflammation and fibrosis in NAFLD, independent of hepatic steatosis [1]. Furthermore, visceral WAT might promote NAFLD by the release of free fatty acids that are delivered directly into the portal vein [2]. There is a close relationship between visceral and liver fat contents in obese and nonobese individuals [3–5]. Lipogenesis is also an important metabolic pathway regulating adipose and hepatic fat accumulation [6].

It is well known that phytochemicals-rich foods like grapes and their products prevent metabolic diseases such as obesity, NAFLD, and insulin resistance. Grape pomace, a byproduct of wine processing, consists mainly of peels, stems, and seeds and accounts for about 20% of the weight of the grapes processed into wine [7]. The antioxidant and anti-inflammatory effects of grape pomace ethanol extract (GPE) in mice fed a high-fat diet (HFD) were reported [8]. In HFD-fed rats, GPE also ameliorated obesity-induced insulin resistance by inhibiting hepatic lipogenic, gluconeogenic, and inflammatory gene expression [9]. However, little is known

about whether GPE can protect against lipid accumulation and inflammation in the WAT and liver of diet-induced obese mice. Therefore, we examined the effects of GPE on the expression profiles of genes related to lipogenesis and inflammation in the WAT and liver, and plasma adipocytokine levels in HFD-fed C57BL/6J mice, which are a good model for diet-induced obesity that display many of the characteristics of the human disease including visceral obesity, hyperglycemia, and hyperinsulinemia [10].

We also hypothesized that combined supplementation of GPE and omija fruit extract (OFE) may synergistically ameliorate visceral fat accumulation, hepatic steatosis, and inflammation in diet-induced obese mice. Fructus Schisandrae, the fruit of omija (*Schisandra chinensis* Baillon), is another phytochemical food which has antioxidant, anti-inflammatory, and antimetabolic properties. Omija has been traditionally used as a tonic, sedative, and antidiabetic agent in Asia and is a main compound of traditional herbal medicine which consists of several herbs [11, 12]. Taeyeumjoweetang, a traditional Korea herbal medicine consisting of eight herbs including omija, suppressed body weight and serum leptin and resistin levels in mice [13] and improved serum and hepatic lipid profiles in HFD-fed mice [14]. In addition, a recent study reported that dibenzocyclooctadiene lignans isolated from *Schisandra chinensis* had a fatty acid synthase inhibitory effect [15].

Accordingly, we investigated the effect of GPE alone or combined with OFE on adiposity, hepatic steatosis, and inflammation in HFD-induced obese mice and the underlying mechanisms based on lipid metabolism and inflammation in the WAT and liver.

2. Materials and Methods

2.1. Preparation of Extracts. Grapes (*Vitis vinifera*, MBA (Muscat Bailey A) species) and omija (*Schisandra chinensis* Baillon) were purchased from Yeongcheon-si (Gyeongsangbuk-do, Korea) and Mungyeong-si (Gyeongsangbuk-do, Korea), respectively. In this study, grape pomace (skin and stem) and omija fruits (Fructus Schisandrae) were used. Samples were prepared by adding 2 L of 80% and 50% ethanol to 100 g of dried grape pomace and omija fruit, respectively; extraction was done at 80°C for 2 h and then cooled. The solution was filtered (Whatman paper no. 2), concentrated with a rotary vacuum evaporator, and stored at −70°C. The final weight of the lyophilized powder of grape pomace ethanol extract (GPE) was 33.9 g (33.9%) and omija fruit ethanol extract (OFE) was 39.7 g (39.7%). Resveratrol is a representative compound in grape pomace and schizandrin is a typical compound in omija. The GPE included 0.2 mg/g resveratrol, 52 mg/g flavonoids, and 6 mg/g catechins (3.3 mg/g catechin, 2.6 mg/g epicatechin). The OFE contained 8 mg/g schizandrin and 7 mg/g flavonoids. The content of flavonoid, resveratrol, and schizandrin of grape pomace and omija extracts was 48 mg/g, 0.2 mg/g, and 55 mg/g, respectively.

2.2. Animal and Diets. Male mice (strain C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 4 weeks of age. The animals were individually housed with a constant temperature (24°C) and 12 h light/dark cycle and fed a pelletized commercial nonpurified diet for 1 week after arrival. The mice were then randomly divided into 3 groups ($n = 10$) and fed the control and experimental diets for 12 weeks: high-fat diet control (HFD, 20% high-fat diet based on AIN-76 diet plus 1% cholesterol, w/w), grape pomace extract (GPE, HFD combined with 0.5% grape pomace extract, w/w), and the combined extracts of grape pomace and omija fruit (GPE + OFE, HFD combined with 0.5% grape pomace extract, and 0.05% omija fruit extract, w/w). The composition of the diets is presented in Table 1. The mice had free access to food and distilled water during the experimental period. Their food intake and body weight were measured daily and weekly, respectively.

At the 12th week, mice were anaesthetized with diethyl ether and sacrificed after 12 h of fasting. Blood was taken from the inferior vena cava and then centrifuged at 1000 ×g for 15 min at 4°C, and the plasma was separated to analyze plasma biomarkers. After blood collection, the liver and adipose tissues were promptly removed, rinsed, weighed, frozen in liquid nitrogen, and stored at −70°C. This animal study protocol was approved by the Ethics Committee for animal studies at Kyungpook National University, Republic of Korea.

2.3. Plasma Adipocytokine and Aminotransferase Levels. To measure the plasma adiponectin and adipsin levels, we used a quantitative sandwich enzyme immunoassay kit (ELISA kit, Millipore, MA, USA). The levels of plasma cytokines (interleukin (IL)-6, monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor (TNF)-α), leptin, resistin, and plasminogen activator inhibitor-1 (PAI-1) were determined with a multiplex detection kit from Bio-Rad (Hercules, CA). All samples were assayed in duplicate and analyzed with a Luminex 200 Labmap system (Luminex, Austin, TX). Data analyses were done with Bio-Plex Manager software version 4.1.1 (Bio-Rad, USA). The plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using enzymatic kits (Asan, Seoul, Republic of Korea).

2.4. Plasma and Hepatic Lipid Contents. Plasma lipid concentrations were determined with commercially available kits: total cholesterol, triglyceride, HDL-cholesterol (Asan, Seoul, Republic of Korea), free fatty acids, and phospholipids (Wako Chemicals, Richmond, VA, USA). The hepatic lipids were extracted using the method of Folch et al. [16] and hepatic lipid levels were analyzed with the same enzymatic kits used in the plasma analyses.

2.5. RNA Extraction and Real-Time Quantitative PCR Analysis. Total RNA was isolated from the liver and WAT using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's instructions. DNase digestion was used to remove any DNA contamination, and RNA was reprecipitated in ethanol to ensure no phenol contamination. The RNA purity and integrity were evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Equal amounts of RNA

Table 1: Composition of experimental diets (unit: % of diet).

Ingredients	CON	GPE	GPE + OFE
Casein	20	20	20
D, L-Methionine	0.3	0.3	0.3
Sucrose	36.996	36.496	36.45
Cellulose	5	5	5
AIN mineral[1]	4.2	4.2	4.2
AIN vitamin[2]	1.2	1.2	1.2
Choline bitartrate	0.2	0.2	0.2
Corn Starch	11.1	11.1	11.1
Lard	17	17	17
Corn oil	3	3	3
Cholesterol	1	1	1
tert-butylhydroquinone	0.004	0.004	0.004
Grape extract		0.5	0.5
Omija extract			0.05
Total	100	100	100

CON: high-fat diet control; GPE: high-fat diet plus grape pomace extract (0.5%, w/w); GPE + OFE: high-fat diet plus grape pomace extract (0.5%, w/w) combined with omija fruit extract (0.05%, w/w). [1]AIN-76 mineral mixture (grams/kg): calcium phosphate 500, sodium chloride 74, potassium citrate 2220, potassium sulfate 52, magnesium oxide 24, manganous carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite 0.01, chromium potassium sulfate 0.55, sucrose 118.03, [2]AIN-76 vitamin mixture (grams/kg): thiamin HCl 0.6, riboflavin 0.6, pyridoxine HCl 0.7, niacin 3, calcium pantothenate 1.6, folic acid 0.2, biotin 0.02, vitamin B_{12} 1, vitamin A (500,000 U/gm) 0.8, vitamin D_3 (400,000 U/gm) 0.25, vitamin E acetate (500 U/gm) 10, menadione sodium bisulfite 0.08, sucrose 981.15.

from each experimental group were pooled to normalize individual differences. Total RNA (1 μg) was reverse-transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen, Germany). Then mRNA expression was quantified by real-time quantitative PCR, using the QuantiTects SYBR green PCR kit (Qiagen, Germany) on the CFX96TM real-time PCR system (Bio-Rad, UK). The sequences of the primers were as follows: ACAT (acetyl coenzyme A acetyltransferase), 5′-AGAAATCAAGCAAAGATCCA-3′ (forward), 5′-AGGAGTCCTTGGGTAGTTGT-3′ (reverse); ACC1 (acetyl CoA carboxylase 1), 5′-GGACAGACTGATCGCAGAGAAAG-3′ (forward), 5′-TGGAGAGCCCCACACACA-3′ (reverse); ACOX1 (acyl CoA oxidase 1), 5′-CCCAACTGTGACTTCCATT-3′ (forward), 5′-GGCATGTAACCCGTAGCACT-3′ (reverse); CD36, 5′-TGGTGGATGGTTTCCTAGCCTTTC-3′ (forward), 5′-TCGCCAACTCCCAGGTACAATC-3′ (reverse); CPT1 (carnitine palmitoyl transferase 1), 5′-ATCTGGATGGCTATGGTCAAGGTC-3′ (forward), 5′-GTGCTGTCATGCGTTGGAAGTC-3′ (reverse); FAS (fatty acid synthase), 5′-CGCTCCTCGCTTGTCGTCTG-3′ (forward), 5′-AGCCTTCCATCTCCTGTCATCATC-3′ (reverse); GAPDH (glyceraldehyde-3-phosphatedehydrogenase), 5′-ACAATGAATACGGCTACAGCAACAG-3′ (forward), 5′-GGTGGTCCAGGGTTTCTTACTCC-3′ (reverse); HMGR (3-hydroxy-3-methyl-glutaryl-CoA reductase), 5′-TTCACGCTCATAGTCGCTGGATAG-3′ (forward), 5′-TGGTTCAATTCTCTTGGACAATCTTC-3′ (reverse); IL-6, 5′-GAGGATACCACTCCCAACAGACC-3′ (forward), 5′-AAGTGCATCATCGTTGTTCATACA-3′ (reverse); LPL (lipoprotein lipase), 5′-CTGACCAAGGATAGTGGGATATAG-3′ (forward), 5′-GGTAACTGAGCGAGACTGTGTCT-3′ (reverse); MCP-1, 5′-TTCCTCCACCACCATGCAG-3′ (forward), 5′-CCAGCCGGCAACTGTGA-3′ (reverse); ME (malic enzyme), 5′-AGGGCACATTGCTTCAGTTC-3′ (forward), 5′-TGTACAGGGCCAGTTTACCC-3′ (reverse); NF-κB (nuclear factor kappa B), 5′-GAAGTGAGAGAGTGAGCGAGAGAG-3′ (forward), 5′-CGGGTGGCGAAACCTCCTC-3′ (reverse); PGC1α (peroxisome proliferator-activated receptor γ co-activator 1 α), 5′-AAGTGTGGAACTCTCTGGAACTG-3′ (forward), 5′-GGGTTATCTTGGTTGGCTTTATG-3′ (reverse); PGC1β (peroxisome proliferator-activated receptor γ co-activator 1 β), 5′-GGTCCCTGGCTGACATTCAC-3′ (forward), 5′-GGCACATCGAGGGCAGAG-3′ (reverse); PPARα (peroxisome proliferator-activated receptor α), 5′-CCTGAACATCGAGTGTCGAATAT-3′ (forward), 5′-GGTCTTCTTCTGAATCTTGCAGCT-3′ (reverse); PPARγ (peroxisome proliferators-activated receptor γ), 5′-ACCACTCGCATTCCTTTGAC-3′ (forward), 5′-CCACAGACTCGGCACTCAAT-3′ (reverse); SCD1 (stearoyl-CoA desaturase 1), 5′-CCCCTGCGGATCTTCCTTAT-3′ (forward), 5′-AGGGTCGGCGTGTGTTTCT-3′ (reverse); TNF-α, 5′-GCAGGTCTACTTTAGAGTCATTGC-3′ (forward), 5′-TCCCTTTGCAGAACTCAGGAATGG-3′ (reverse); and UCP2 (uncoupling protein 2), 5′-ACCAAGGGCTCAGAGCATGCA-3′ (forward), 5′-TGGCTTTCAGGAGAGTATCTTTG-3′ (reverse). Cycle thresholds were determined based on the SYBR green emission intensity during the exponential phase. The fold changes were calculated using the 2-ΔΔCt method; transcripts of GAPDH were also amplified from the samples in order to validate the internal control genes for real-time quantitative PCR detection.

2.6. Hepatic Fatty Acid Oxidation. Enzyme sources were prepared according to the method developed by Hulcher and Oleson [17] with slight modification. Fatty acid β-oxidation (β-oxidation) was measured spectrophotometrically by monitoring the reduction of NAD to NADH in the presence of palmitoyl CoA as described by Lazarow [18], with slight modification. The amount of protein in the enzyme sources was determined with the Bradford method using bovine serum albumin as the standard.

2.7. Histopathological Analysis. The liver and epididymal WAT were fixed in 10% formalin buffer solution and then routinely processed for paraffin embedding. The 4 μm sections of each tissue were stained with hematoxylin eosin (H&E) and the stained tissues were observed under an optical microscope (Zeiss Axioscope, Germany) with a magnifying power of ×200. The epididymal adipocyte size was measured by using Motic Images Plus 2.0 ML (Motic).

2.8. Statistical Analysis. The statistical analyses were performed with the statistical package for social science software program (SPSS Inc., Chicago, IL, USA). Significant

Table 2: Effects of GPE alone or combined with OFE on food intake, body weight, plasma adipokines and lipids levels in HFD-fed mice.

	CON	GPE	GPE + OFE
Food intake (g/day)	3.23 ± 0.08	3.00 ± 0.10	3.21 ± 0.05
Initial body weight (g)	18.95 ± 0.07	18.65 ± 0.45	18.85 ± 0.51
Final body weight (g)	33.74 ± 1.03	31.87 ± 0.81	31.15 ± 0.94
Adiponectin (μg/mL)	9.67 ± 0.27	10.43 ± 0.27	10.41 ± 0.31
Adipsin (μg/m)	0.99 ± 0.04	1.00 ± 0.03	1.08 ± 0.03
Free fatty acid (mmol/L)	0.47 ± 0.04^{a}	0.45 ± 0.04^{a}	0.29 ± 0.04^{b}
Triglyceride (mmol/L)	0.92 ± 0.09	1.03 ± 0.08	0.90 ± 0.03
Phospholipid (mmol/L)	2.24 ± 0.06	2.39 ± 0.08	2.27 ± 0.04
Total cholesterol (mmol/L)	4.21 ± 0.16	4.66 ± 0.28	4.16 ± 0.18
HDL cholesterol (mmol/L)	0.76 ± 0.06^{a}	0.96 ± 0.09^{b}	0.84 ± 0.03^{ab}
HTR (%)	17.99 ± 1.00	20.30 ± 0.89	20.31 ± 0.59
AI	4.69 ± 0.34^{a}	4.00 ± 0.21^{b}	3.96 ± 0.15^{b}

Data are mean ± SE ($n = 10$). ^{ab}Means not sharing a common letter are significantly different among groups at $P < 0.05$. CON: high-fat diet control; GPE: high-fat diet plus grape pomace extract (0.5%, w/w); GPE + OFE: high fat diet plus grape pomace extract (0.5%, w/w) combined with omija fruit extract (0.05%, w/w); HTR: HDL cholesterol/total cholesterol; AI atherogenic index; (total-cholesterol − HDL cholesterol)/HDL cholesterol.

differences between the means were determined by one-way ANOVA. Duncan's multiple-range test was performed if differences were identified between the groups at $P < 0.05$. All data are expressed as the means with their standard error of the mean.

3. Results

3.1. Food Intake, Body Weight Gain, and WAT Weight and Size. Supplementation with GPE did not significantly reduce body weight gain, WAT weight, and WAT cell size in mice fed a HFD (Figures 1(a)–1(c)). However, mice supplemented with GPE + OFE showed a significantly lower body weight gain compared to the HFD control (Figure 1(a)). Furthermore, supplementation with GPE + OFE markedly reduced the weight of visceral WAT (including epididymal, perirenal, retroperitoneal, and mesenteric WAT), subcutaneous WAT, scapular WAT, and total WAT as well as epididymal adipocyte size in HFD-fed mice (Figures 1(b) and 1(c)). There was no significant change in food intake among the groups (Table 1).

3.2. Plasma Adipokine Levels. Similar to the results for body weight gain and body fat, supplementation with GPE + OFE significantly lowered plasma leptin and PAI-1 levels compared to the HFD control group (Figure 1(d)). Furthermore, the combination of GPE and OFE significantly lowered plasma TNF-α, IL-6, and MCP-1 levels in the HFD-fed mice. The plasma TNF-α level was also lowered in the GPE group compared to the HFD control group. Mice supplemented with GPE + OFE exhibited a 24% lower plasma resistin level compared to the HFD control mice, although the differences were not statistically significant. Plasma adiponectin and adipsin levels were not significantly altered by GPE alone or with OFE (Table 1).

3.3. Expression of Genes Involved in Adipogenesis and Inflammation in Epididymal WAT. We investigated the potential mechanisms by which the combination of GPE and OFE might attenuate the HFD-induced activation of adipogenesis in the epididymal WAT of mice (Figure 1(e)). Supplementation with GPE alone or with OFE significantly downregulated the mRNA level of adipogenic transcription factor, PPARγ, in the epididymal WAT of HFD-fed mice. Additionally, the mRNA levels of several key adipogenic target genes, FAS, ME, and LPL, were significantly downregulated in the GPE + OFE-supplemented mice compared to the HFD control mice. Mice supplemented with GPE also showed lower mRNA levels of adipogenic genes, FAS (26%), ME (27%), and LPL (18%), compared to the HFD control mice, but the differences were not statistically significant.

Next, the mRNA expression of key genes involved in inflammation was measured in the epididymal WAT of mice (Figure 1(e)). Supplementation with GPE resulted in a significantly lower NF-κB mRNA level in epididymal WAT, and it tended to lower the epididymal TNF-α and MCP-1 mRNA levels by 33% and 23%, respectively, compared to the HFD control group. GPE + OFE also significantly downregulated the mRNA levels of proinflammatory cytokine genes, such as TNF-α, IL-6, and MCP-1, as well as transcription factor, NF-κB, in epididymal WAT compared to the HFD control mice.

3.4. Plasma and Hepatic Lipid Levels. There were no significant differences in plasma triglyceride, phospholipid, and total-cholesterol levels among the groups (Table 2). However, mice supplemented with GPE showed a significantly higher level of plasma HDL cholesterol and a lower atherogenic index (AI) compared to the HFD control mice. Supplementation with GPE + OFE also tended to increase the plasma HDL-cholesterol level and significantly lower the AI value compared to the control group. Furthermore, the plasma free fatty acid level was significantly lowered in the GPE + OFE group only, but not in the GPE group, compared to the HFD control group.

The contents of hepatic free fatty acid, triglyceride, and cholesterol were significantly lower in the GPE + OFE group than in the control group, whereas GPE alone had no

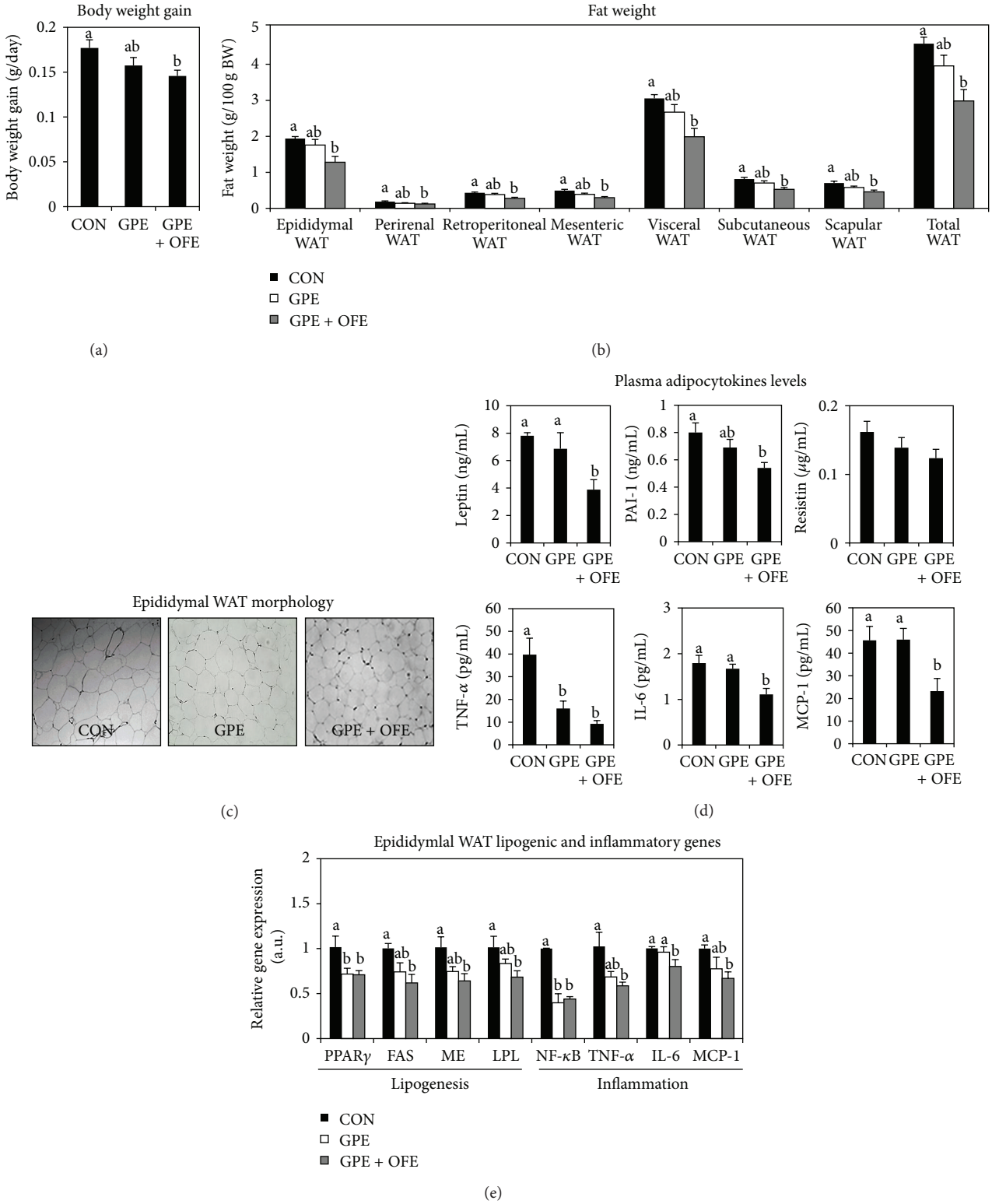

Figure 1: Effect of GPE alone or combined with OFE on body weight gain (a), body fat weight (b), epididymal WAT morphology (c), plasma adipocytokine levels (d) and epididymal WAT lipogenic and inflammatory gene expressions (e) in HFD-fed mice. (a), (b), (d), and (e) Data are the Means ± SE ($n = 10$). [ab]Means not sharing a common letter are significantly different among the groups at $P < 0.05$. (c) Representative photomicrographs of epididymal WAT are shown at ×200 magnification ($n = 10$). CON: high-fat diet control; GPE: high fat diet plus grape pomace extract (0.5%, w/w); GPE + OFE: high fat diet plus grape pomace extract (0.5%, w/w) combined with omija fruit extract (0.05%, w/w); WAT: white adipose tissue; PPARγ: peroxisome proliferator-activated receptor γ; FAS: fatty acid synthase; ME: malic enzyme; LPL: lipoprotein lipase; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; MCP-1: monocyte chemotactic protein-1.

significant effect on these hepatic lipid contents (Figure 2(a)). Morphological analysis of the liver also indicated that the combination of GPE and OFE supplementation markedly decreased lipid accumulation shown by the decreases in both the number and size of hepatic lipid droplets compared to the HFD control group, and the liver weight in GPE + OFE was lower than that in the control group (Figures 2(b) and 2(c)). Overall, dietary GPE + OFE supplementation can ameliorate HFD-mediated hepatic steatosis in diet-induced obese mice.

3.5. Hepatic Lipogenic and Inflammatory Gene Expression, Fatty Acid Oxidation, and Plasma AST and ALT. We determined the mRNA expression of genes involved in lipogenesis as well as fatty acid oxidation in the liver to investigate how GPE + OFE ameliorates hepatic fat accumulation (Figures 2(d) and 2(e)). The mRNA expressions of genes for fatty acid uptake and *de novo* fatty acid synthesis, including CD36, FAS, ACC1, SCD1, ME, and LPL, were markedly lower in the GPE + OFE-supplemented mice than in the HFD control mice. Furthermore, mRNA expressions of key cholesterol regulating genes (HMGR and ACAT) as well as fatty acid oxidation-related genes (ACOX1 and UCP2) were significantly lowered in the GPE + OFE group than in the control mice. In contrast, hepatic fatty acid β-oxidation was significantly elevated in both the GPE and GPE + OFE groups than that of the control group.

Next, we explored the effect of GPE alone or with OFE on hepatic inflammatory gene expression (Figure 2(f)). The mRNA expression of the proinflammatory cytokines, such as IL-6 and TNF-α, was significantly downregulated in the liver of the GPE + OFE mice compared to the control mice. GPE + OFE-supplemented mice also showed a significant decrease in hepatic NF-κB mRNA expression, but GPE alone did not significantly alter the expression of these proinflammatory genes in the liver.

We also investigated whether the GPE + OFE can protect the liver from HFD-induced liver damage (Figure 2(g)). Supplementation with GPE + OFE group significantly reduced plasma ALT and AST levels compared to the HFD control mice. Mice supplemented with GPE also exhibited 7% and 14% lower plasma ALT and AST levels compared to the HFD control mice although differences were not statistically significant. These results indicate that HFD-associated liver dysfunction could be ameliorated by GPE + OFE.

4. Discussion

The present study first examined whether the long-term supplementation of GPE alone or with OFE could induce protective effects against HFD-induced obesity and NAFLD in C57BL/6J mice. To investigate the underlying mechanisms, we focused on the expression profiles of genes related to lipogenesis and inflammation in the WAT and liver, and on the levels of plasma adipocytokines. In the present study, we demonstrated that the combination of GPE and OFE led to a favorable effect on adiposity, hepatic steatosis, and inflammation in HFD-induced obese mice, although GPE alone did not significantly decrease body weight, body fat, and hepatic lipid accumulation.

A previous study reported that GPE supplementation (approximately 450 mg/kg body weight/day) did not significantly suppress HFD-induced body weight gain in rats [9]. Recently, Hogan et al. [8] also demonstrated that supplementation with GPE (approximately 250 mg/kg body weight/day) in a HFD for 12 weeks did not affect body weight gain but reduced the levels of proinflammatory maker and C-reactive protein in the plasma of mice. In agreement with these previous studies, we observed that dietary GPE (approximately 470 mg/kg body weight/day) did not induce a significant lowering effect on body weight gain and adiposity, but the plasma TNF-α level and epididymal NF-κB mRNA expression were significantly lower in the GPE group than those in the control group, suggesting dietary GPE has a potential antiinflammatory effect.

In contrast to GPE alone, the combination of GPE and OFE significantly lowered body weight gain, WAT weight and WAT adipocyte size in mice fed a HFD. The decrease in WAT weight, and adipocyte size observed in the GPE + OFE-supplemented obese mice was associated with the downregulated mRNA expression of genes involved in *de novo* fatty acid syntheses, FAS and ME, in epididymal WAT. FAS is a key enzyme responsible for *de novo* biosyntheses of long-chain fatty acids from acetyl CoA and malonyl CoA in the presence of NADPH, and ME generates NADPH to be consumed in fatty acid syntheses. Expression of both enzymes is regulated at the transcriptional level and is sensitive to nutritional and hormonal regulation, [19, 20]. In addition, the decrease in mRNA expression of LPL and PPARγ could also contribute to the amelioration of adiposity with supplementation of GPE and OFE in HFD-fed mice. LPL is expressed at high levels in adipose tissue and hydrolyzes circulating triglyceride-rich lipoproteins (very low-density lipoproteins and chylomicrons) to generate free fatty acids, which are subsequently reesterified for storage as triglycerides in adipocytes [21]. The level of LPL mRNA was significantly increased in the WAT of HFD-fed mice and obese subjects [22, 23], whereas WAT-specific LPL deficiency diminished body weight and fat mass in *ob/ob* mice although it increased endogenous fatty acid syntheses in WAT [24]. PPARγ is a main transcription factor that regulates many adipocyte genes encoding proteins and enzymes involved in adipogenesis and lipid metabolism, including fatty acid syntheses, fatty acid uptake and storage [25]. Collectively, the decreased mRNA expression of adipogenic genes (FAS, ME, and LPL) and their transcription factor (PPARγ) in response to GPE plus OFE seemed to contribute to a significant reduction in body fat accumulation in the HFD-induced obese mice. Similarly, a traditional Korean herbal medicine that includes omija (Taeumjowitang) significantly lowered body weights, body fat, and serum leptin level in HFD-fed mice [13] and obese Korean children [26], and another pilot study that is evaluating the effect of Taeumjowitang on obesity in Korean adults is currently in progress [27]. We also found that a 12-week dietary supplementation with Taeumjowitang at two doses (1.5%, 3%, w/w) dose-dependently lowered body weight and body fat mass in mice fed a HFD (60% kcal from fat based on AIN-93G diet) (Choi J. Y. and Choi M. S., unpublished data 2013). Furthermore, one preliminary

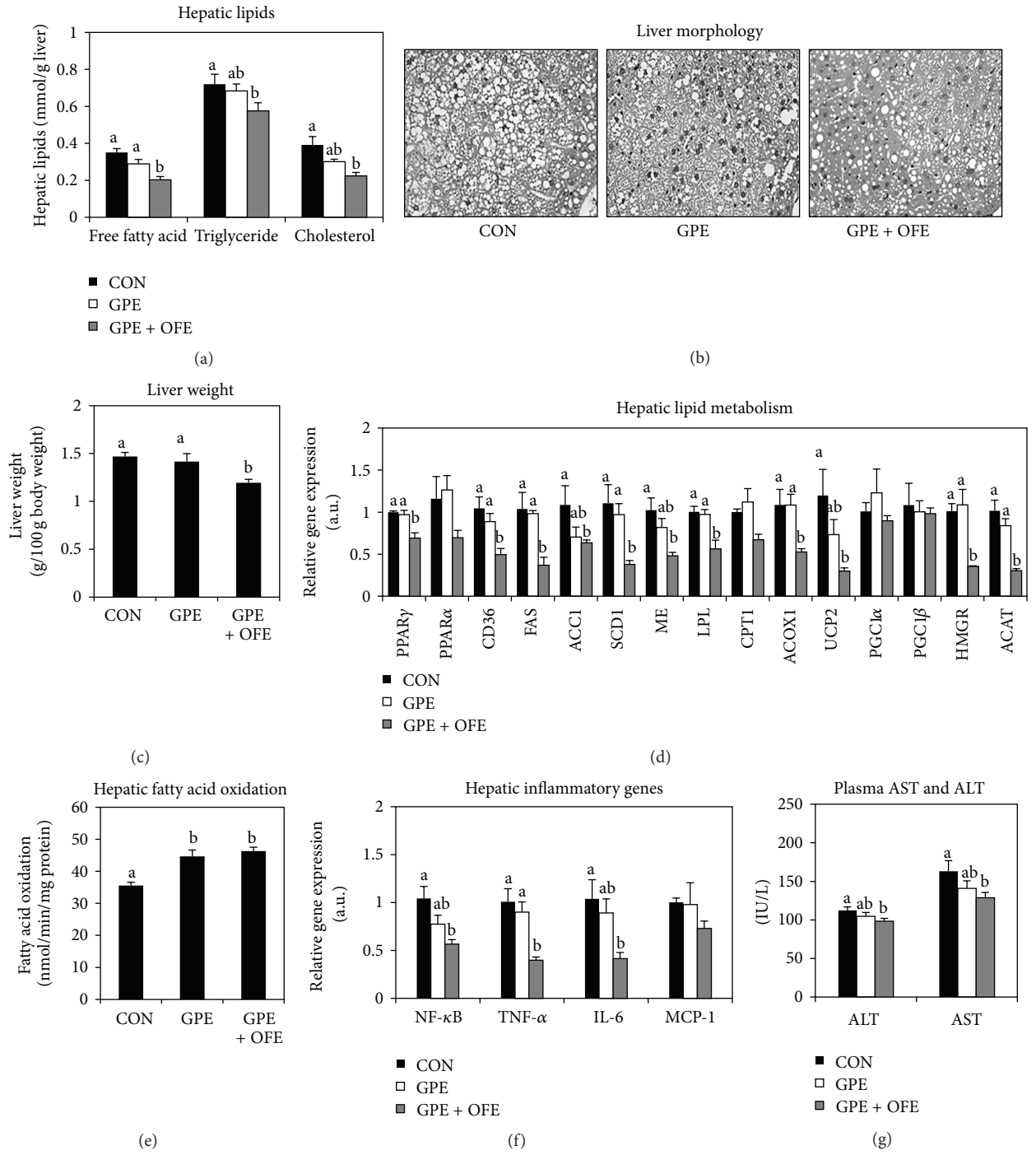

Figure 2: Effect of GPE alone or combined with OFE on hepatic lipid contents (a), liver morphology (b), liver weight (c) and hepatic lipogenic and inflammatory gene expressions and fatty acid oxidation (d–f) in HFD-fed mice. (a), (c)–(f) Data are the Means ± SE ($n = 10$). [ab]Means not sharing a common letter are significantly different among the groups at $P < 0.05$. (b) Representative photomicrographs of the liver are shown at ×200 magnification ($n = 10$). CON: high-fat diet control; GPE: high fat diet plus grape pomace extract (0.5%, w/w); GPE + OFE: high fat diet plus grape pomace extract (0.5%, w/w) combined with omija fruit extract (0.05%, w/w); WAT: white adipose tissue; PPARγ, peroxisome proliferator-activated receptor γ; FAS: fatty acid synthase; ME: malic enzyme; LPL: lipoprotein lipase; HMGR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; ACAT: acyl-CoA cholesterol acyl transferase; NF-κB: nuclear factor-κB; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; MCP-1: monocyte chemotactic protein-1.

study in exercise-trained rats fed a AIN-76 semisynthetic diet containing two doses (0.002 and 0.006 g/kg body weight) of OFE for 6 weeks demonstrated beneficial effects on body fat accumulation in a dose-dependent manner (Kim Y. J., Jung U. J., and Choi M. S., unpublished data 2010-2011). The body fat lowering effect of OFE was related to the decreased activity of adipose enzymes involved in fatty acid syntheses (fatty acid synthase and malic enzyme). Notably, the dose of OFE tested in the experiment using exercise-trained rats (0.002 g/kg body weight and 0.006 g/kg body weight) was lower than that of the present experiment using high-fat diet mice (0.05 g/kg body weight). However, the net effect of OFE needs to be elucidated in order to validate a possible mechanism regarding the synergistic action of GPE plus OFE in diet-induced obese mice, which is in progress at our laboratory presently.

Along with its active role in regulating energy balance, WAT secretes a variety of adipocytokines that have important physiologic functions, including cytokines (TNF-α and IL-6), chemokines (MCP-1), leptin, PAI-1, resistin, adiponectin and adipsin [28]. Leptin is principally produced by adipocytes and PAI-1 is also largely produced by visceral adipocytes [29]. In addition, several proinflammatory cytokines and chemokines such as IL-6, TNF-α and MCP-1 are secreted by adipocytes (as well as nonadipocyte cells such as macrophages), thus contributing to the chronic inflammatory state often observed in obesity [30]. The circulating levels of several adipocytokines, such as TNF-α, IL-6, MCP-1, leptin, and PAI-1, are elevated in obesity and are reduced with weight loss [31–33]. In particular, visceral WAT from obese subjects has been found to secrete higher levels of TNF-α, IL-6, MCP-1, leptin, and PAI-1 but lower levels of adiponectin compared to lean subjects [34–37]. We found that supplementation with GPE + OFE significantly lowered the plasma leptin, PAI-1, MCP-1, TNF-α and IL-6 levels compared to the control group although it did not alter the plasma adiponectin level. In addition, GPE plus OFE significantly downregulated mRNA expression of proinflammatory cytokines (TNF-α, IL-6) and chemokines (MCP-1) as well as its transcription factor NF-κB in epididymal WAT. There was a strong positive correlation between circulating and adipose tissue levels of IL-6 and TNF-α in obese individuals, which suggested that circulating proinflammatory cytokines originate from adipose tissue [38]. MCP-1 mRNA expression was also increased in the epididymal WAT of diet-induced obese mice, along with the plasma MCP-1 level [39]. The transcription factor NF-κB is well known to up-regulate the mRNA expression of many inflammation-related genes, including TNF-α, IL-6 and MCP-1 [40].

NAFLD is characterized by the accumulation of fat and inflammatory changes in the liver. Visceral adiposity is closely related to NAFLD [41] because free fatty acids and proinflammatory factors released from visceral WAT are directly transported to the liver by the portal vein and may contribute to hepatic steatosis and inflammation [42]. Higher concentrations of circulating free fatty acids are observed in obese persons and animals, particularly those with abdominal obesity [43]. Excess free fatty acid availability in plasma leads to increased hepatic free fatty acid uptake and syntheses of triglyceride, which can be stored as lipid droplets within hepatocytes. In subjects with NAFLD, nearly 60% of the hepatic triglycerides were derived from circulating free fatty acid in a fasting state, suggesting plasma free fatty acid is the main contributor to triglyceride accumulation in the liver [43]. Interestingly, in the present study, the supplementation of GPE + OFE significantly decreased the plasma free fatty acid level as well as hepatic free fatty acid and triglyceride contents in HFD-fed mice. The hepatic free fatty acid level was higher in subjects with morbid obesity or alcoholic liver disease compared to controls [44]. Larter et al. [45] suggested that hepatic free fatty acid accumulation itself is a lipotoxic insult for liver injury in fatty liver disease. We also found that the GPE + OFE diet significantly downregulated mRNA expression of lipogenic transcription factor PPARγ and their target genes, CD36, FAS, ACC1, SCD1, ME and LPL, in the liver. The expression of genes involved in fatty acid oxidation (ACOX1 and UCP2) was also decreased in GPE + OFE group compared to the control group, which seemed to be meditated by adaptation process of energy metabolism. Similar to our results, genetic ablation of PPARγ in *ob/ob* mice significantly downregulated expression of hepatic genes involved in fatty acid β-oxidation (PPARα, ACOX, and UCP2) as well as fatty acid uptake (CD36) and syntheses (FAS, SCD1 and LPL) [46]. Furthermore, GPE + OFE feeding markedly decreased hepatic cholesterol accumulation, in part, by suppressing mRNA expression of hepatic HMGR and ACAT which are rate-limiting enzymes of cholesterol syntheses and esterification, respectively. Accordingly, a decrease in plasma free fatty acids and hepatic fatty acid uptake, *de novo* fatty acid and cholesterol syntheses may partially explain the improvement of hepatic lipid accumulation and the subsequent lipid droplet formation observed in GPE + OFE-supplemented obese mice. Although the mRNA expression of fatty acid oxidation-related genes such as ACOX1 and UCP2 in liver was downregulated in GPE + OFE-supplemented mice compared to that in the control mice, the hepatic fatty acid β-oxidation was activated by GPE alone and with OFE, indicating presence of the posttranslational regulation. One possibility is that GPE with or without OFE may promote fatty acid β-oxidation by regulating the ratio of NADH/NAD^+ and acetyl CoA/CoA, since an increase in the NADH/NAD^+ or acetyl CoA/CoA ratios results in inhibition of fatty acid β-oxidation [47]. To clearly understand whether GPE alone and GPE with OFE regulate the level of the products formed during β-oxidation, further experiments are needed to be performed.

Referring to the previously ment coned, proinflammatory factors from portal circulation, potentially produced in visceral WAT, might affect hepatic steatosis and inflammation [42]. Visceral WAT was independently associated with hepatic inflammation and fibrosis in NAFLD subjects and serum IL-6 levels, which correlated with visceral fat, independently predicting an increase in hepatic inflammation [1]. Leptin is also reported to be a mediator of liver fibrosis after chronic liver injury in mouse models [48]. The liver can also produce and secrete inflammatory cytokines. Cai et al. [49] suggested that the presence of hepatic steatosis is closely related to chronic hepatic inflammation through NF-κB activation and downstream cytokine production. Selective

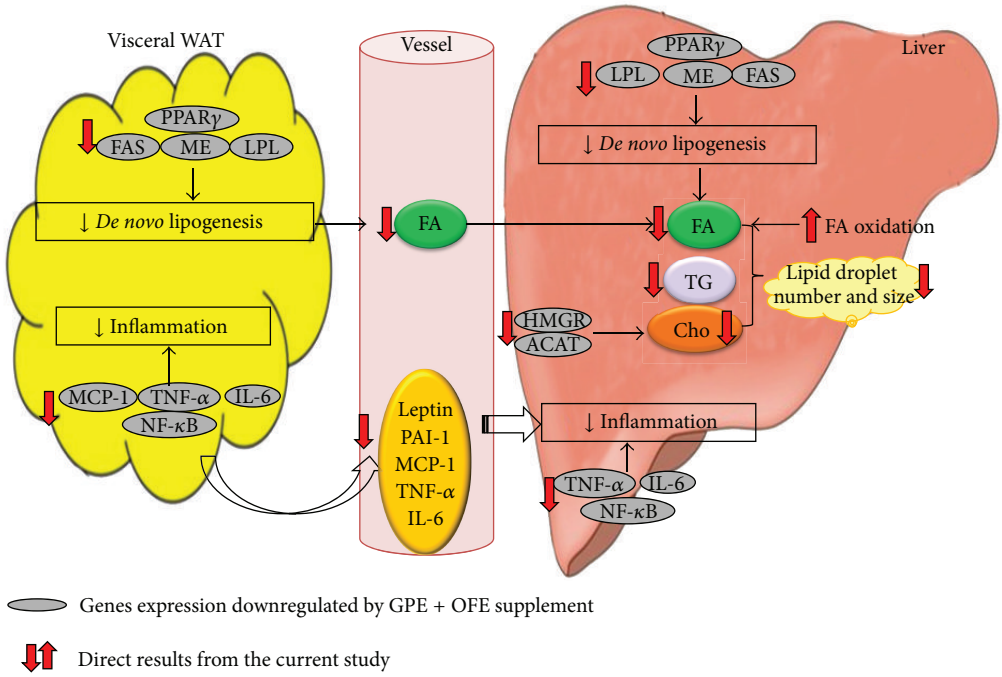

Figure 3: Schematic of the proposed mechanism underlying the protective effect of GPE combined with OFE on lipid metabolism and inflammation in the liver and WAT adipose tissue of HFD-fed mice. Supplementation with GPE + OFE significantly lowered body weight gain and body fat mass by partly suppressing mRNA expression of lipogenic genes (FAS, ME, and LPL) and its transcription factor (PPARγ) in epididymal WAT. The mRNA expression of hepatic genes involved in fatty acid and cholesterol syntheses (PPARγ FAS, ME, LPL, HMGR, and ACAT) was also downregulated by supplementation with GPE + OFE, whereas GPE + OFE supplementation activated hepatic fatty acid oxidation, leading to decreased hepatic lipid accumulation. Furthermore, the supplementation of GPE + OFE significantly decreased the levels of plasma adipocytokines (leptin, PAI-1, MCP-1, TNF-α, and IL-6) as well as the mRNA expression of proinflammatory transcription factor, NF-κB, and its target genes, including MCP-1, TNF-α and IL-6, in the liver and epididymal WAT, which may be related to the improvement in obesity and NAFLD. ACAT: acyl-CoA:cholesterol acyltransferase; Cho: cholesterol; FA: fatty acid; FAS: fatty acid synthase; HMGR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase; LPL: lipoprotein lipase; IL-6: interleukin-6; MCP-1: monocyte chemotactic protein-1; ME: malic enzyme; NF-κB: nuclear factor-κB; PPARγ: peroxisome proliferator-activated receptor γ; TG: triglyceride; TNF-α: tumor necrosis factor-α.

hepatocellular activation of NF-κB increased production of hepatic inflammatory cytokines such as IL-6 and TNF-α in mice to a similar extent as observed in HFD-fed obese mice, whereas liver-specific NF-κB inhibition prevented HFD-induced inflammatory gene expression [49]. IL-6 is the inflammatory cytokine that is synthesized in the liver as well as the WAT and increases in NAFLD [50]. TNF-α also plays a major role in the development of NAFLD by upregulating lipogenic gene expression, increasing mitochondrial generation of reactive oxygen species, promoting hepatocyte apoptosis and recruiting inflammatory cells to the liver [51, 52]. The inhibition of TNF-α improved NAFLD in HFD-fed *ob/ob* mice [53], while TNF-α receptor-deficient mice protected against hepatic steatosis [54]. In the present study, similar to WAT, a combination of GPE and OFE significantly downregulated the proinflammatory transcription factor NF-κB and its target genes, TNF-α and IL-6, in the liver which may be one potential mechanism for improving NAFLD in HFD-induced obese mice. These protective effects against NAFLD were also supported by significant decreases in plasma levels of ALT and AST in GPE + OFE-supplemented obese mice, since the elevated liver aminotransferase (ALT and AST) is positively correlated to 90% patients with nonalcoholic steatohepatitis, characterized by a liver lipid accumulation combined with hepatic inflammation [55].

5. Conclusions

The present study demonstrated that combined supplementation with GPE and OFE significantly ameliorated adiposity and hepatic steatosis more than the responses to GPE alone. It seemed that the expression of genes involved in the multiple steps of lipid accumulation and inflammation in the liver and WAT was downregulated in response to the GPE plus OFE diet (Figure 3). These findings provide information on the molecular mechanisms by which the combination of GPE and OFE influences the regulation of body fat and hepatic fat accumulation and inflammation, and further suggest that GPE plus OFE may have a potential use for regulating adiposity and NAFLD in obese mice.

Authors' Contribution

Su-Jung Cho and Un Ju Jung contributed equally to this work.

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Research Article

Pharmacometrics of 3-Methoxypterostilbene: A Component of Traditional Chinese Medicinal Plants

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3-Methoxypterostilbene is a naturally occurring stilbene with potential in the treatment of diabetes. The preclinical pharmacokinetics and pharmacodynamics of 3-methoxypterostilbene were evaluated in the present study. The right jugular veins of male Sprague-Dawley rats were cannulated. The rats were dosed 10 mg/kg or 100 mg/kg of 3-methoxypterostilbene intravenously (IV) or orally (PO), respectively. Serum and urine samples were analyzed using a previously validated reversed-phase HPLC method. Serum AUC, serum $t_{1/2}$, urine $t_{1/2}$, Cl_{total}, and Vd for IV dosing were 48.1 ± 23.8 μg/h/mL, 18.9 ± 10.9 h, 9.54 ± 1.51 h, 47.8 ± 23.7 L/h/kg, and 5.11 ± 0.38 L/kg, respectively (mean ± SEM, $n = 4$) . Serum AUC, serum $t_{1/2}$, urine $t_{1/2}$, Cl_{total}, and Vd for PO dosing were 229 ± 44.6 μg/h/mL, 73.3 ± 8.91 h, 20.6 ± 3.01 h, 0.48 ± 0.008 L/h/kg, and 52.0 ± 10.5 L/kg, respectively (mean ± SEM, $n = 4$). Bioavailability of the stilbene was determined to be 50.6% ± 10.0%. A 3-methoxypterostilbene glucuronidated metabolite was detected in both serum and urine. 3-Methoxypterostilbene exhibited antidiabetic activity including α-glucosidase and α-amylase inhibition as well as concentration-dependent antioxidant capacity similar to resveratrol. 3-Methoxypterostilbene also exhibited anti-inflammatory activity. 3-Methoxypterostilbene appears to be a bioactive compound and may be useful in reducing postprandial hyperglycemia.

1. Introduction

3-Methoxypterostilbene (*trans*-3,3′-5-trimethoxy-4′-hydroxystilbene), $C_{17}H_{18}O_4$, MW 286.324 g/mol (Figure 1), is a naturally occurring stilbene [1, 2] that can also be easily synthesized using simple combinatorial synthesis [3–7]. 3-Methoxypterostilbene is a structural analogue of resveratrol, which has demonstrated a myriad of potential prohealth effects including anticancer, cardioprotective, anti-inflammatory, neuroprotective, and antiobesity properties [8]. However, it differs from resveratrol in its physicochemical properties. The predicted octanol water partition coefficient (XLogP) for 3-methoxypterostilbene is 3.54 ± 0.49 [9] and the experimentally determined XLogP for resveratrol is 1.53 ± 0.01 [10].

3-Methoxypterostilbene has been isolated in two plants used in traditional Chinese medicine. The compound has been found in *Sphaerophysa salsula* (also known as *Swainsona salsula*), a shrub called "ku ma du," used for the treatment of hypertension [1]. 3-Methoxypterostilbene has also been found as an aglycone of a stilbene glycoside in the commonly used *Rheum palmatum* (Chinese rhubarb), called "da huang," used to treat digestive disorders [2].

Due to 3-methoxypterostilbene's structural similarity to resveratrol and its presence in traditional Chinese medicinal plants, 3-methoxypterostilbene may also possess potential health benefits. However, information in the literature on the bioactivity of 3-methoxypterostilbene is scant. In a report seeking to identify biologically active piceatannol (another structural analogue of resveratrol) analogs with greater stability than piceatannol, 3-methoxypterostilbene was reported to demonstrate significantly greater activity in a 9 KB cytotoxicity assay as well as a crown-gall plant antitumor (potato disk) assay than piceatannol [3]. 3-Methoxypterostilbene proved to be as effective as resveratrol in the inhibition of bacterial lipopolysaccharide-induced tissue factor expression in human peripheral blood mononuclear cells in a

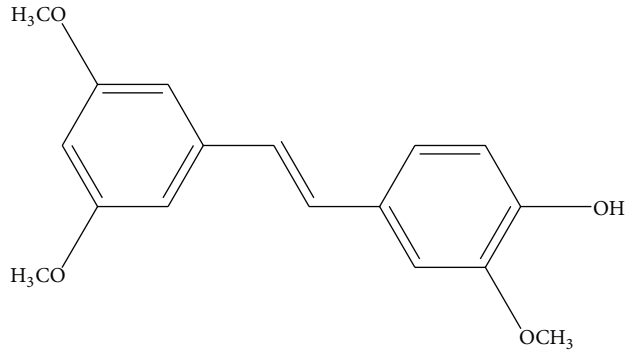

Figure 1: Chemical structure of 3-methoxypterostilbene.

study investigating resveratrol derivatives and coronary heart disease [11]. Several studies have also demonstrated that 3-methoxypterostilbene possesses greater apoptotic-inducing activity than resveratrol in human leukemia-derived HL60 cells [4, 12, 13].

With the ever-increasing natural products canon of knowledge and growing concern for obesity-related diseases, there has been much focus on the use of natural products to aid in the management and treatment of type 2 diabetes. Resveratrol has been extensively studied in animals for its potential to treat obesity and type 2 diabetes [8]. Resveratrol appears to be able to increase insulin sensitivity in various animal models of insulin resistance [14–18]. Additionally, animal studies using models that consisted of genetically obese rats and mice with dietary induced obesity or chemically induced diabetes found that resveratrol reduced blood glucose levels, which is important in the management of type 2 diabetes and prediabetic patients [14, 15, 19–25]. Thus, structural analogues of resveratrol may also possess antidiabetic properties similar to those of resveratrol.

To our knowledge, there have been no studies evaluating the pharmacokinetics, disposition, or the *in vivo* metabolism of 3-methoxypterostilbene other than that of a single rat previously reported by Martinez et al. [9]. The objectives of the present study are to examine the pharmacokinetic disposition of 3-methoxypterostilbene as well as its *in vivo* metabolism and antidiabetic properties. To facilitate this, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for quantification of 3-methoxypterostilbene in biological matrices using ultraviolet detection [9]. Additionally, the objectives of this study were to investigate select biological activities of 3-methoxypterostilbene and perform a content analysis on commercially available dried traditional Chinese herbs reported to contain 3-methoxypterostilbene using the previously validated RP-HPLC method. For the first time, to our knowledge, the preclinical pharmacokinetics, antioxidant activity, cyclooxygenase-1 and -2 (COX-1 and -2) inhibition, and α-glucosidase and α-amylase inhibitory activity of 3-methoxypterostilbene are reported.

2. Materials and Methods

2.1. Chemicals and Reagents. 3-Methoxypterostilbene was provided by the Sabinsa Corporation (Piscataway, NJ, USA) and pinosylvin was purchased from Sequoia Research Products Ltd. (Oxford, UK). HPLC-grade acetonitrile and water were purchased from J. T. Baker (Phillipsburg, NJ, USA). β-Glucuronidase type IX A (β-glucuronidase), poly(ethylene glycol) (PEG) 400, dimethyl sulfoxide (DMSO), α-glucosidase from *Saccharomyces cerevisiae*, 4-nitrophenyl α-D-glucopyranoside, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), resveratrol, ibuprofen, etodolac, and α-amylase from porcine pancreas type VI-B were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amylase HR reagent was purchased from Megazyme International Ireland (Wicklow, Ireland). β-Glucosidase from almonds was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dried *Swainsona salsula* extract was provided by DaXingAnLing Snow Lotus Herb Bio-technology Co., Ltd. (Heilongjiang, China) and dried da huang (Chinese rhubarb) was purchased from Kwok Shing Ent. Ltd. (Scarborough, ON, Canada). The antioxidant activity kit and cyclooxygenase-1 and -2 inhibitor screening kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

2.2. Chromatographic System and Conditions. The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of two LC-10A pumps, a SIL-10AF autoinjector, a SPD-M10A photodiode array detector, and a SCL-10A system controller. Data collection and integration were achieved using Shimadzu EZ Start Class VP (version 7.4) software. A Phenomenex Luna $C_{18}(2)$ (5 μm, 250 × 4.60 mm) column was used. The mobile phase consisted of acetonitrile and water (62 : 38 v/v) that were filtered and degassed under reduced pressure prior to use. Pinosylvin was used as the internal standard. Isocratic separation at ambient temperature using a flow rate of 1.05 mL/min was employed. Ultraviolet detection was carried out at 327 nm. Validation indicated that the precision of the assay was <12% (RSD) and was within 12% at the limit of quantification (LOQ) (0.05 μg/mL). The bias of the assay was <15% and was within 13% at the LOQ [9].

2.3. Animals and Surgical Procedures. Male Sprague-Dawley rats (≈200 g) were obtained from Simonsen Laboratories (Gilroy, CA, USA) and allowed food (Purina Rat Chow 5001) and water *ad libitum* upon arrival to the vivarium. Rats were housed in a temperature- and humidity-controlled facility with a 12 h light/dark cycle. Prior to the first day of the pharmacokinetic experiment, the rats were anesthetized using isoflurane (IsoFlo, Abbott Laboratories, Abbot Park, IL, USA) coupled with an oxygen regulator, and monitored by pedal reflex and respiration rate to maintain a surgical plane of anesthesia. The right jugular veins of the rats were cannulated with sterile silastic cannulas (Dow Corning, Midland, MI, USA). After cannulation, Intramedic PE-50 polyethylene tubing (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was exposed through the dorsal

skin. The cannulas were flushed with nonheparinized 0.9% sterile saline solution. The animals were placed in metabolic cages to recover and fasted overnight.

Animal ethics approval was obtained from the University of Manitoba Office of Research Ethics and Compliance.

2.4. Dosages. No pharmacokinetics studies are reported in the literature for 3-methoxypterostilbene. Despite the paucity of 3-methoxypterostilbene pharmacokinetic studies in the literature, studies on other stilbenes have reported intravenous (IV) dosage ranges from 10 to 20 mg/kg [26–30] and from 50 to 300 mg//kg for oral (PO) dosage [27, 31, 32]. In keeping with the literature, doses of 10 mg/kg IV and 100 mg/kg PO were chosen.

2.5. Pharmacokinetic Study. Male Sprague-Dawley rats (n = 8, average weight 200 g) were cannulated as described in the Animals and Surgical Procedures section. The animals were placed in metabolic cages following surgery where they were recovered overnight and fasted for 12 h prior to dosing. On the day of the experiment, the animals were dosed with 3-methoxypterostilbene in PEG 400 either IV (10 mg/kg, n = 4) or PO (100 mg/kg, n = 4). After dosing, a series of whole blood samples (0.5 mL) were collected at 0, 1, and 15 min, and 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 h and 0, 0.25, 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 h for IV and PO dosed rats, respectively. The cannulas were flushed with 0.5 mL 0.9% nonheparinized saline solution after each sample collection. The samples were collected into regular 2.0 mL Eppendorf tubes, centrifuged at 10,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA), and the serum was collected. The serum was divided into two 0.1 mL fractions and placed into regular 2.0 mL Eppendorf tubes labeled as free and total serum samples. Samples were stored at −20°C until analyzed. Urine samples were also collected at 0, 2, 6, 12, 24, 48, and 72 h following 3-methoxypterostilbene administration. The volumes of urine produced by the rats were recorded, and two 0.1 mL aliquots were collected into separate prelabeled regular polypropylene microcentrifuge tubes and labeled as free and total urine samples and stored at −20°C until analyzed. At 72 h after dose, the animals were euthanized exsanguinated, and serum was collected.

2.6. Serum and Urine Sample Preparation. Serum and urine samples (0.1 mL) were run in duplicate with or without the addition of 20 μL of 500 U/mL β-glucuronidase and incubated in a shaking water bath at 37°C for 2 h to liberate any glucuronide conjugates [33]. The proteins present in the serum samples were precipitated using 1 mL of −20°C acetonitrile. Urine and serum samples were vortexed (Vortex Genie-2, VWR Scientific, West Chester, PA, USA) for 30 s and centrifuged at 10,000 rpm for 5 min. The supernatants were transferred to new, labeled 2.0 mL Eppendorf microcentrifuge tubes. The samples were evaporated to dryness by a stream of nitrogen gas. The residues were reconstituted with 200 μL of mobile phase, vortexed for 30 s, and centrifuged at 10,000 rpm for 5 min. The supernatants were transferred to HPLC vials, and 100 μL was injected into the HPLC system for each sample.

β-Glucuronidase from *E. coli* type IX-A acts to specifically cleave the sugar moiety attached to the parent compound from the 3-methoxypterostilbene glucuronide back into the aglycone (3-methoxypterostilbene). The samples which did not undergo enzymatic hydrolysis (free samples) were utilized to determine the concentration of the aglycone, whereas the samples that did undergo enzymatic hydrolysis (total samples) were used to determine the concentration of the aglycone originally present in the sample in addition to the concentration of the glucuronidated metabolite cleaved back to 3-methoxypterostilbene. Therefore, by subtracting the free sample concentration from the total sample concentration, the concentration of the glucuronidated metabolite can be calculated without the use of an additional chromatographic run and analysis.

2.7. Pharmacokinetic Analysis. Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (SEM) were calculated for each group. Samples were analyzed using WinNonlin software (version 1.0; Pharsight Corporation, Mountain View, CA, USA) to calculate the pharmacokinetic parameters. The rats' concentrations versus time points were subjected to a noncompartmental model. The apparent elimination rate constant (KE) was estimated from the slope of the log-linear phase of declining concentration versus time plot. The half-life and rate of eliminated were determined by applying the previously described software with the specified parameters. The renal clearance was calculated by multiplying the fraction of compound excreted unchanged with total body clearance. The plasma half-life ($t_{1/2}$) was determined using the following equation: $t_{1/2}$ = 0.693/KE. To determine the fraction of unchanged 3-methoxypterostilbene excreted (f_e) in urine, the total amount of urine was divided by the total dose administered. The renal clearance ($\mathrm{CL}_{\mathrm{renal}}$) was determined by the equation: $\mathrm{CL}_{\mathrm{renal}} = f_e \times \mathrm{CL}_{\mathrm{total}}$.

2.8. Content Analysis of Dried Traditional Chinese Medicinal Plants. 3-Methoxypterostilbene has been reported to be present in two plants used in traditional Chinese medicine—*Sphaerophysa salsula* (*Swainsona salsula*) and *Rheum palmatum* (Chinese rhubarb) [1, 2]. Dried *S. salsula* extract and dried Chinese rhubarb are both commercially available products. Chinese rhubarb was frozen under liquid nitrogen and then ground into a fine powder. *S. salsula* extract was already in powdered form. Two 0.1 g of each product were measured and placed into 2.0 mL Eppendorf tubes. 1.5 mL methanol was added to each tube for extraction. Tubes were vortexed for 30 seconds, agitated for 3 h, and centrifuged at 10,000 rpm for 5 min. One of the duplicates was treated to extract only aglycones (free) and the second of the duplicates was treated to cleave any glycosides to aglycones (total) by using β-glucosidase from almonds. The supernatants from the free samples were transferred into new 2.0 mL Eppendorf tubes, and 50 μL of internal standard, pinosylvin, was added. Samples were vortexed for 30 s, dried to completion under

a stream of nitrogen gas, and stored at −20°C until analysis. The supernatants of the total samples were transferred to new 2.0 mL Eppendorf tubes, dried to completion under a stream of compressed nitrogen gas, and reconstituted with PBS (200 μL at pH 7.4). 20 μL of β-glucosidase (750 U/mL in PBS at pH 7.4) was added and samples were incubated for 48 h at 37°C in a shaking water bath. β-Glucuronidase acts by cleaving the glycosidic sugar moieties frequently present in plant extracts as previously described [29]. Acetonitrile (1 mL) was added to stop the enzymatic reaction, followed by the addition of internal standard (50 μL). Samples were centrifuged at 10,000 rpm for 5 min and the supernatant was dried to completion under a stream of compressed nitrogen gas. Both free and total samples were reconstituted in mobile phase (200 μL), and 100 μL was injected into the HPLC under the same conditions previously described.

2.9. Antioxidant Capacity Determination. The antioxidant capacities of 3-methoxypterostilbene and resveratrol were measured through an assay that relied on the inhibition of the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin. The amount of $ABTS^{\bullet+}$ can be monitored spectrophotometrically. The degree of suppression of absorbance caused by the compound of interest is proportional to the concentration of $ABTS^{\bullet+}$, which is expressed as Trolox equivalents (μg/mL). For this assay, 3-methoxypterostilbene and resveratrol were dissolved in DMSO on the day of the experiment to yield concentrations of 1, 5, 10, 50, and 100 μg/mL. No additional dilution was employed. To run the assay, 10 μL of sample was combined with 10 μL metmyoglobin and 150 μL chromogen. Then, 40 μL of hydrogen peroxide working solution was added within 1 min to all the samples. The plate was covered and incubated on a shaker for 5 min at room temperature 923 ± 1°C), and the absorbance was measured at 750 nm using the Synergy HT multiwell plate reader and Gen5 data analysis software (Biotek Instruments Inc., Winooski, VT, USA). The assay was performed in quadruplet. For more information regarding the assay protocol, please refer to the instructions for the kit (Antioxidant Assay Kit from Cayman Chemical—Cat. no. 709091).

2.10. Cyclooxygenase Inhibition Determination. The inhibition of COX-1 and -2 by 3-methoxypterostilbene was measured through the use of a three-day commercial assay kit. The assay relied on the quantification of the prostanoid product based on enzyme immunoassay (EIA), which uses a nonspecific antibody for prostaglandins (PGs). The constant concentration of the PG-AChE-tracer and the varying concentrations of the PGs available to bind to the antiserum is inversely proportional to the concentration of free PGs in the well. For this assay, 3-methoxypterostilbene was dissolved in DMSO over the concentration range of 1–250 μg/mL. Ibuprofen and etodolac, COX-2 preferential NSAIDs, were dissolved in DMSO and used as controls over the concentration range of 1–250 μg/mL. Reagents were prepared according to the manufacturer's instructions that accompanied the kit. The ELISA plate for this purpose was read at an absorbance of 415 nm within 10 minutes at room temperature (23 ± 1°C) using the Synergy HT multiwell plate reader and Gen5 data analysis software (Biotek Instruments Inc., Winooski, VT, USA). The assay was performed in quadruplet. For more information regarding the assay protocol, please refer to the instructions for the kit (COX Inhibitor Screening Assay Kit from Cayman Chemical—Cat. no. 560131).

2.11. α-Glucosidase Inhibition Determination. Inhibition of α-glucosidase was determined through a colorimetric assay adapted and modified from the method presented by Tadera et al. [34]. The assay uses *p*-nitrophenyl-α-D-glucopyranoside (PNPG), which is hydrolyzed specifically by α-glucosidase into a yellow-colored product (*p*-nitrophenol). The absorbance at 410 nm of liberated *p*-nitrophenol was measured. For this assay, 3-methoxypterostilbene and resveratrol were dissolved in DMSO to create concentration ranges from 0 to 200 μg/mL. 160 μL of 100 mM phosphate buffer (pH 6.8), 25 μL of 20 mM PNPG in phosphate buffer, and 10 μL of stilbenes in DMSO were added to wells of a 96-well plate (10 μL DMSO was added to the control wells). The plate was incubated at 30°C for 5 min and then 10 μL of the buffer containing 0.02 mg/mL of enzyme was added to each well. The plate was further incubated for 5 min. 20 μL of 3.25 M sodium hydroxide was added to each well to stop the reaction. The plate was immediately read at an absorbance of 410 nm at room temperature (23 ± 1°C) using the Synergy HT multiwell plate reader and Gen5 data analysis software (Biotek Instruments Inc., Winooski, VT, USA). The assay was performed in sextuplicate.

Inhibition (%) was calculated as $((A - B)/A) \times 100$, where A was the average absorbance of the control wells and B was the absorbance of the wells containing stilbenes.

2.12. Alpha-Amylase Inhibition Determination. Inhibition of α-amylase was determined through a colorimetric assay also adapted and modified from the method presented by Tadera et al. [34]. A synthetic substrate, nonreducing-end-blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) commercially prepared as amylase HR reagent, which is hydrolyzed specifically by α-amylase into *p*-nitrophenyl maltosaccharide is employed. The α-glucosidase present in the assay then converts the new substrate into *p*-nitrophenol and absorbance at 410 nm is measured as previously stated in the α-amylase assay. 3-methoxypterostilbene and resveratrol were dissolved in methanol to create concentration ranges from 0 to 200 μg/mL. 100 μL of amylase HR reagent (prepared following directions accompanying the reagent, Megazyme Amylase HR Reagent, Cat. no. R-AMHR4), and 40 μL of stilbene in methanol were added to a 96-well plate (40 μL of methanol was added to the control wells). The plate was incubated for 5 minutes at 37°C and then 60 μL of 0.1 mg/mL α-amylase in 0.1 M HEPES buffer (pH 6.9) was added to the reaction mixture. After further incubation at 37°C, for 10 min, 20 μL of 3.25 M sodium hydroxide was added to each well to stop the reaction. The liberated *p*-nitrophenol was determined and the percent inhibition calculated as

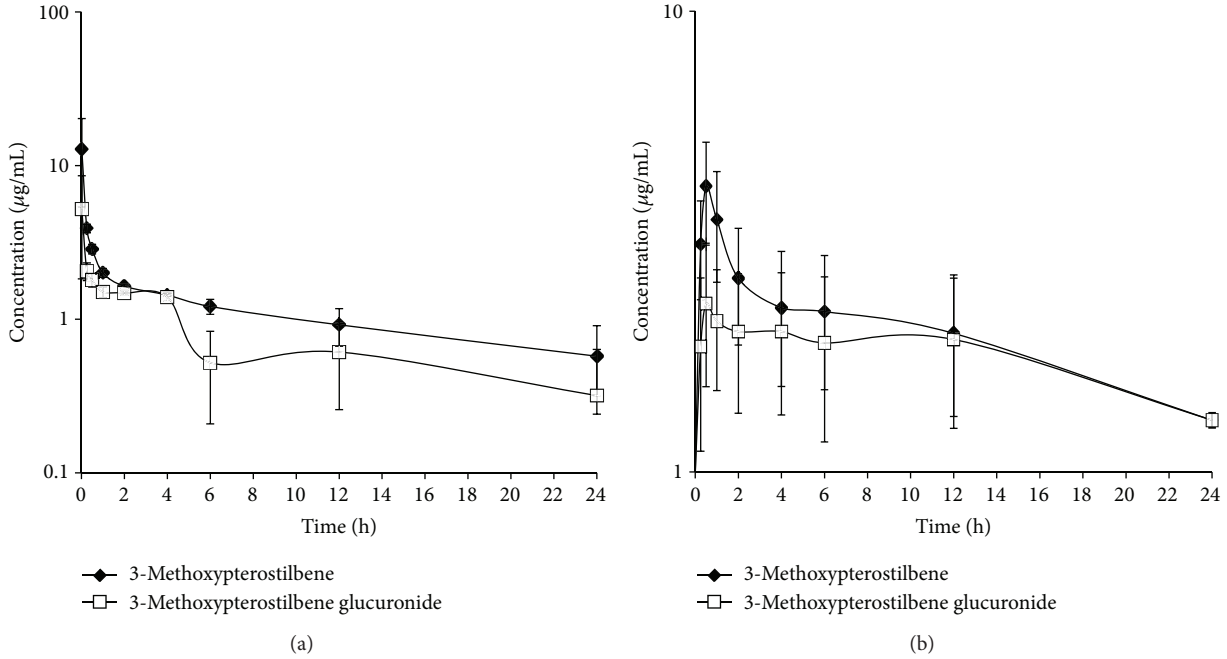

Figure 2: (a) 3-Methoxypterostilbene disposition in serum following intravenous administration. (b) 3-Methoxypterostilbene disposition in serum following oral administration ($n = 4$, mean ± SEM).

described in the assay for α-glucosidase activity previously described. The assay was performed in sextuplicate.

2.13. Statistical Analysis. Compiled data were presented as mean and standard error of the mean (mean ± SEM). Where possible, the data were analyzed for statistical significance using Minitab 15 statistical software (Minitab, Inc., State College, PA, USA). Student's t-test was employed for unpaired samples with a value of $P < 0.05$ being considered statistically significant.

3. Results and Discussion

3.1. Pharmacokinetic Study. Standard curves established linearity over the concentration range studied for the serum and urine samples. Chromatograms showed no interference from endogenous components. Total samples (incubated with β-glucuronidase from *Escherichia coli* type IX-A) verified the presence of a glucuronidated metabolite based on the increase in 3-methoxypterostilbene (aglycone parent compound) concentrations after enzymatic hydrolysis in both serum and urine. Glucuronidation of 3-methoxypterostilbene parallels previous rat and human studies with resveratrol existing predominately in its conjugated form in both plasma and urine [35].

The serum concentration versus time profile for IV-dosed 3-methoxypterostilbene demonstrates a rapid decline in concentration in the first hour, representing a distribution phase, which was followed by a steady elimination up to 24 hours, after which the serum concentrations were below detectable concentrations (0.05 μg/mL) (Figure 2(a)). 3-Methoxypterostilbene dosed PO displayed rapid absorption with an average T_{max} of 30 minutes (Figure 2(b)). The glucuronidated metabolite in both routes of administration appeared to display multiple peaking which is suggestive of enterohepatic recycling as indicated by an increase in serum concentration around 4 h after dose. Enterohepatic recycling has previously been reported for resveratrol [27, 36].

Table 1: Pharmacokinetic parameters of 3-methoxypterostilbene in the rat.

Pharmacokinetic parameter	Intravenous Mean ± SEM	Oral Mean ± SEM
AUC_{inf} (μg·h/mL)	48.1 ± 23.8	229 ± 44.6
Vd_{β} (L/kg)	5.11 ± 0.380	52.0 ± 10.5
$CL_{hepatic}$ (L/h/kg)	47.1 ± 23.3	0.480 ± 0.0800
CL_{renal} (L/h/kg)	0.760 ± 0.460	0.0100 ± 0.000
CL_{total} (L/h/kg)	47.8 ± 23.7	0.480 ± 0.0800
f_e (%)	1.64 ± 0.950	1.24 ± 0.160
$t_{1/2}$ (h) serum	18.9 ± 10.9	73.3 ± 8.91
$t_{1/2}$ (h) urine	9.54 ± 1.51	20.6 ± 3.01
MRT (h)	26.0 ± 15.0	105 ± 13.1
Bioavailability (F%)	100	50.6 ± 10.0

Table 1 summarizes the pharmacokinetic parameters exhibited by 3-methoxypterostilbene at an IV dose of 10 mg/kg and a PO dose of 100 mg/kg. Noncompartmental analysis in WinNonlin software (ver. 1.0) was used to model both serum and urine data. The total serum clearance of 3-methoxypterostilbene was determined to be 47.8 ± 23.7 L/h/kg for IV dosing and 0.480 ± 0.0800 for PO dosing. The mean fraction excreted in urine unchanged (f_e) was

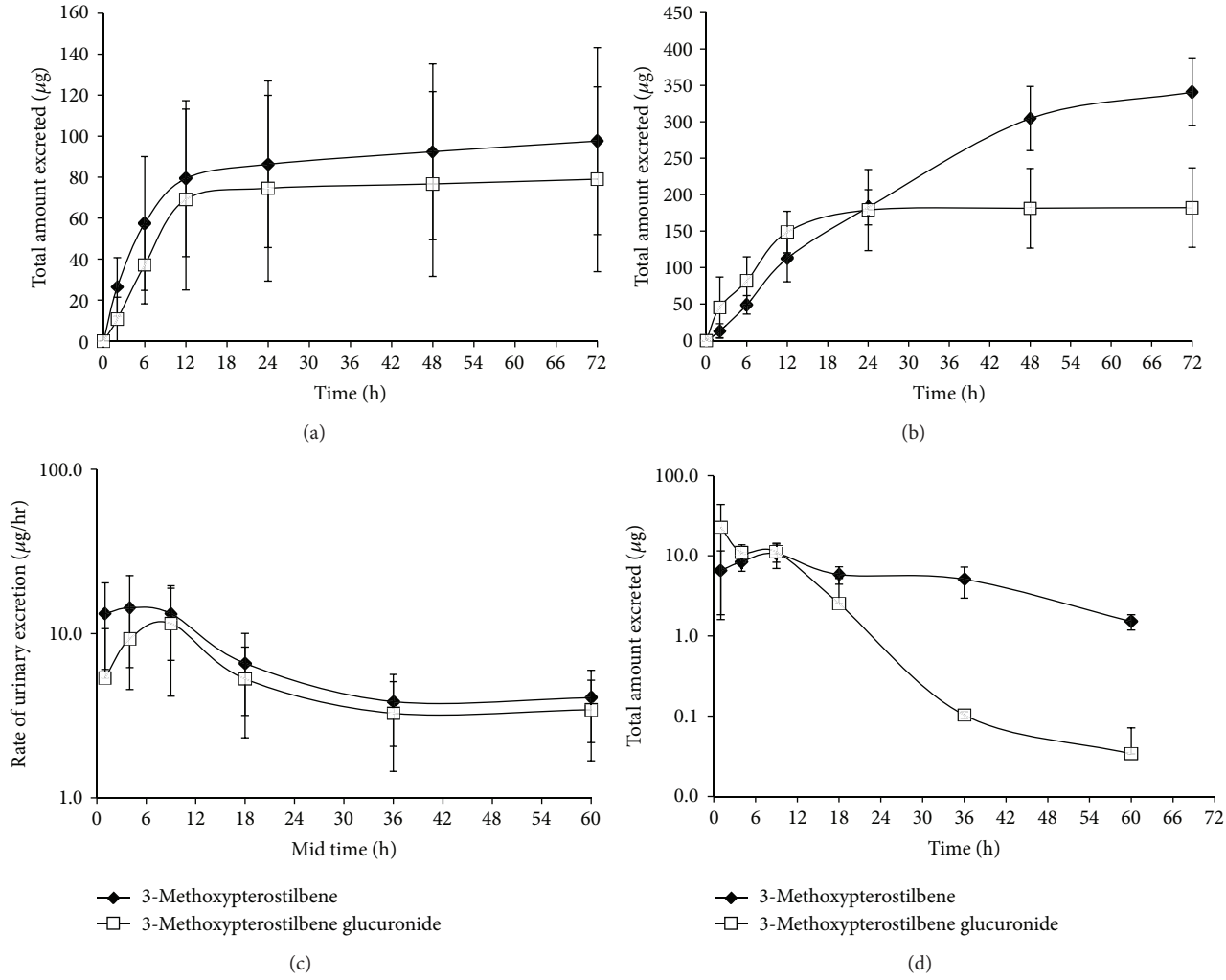

Figure 3: (a) Cumulative 3-methoxypterostilbene and glucuronidated metabolite (μg) excreted in urine over 72 h after intravenous administration. (b) Cumulative 3-methoxypterostilbene and glucuronidated metabolite (μg) excreted in urine over 72 h after oral administration. (c) Rate of excretion (μg/h) of 3-methoxypterostilbene and glucuronidated metabolite in urine over 72 h after intravenous administration. (d) Rate of excretion (μg/h) of 3-methoxypterostilbene and glucuronidated metabolite in urine over 72 h after oral administration (n = 4 mean ± SEM).

1.64 ± 0.950% for IV and 1.24 ± 0.160% for PO, indicating that 3-methoxypterostilbene is mainly excreted via nonrenal routes. Renal clearance (CL_{renal}) was measured at 0.760 ± 0.46 L/h/kg for IV and 0.0100 ± 0.00100 L/h/kg for PO, and hepatic clearance ($CL_{hepatic} = CL_{total} - CL_{renal}$) was determined to be 47.1 ± 23.3 L/h/kg for IV and 0.480 ± 9.0800 L/h/kg for PO assuming that nonrenal clearance is hepatic clearance. The volume of distribution of 3-methoxypterostilbene is 5.11 ± 0.380 L/kg IV and 52.0 ± 10.0 L/kg PO, which is greater than total body water, suggesting that 3-methoxypterostilbene is highly distributed into tissues. The mean area under the curve (AUC), representing the total amount of exposure in the serum over time, was 48.1 ± 23.8 μg·h/mL for IV and 229 ± 44.6 μg·h/mL for PO. The serum concentration of 3-methoxypterostilbene declined very slowly with a mean elimination half-life of 18.9 ± 10.9 h for IV and 73.3 ± 8.91 h for PO. The oral bioavailability for 3-methoxypterostilbene was determined to be 50.6%0020 ± 10.0%.

Reported bioavailability of resveratrol in rats ranges from 20 to 38.8% [27, 32] and <1% in humans [37] with a wide variability in pharmacokinetic parameters among individuals [38]. Species-dependent rapid conjugation with higher glucuronidation rates and affinity in humans may limit stilbene bioavailability and show expressed differences in pharmacokinetics between rodent and human studies. Poor bioavailability in humans is a potential limitation in the use of resveratrol as a therapeutic agent, hence the interest in structural analogs. 3-Methoxypterostilbene bioavailability in rats has been determined to be 50.6% ± 10.0%. The melting point range of 3-methoxypterostilbene was experimentally determined to be 88.5–91.2°C. The reported melting point range of resveratrol is 253–255°C [39]. Therefore, 3-methoxypterostilbene has a lower crystallinity than

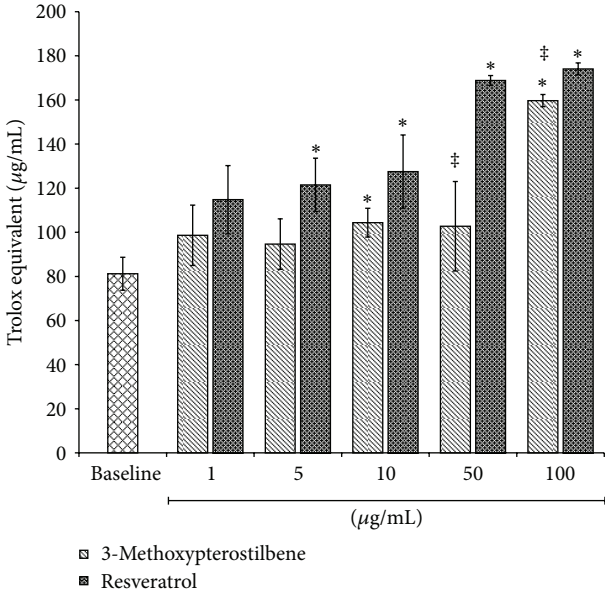

Figure 4: Antioxidant capacity ($n = 4$, mean ± SEM) of 3-methoxypterostilbene and resveratrol at 1, 10, 50, and 100 μg/mL dissolved in DMSO. *Significantly greater than baseline (DMSO) sample ($P < 0.05$). ‡Significantly different from resveratrol at the same concentration ($P < 0.05$).

resveratrol. The low crystallinity of 3-methoxypterostilbene is responsible for its increased dissolution over resveratrol. Furthermore, the aqueous solubility of resveratrol is ~30 mg/mL [40] and the predicted aqueous solubility, using AlogPs [41] of 3-methoxypterostilbene is 8–88 mg/L. The increased bioavailability of 3-methoxypterostilbene in rats compared to resveratrol is likely due to its lower crystallinity resulting in enhanced dissolution over resveratrol and may be in part due to differences in solubility and absorbance mechanisms which will be examined in further studies. The increased bioavailability of 3-methoxypterostilbene over resveratrol in rats may extend to the greater bioavailability of 3-methoxypterostilbene than that of resveratrol in humans.

Analysis of urine samples for both routes of administration displayed the presence of the parent compound, 3-methoxypterostilbene and the glucuronidated metabolite previously identified in the serum (Figures 3(a) and 3(b)). The total cumulative urinary excretion plot (Figure 3(a)) indicates that 3-methoxypterostilbene is excreted predominantly in the aglycone form for PO administration and almost equally in the aglycone and glucuronidated metabolite form for IV dosing. The glucuronidated metabolite appears to be mostly excreted by 12 h after dose for both routes of administration while 3-methoxypterostilbene (aglycone) appeared to be predominately excreted by 12 h after dose for IV but steadily increased in excretion even at 72 h after dose for PO administration. The half-life of 3-methoxypterostilbene in urine was determined to be 9.54 ± 1.41 h for IV and 20.6 ± 3.01 h for PO. The rate of urinary excretion plot (Figure 3(b)) indicates that 3-methoxypterostilbene and its glucuronidated metabolite have similar rates of excretion as indicated by their parallel slope (−KE/2.303) for IV administration but the glucuronidated metabolite appears to have a greater rate of excretion over the aglycone after PO administration.

The total dose of 3-methoxypterostilbene administered was 10 mg/kg for IV and 100 mg/kg PO. The average weight of the rats in this experiment was ~200 g. Each rat received ~2 mg of 3-methoxypterostilbene IV and 20 mg PO. The plots of cumulative amount excreted in urine for both the aglycone and glucuronidated metabolite forms excreted (~98 μg and 80 μg, resp., for IV and 341 μg and 182 μg, resp., for PO) are very small compared to the overall dose administered (~2 mg and 20 mg for IV and PO, resp.). This further suggests that 3-methoxypterostilbene is eliminated predominately by nonrenal routes. As previously mentioned, f_e was 1.64 ± 0.950% for IV and 1.24±0.160% for PO, and therefore CL_{renal} was 0.760 ± 0.460 L/h/kg for IV and 0.0100 ± 0.00100 L/h/kg for PO. Excretion via nonrenal routes for 3-methoxypterostilbene agrees with literature reports of nonrenal excretion of other stilbenes [27, 30].

3.2. Content Analysis of Dried Traditional Chinese Medicinal Plants. Evaluation of the *S. salsula* extract and dried *R. palmatum* indicated that only the *S. salsula* extract contained detectable levels of 3-methoxypterostilbene. The aglycone concentration for the *S. salsula* extract was determined to be 0.842 μg/g and the total concentration of 3-methoxypterostilbene (aglycone and glycoside) was determined to be 0.853 μg/g indicating that 3-methoxypterostilbene exists primarily in its aglycone form in *S. salsula* extract. Despite the report that 3-methoxypterostilbene exists as an aglycone of a stilbene glycoside in *R. palmatum* [2], the compound was not detected as an aglycone or glycoside in the commercially available dried Chinese rhubarb. It is suspected that 3-methoxypterostilbene may be detectable in other commercially available Chinese rhubarb samples and that plant variation likely accounts for the lack of detection in this sample.

3.3. Antioxidant Capacity of 3-Methoxypterostilbene. Figure 4 reports the antioxidant capacity of 3-methoxypterostilbene in units of Trolox equivalents (μg/mL). The baseline (DMSO only) samples have a low antioxidant capacity (81.2 ± 7.47 μg/mL or 0.325 ± 0.0299 mM). 3-Methoxypterostilbene demonstrates a modest concentration-dependent antioxidant activity with an antioxidant capacity at 1 μg/mL of 98.6 ± 13.7 μg/mL (0.394 ± 0.0546 mM) Trolox equivalents and a capacity at 100 μg/mL of 174 ± 2.70 μg/mL (0.695 ± 0.00940 mM) Trolox equivalents. This indicated that 3-methoxypterostilbene prevents oxidation at comparable levels to Trolox, if not better (as seen at lower concentrations). 3-Methoxypterostilbene demonstrated significantly greater activity to the baseline samples from 10 to 100 μg/mL ($P < 0.05$) and only showed significant difference from resveratrol activity at the two highest concentrations tested, 50 and 100 μg/mL ($P < 0.05$).

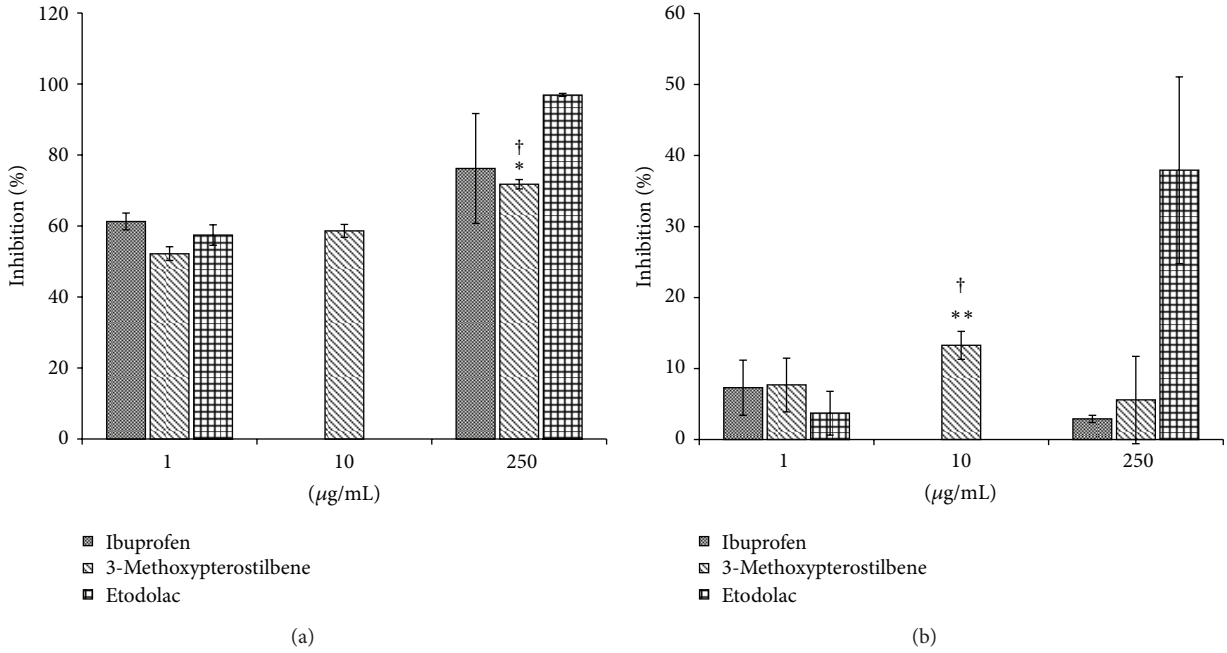

Figure 5: (a) Cycloxoygenase I inhibition activity of 3-methoxypterostilbene at 1, 10, and 250 μg/mL. (b) Cycloxoygenase II inhibition activity of 3-methoxypterostilbene at 1, 10, and 250 μg/mL. *Significantly greater activity than 1 μg/mL ibuprofen ($P < 0.05$). **Significantly greater activity than 250 μg/mL ibuprofen ($P < 0.05$). †Significantly greater activity than 1 μg/mL etodolac ($P < 0.05$). ‡Significantly greater activity than 250 μg/mL etodolac ($P < 0.05$) ($n = 4$, mean ± SEM).

The ability of 3-methxoypterostilbene to work as an antioxidant is of paramount importance as the wide range of health benefits of polyphenols are thought to result at least in part from their antioxidant capacity. For example, the ability of resveratrol to limit the start and progression of atherosclerosis is associated with the compound's ability to inhibit lipid oxidation of polyunsaturated fatty acids [42]. It is likely that the health benefits of 3-methoxypterostilbene will also be associated with its antioxidant capacity. In the literature, it is well known that the number and position of the hydroxyl groups on stilbenes are critical for bioactivity and *t* antioxidant activity [43–46]. A computer model of antioxidant activity of hydroxystilbenes suggests that 3-methoxypterostilbene is one of the most potent 3,4 hydroxystilbenes modeled and has greater potency than resveratrol [47].

3.4. Cyclooxygenase Inhibitory Activity. Figure 5 reports the cyclooxygenase inhibitory activity of 3-methoxypterostilbene and two NSAIDs: etodolac and ibuprofen. 3-Methoxypterostilbene appears to have greater activity against COX-1 than COX-2. Figure 5(a) demonstrates the positive concentration-dependent inhibitory activity of 3-methoxypterostilbene against COX-1. While 3-methoxypterostilbene did not demonstrate significantly greater ($P < 0.05$) COX-1 inhibition activity than the two NSAIDs at lower concentrations, the high concentration (250 μg/mL) of 3-methoxypterostilbene demonstrated significantly greater activity ($P < 0.05$) than the NSAIDs at low doses. Figure 5(a) details the COX-2 inhibitory activity of 3-methoxypterostilbene. At the concentration of 10 μg/mL, 3-methoxypterostilbene demonstrated statistically greater inhibition ($P < 0.05$) than etodolac at 1 μg/mL and ibuprofen at 250 μg/mL. Several stilbenes, including resveratrol, are known to be preferential COX-2 inhibitors [28].

3.5. Antidiabetic Activity

3.5.1. Alpha-Glucosidase Activity. Figure 6 reports the alpha-glucosidase inhibition of 3-methoxypterostilbene and resveratrol. Resveratrol shows a clear positive concentration-dependent inhibition relationship whereas 3-methoxypterostilbene does not appear to exhibit greater inhibition at higher concentrations. α-Glucosidase inhibition activity is only statistically different ($P < 0.05$) between the two stilbenes at the two highest concentrations tested (100 and 200 μg/mL).

α-Glucosidase is an enzyme found in the small intestine which hydrolyzes 1,4-α-bonds of disaccharides into glucose. Inhibition of α-glucosidase suppresses postprandial hyperglycemia by lowering the rate of glucose absorption via delayed carbohydrate digestion and extended digestion time. α-Glucosidase inhibitors are useful in maintaining glycemic control of prediabetic and type two diabetic patients. The lower concentrations of 3-methoxypterostilbene tested may be biologically achievable resulting in moderate inhibition of α-glucosidase comparable to that of resveratrol, and reduction of postprandial hyperglycemia could be seen.

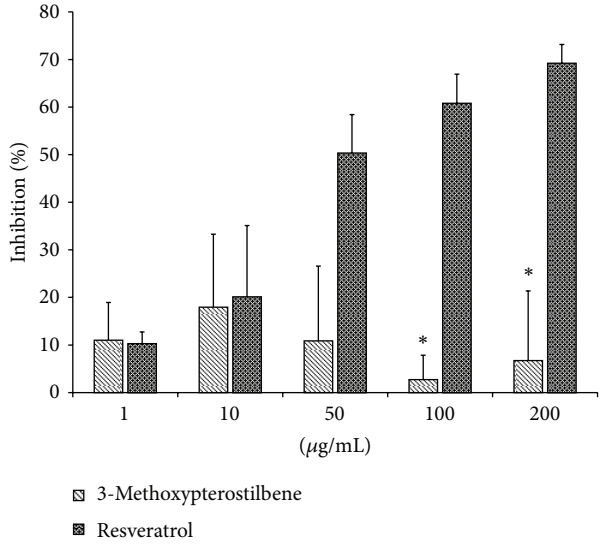

Figure 6: Alpha-glucosidase inhibition activity of 3-methoxypterostilbene and resveratrol ($n = 6$, mean ± SEM). *Significantly different from resveratrol at the same concentration ($P < 0.05$).

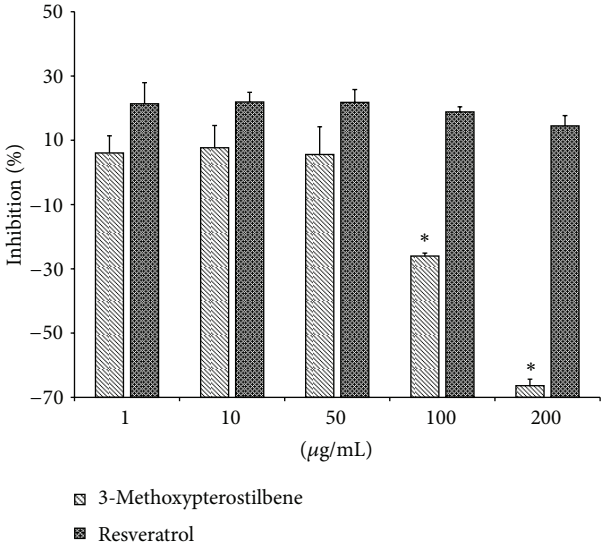

Figure 7: Alpha-amylase inhibition activity of 3-methoxypterostilbene and resveratrol ($n = 6$, mean ± SEM). *Significantly different from resveratrol at the same concentration ($P < 0.05$).

3.5.2. Alpha-Amylase Activity. α-Amylase inhibition activity by 3-methoxypterostilbene and resveratrol is shown in Figure 7. Both resveratrol and 3-methoxypterostilbene display relatively weak inhibition of α-amylase, and both stilbenes show a slightly negative concentration inhibition relationship. At the highest concentrations tested, 3-methoxypterostilbene appears to increase activity of α-amylase. At lower, biologically relevant concentrations (1-50 μg/mL), there is no significant difference between inhibition activity of 3-methoxypterostilbene and resveratrol.

In humans, α-amylase is an enzyme predominately found in the pancreas and saliva. Like α-glucosidase, α-amylase hydrolyzes α-1,4-glycosidic bonds but acts on polysaccharides. Inhibition of α-amylase also reduces postprandial hyperglycemia, and α-glucosidase inhibitors may be used to treat type 2 diabetes. The modest inhibitory activity of 3-methoxypterostilbene at biologically relevant levels suggests that it may be as effective as resveratrol at helping reduce postprandial hyperglycemia.

4. Conclusions

In summary, the pharmacokinetics in rats and the *in vitro* metabolism of 3-methoxypterostilbene was evaluated for the first time. 3-methoxypterostilbene demonstrates improved bioavailability compared to resveratrol. 3-Methoxypterostilbene demonstrated antioxidant activity comparable to resveratrol at biologically relevant concentrations. This stilbene also showed strong COX-1 inhibition comparable to two NSAIDs and moderate inhibition of COX-2. 3-Methoxypterostilbene demonstrated moderate antidiabetic activity via inhibition of α-glucosidase and α-amylase comparable to resveratrol. Further exploration of the pharmacodynamics of 3-methoxypterostilbene is under way to demonstrate utility in reducing postprandial hyperglycemia, adiposeness, cardiac hypertrophy and inflammation.

Conflict of Interests

The authors declare no financial conflict of interest.

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Review Article

Significance of Kampo, Japanese Traditional Medicine, in the Treatment of Obesity: Basic and Clinical Evidence

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The cause of obesity includes genetic and environmental factors, including cytokines derived from adipocytes (adipo-cytokines). Although drug therapy is available for obesity, it is highly risky. Our main focus in this review is on the traditional form of Japanese medicine, Kampo, in the treated of obesity. Two Kampo formulas, that is, bofutsushosan (防通散) and boiogito (防己黄耆), are covered by the national health insurance in Japan for the treatment of obesity. Various issues related to their action mechanisms remain unsolved. Considering these, we described the results of basic experiments and presented clinical evidence and case reports on osteoarthritis as examples of clinical application of their two Kampo medicine. Traditional medicine is used not only for treatment but also for prevention. In clinical practice, it is of great importance to prove the efficacy of combinations of traditional medicine and Western medicine and the utility of traditional medicine in the attenuation of adverse effects of Western medicine.

1. Background

1.1. Historical Background. Until the 20th century, Japanese herbal medicine (Kampo medicine) had not been recognized as a useful tool for the treatment of obesity. Since the latter half of the 20th century, great attention has been paid to upper-body obesity or visceral obesity as a risk factor for several lifestyle-related diseases. In the 1980s and 1990s, several investigators proposed that dyslipidemia, high blood glucose, and high blood pressure were important risks for cardiovascular diseases due to metabolic disorders [1–3].

These days, this combination of disorders (i.e., dyslipidemia, hypertension, and hyperglycemia) is indicative of "metabolic syndrome." The initial cause of the metabolic syndrome or abdominal obesity is likely to be visceral fat accumulation (which is regulated by both genetic and environmental factors). It is generally recognized that the accumulation of visceral fat causes the secretion of adiponectin to decrease and the so-called bad adipocytokines (plasminogen activator inhibitor-1 [PAI-1] [4–6], tumour necrosis factor-α [TNF-α] [7–9], angiotensinogen, etc.) to increase. Proper diet and exercise are the basic therapeutic approaches to reduce abdominal obesity. Medications are considered afterwards.

1.2. Antiobesity Medication. Only one antiobesity medication orlistat (Xenical) is currently approved by the Food and Drug Administration (FDA) for long-term use [10–15]. It reduces intestinal fat absorption by inhibiting pancreatic lipase. Rimonabant (Acomplia), a second drug, works via specific blockade of the endocannabinoid system. Its development is originated from the knowledge that cannabis smoking often increases hunger, which is often referred to as "the munchies". Its use in the treatment of obesity had been approved in Europe but not in the USA or Canada due to safety concerns [16–20]. In October 2008, the European Medicines Agency recommended the suspension of the sale of rimonabant as the risks seemed to be greater than the benefits [21]. In October

2010, sibutramine (Meridia), which acts in the brain to inhibit neurotransmitters deactivation and thereby appetite, was withdrawn from the USA and Canadian markets due to cardiovascular concerns [11, 22]. Because of their potential side effects, anti-obesity drugs should only be prescribed for obesity when the benefits outweigh the risks of treatment [23, 24].

Hasani-Ranjbar et al. [25] have reported a systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity. This review focuses on the efficacy and safety of effective herbal medicines in the management of obesity in humans and animals. Of the publications identified in the initial database, 915 results were identified and reviewed, and a total of 77 studies were included (19 human and 58 animal studies). Studies with *Cissus quadrangularis, Sambucus nigra, Asparagus officinalis, Garcinia atroviridis*, and ephedra and caffeine, Slimax (extract of several plants including *Zingiber officinale* and bofutsushosan) showed a significant decrease in body weight. In 41 animal studies, significant weight loss or inhibition of weight gain was found. No significant adverse effects or mortality were observed except in studies with supplements containing ephedra, caffeine, and bofutsushosan.

Given this background, our focus will be on Kampo as the treatment for obesity [26, 27].

2. The Status of Kampo Medicine in the Treatment of Obesity

2.1. The Definition of Obesity. In 1997, the World Health Organization (WHO), in cooperation with the International Obesity Task Force (IOTF), defined normal weight as body mass index (BMI) from 18.5 to <25 kg/m^2 and obesity as BMI ≥25 kg/m^2. According to the Japan Society for the Study of Obesity criteria, obesity disease is diagnosed when individuals have obesity-associated disease or visceral fat area on abdominal CT scan equal to or greater than 100 cm^2 [28].

2.2. Kampo Medicine Provided under Health Insurance in Japan. The Kampo medicine of "traditional medical treatment" in Japan is in special environment. In a covered-medical-services system of Japan, getting a medical license for per the Forney Western medicine is mandate to prescribe Kampo medicine. Medical treatment is performed with the same stance as Western medicine. However, it is very difficult to understand Kampo medicine. Terasawa has indicated the outline of Kampo medicine to the first issue of this journal [29–31]. The universal healthcare system was established in Japan in 1961, and 4 Kampo extracts were approved as prescribable medications in 1967. That number is now 148. The application increased, and according to a survey by the Journal Nikkei Medical, more than 70% of physicians prescribe Kampo drugs today [32]. Physicians in Japan are entitled to prescribe both Western and Kampo medicines. Among the 148 Kampo formulas, only 2 are for the treatment of obesity. The concept of obesity is not described in classical Kampo. However, eating too much sweet food has been described as the cause of diabetes. On the other hand, how to manage and treat lifestyle-related disease is well described in classical Kampo medicine. In this paper, we focus on the two Kampo formulations used in the treatment of obesity, bofutsushosan (防風通聖散) and boiogito (防己黄耆湯).

2.3. Bofutsushosan. Chemical composition and HPLC fingerprint are shown in Figures 1 and 2. Bofutsushosan is indicated for the relief of the following symptoms in patients with thick subcutaneous abdominal fat and a tendency toward constipation: hypertension (palpitation, shoulder stiffness, and hot flushes), obesity, swelling, and constipation. The usual adult dose is 7.5 g/day orally in 2 or 3 divided doses before or between meals. The dosage may be adjusted according to the patient's age, body weight, and symptoms. Bofutsushosan should be administered with care in patients with the following conditions: (1) diarrhea or soft feces (these symptoms may be aggravated), (2) a weak gastrointestinal tract (anorexia, epigastric distress, nausea, vomiting, abdominal pain, soft feces, and diarrhea may occur), (3) anorexia, nausea, or vomiting (these symptoms may be aggravated), (4) a period of weakness after disease or with a greatly weakened constitution (adverse reactions are likely to occur, and the symptoms may be aggravated by treatment), (5) a marked tendency to sweat (excess sweating and/or generalized weakness may occur), (6) cardiovascular disorders including angina pectoris and myocardial infarction or those with a history of such disorders, (7) severe hypertension, (8) severe renal dysfunction, (9) dysuria, and (10) hyperthyroidism. The diseases and symptoms mentioned in (6)–(10) may be aggravated by treatment. The source is *Manbyo-kaishun-Chufumon* (万病回春, 中風門). The prescription was used in Ikkando (一貫堂) medicine, which was systematized by Dohaku Mori (1867–1931). Bofutsushosan is standard for apoplectic patients, whose appearance suggests they are at risk of future cerebral hemorrhage, and are slightly obese, have a paunch, and have a sturdy build with pale yellow skin color. Bofutsushosan is used for people with solid build, slight obesity, thick abdominal subcutaneous fat, strong intestines, and good appetite. They are more prone to constipation who suffer inflammation in the nose and throat because the whole body is susceptible to heating up, and they tend to have high blood pressure with sensitivity to heat.

2.4. Boiogito. Chemical composition and HPLC fingerprint are shown in Figures 3 and 4. Boiogito is indicated for the relief of the following symptoms in white-complexioned, soft-muscled, flabby patients who are easily fatigued, perspire profusely, do not excrete enough urine, and develop edema in the lower limbs, knee joint swelling, and pain: nephritis, nephrosis, nephropathy of pregnancy, hydrocele testis, obesity, arthritis, carbuncles, furuncles, myositis, edema, dermatosis, hyperhidrosis, and menstrual irregularity. The usual adult dose is 7.5 g/day orally in 2 or 3 divided doses before or between meals. The dosage may be adjusted according to the patient's age and body weight and symptoms. (1) When this product is used, the patient's "*Sho*" (証, patterns) should be

Description	Bofutsushosan extract granules for ethical use	
Composition	7.5 g of TSUMURA bofutsushosan extract granules contains 4.5 g of a dried extract of the following mixed crude drugs	
	JP scutellaria root	2.0 g
	JP glycyrrhiza	2.0 g
	JP platycodon root	2.0 g
	JP gypsum	2.0 g
	JP atractylodes rhizome	2.0 g
	JP rhubarb	1.5 g
	JP schizonepeta spike	1.2 g
	JP gardenia fruit	1.2 g
	JP peony root	1.2 g
	JP cnidium rhizome	1.2 g
	JP Japanese angelica root	1.2 g
	JP mentha herb	1.2 g
	JP saposhnikovia root	1.2 g
	JP *Ephedra* herb	1.2 g
	JP forsythia fruit	1.2 g
	JP ginger	0.3 g
	Talc	3.0 g
	Anhydrous mirabilitum	0.7 g
	(JP: the Japanese pharmacopoeia)	
Inactive ingredients	JP light anhydrous silicic acid JP magnesium stearate JP lactose hydrate	

Figure 1

taken into account. The patient's progress should be carefully monitored, and if symptoms/findings do not improve, continuous treatment should be avoided. (2) Since this product contains glycyrrhiza (甘草, kanzo), careful attention should be paid to the serum potassium level, blood pressure, and so forth, and if any abnormality is observed, administration should be discontinued. (3) When this product is coadministered with other Kampo preparations (Japanese traditional herbal medicines) and so forth, attention should be paid to duplication of crude drug contents. Sho: the term "Sho" refers to a particular pathological status (pattern of symptoms) determined by Kampo diagnosis. The pattern is based on the patient's constitution, symptoms, and so forth. Kampo preparations (Japanese traditional herbal medicines) should be used after their suitability for "Sho" has been confirmed. The source is the *Kinkiyoryaku* (金匱要略): convulsion, dampness, and heatstroke diseases—water qi diseases (痙湿暍病篇·水氣病編). It says "boiogito is chiefly used for people suffering neuralgia, floating pulse, heavy body, and sweating with aversion to wind. The external signs for boiogito include wind edema and floating pulse. The patient may appear to have a sweaty head, but no other external signs, except the lower body, feels heavy with edema extending to the groin, making bending and extending difficult, and yet suffer no ill effects above the low back." In other words, it is used for patients with the so-called flabby constitution, pale complexion, proneness to fatigue, sweatiness, and decreased urine output. Boiogito is effective for patients who often suffer arthralgia or low back pain, who are susceptible to edema and sensitive to cold, who demonstrate the so-called "frog belly" when lying down, and who have abdominal skin that shows dimpling, softness (deficiency *Nankyo*) when pinched, and flabbiness. Boiogito is more often used in women than men. Patients are pale, plump, flabby, and heavy; their demeanor is listless; they shy away from cleaning and cooking, move infrequently, and eat little. Patients generally pass stools daily, have low menstrual flow, and may complain of irregular menses. They readily perspire, and in summer, their perspiration is profuse. Edema develops in the legs to the degree that shoes and socks are tight by the end of the day.

2.5. Other Prescriptions. Daisaikoto (大柴胡湯) is used for obese patients with muscular build and robust appetite owing to their active lifestyle. When faced with stress or unpleasantness, patients readily develop liver qi depression, irritability, irascibility, a bitter taste in the mouth, and blood congestion in the eye. Transverse invasion of liver qi into the stomach upsets splenogastric function and abnormally promotes appetite, which in turn adds to the obesity [30]. Tokakujokito (桃核承氣湯) and keishibukuryogan (桂枝茯苓丸) are commonly used for women with sudden weight gain in menopause. In patients with blood stasis (Oketsu), the blood rises to the face turning the face and mucous membranes of the tongue and lips red. Venous engorgement and telangiectasia of the skin and mucous membranes is associated with dry, rough skin, lower abdominal bloating, resistance, and tenderness and autonomic symptoms such as upper heat and lower cold (*Hienobose*). Patients also often complain of numbness and pain in various parts of the body.

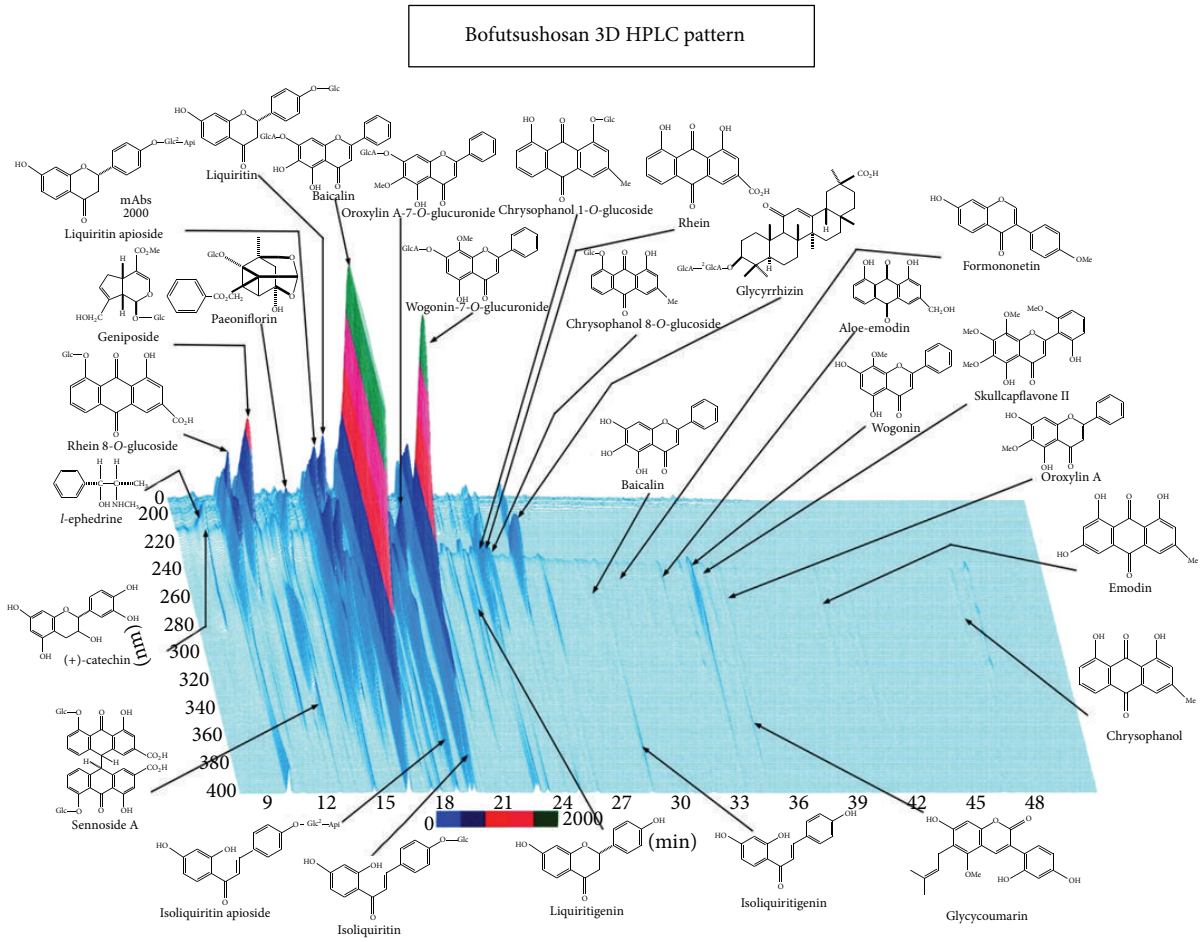

Figure 2

Description	Boiogito extract granules for ethical use	
Composition	7.5 g of TSUMURA boiogito extract granules contains 3.75 g of a dried extract of the following mixed crude drugs	
	JP astragalus root	5.0 g
	JP sinomenium stem	5.0 g
	JP atractylodes lancea rhizome	3.0 g
	JP jujube	3.0 g
	JP glycyrrhiza	1.5 g
	JP ginger	1.0 g
	(JP: the Japanese pharmacopoeia)	
Inactive ingredients	JP light anhydrous silicic acid JP magnesium stearate JP lactose hydrate	

Figure 3

3. Basic Research of Kampo

3.1. Introduction of Basic Research. Numerous investigators are attempting to clarify how Kampo exerts its effects. However, many issues need to be resolved before the mechanisms are clearly understood. First, as shown by the data on HPLC, many of the components of Kampo have effects. Also, HPLC analysis does not indicate volatile-element composition. Components may chemically react when mixed. Thus, it is hard to show which of the components in Kampo the active

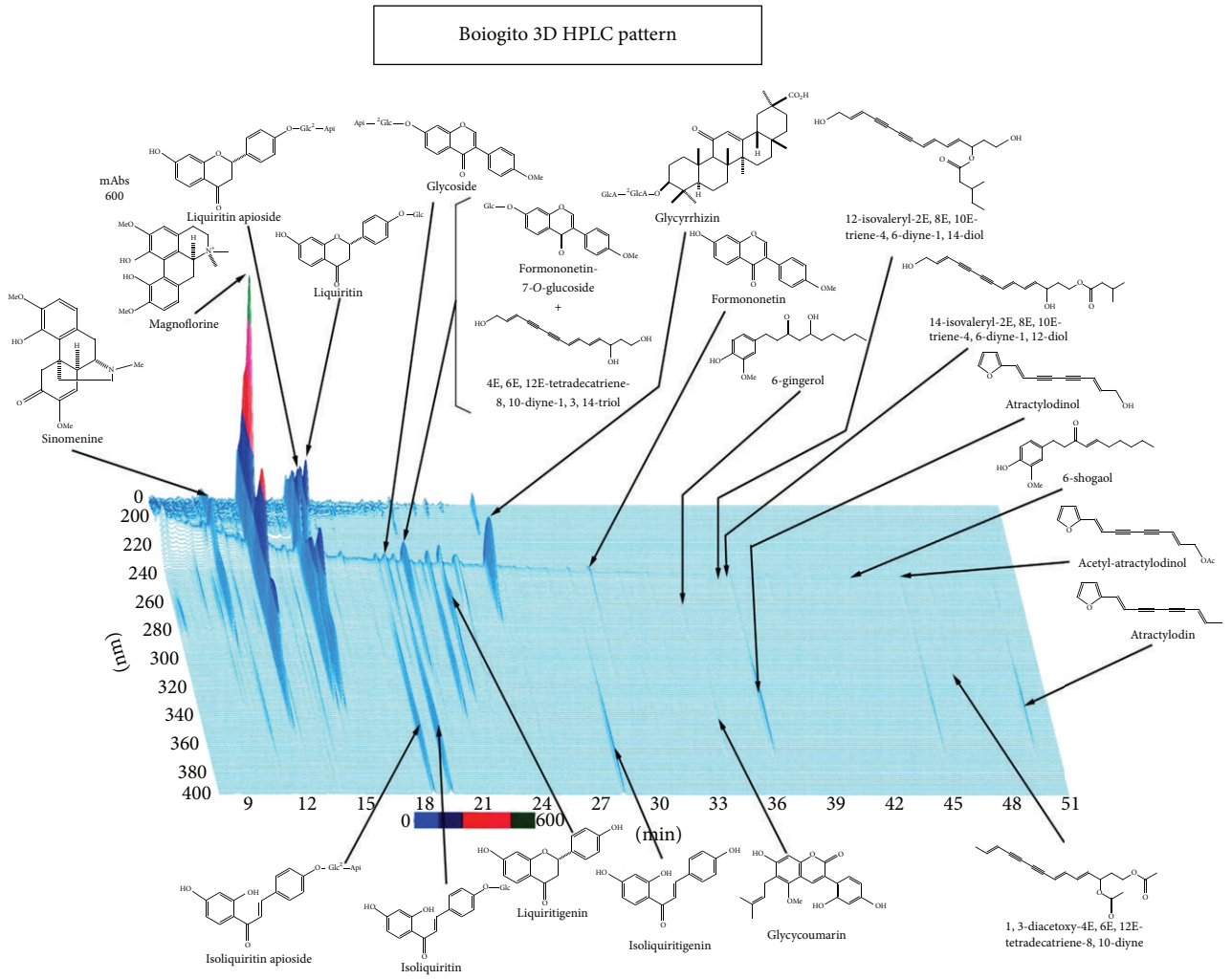

Figure 4

components are. Second, Kampo are components considered to be prodrugs, which exert their effects after being metabolized by the human body. On the other hand, some exert their effects immediately after oral administration.

3.2. Bofutsushosan. *Ephedra* herb (麻黄, mao) is one of the most important natural remedies in Kampo. Ephedrine, a main component of *Ephedra* herb, activates adrenalin receptors in sympathetic nerves, leading to increased production of cAMP and in turn to increased heat production from brown adipose tissue [33–35]. Nakayama et al. have reported bofutsushosan that seems effective in the activities of antiobesity, antihyperlipidemia, and antihyperlipids in liver cytoplasm [33]. In France, ephedrine combined with caffeine has been used for the treatment of obesity, and the action mechanism is considered to be phosphodiesterase inhibition [36]. Yoshida et al. have reported the antiobesity action of bofutsushosan in monosodium glutamate (MSG) obese mice [34]. The aim was to investigate whether the antiobesity action of bofutsushosan is due to the stimulation of brown adipose tissue thermogenesis and inhibition of phosphodiesterase activity. Bofutsushosan works by stimulating BAT thermogenesis and inhibiting phosphodiesterase activity in mice. Akagiri et al. have reported that bofutsushosan, an oriental herbal medicine, attenuates the weight gain of white adipose tissue and the increased size of adipocytes associated with the increase in their expression of uncoupling protein 1 in high-fat diet-fed male KK/Ta mice. Bofutsushosan decreases the weight and size gains of WAT along with upregulating UCP1 mRNA in WAT in high-fat diet-fed mice [37]. Shimada et al. have reported preventive effects of bofutsushosan on obesity and various metabolic disorders. In the TSOD mice treated with bofutsushosan, body weight gain and visceral/subcutaneous fat accumulation were significantly suppressed. Biochemical parameters in plasma (glucose, TC, insulin, and tumor necrosis factor-alpha level) were significantly suppressed, and abnormal glucose tolerance, elevation of blood pressure, and peripheral neuropathy accompanying progression of metabolic disorders were also significantly suppressed [27].

3.3. Boiogito. There are few basic experimental studies on boiogito. Shimada et al. have reported preventive effect of boiogito on metabolic disorders in the TSOD mouse,

a model of spontaneous obese type II diabetes mellitus. boiogito is effective as an antiobesity drug for the "asthenic constitution" type in which subcutaneous fat accumulates but cannot be expected to exert a preventive effect against various symptoms of metabolic syndrome that are based on visceral fat accumulation [26]. We previously reported that boiogito had antiobesity action in ovariectomized rats [38]. In this experiment, the antiobesity properties of boiogito were evaluated in ovariectomized rats by measuring changes in levels of serum cytokines and fat cell adipocytokines. After treatment with boiogito for 6 weeks (20-week-old rats), there was a significant weight decrease compared to the control group, a significant dose-dependent increase in serum tumor-necrosis-factor- (TNF-)α level, and a significant increase in adipose-tissue TNF-α level, suggesting that boiogito contributes to weight gain inhibition via the secretion of TNF-α by fat cells. On the other hand, peroxisome proliferator-activated receptor-γ and adiponectin protein levels did not differ significantly between experimental and control groups; the levels of their corresponding mRNAs tended to increase dose dependently, and the level of resistin did not change significantly.

Our previous study using rat preadipocytes suggests that bofutsushosan and boiogito inhibit differentiation and proliferation of white adipocytes through distinct mechanisms [39]. Three Kampo medicines, boiogito, bofutsushosan, and orengedokuto used for treatment of obesity were investigated to determine their effects on adipogenesis in cultured rat white adipocytes. Administration of the three extracts (1–100 mg/mL) suppressed adipogenesis in a concentration-dependent manner without any cytotoxicity. The three herbal extracts were found to have the potential to prevent adipogenesis in rat white adipocytes. Different mechanisms modulating gene expression levels were involved.

4. Clinical Application of Kampo

4.1. Introduction of Evidence-Based Medicine (EBM). Two randomized controlled trials (RCTs) of bofutsushosan show its potential as an antioxidant. Ogawa et al. conducted a double-blind (DB) RCT on the effect of bofutsushosan on the lag time of low-density lipoprotein (LDL) oxidation in healthy individuals [40]. Antioxidants are present in herbs or crude herbal formulations. The effect of bofutsushosan on *ex vivo* LDL oxidation lag time was studied in healthy human subjects. Although bofutsushosan had no detectable systemic antioxidative effects, *ex vivo* results suggested its antioxidative effect on LDL oxidation. The RCT by Hioki et al. demonstrated the effect and safety of bofutsushosan in Japanese obese subjects [41]. The aim was to determine whether bofutsushosan could decrease visceral adiposity and insulin resistance. They concluded that bofutsuhosan could be a useful herbal medicine in treating obesity with impaired glucose tolerance.

4.2. Case of Knee Osteoarthritis. After receiving sufficient medical treatment from an orthopedist, and also in order to raise a patient's quality of life (QOL), they introduce to a Kampo medicine medical specialist. Knee osteoarthritis is a degenerative disease of the knee joint that is more common in people older than 40 years and in women. The most important characteristic of knee osteoarthritis is the degeneration of the knee joint articular cartilage, causing decreased QOL in affected people. Obesity is one of the most important causes of osteoarthritis. We report the two cases of obese patients who showed marked improvement in osteoarthritis-related clinical symptoms as a result of Kampo treatment.

Case 1 (a 64-year-old female). Chief complaints: bilateral articular pain in the knee. Past history: hypertension, hyperlipidemia, obesity, and osteoarthritis. Present illness: from the age of 42 year, she underwent dietary therapy for obesity, which was not remarkably effective. She was referred to our department for Kampo therapy on May 12, 2004. Physical findings: body height 143 cm, body weight 76.4 kg, and BMI 37.4 kg/m^2. Oriental medical diagnosis: interior heat excess pattern (eight principles classification), sunken excessive pulse (pulse diagnosis), yellow slimy tongue fur (tongue diagnosis), and excessive abdominal strength, slight gastric stuffiness, and paunch (abdominal examination); therefore, bofutsushosan (TJ-62) 7.5 g t.i.d. was prescribed (pattern-based diagnosis). Clinical course: body weight was decreased by 4 kg (from 76 kg to 72 kg) in 14 days after the start of bofutsushosan (TJ-62) 7.5 g t.i.d. There was a remarkable improvement in leg edema and bowel movement. On day 28, her body weight was 70 kg, and she no longer needed a painkiller prescribed by her orthopedist for knee osteoarthritis.

Case 2 (a 76-year-old female). Chief complaints: poor condition of the knee. Past history: hyperlipidemia and osteoarthritis. Present illness: she had been treated for hyperlipidemia by her family doctor for a long time. four years ago, she received a diagnosis of knee osteoarthritis and was treated accordingly. She visited our department to receive Kampo treatment on April 20, 2007. Physical findings: body height 151 cm, body weight 60.3 kg, and BMI 26.4 kg/m^2. Oriental medical diagnosis: eight network classification: imaginary cold proof back. Pulse diagnosis: slightly floating. Tongue diagnosis: wet, frank color, and thin white moss. Abdominal examination: belly force: imaginary frog belly leg edema (+) and Based on sui testimony diagnosis, the treatment with boiogito (TJ-20) 7.5 g t.i.d. was initiated. Clinical course: two weeks after the administration of boiogito, knee joint pain was improved, and body weight reduced 2 kg (from 60 to 58 kg). One month after administration, she no longer needed the painkiller prescribed by her orthopedist.

In Case 1, the diagnosis was the hyperfunctioning type of febrile syndrome of the viscera (according to the four paired parameters of Kampo diagnosis) and the so-called "muscular type," which is associated with constipation and dizziness. Bofutsushosan was prescribed because of her marked obesity (BMI 37.4), and it proved to be very effective. In Case 2, the diagnosis was the hypofunctioning type of febrile syndrome of the viscera (according to the four paired parameters of

Kampo diagnosis) and the so-called "white complexion and flabby body type." She had edema and excessive sweating with weak stomach. We prescribed boiogito. Without body movement, no energy is consumed. The lack of exercise reduces the amount of muscle producing energy and, in turn, basal metabolism leading to resistance of the body to energy consumption. Notably, the lack of exercise impacts the reduction of basal metabolism more than it does with the reduction of energy consumption. It is generally accepted that environmental factors as well as genetic factors influence the development of obesity. Still, it is not easy to manage obesity. Lifestyle modifications, such as dietary and exercise interventions, are hard to follow. For obese subjects with knee problems, walking exercise is practically impossible. Thus, any suggestion by family members or others that exercise is needed could impose a mental burden. Based on the findings in the above-mentioned two cases, we suggest that the herbal treatment may trigger the awareness of weight loss. Majima et al. have reported the effect of the Japanese herbal medicine, Boiogito, on the osteoarthritis of the knee with joint effusion. boiogito have a possibility for a treatment modality for joint effusion with osteoarthritis of the knee [42].

5. Conclusion

Modern Western medicine is the official medicine practiced in every country. In Kampo medicine as well as other traditional medicines, different formulas have been prescribed for patients with the same disease, and diagnosis has been made by considering the constitution and condition of each patient. Such an individualized treatment has been successful in patients without any particular abnormalities of laboratory data. WHO is rigorously trying to incorporate complementary medicine into conventional medicine, emphasizing the importance of traditional medicine. Kampo is expected to be applied not only to therapeutics but also to disease prevention. In clinical practice, the usefulness of Kampo in combination with Western medicine is to be confirmed.

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Research Article

Boehmeria nivea Stimulates Glucose Uptake by Activating Peroxisome Proliferator-Activated Receptor Gamma in C2C12 Cells and Improves Glucose Intolerance in Mice Fed a High-Fat Diet

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We examined the antidiabetic property of *Boehmeria nivea* (L.) Gaud. Ethanolic extract of *Boehmeria nivea* (L.) Gaud. (EBN) increased the uptake of 2-[*N*-(nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose in C2C12 myotubes. To examine the mechanisms underlying EBN-mediated increase in glucose uptake, we examined the transcriptional activity and expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), a pivotal target for glucose metabolism in C2C12 myotubes. We found that the EBN increased both the transcriptional activity and mRNA expression levels of PPAR-γ. In addition, we measured phosphorylation and expression levels of other targets of glucose metabolism, such as AMP-activated protein kinase (AMPK) and protein kinase B (Akt/PKB). We found that EBN did not alter the phosphorylation or expression levels of these proteins in a time- or dose-dependent manner, which suggested that EBN stimulates glucose uptake through a PPAR-γ-dependent mechanism. Further, we investigated the antidiabetic property of EBN using mice fed a high-fat diet (HFD). Administration of 0.5% EBN reduced the HFD-induced increase in body weight, total cholesterol level, and fatty liver and improved the impaired fasting glucose level, blood insulin content, and glucose intolerance. These results suggest that EBN had an antidiabetic effect in cell culture and animal systems and may be useful for preventing diabetes.

1. Introduction

Diabetes is a serious health issue that affects the life span of humans. Of those diagnosed with diabetes, approximately 5–10% have type 1 diabetes, which is characterized by the loss of insulin production in pancreatic beta-cells, whereas 90–95% have type 2 diabetes, which is characterized by insulin resistance. Although various drugs have been developed for diabetes treatment, their side effects remain obstacles to their use. Natural ingredients are widely distributed in the plant kingdom. They have been traditionally used to treat a variety of human diseases, including metabolic disorders. It is believed that natural ingredients are safer than synthetic compounds because they have been used for a long time [1, 2]. The results of a number of studies conducted by other researchers and our previous study also suggested that naturally occurring compounds could exert beneficial health effects in the treatment of diabetes by modulating cellular signaling pathways [2–5]. Among signaling molecules, peroxisome proliferator-activated receptor gamma (PPAR-γ) regulates fatty acid and glucose metabolism. PPAR-γ has been implicated in the pathology of obesity and diabetes [3–5]. PPAR-γ agonists such as glitazone have been used to treat hyperglycemia [6]. In addition, PPAR-γ agonists derived from natural herbs may prevent diabetes. For example, *Cornus kousa* F. Buerger ex Miquel, a medicinal plant, increases PPAR-γ activity and stimulates glucose uptake. In addition, *Aegle marmelos* fruit aqueous extract, *Syzygium aromaticum* flower bud (clove) extract, and *Sambucus nigra* (elderflower) extract exert antidiabetic effects by increasing PPAR-γ activation or expression [7–9]. AMP-activated protein kinase (AMPK) and Akt protein are other important

signaling molecules in glucose homeostasis. AMPK is an insulin-independent regulator of glucose uptake. By contrast, the PI3 kinase/Akt pathway is an insulin-dependent regulator of glucose uptake. Thus, AMPK and Akt are also therapeutic targets for metabolic disorders such as obesity and diabetes [10, 11].

Boehmeria nivea (L.) Gaud., a flowering plant in the nettle family Urticaceae, has been widely grown in east Asian countries such as Korea, India, and China. The edible parts of this plant, the leaves and roots, have been reported to have anti-inflammatory, antioxidant, and antifungal effects [12, 13]. However, the antidiabetic effect of *B. nivea* has not been clearly elucidated. Therefore, in this study we evaluated the antidiabetic potential of ethanol extract of *B. nivea* (EBN) and its signaling mechanisms by using *in vitro* and *in vivo* approaches.

2. Materials and Methods

2.1. Materials. An authenticated *B. nivea* sample was provided by a public officer from the Seocheon County Office (Seocheon, Republic of Korea), where a voucher specimen has been deposited. C2C12 and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Welgene (Daegu, Republic of Korea). Horse serum was purchased from Life Technologies (Seoul, Republic of Korea). 2-[*N*-(Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Invitrogen (Carlsbad, CA, USA). PPAR-γ and glyceraldehyde phosphate dehydrogenase (GAPDH) primers were designed based on sequence data from the NCBI database and were purchased from Bioneer (Daejeon, Republic of Korea). Phospho-AMP-activated protein kinase (pAMPK), phospho-Akt (pAKT), PPAR-γ, and AMPK were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin and horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Extraction and Lyophilization. High-quality *B. nivea* specimens were selected and mixed by a specialist, and the ethanol extract was prepared. Leaves of *B. nivea* were pulverized and extracted with 70% ethanol by shaking for 24 h at 25°C, and the precipitates were removed by centrifugation at 8,000 ×g for 30 min (Beckman, USA). Supernatants were lyophilized using a freeze dryer (Il Shin, Dongducheon, Republic of Korea). The yield of ethanol extract from the leaves of *B. nivea* was 10.0% (w/w). Ethanol extract from the leaves of *B. nivea* (EBN) was dissolved in distilled water and sterilized by passage through a 0.45 μm Millipore filter unit for use in the experiments.

2.3. Muscle Cell Differentiation and Glucose Uptake Assay. C2C12 cells were cultured in DMEM containing 10% FBS. The cells were maintained at 37°C in a humidified 5% CO_2 environment. After the cells reached confluency, the medium was changed to DMEM supplemented with 2% horse serum, until the cells were entirely differentiated. For the experiments, the cells were starved in low-glucose serum-free DMEM for 12 h and then treated with or without 50 μM 2-NBDG or with 2-NBDG with EBN at the indicated concentrations (200, 400, 800, and 1200 μg/mL) for 24 h. Cellular uptake of 2-NBDG was measured using a fluorometer at excitation and emission wavelengths of 465 and 540 nm, respectively.

2.4. PPAR-γ Transcriptional Activity Assay. PPAR-γ transcriptional activity was measured as described previously [14]. HEK293 cells were cultured in DMEM containing 10% FBS. Cells were transiently transfected with 1 μg of total DNA (expression plasmids for PPAR-γ, retinoid X receptor α (RXRα), PPAR response elements (PPREs), and β-galactosidase) by using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After transfection, the cells were treated with EBN at the indicated concentrations (200, 400, 800, and 1200 μg/mL) in the absence or presence of rosiglitazone, a PPAR-γ agonist, for 24 h. PPAR-γ transcriptional activity was examined using a luciferase reporter gene assay with the Luciferase Assay System (Promega, Madison, WI, USA) and was normalized to the β-galactosidase activity.

2.5. RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction. Differentiated C2C12 cells were treated with various concentrations of EBN for 6 h, and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from isolated RNA and was amplified by polymerase chain reaction (PCR) in a PCR thermal cycle device by using specific primers: PPAR-γ (sense), 5′-ACC ACT CGC ATT CCT TTF AC-3′; PPAR-γ (antisense), 5′-TCA GCG GGA AGG ACT TTA TG-3′; β-actin (sense), 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′; and β-actin (antisense), 5′-GGA TGC CAC AGG ATT CCA TAC CCA-3′.

2.6. Protein Extraction and Western Blot Analysis. Total protein was extracted from EBN-stimulated cells by using lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, a protease-inhibitor cocktail, and a phosphatase-inhibitor cocktail). Equal amounts of protein (30 μg) were separated using 10% SDS-PAGE and were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) for 1 h and was incubated in primary antibody diluted in TBS. After washing with TBST (TBS with 0.1% Tween 20), the membrane was incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Blots were developed with an enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, UK).

2.7. Animal Experiments. Male C57BL/6J mice (age, 3 weeks) were obtained from Nara Biotech (Seoul, Republic of Korea) and housed under a 12 h light/12 h dark cycle in a temperature- and humidity-controlled room (24°C ± 1°C at 50% relative humidity). After adaptation for 1 week, the mice were freely fed a 10% fat normal diet (ND, D12450B, Research

Diets, New Brunswick, NJ, USA), a 60% kcal high-fat diet (HFD, D12492, Research Diets, New Brunswick, NJ, USA), or a 60% kcal high-fat diet plus 0.5% EBN (HFD + 0.5% EBN) for 9 weeks. Food intake and body weight were measured every week. After 9 weeks, the mice were fasted overnight and then killed. The blood samples were collected from the orbital vein. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute.

2.8. Glucose Tolerance Test. The glucose tolerance test (GTT) was performed at 8 weeks. After overnight fasting, mice were intraperitoneally administered glucose (1 g/kg of body weight), and blood was collected from the tail vein every 30 min from 0 min to 150 min after injection. Blood glucose levels were examined by an Accu-Chek glucometer (Roche, Basel, Switzerland).

2.9. Quantitation of Serum Total Cholesterol and Insulin Levels. Fasting serum levels of total cholesterol (TC) and insulin were determined by enzymatic methods using commercial kits (Asan Pharm, Seoul, Republic of Korea).

2.10. Histopathology. Liver tissue was fixed in 4% buffered formalin and cut into 4 μm thick sections. The sections were stained with hematoxylin and eosin (H&E) and examined by microscopy. Fat content was scored semiquantitatively with the following parameters, as described previously [15]: 0 = no fat; 1+ = <25%, 2+ = 25–50%, 3+ = 51–75%, 4+ = 76–95%, and 5+ = 100%.

2.11. Cytotoxicity Test. Cells were starved with low-glucose, serum-free DMEM for 12 h and treated with EBN at the indicated concentrations (200, 400, 800, and 1200 μg/mL) for 24 h. The medium was removed, and the cells were incubated with 100 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA) in PBS at 5 mg/mL for 4 h. The absorbance was measured using an ELISA reader at 540 nm.

2.12. Statistical Analysis. The data have been expressed as the mean ± standard deviation (SD) values of at least 3 independent experiments. Statistical analyses were performed using SPSS version 9.0 (SPSS Inc., Chicago, IL, USA). Differences between means were evaluated using two-way analysis of variance (ANOVA) followed by the Bonferroni test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. EBN Stimulates Glucose Uptake in C2C12 Myotubes. We first performed the 2-NBDG uptake assay to examine the antidiabetic activity of EBN in C2C12 myotubes. EBN significantly increased 2-NBDG uptake in a dose-dependent manner (Figure 1(a)). Cytotoxic effects of EBN were not observed below 1200 μg/mL (Figure 1(b)). This result suggests that EBN increases glucose uptake in myotubes.

Table 1: Effect of EBN on high-fat diet-induced body weight changes, serum cholesterol, and insulin.

	ND	HFD	EBN
Initial body weight (g)	20.3 ± 0.9	20.0 ± 1.1	20.1 ± 0.9
Final body weight (g)	28.8 ± 3.0	40.6 ± 3.8[a]	35.7±4.6[b]
Serum			
Total cholesterol (mg/dL)	172 ± 14	209 ± 9[a]	196 ± 9[b]
Insulin (ng/mL)	0.331 ± 0.112	1.22 ± 0.21[a]	0.812 ± 0.201[b]

ND: normal diet; HFD: high-fat diet; EBN: high-fat diet plus 0.5% EBN. Data are expressed as mean ± SD ($n = 10$). Statistical significance: [a]$P < 0.05$ for the ND versus HFD; [b]$P < 0.05$ for the HFD versus HFD + 0.5% EBN.

3.2. Activation of PPAR-γ, rather than AMPK and Akt, Is Involved in EBN-Stimulated Glucose Uptake. To determine the molecular mechanisms underlying EBN-stimulated glucose uptake, we measured the activities of PPAR-γ, AMPK, and Akt. PPAR-γ is a critical target of a number of insulin-sensitizing drugs [3–5]. Treatment with EBN significantly increased the transcriptional activity of PPAR-γ in HEK293 cells (Figure 2(a)). Under the same conditions, 25 μM rosiglitazone was used as a positive control for PPAR-γ activation. In addition, we measured the expression of PPAR-γ in EBN-treated C2C12 myotubes. EBN increased the expression of PPAR-γ in C2C12 myotubes (Figure 2(b)). Then, we examined whether AMPK and Akt signaling pathways were involved in EBN-stimulated glucose uptake. AMPK is another target for the antidiabetic effect of metformin, and Akt is a critical mediator of the insulin-sensitizing effect [10, 11]. EBN did not increase the phosphorylation or expression of AMPK and Akt in a dose- and time-dependent manner in C2C12 myotubes (Figures 2(c) and 2(d)). 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) was used as a positive control for AMPK activation. These results indicate that EBN stimulates glucose uptake, at least in part through activation of PPAR-γ. AMPK and Akt signaling pathways, however, were not involved in EBN-stimulated glucose uptake in C2C12 myotubes.

3.3. Administration of EBN Improves Body Weight, Body Fat Mass, Liver Fat Content, and Serum TC Levels in Mice Fed a HFD. We performed an *in vivo* experiment to confirm the antidiabetic effect of EBN. The initial body weights of mice in each group were not statistically different (Table 1). The final body weights were lower in the EBN group (Table 1). The body weights in the HFD group were higher than those in the ND group (Figure 3(a)). The body weights of mice in the HFD + 0.5% EBN group were lower than those in the HFD group. The total food intake was not statistically different in each group (Figure 3(b)). In addition, we assessed body fat mass by using computed tomography (CT) imaging and found that the whole body fat mass was higher in the HFD group than in the ND group, which is shown in red in Figure 3(c). Administration of 0.5% EBN significantly reduced the whole body fat mass in mice fed a HFD (Figure 3(c)). Further, serum cholesterol levels were significantly lower in the HFD + 0.5% EBN group than in the HFD group (Table 1). Moreover, the concentration of fat in the liver tissue was significantly

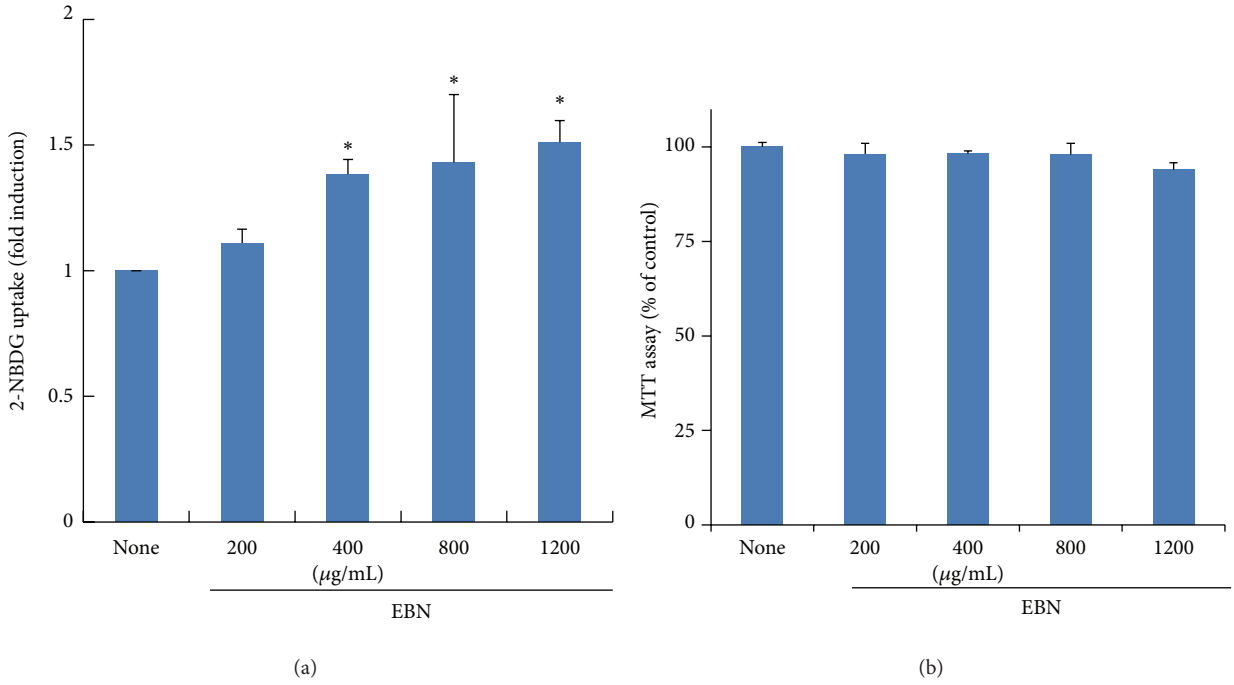

Figure 1: Effect of EBN on glucose uptake in C2C12 myotubes. Differentiated C2C12 cells were treated with EBN and 2-[*N*-(nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) for 24 h. Then, the 2-NBDG assay was performed as described in Section 2 (a). After treatment of EBN for 24 h in C2C12 myotubes, the cytotoxicity was measured by MTT assay as described in Section 2. The result of MTT assay was expressed as percentage of control (None) (b). Data are expressed as mean ± standard deviation (SD); $n = 3$. $^*P < 0.05$ versus None.

lower in the HFD + 0.5% EBN group than in the HFD group. The histopathology scores also showed that 0.5% EBN significantly decreased fat accumulation stimulated by HFD (Figure 3(d)).

3.4. Effect of EBN on Fasting Glucose Levels and Glucose Intolerance. We determined fasting glucose and insulin levels and performed a GTT to determine the antidiabetic effect of EBN in mice fed a HFD. The fasting glucose level was significantly higher in the HFD group than in the ND group. The fasting glucose level was lower in the HFD + 0.5% EBN group than in the HFD group (Figure 4(a) and Table 1). Compared to the ND group, the HFD group showed an abnormal increase in insulin levels, but the insulin levels significantly decreased in the HFD + 0.5% EBN group (Table 1). The GTT test showed that the HFD led to glucose intolerance. Administration of 0.5% EBN markedly decreased the glucose intolerance induced by the HFD (Figures 4(b) and 4(c)). These results show that EBN improves glucose tolerance and insulin resistance.

4. Discussion

In this study, we showed that EBN exerts an antidiabetic effect in C2C12 myotubes and in a mouse model of a HFD. In addition, we showed that PPAR-γ activation, rather than AMPK and Akt signaling, was associated with the antidiabetic activity of EBN. Our findings demonstrate that EBN has antidiabetic and antiobesity effects *in vitro* and *in vivo*. Compared to the findings of a recent study that showed that *B. nivea* inhibits alpha-glucosidase or beta-glucosidase, our findings better substantiate the antidiabetic effect of EBN [16]. Although the recent study showed the glucosidase inhibitory effect of *B. nivea in vitro*, it did not describe the mechanisms underlying this effect; our study is based on these data, and we performed experiments to confirm the antidiabetic effects of *B. nivea*. Through cell and animal experiments, we demonstrated the precise antidiabetic efficacy of *B. nivea* and the mechanisms underlying the effect. The total phenolic content of EBN was 3640 mg/100 g. Several phenolic compounds such as rutin (46.48 mg/100 g), chlorogenic acid (1.96 mg/100 g), luteolin-7-glucoside (11.29 mg/100 g), naringin (1.13 mg/100 g), hesperidin (23.69 mg/100 g), and tangeretin (1.59 mg/100 g) have already been found in EBN [17].

We showed that EBN-stimulated glucose uptake is accompanied by PPAR-γ activation and expression but is not associated with AMPK and Akt activation. PPAR-γ participates in glucose uptake, and PPAR-γ agonists increase the sensitivity of insulin receptors [3–5]. In addition to thiazolidinedione, a PPAR-γ agonist that is used clinically, naturally occurring ingredients or compounds have also been used as PPAR-γ agonists to significantly improve insulin resistance [3, 18, 19]. For example, *Cornus kousa* F. Buerger ex Miquel, a medicinal plant, increases PPAR-γ ligand binding activity and stimulates glucose uptake by an AMPK-independent mechanism [7]. A number of studies have shown that

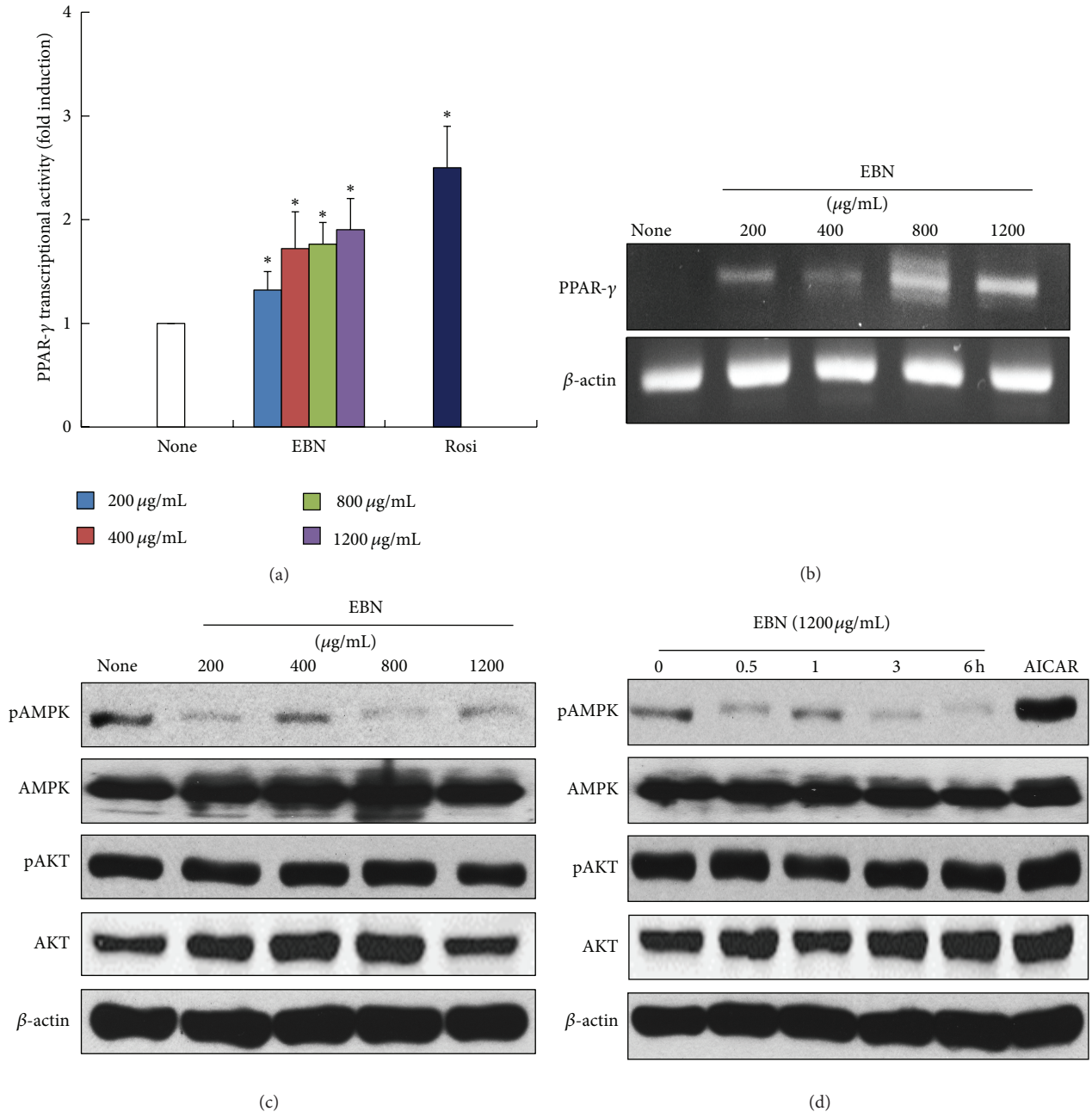

Figure 2: The effect of EBN on transcriptional activity and expression of peroxisome proliferator-activated receptor gamma. HEK293 cells were transiently transfected with luciferase construct containing peroxisome proliferator-activated receptor gamma (PPAR-γ), retinoid X receptor (RXRα), PPAR-response element (PPRE), and β-galactosidase. Then, the cells were treated with ethanol extract of *Boehmeria nivea* (EBN) (200, 400, 800, and 1200 μg/mL) for 24 h. Luciferase assay was performed, and the activity was normalized with that of β-galactosidase activity (a). The differentiated C2C12 cells were exposed to EBN in a dose-dependent manner for 6 h. The mRNA level of PPAR-γ and GAPDH was measured by reverse transcriptase-polymerase chain reaction (RT-PCR) (b). Differentiated C2C12 cells were treated withEBN in a dose- and time-dependent manner. AMP-activated protein kinase (AMPK) and Akt expressions were determined by western blot analysis ((c), (d)). 1 mM AICAR was used as positive control for AMPK activation. Data are expressed as mean ± standard deviation (SD); $n = 3$. Statistical significance: $^{*}P < 0.05$ for the none versus EBN or rosiglitazone.

aqueous extract from the fruits of *Aegle marmelos*, *Syzygium aromaticum* flower bud (clove) extract, and *Sambucus nigra* (elderflower) extract improve insulin resistance by increasing PPAR-γ activation or expression [8, 9]. Therefore, consistent with previous studies, PPAR-γ activation and expression could explain the antidiabetic effect of EBN observed in our study. AMPK and Akt were not altered by EBN under our experimental conditions. AMPK is a key regulator of glucose metabolism and is a target of metformin, an antidiabetic drug [10]. Like PPAR-γ, AMPK is a target for naturally occurring

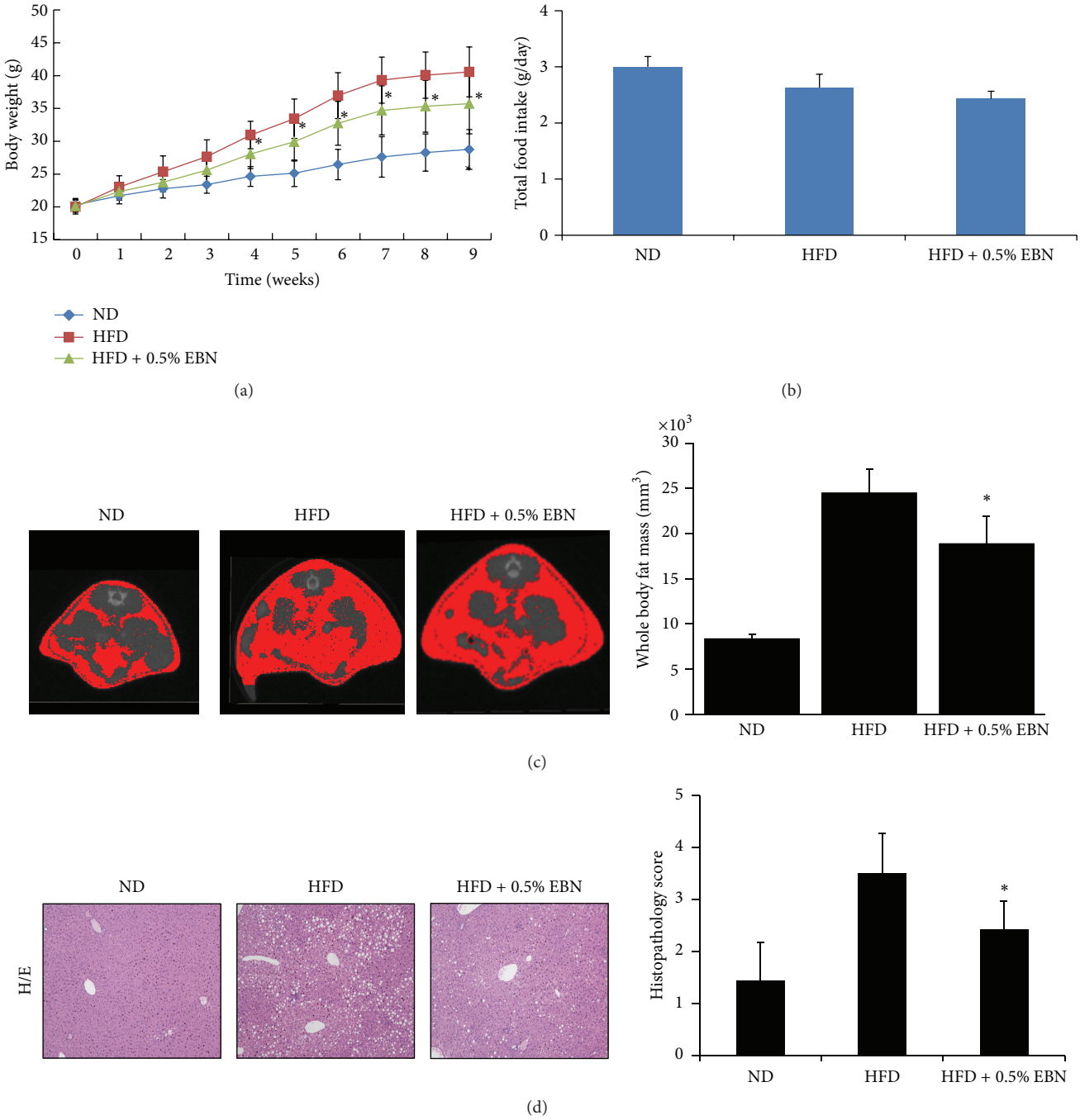

Figure 3: The effect of EBN on body weight, total food intake, and accumulation of fat in the liver of mice fed a high-fat diet. Mice were fed normal diet (ND), high-fat diet (HFD), and high-fat diet with 0.5% EBN (HFD + 0.5% EBN). Body weight and food intake were measured every week ((a), (b)). Body fat mass was assessed using computed tomography (CT) during the 8 weeks (c). In addition, we measured the accumulation of fat in the liver as described in Section 2 (d). Data are expressed as the mean ± standard deviation (SD) ($n = 10$). Statistical significance: $^*P < 0.05$ for the HFD versus HFD + 0.5% EBN.

ingredients or compounds for the prevention of diabetes [20]. Insulin-dependent PI3K/Akt signaling can also improve diabetes by activating insulin receptor [21]. Once insulin binds to insulin receptor, insulin receptor substrate 1 (IRS-1) is activated. Activated IRS-1 triggers insulin signaling cascades involving PI3K, phosphoinositide-dependent kinase-1 (PDK-1), and Akt and increases glucose transporter 4 (Glut-4) translocation and glucose uptake [22]. EBN treatment did not alter insulin-dependent PI3K/Akt signaling and AMPK signaling (Figures 2(c) and 2(d)). These results indicate that EBN-stimulated glucose uptake is mediated by PPAR-γ, which suggests that EBN does not mimic the effect of insulin

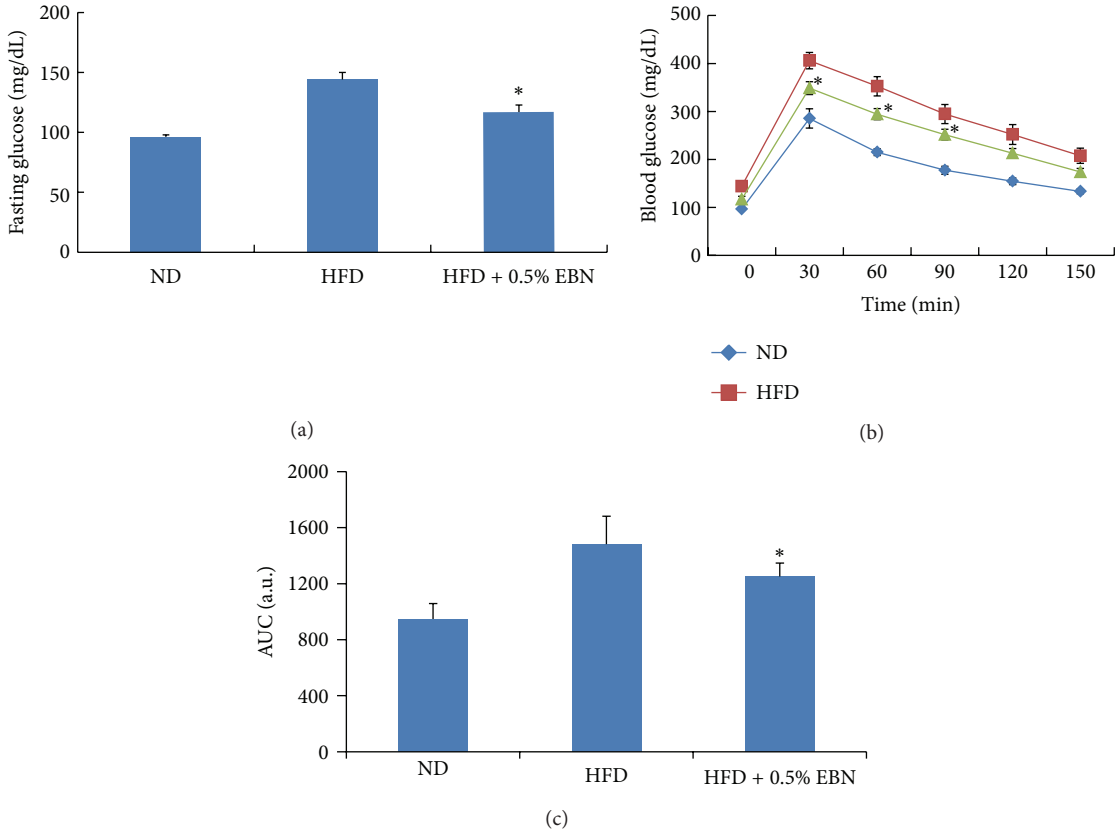

FIGURE 4: The effect of EBN on fasting glucose levels and glucose intolerance in mice fed a high-fat diet. Mice were starved for 12 h and blood was drawn from the orbital vein, and we measured the fasting glucose levels as described in Section 2 (a). Glucose tolerance test was performed at the end of 8 weeks. Mice were injected with glucose (1 g/kg of body weight), and blood glucose levels were examined every 30 min from 0 min to 150 min after injection (b). Area under curve (AUC) during glucose tolerance test (c). Data are expressed as mean ± standard deviation (SD) ($n = 10$). Statistical significance: $^*P < 0.05$ for the high-fat diet (HFD) versus HFD plus 0.5% EBN.

through the insulin receptor or the effect of metformin on AMPK activation. To our knowledge, this is the first study to show the antidiabetic effect and mechanisms of EBN. However, it is still unclear how EBN acts as a PPAR-γ agonist. One possibility, hinted at by early reports, is that PPAR-γ agonists stimulate glucose uptake by increasing Glut4 expression and mRNA levels [23]. In addition, elevated levels of PPAR-γ transcript are accompanied by enhanced Glut-4 transcription and glucose uptake, suggesting that glucose uptake and Glut-4 transcription are induced through the activation of PPAR-γ by PPAR-γ agonists [24]. Therefore, the antidiabetic effect of EBN may be due to increased PPAR-γ and Glut-4 transcription. However, further studies are required to explain the cascades downstream from EBN-mediated activation of PPAR-γ that stimulate glucose uptake.

Administration of 0.5% EBN significantly reduced body weight, body fat mass, liver fat content, and serum total cholesterol levels. In addition, EBN significantly improved HFD-induced fasting glucose levels and glucose tolerance. A number of studies have described a link between HFD-induced obesity and metabolic disorders, including diabetes, hyperlipidemia, and hypercholesterolemia [25, 26]. Obesity causes insulin resistance and thus results in type 2 diabetes. We have shown that a HFD accelerated obesity and glucose intolerance. Administration of EBN dramatically improved the glucose intolerance caused by a HFD. Sancheti et al. showed that the root extract of *B. nivea* exerts an antidiabetic effect against streptozotocin- (STZ-) induced diabetes in rats [16]. In fact, the STZ-induced diabetic model is generally used to identify antidiabetic drugs or ingredients that offset type 1 diabetes. In the present study, we investigated the antidiabetic effect of the leaf extract of *B. nivea*. We used the leaf extract because the leaves of *B. nivea* have traditionally been ingested and used for the prevention of certain diseases in Oriental countries. The results reported by Sancheti et al. on the antidiabetic effect of the root extract of *B. nivea* in a type 1 diabetes model indicated that *B. nivea* may be effective for the prevention of metabolic disorders such as diabetes, obesity, and fatty liver [27]. As expected, we found that EBN was effective in obesity-induced type 2 diabetes mice. Taken together, these findings indicate that the preventative effects of EBN are applicable to obesity-induced insulin resistance similar to that in type 2 diabetes. In addition, the serum levels of total cholesterol and the fat content of the liver decreased in the EBN-treated group. An elevated total cholesterol level is a risk factor for obesity and cardiovascular diseases. Previous

papers have also reported that a number of ingredients extracted from plants exert a cholesterol-lowering effect by lowering the absorption of cholesterol and increasing fecal sterol excretion [28, 29]. In addition, several compounds found in plants inhibit cholesterol synthesis and reduce low-density lipoprotein- (LDL-) cholesterol levels in the plasma [30, 31]. Sancheti et al. have also reported that *B. nivea* exerts an inhibitory effect on cholinesterase *in vitro*, suggesting that *B. nivea* has the potential to lower cholesterol levels [16]. Cholinesterase plays an important role in the metabolism of lipids. Elevation of the serum cholinesterase level is accompanied by high serum cholesterol levels [16]. Thus, the cholesterol-lowering effect of EBN may be due to increased cholesterol excretion or decreased cholesterol synthesis in the liver. The cholesterol lowering effect of EBN may also be due to reduced serum levels of cholinesterase. However, the mechanisms by which EBN lowers serum cholesterol levels should be elucidated further in future studies.

5. Conclusions

Our study showed that EBN exerted an antidiabetic effect by targeting PPAR-γ signaling in myotubes. In addition, EBN improved the abnormal increase in body weight, liver fat, and serum cholesterol level observed in HFD-fed mice. Therefore, *B. nivea* may be useful for preventing diabetes. However, additional studies are required to explain the precise signaling cascades underlying the antidiabetic effect of *B. nivea* in both cell and animal models.

Acknowledgments

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Research Article

In Vitro Evaluations of Cytotoxicity of Eight Antidiabetic Medicinal Plants and Their Effect on GLUT4 Translocation

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Despite the enormous achievements in conventional medicine, herbal-based medicines are still a common practice for the treatment of diabetes. *Trigonella foenum-graecum*, *Atriplex halimus*, *Olea europaea*, *Urtica dioica*, *Allium sativum*, *Allium cepa*, *Nigella sativa*, and *Cinnamomum cassia* are strongly recommended in the Greco-Arab and Islamic medicine for the treatment and prevention of diabetes. Cytotoxicity (MTT and LDH assays) of the plant extracts was assessed using cells from the liver hepatocellular carcinoma cell line (HepG2) and cells from the rat L6 muscle cell line. The effects of the plant extracts (50% ethanol in water) on glucose transporter-4 (GLUT4) translocation to the plasma membrane was tested in an ELISA test on L6-GLUT4myc cells. Results obtained indicate that *Cinnamomon cassia* is cytotoxic at concentrations higher than 100 μg/mL, whereas all other tested extracts exhibited cytotoxic effects at concentrations higher than 500 μg/mL. Exposing L6-GLUT4myc muscle cell to extracts from *Trigonella foenum-graecum*, *Urtica dioica*, *Atriplex halimus*, and *Cinnamomum verum* led to a significant gain in GLUT4 on their plasma membranes at noncytotoxic concentrations as measured with MTT assay and the LDH leakage assay. These findings indicate that the observed anti-diabetic properties of these plants are mediated, at least partially, through regulating GLUT4 translocation.

1. Introduction

Traditional Greco-Arab and Islamic herbal-based preparations have gained enormous popularity in the Arab world as well as Islamic world over the past three decades. This widespread use and popularity have also brought some concerns and fears over professionalism of traditional healers, quality, efficacy, and safety of the "natural" products available on the market [1]. According to ethnopharmacological surveys there are at least 2,600 plant species in the Middle Eastern region. About 700 of these plants are noted in medieval Greco-Arab and Islamic medical books for their therapeutical use [2]. More than 450 of these plants have continued to be employed in the treatment and prevention of human diseases in most Arab-Islamic countries. Safety assessment of herbal products has often been neglected since prolonged and apparently safe use usually is considered as an evidence of its natural safety. Another very important factor is the belief that these medicines are prepared according to the principles of the Greco-Arab medicine that builds the basis for the modern Western medicine. Therefore, most producers and caregiver institutions of Arab herbal medicines are named after the famous scholars Ibn Sina (Avicenna, 980–1037), Al-Razes (Rhazes, 864–930), and Al-Kindi (Alkindus, 800–873) [3]. However, a prolonged traditional and apparently safe usage is not always a reliable guarantee of safety since it is difficult for traditional practitioners to monitor or to detect delayed effects (e.g., carcinogenicity, mutagenicity), rare adverse effects, and adverse effects arising from long-term use. Adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions or uses might result in life-threatening or lethal side effects. Most reports concerning toxic effects of herbal medicines are associated with hepatotoxicity. Yet, reports of other toxic effects including kidney, nervous system, blood, cardiovascular and dermatologic effects, mutagenicity, and carcinogenicity have also been published in the biomedical literature [3]. Several reasons

can cause herbals toxicity, such as, lack of pharmaceutical-level quality control at all stages of production; confusing nomenclature and inaccurate plant identification; variations in levels of active ingredients in different plant parts, plants harvested at different times or stages of development; or the geography, weather, soil, and other conditions specific to the plants growth and development. The complex chemical mixtures of plants and interactions with other herbs, drugs, adulterants, or contaminants; accidental or deliberate unprofessional, unwise, or careless practitioner treatments or recommendations; or incorrect patient use also contribute to safety issues and increase the risk of adverse reactions. Moreover, contamination of herbals with microorganisms, fungal toxins such as aflatoxin, pesticides, heavy metals, and synthetic drugs has been described [4]. Herbal medications are usually mixtures of several ingredients or plants harvested during different seasons and extracted through variable procedures, which makes the identification of both the pharmacologically active and toxic compounds difficult [5].

Diabetes is metabolic disease usually caused by a combination of hereditary and environmental factors, which result in hyperglycemia and other classical symptoms, especially polyuria, polydipsia, and polyphagia. Eventually, hyperglycemia leads to serious damage in blood vessels and the nerves as well as blurred vision and irritability. According to the World Health Organization (WHO), the number of people with diabetes will be doubled within less than 30 years. The prevalence of diabetes is the highest in the Middle East, where the number of diabetic subjects reached 15.2 million in 2000. The number of diabetic in the Middle East will almost triple by 2030 (from 15.2 million in 2000 to about 42.6 millions in 2030). This rapid increase is due to population growth, aging, urbanization, increasing prevalence of obesity, and physical inactivity. Diabetes has been recognized by medieval Greco-Arab physicians and its main symptoms were known by the increased thirst, frequent urination, and tiredness. Greco-Arab physicians and practitioners had used series of medicinal plants for treating these combined symptoms. In addition to several instructions for consumption of specific food, mild exercises were recommended. For example, Avicenna (980–1037), a renowned physician of the Golden Ages of the Arab-Islamic civilization, described diabetes in his book *The Canon of Medicine*. He mentioned gangrene and collapse of sexual function as a complication of this disease. Avicenna and other Greco-Arab physicians recommended the use of various medicinal plants for the treatment of diabetes. Fenugreek, walnut, salt bush, olive nettle, garlic, onion, black seed, and cinnamon are just a few of the medicinal plants that are strongly recommended as antidiabetic and antioxidant [6–8]. Bioactive ingredients of some of these plants have been investigated for their efficacy in treating various human diseases. The antidiabetic mechanisms of a plant extract are usually insulin sensitizer, insulin mimics, insulin secretagogues, and inhibitors of intestinal carbohydrate digestion and absorption. Insulin sensitizers include plants that increase glucose uptake and disposal by muscle, fat, and hepatic cells as well as those that regulate hepatic glycogen metabolism. In this category, garlic and onion decrease blood glucose levels by normalizing liver hexokinase and glucose-6-phosphatase activity [9]. Black seed and cinnamon were suggested to have insulin mimetic properties, through enhancing insulin signaling pathway independently of insulin [10].

In the present *in vitro* study we tested the safety of *Trigonella foenum-graecum* (fenugreek), *Atriplex halimus* (salt bush), *Olea europaea* (olive), *Urtica dioica* (nettle), *Allium sativum* (garlic), *Allium cepa* (onion), *Nigella sativa* (black seed), and *Cinnamomon cassia* (cinnamon). Furthermore, the effects of these plant extracts on glucose transporter-4 (GLUT4) translocation to plasma membrane was tested on L6 muscle cells stably expressing myc-tagged GLUT4 (L6-GLUT4myc) using cell-ELISA test. Glucose uptake into skeletal muscle is mediated by the facilitative hexose transporter, GLUT4, a membrane protein that continuously cycles between intracellular stores and the plasma membrane (PM). Insulin primarily promotes the rate of GLUT4 exocytosis and fusion with the PM, a process termed GLUT4 translocation that results in a gain in surface GLUT4 [11]. Results obtained in the present *in vitro* study indicate that 50% ethanol extracts from *Trigonella foenum-graecum*, *Urtica dioica*, *Atriplex halimus*, and *Cinnamomun verum* led to a significant gain in GLUT4 translocation at noncytotoxic concentrations as measured with MTT assay and the LDH leakage assay. These findings indicate that GLUT4 translocation is involved in the observed antidiabetic properties of these medicinal plants.

2. Materials and Methods

2.1. Materials. Fetal bovine serum, α-MEM (modified Eagle's medium), D-MEM standard culture medium, and all other tissue culture reagents used were purchased from biological industries (Beit Haemek, Israel). Horseradish-peroxidase-(HRP-) conjugated goat anti-rabbit antibodies were obtained from Promega (Madison, WI, USA). Polyclonal anti-myc (A-14) and other standard chemicals were purchased from Sigma.

2.2. Plant Extract Preparation. *Trigonella foenum-graecum* (seeds), *Atriplex halimus* (leaves and stem), *Olea europaea* (leaves), *Urtica dioica* (leaves and stem), *Allium sativum* (bulb), *Allium cepa* (bulb), *Nigella sativa* (seeds), and *Cinnamomon cassia* (bark) were purchased from Al Alim-Medicinal Herb Center, Zippori, Israel. Fifteen grams of the hand grinded plant material were added to 100 mL of 50% ethanol in double distilled water and heated for 15 minutes at 60°C under stirring. Extract supernatants obtained were passed through a 0.2 μm filter and stored in aliquots at −80°C for further experimental work.

2.3. Cell Culture. Cells from the HepG2 cell line were purchased from ATCC (HB-8065). Cells from the rat L6 muscle cell line, stably expressing myc-tagged GLUT4 (L6-GLUT4myc), being a kind gift from Prof. Amira Klip (The Hospital for Sick Children, Toronto, Canada), were maintained in myoblast monolayer culture. All cells were grown under an atmosphere of 95% air and 5% CO_2 in α-MEM (L6 cells) or D-MEM (HepG2 cells) supplemented with

10% fetal calf serum (FCS), 1 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

2.4. MTT Assay. The MTT assay is based on the protocol described for the first time by Mosmann [12]. The assay was optimized for the cell lines used in the experiments. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was applied to assess cell viability as described in [13]. Cells (2×10^4/well) were plated in 100 μL of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. Plant extracts were added at increasing concentrations (0–2 mg/mL) for 24 h. The cells medium was replaced with 100 μL fresh medium/well containing 0.5 mg/mL MTT and cultivated for another 4 h darkened in the cells incubator. The supernatant was removed and 100 μL isopropanol/HCl (1 mM HCl in 100% isopropanol) were added per well. The absorbance at 570 nm was measured with microplate reader (Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

$$\text{Percent viability} = \left(\frac{\text{A 570 nm of plant extract treated sample}}{\text{A 570 nm of nontreated sample}}\right) * 100. \quad (1)$$

2.5. Lactate Dehydrogenase Assay (LDH). LDH, a cytoplasmic enzyme, release is the consequence of cell membrane rupture. Activity of LDH released to the cell culture medium was monitored following the formation of formazan by coupled enzymatic reaction at 500 nm according to the manufacture kit (Promega). Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to the LDH activity released in the control cells. HepG2 and L6-GLUT4myc cells (2×10^4/well) were plated in 100 μL of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. After cell attachment (24 h) cells were treated with increasing concentrations of the plant extracts (0–2 mg/mL). The extracellular LDH activity was measured in the medium after 24 h. Therefore, 50 μL from each well was transferred to a new 96 well plate; the enzyme reaction was carried out according to the manufacture kit (CytoTox 96, Promega). All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

$$\text{Percent viability} = \left(\left(\frac{\text{A 492 nm of plant extract treated sample}}{\text{A 492 nm of control}}\right) * 100\right) - 100. \quad (2)$$

2.6. Determination of Surface GLUT4myc. Surface myc tagged GLUT4 was measured in intact, nonpermeabilized cells as previously described [14] using anti-myc antibody followed by secondary antibody conjugated to horseradish peroxidase. Cells grown in 24-well plates for one day followed by addition of the plant extracts for 24 h. Serum starved for 3 h were treated without or with 100 nM insulin for 20 min, washed twice with ice-cold PBS, fixed for 10 min with 3% paraformaldehyde, blocked 10 min with 3% (v/v) goat serum and reacted with polyclonal anti-mycantibody (1 : 200) for 1 h at 4°C, washed 10 times with PBS and reacted with horseradish peroxidase-bound goat anti-rabbit secondary antibody (1 : 1000) for 1 h at 4°C, and washed 15 times with PBS. Cells were then incubated with 1 mL *o*-phenylenediamine dihydrochloride reagent and allowed to develop for 20–30 min in the linear range in the dark at room temperature. The reaction was stopped with 1 mL/well of 3 N HCl. Supernatants were collected and absorbance was measured at 492 nm. Background absorbance obtained in the absence of anti-mycantibody was subtracted from all values.

2.7. Statistical Analysis. Error limits cited and error bars plotted represent simple standard deviations of the mean. When comparing different samples, results were considered to be statistically different when $P < 0.05$ (Student's t-test for unpaired samples).

3. Results and Discussion

Cytotoxic effects of 50% ethanol/water extracts of *Trigonella foenum-graecum*, *Atriplex halimus*, *Olea europaea*, *Urtica dioica*, *Allium sativum*, *Allium cepa*, *Nigella sativa*, and *Cinnamomon cassia* were evaluated in cells from the human hepatocellular carcinoma (HepG2) and cells from the rat L6 muscle cell line, stably expressing myc-tagged GLUT4 (L6-GLUT4myc), using the MTT assay and the LDH leakage assay. These two tests are widely used in *in vitro* toxicology studies. They are used for the detection of cytotoxic and other negative effects on cell viability following exposure to test materials. In general, in order to increase the reliability of the results obtained and to avoid overestimation or underestimation of the toxicity of the plant extracts, more than one assay should be used to determine cell viability in *in vitro* studies. Therefore, the MTT assay and the LDH leakage assay (Figure 1) were used here. Cells were treated with increasing concentrations of the plant extracts (up to 4 mg/mL) for 24 h. The MTT results were used to determine the EC_{50} values (Table 1).

The involvement of glucose transporter (GLUT4) in the observed antidiabetic effects of tested eight medicinal plants was evaluated applying the GLUT4 translocation assay. Insulin increases GLUT4 translocation to the surface of myoblasts, where it mediates the increase in glucose uptake [11]. To examine the contribution of the plants extract to GLUT4 localization on the plasma membrane, the extracts were added to the L6-GLUT4myc cells in the presence or absence of insulin, and GLUT4myc translocation to the plasma membrane was tested as described in the Material and Methods section. Herein we will present and discuss results obtained for the eight plants examined.

Table 1: GLUT4 translocation and EC_{50} of plant extracts in cells from HepG2 and L6myc cell lines. Data given represent the mean ± SEM from three independent experiments carried out in triplicates.

Plant name	Part used	Cell type	EC_{50} (mg/mL)	Cell surface* GLUT4myc
Allium sativum	Bulb	L6myc	>2	−
		HepG2	>2	
Allium cepa	Bulb	L6myc	>2	−
		HepG2	>2	
Trigonella foenum	Seeds	L6myc	>2	++
		HepG2	>2	
Olea europaea	Leaves	L6myc	0.79 ± 0.036	−−
		HepG2	>2	
Nigella sativa	Seeds	L6myc	>2	+
		HepG2	1.47 ± 0.49	
Urtica dioica	Leaves and stem	L6myc	0.73 ± 0.09	++
		HepG2	0.76 ± 0.13	
Atriplex halimus	Leaves and stem	L6myc	1.72 ± 0.22	++
		HepG2	0.8 ± 0.3	
Cinnamomon cassia	Bark	L6myc	0.39 ± 0. 01	+
		HepG2	0.12 ± 0. 01	

*Gain in GLUT4 on plasma membranes at noncytotoxic concentrations, (−) no effect, (−−) decreased GLUT4 translocation, (+) slightly increased the GLUT4 translocation, (++) high GLUT4 translocation.

3.1. Allium sativum (Garlic) and Allium cepa (Onion). *Allium sativum* and *Allium cepa* are widely used in the prevention and treatment of various diseases, including but not limited to infections, cancer, and diabetes [15]. Both, the garlic cloves as well as onion bulbs share many similar active compounds (e.g., allyl propyl and diallyl sulfide), decrease blood glucose levels also by normalizing liver hexokinase and glucose-6-phosphatase activities [9], and increase insulin secretion from the pancreas. However, excessive onion and garlic consumption might lead to harmful effects on the body [15]. This observation was confirmed in the present study. As shown in Figure 1(a), no significant reduction in cell viability of HepG2 and L6myc cell lines was seen at any concentration up to 1 mg/mL as assessed with MTT and LDH assays. Reduction of cell viability was seen at very high concentrations, which confirms the description of [15]. Regarding their reported hypoglycemic properties, our results indicate that the antidiabetic properties of these two plants are not mediated through GLUT4 translocation to the PM. As shown in Figures 2(a) and 2(b), garlic and onion extracts did not alter the amount of surface-exposed GLUT4myc in the basal (unstimulated) state and even had slightly decreased GLUT4myc translocation to the plasma membrane in the insulin stimulated state when cells were treated with 1 mg/mL of the plants extracts.

3.2. Trigonella foenum-graecum (Fenugreek). *Trigonella foenum-graecum* has been used to treat diabetes and sore throats and in poultices used to treat sores and abscesses. Recent investigations into the medicinal properties of fenugreek suggest its importance not only as a preventive for chronic diseases such as diabetes, but also for enhancing normal physiological processes, especially with respect to athletic performance. Fenugreek seeds are very rich in dietary fibers that modulate delaying the absorption of sugar and cholesterol from the intestines, thus protecting against diabetes, heart disease, and obesity [16].

Clinical and experimental studies show positive effect of the fenugreek seeds in the metabolism of glucose in the body. Fenugreek seeds contain a gel-like soluble fiber which combines with bile acid and lowers triglyceride and LDL cholesterol levels. To maximize their medicinal effect, fenugreek seeds are chopped finely and served as a flavorful or soaked in water for overnight. The nicotinic acid, alkaloid trigonelline, and coumarin contained by defatted section of the seed of fenugreek prove to be the responsible active ingredient for its antidiabetic properties [17, 18]. In clinical trials, low doses had no significant effect on fasting blood glucose (FBG) levels of diabetic subjects. However, higher dose (100 mg/kg body weight) of defatted seed powder for 10 days improved FBG values. Several active ingredients were purified from fenugreek seeds. Some of them isolated from fenugreek seeds (e.g., trigonelline and nicotinic, GII) have antidiabetic properties. Treatment of the moderately diabetic rabbits with a novel active ingredient named GII (100 mg/kg body weight for 3 weeks) reduced fasting blood glucose to nearly normal [19]. To assess the functional relevance of *Trigonella foenum-graecum* extract to GLUT4 traffic, we explored the effects of plant ethanol/water (50%, 50%) extract on GLUT4myc translocation to the PM. GLUT4myc translocation to the cell surface was almost doubled in the presence of 1 mg/mL of *Trigonella foenum-graecum* extract. Insulin stimulated GLUT4myc translocation was slightly increased from 170% in the nontreated cells to about 200% and 230% in the cells treated with 0.5 and 1 mg/mL extract, respectively (Figure 2(c)). Results obtained here indicate that *Trigonella foenum-graecum* extract led to a significant gain in GLUT4 on L6 plasma membrane at noncytotoxic concentrations

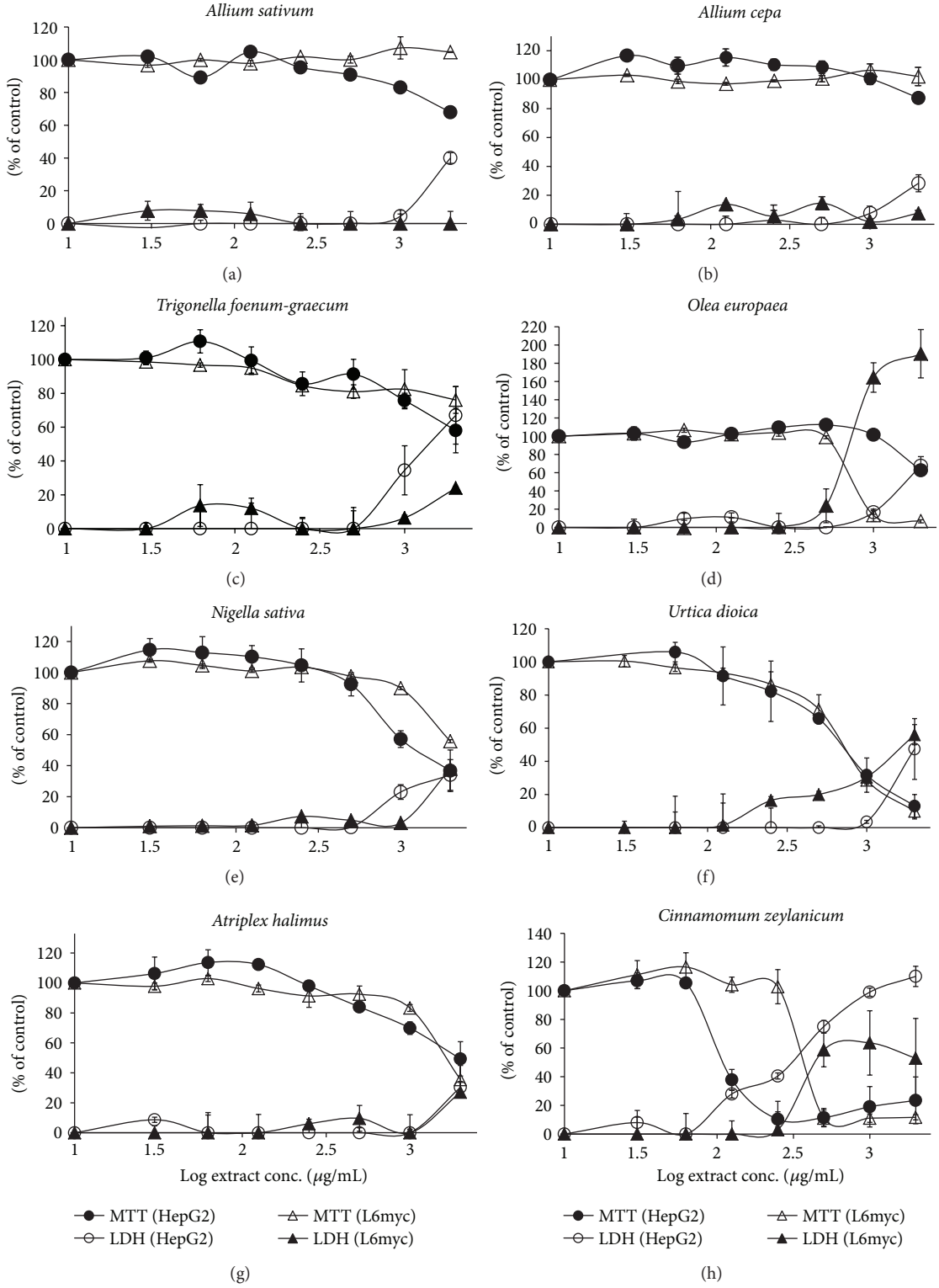

Figure 1: Cytotoxic effects of plant extracts tested on cells from HepG2 and L6myc cell lines. L6-GLUT4myc cells and HepG2 cells were seeded in 96-well plate (20,000 cells/well), exposed to *Allium sativum* (a), *Allium cepa* (b), *Trigonella foenum-graecum* (c), *Olea europaea* (d), *Nigella Sativa* (e), *Urtica pilulifera* (f), *Atriplex halimus* (g), and *Cinnamomum zeylanicum* (h) for 24 h. Cytotoxicity was measured by LDH leakage assay and MTT assay. Each point represents the mean of the data from three independent experiments; bars represent the standard error (S.E) relative to the control. In order to decrease the y-axis scale in the LDH presented calculated results, a 100% (control value) was subtracted from all the results shown.

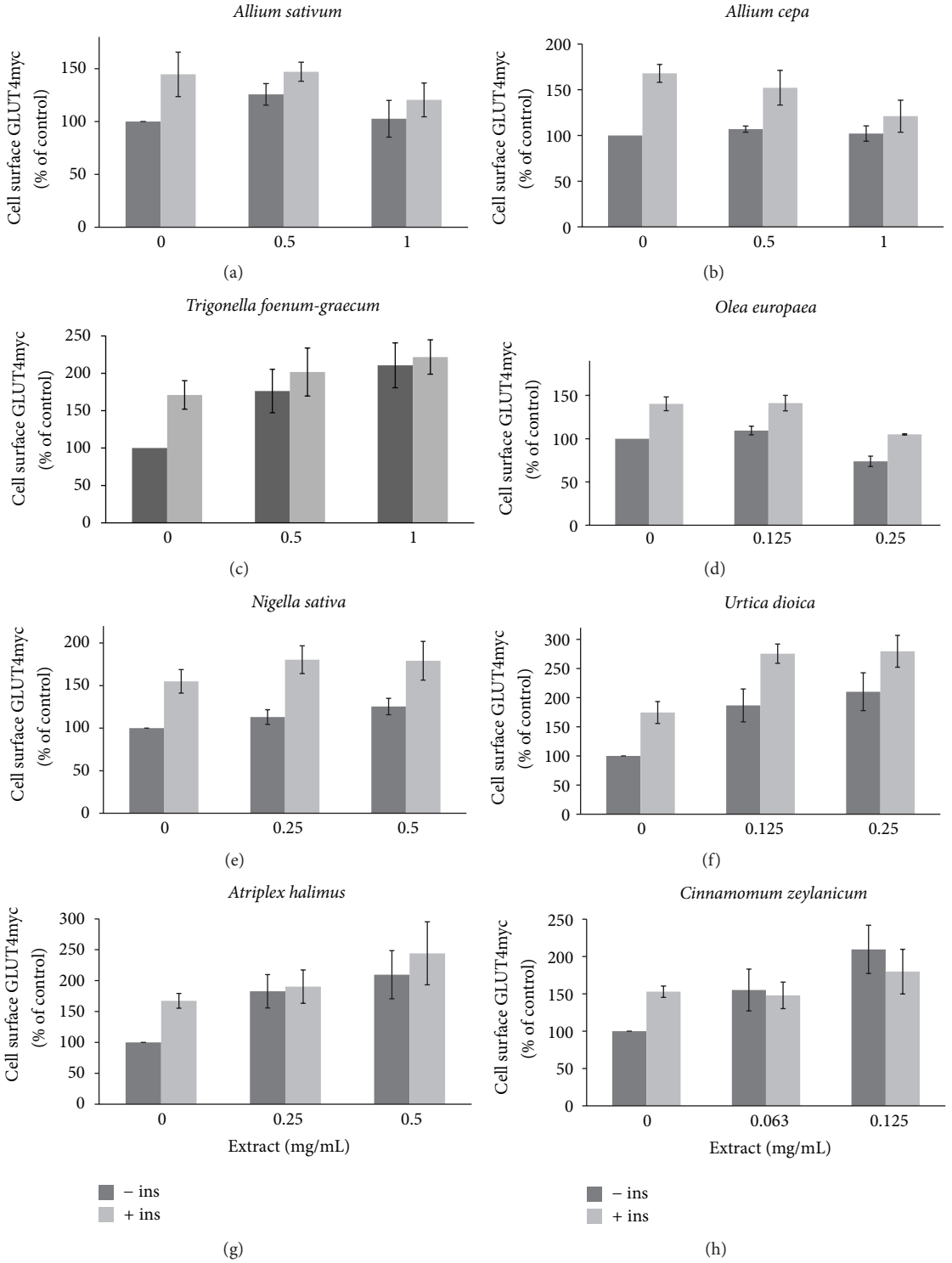

Figure 2: Effect of plant extracts on GLUT4 translocation. L6-GLUT4myc cells were seeded in 24-well plate (100,000 cells/well) and exposed to *Allium sativum* (a), *Allium cepa* (b), *Trigonella foenum-graecum* (c), *Olea europaea* (d), *Nigella Sativa* (e), *Urtica pilulifera* (f), *Atriplex halimus* (G), and *Cinnamomum zeylanicum* (h) for 24 h. Serum depleted cells were treated without or with 100 nM insulin for 20 min at 37°C and surface myc-tagged GLUT4 density was quantified using the antibody coupled colorimetric assay. Shown are the means ± S.E relative to basal nontreated cells from three independent experiments (each has three triplicates).

(up to 1 mg/mL) as measured with MTT assay and the LDH leakage assay. These findings indicate that antidiabetic properties of *Trigonella foenum-graecum* extract are mediated, at least partially, through GLUT4 translocation. In addition to its beneficial antidiabetic effects, no cytotoxic effects were seen with fenugreek up to relatively high concentrations of 1.7 mg/mL (Figure 1(c)). The EC_{50} value of Fenugreek was found to be higher than 2 mg/mL for the two cell lines tested (Table 1).

3.3. Olea europaea (Olive Leaf). *Olea europaea* is one of the most commonly known medicinal plants that have been used for centuries within Greek countries, Arab countries, and others. The major active compounds of olive leaf are oleuropeoside, hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside [20]. Oleuropein and phenolics disclosed distinct hypoglycaemic effects (at a dose of 16 mg/kg, together with hypotensive and hypolipidemic properties). They were reported to posses antioxidant capacity as well as antimicrobial [21]. Various beneficial effects are attributed to olive leaf extracts. Clinical evidence has proven the blood pressure lowering effects of olive leaf extract [22] as well as antiaging, antioxidant immunostimulatory and antibiotic (antibacterial, antifungal, and anti-inflammatory) effects. Said et al., 2008 [23] used powdered olive leaf (in a mixture with *Juglans regia*, *Urtica dioica*, and *Atriplex halimus*) and reported that this mixture decreases glucose absorption from the intestine and lowers blood glucose levels in rats and diabetic subjects. No significant effects on the rate of GLUT4 translocation were seen here when cells treated with 125 μg/mL extract. Interestingly, however, exposing L6-GLUTmyc cells to 250 μg/mL significantly decreased GLUT4 translocation to the PM from 100% to 80% in the basal state (without insulin stimulation) and from 150% to 105% in insulin stimulated cells (Figure 2(d) and Table 1). It seems that the above mentioned antidiabetic effects of olive extracts are mediated by distinct mechanisms than GLUT4 translocation system. In addition, no cytotoxic effects were observed with concentrations up to 0.8 mg/mL as measured with MTT and LDH assays (Figure 1(d) and Table 1).

3.4. Nigella sativa (Black Seed). *Nigella sativa* is one of the most commonly used medicinal plants that have been used for centuries as a spice as well as a protective and curative remedy for numerous diseases. Potential toxicity of the fixed oil of the seeds was investigated in mice and rats through determination of LD_{50} values and examination of possible biochemical, hematological and histopathological changes. LD_{50} values, obtained by single doses (acute toxicity) in mice, were 28.8 mL/kg body with oral administration and 2.06 mL/kg body with intraperitoneal administration. Chronic toxicity was studied in rats treated daily with an oral dose of 2 mL/kg body wt for 12 weeks. Changes in key hepatic enzymes levels, including ALT, AST, and GSH, and histopathological modifications (heart, liver, kidneys, and pancreas) were not observed in rats treated with *Nigella sativa* oil after 12 weeks of treatment. Negative effects of *Nigella sativa* were reported by Khader et al., (2007) who tested toxicological properties and potential antimutagenic effects of *Nigella sativa* aqueous extracts using primary rat hepatocyte cultures against N-methyl-N-nitro-N-nitrosoguanidine (MNNG). They found that *Nigella sativa* led to a significant increase of chromosomal aberrations in the case of pretreatment [24]. Thymoquinone, dithymquinone, thymohydroquinone, and thymol, are the main active compounds responsible for the therapeutic effects of *Nigella sativa* seeds. Many scientific reports addressed the antidiabetic effects of plant mixtures containing *Nigella sativa*. These studies revealed that the blood glucose lowering effect was due to the inhibition of hepatic gluconeogenesis. For instance, an aqueous extract of a plant mixture containing *Nigella sativa* was found to lower the blood glucose level significantly after oral administration [25]. Furthermore, intraperitoneal administration of *Nigella sativa* seed's oil produced a significant hypoglycemic effect in normal and alloxan-induced diabetic rabbits. Similar results were seen in rats treated with plants mixture including *Nigella sativa* [26]. Another study with Streptozotocin- and Nicotinamide-induced diabetes mellitus in hamsters revealed that four weeks of treatment with *Nigella sativa* oil result in significant decrease in blood glucose level together with significant increase in serum albumin level [27]. These findings indicate that the hypoglycemic effect of *Nigella sativa* oil is, at least partially, mediated by a stimulation of beta cells coincident with an increase in serum insulin level and possess insulinotropic properties in type II like model. In another study, the hypoglycemic effect of *Nigella sativa* was supposed to be mediated by extrapancreatic actions rather than by stimulated insulin release [28, 29]. Results obtained here indicate that 50% ethanol extracts of 0.25 and 0.5 mg/mL *Nigella sativa* did not affect significantly the GLUT4 translocation in the presence of insulin and slightly increased it (about 20%) in the basal state (Figure 2(e)). Figure 1(a) depicts the MTT and LDH assays results of the treatment of HepG2 and L6myc cells with *Nigella sativa* extract, which was found to be safe up to concentrations of 500 μg/mL. The EC_{50} values for this extract were seen at 1470 μg/mL and higher than 2000 μg/mL for HepG2 and L6myc cell lines, respectively (Table 1). Taken together, the data emerged from these studies indicate that *Nigella sativa* is not toxic, as evidenced by high LD_{50} values, hepatic enzymes stability, and organ integrity, suggesting a wide margin of safety for the therapeutic doses of *Nigella sativa* extracts.

3.5. Urtica dioica (Nettle). Its Leaf has a long history as an herbal remedy and nutritious addition to the diet. Nettle leaves are a rich source of essential amino acids, ascorbic acid, several mineral elements and vitamins, such as iron, provitamin A and vitamin C [30]. Nettle is believed to be anticarcinogenic, antiulcer, antioxidant, anti-inflammatory, immune suppressive, and antirheumatoid [31]. There is also evidence that nettle extracts possess hypoglycaemic properties and improve glucose tolerance [23]. Consistently, exposing L6-GLUT4myc cells to 125 and 250 μg/mL of nettle extracts almost doubled GLUT4 translocation to the PM in the basal state and increased about 1.6 fold in the insulin stimulated state (Figure 2(f) and Table 1). Nettle extract was

safe to use in the cell lines tested up to 500 μg/mL in L6 and in HepG2 cell lines (Figure 1(f) and Table 1).

3.6. Atriplex halimus (Saltbush). *Atriplex halimus* is well known and extensively used to treat diabetes especially in the Middle East. An animal model for diabetogenesis and obesity proved that this plant is an extremely effective antidiabetic herb and shows an insulin potentiating effect. Sand rats were fed diets composed of standard laboratory animal chow with or without *Atriplex halimus*. Saltbush lowered the blood glucose levels and enhanced insulin secretion in animals fed with it [32]. Said et al., showed that saltbush (in a mixture with the leaves of *Juglans regia*, *Urtica dioica*, and *Olea europaea*) is effective in lowering blood glucose levels in diabetic patients. In addition, *in vitro* experiments showed that the mixture facilitates glucose entry into yeast cells during anaerobic fermentation. This observation was attributed to an effect of *Atriplex halimus* content in the mixture [23]. Our results support the previous reported studies and shows that the main antidiabetic mechanism of the saltbush is increasing GLUT4 translocation to the plasma membrane, thereby increasing glucose uptake to the muscle, liver, and fat cells. Exposing L6-GLUT4myc cells to 0.25 and 0.5 mg/mL saltbush extracts almost doubled GLUT4 translocation in the basal state. When treated with insulin, GLUT4 translocation was not altered in the presence of 0.25 mg/mL Saltbush extract but was increased from 160% to 230% in the presence of 0.5 mg/mL of the plant extract (Table 1 and Figure 2(g)). Saltbush seems to be extremely safe for intake as its EC_{50} was about 2 mg/mL (Table 1 and Figure 1(g)).

3.7. Cinnamomun verum (Cinnamon). *Cinnamomun verum* possesses antioxidant properties that help to reduce the damaging effects of diabetes [33]. Cinnamon is also believed to increase both insulin secretion and the body's cells' sensitivity to insulin via its active ingredient, methyl hydroxy chalcone polymer (MHCP). These two ways of action improve the efficiency of insulin and increase the conversion of consumption of glucose. Results obtained here demonstrate that GLUT4myc translocation to the cell surface was increased by 1.5- and 2-fold in the basal state in the presence of 62 and 125 μg/mL of cinnamon extract, respectively. Insulin stimulated GLUT4myc translocation to the PM was not affected when the L6-GLUT4myc cells were treated with 62 μg/mL, but was slightly increased when treated with 125 μg/mL (Figure 2(h) and Table 1). Results obtained here illustrate efficacy of *Cinnamomun verum* in enhancing GLUT4 translocation to the PM. Cytotoxic effects of *Cinnamomun verum* extracts were seen at concentrations higher than 100 μg/mL for HepG2 cells and higher than 250 μg/mL for L6myc cells as assessed with MTT and LDH assays (Figure 1(h) and Table 1). The EC_{50} of *Cinnamomun verum* was around 100 μg/mL for HepG2 cells and around 390 μg/mL for L6myc cells (Table 1). This cytotoxic effects must be taken into consideration when applying *Cinnamomun verum* as therapeutic or prophylactic agent.

4. Conclusions

Trigonella foenum-graecum, *Atriplex halimus*, *Olea europaea*, *Urtica dioica*, *Allium sativum*, *Allium cepa*, *Nigella sativa*, and *Cinnamomon cassia* are widely used in the Greco-Arab and Islamic medicine for their antidiabetic and antioxidant properties. Safety assessment of herbal products has often been neglected since prolonged and apparently safe use is usually considered as an evidence of its nature's source. Results obtained here indicate that *Cinnamomon cassia* is cytotoxic at concentrations higher than 100 μg/mL, whereas the other extracts exhibited cytototoxic effects at concentrations higher than 500 μg/mL. *Trigonella foenum-graecum*, *Urtica dioica*, *Atriplex halimus* and *Cinnamomun verum* induced a significant gain in GLUT4 translocation in muscle cells PM at noncytotoxic concentrations. These findings indicate that antidiabetic properties of these medicinal plants are mediated, at least partially, through mediating GLUT4 translocation to the PM.

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Research Article

Effect of Berberine on PPARα/NO Activation in High Glucose- and Insulin-Induced Cardiomyocyte Hypertrophy

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Rhizoma coptidis, the root of *Coptis chinensis Franch*, has been used in China as a folk medicine in the treatment of diabetes for thousands of years. Berberine, one of the active ingredients of *Rhizoma coptidis*, has been reported to improve symptoms of diabetes and to treat experimental cardiac hypertrophy, respectively. The objective of this study was to evaluate the potential effect of berberine on cardiomyocyte hypertrophy in diabetes and its possible influence on peroxisome proliferator-activated receptor-α (PPARα)/nitric oxide (NO) signaling pathway. The cardiomyocyte hypertrophy induced by high glucose (25.5 mmol/L) and insulin (0.1 μmol/L) (HGI) was characterized in rat primary cardiomyocyte by measuring the cell surface area, protein content, and atrial natriuretic factor mRNA expression level. Protein and mRNA expression were measured by western blot and real-time RT-PCR, respectively. The enzymatic activity of NO synthase (NOS) was measured using a spectrophotometric assay, and NO concentration was measured using the Griess assay. HGI significantly induced cardiomyocyte hypertrophy and decreased the expression of PPARα and endothelial NOS at the mRNA and protein levels, which occurred in parallel with declining NOS activity and NO concentration. The effect of HGI was inhibited by berberine (0.1 to 100 μmol/L), fenofibrate (0.3 μmol/L), or *L*-arginine (100 μmol/L). MK886 (0.3 μmol/L), a selective PPARα antagonist, could abolish the effects of berberine and fenofibrate. N^G-nitro-*L*-arginine-methyl ester (100 μmol/L), a NOS inhibitor, could block the effects of *L*-arginine, but only partially blocked the effects of berberine. These results suggest that berberine can blunt HGI-induced cardiomyocyte hypertrophy *in vitro*, through the activation of the PPARα/NO signaling pathway.

1. Introduction

Diabetes mellitus (DM) is a common disease worldwide and its prevalence has increased in recent decades. Patients with type 2 DM are at two-to-five fold higher risk for developing cardiomyopathy, compared with age-matched patients without DM [1, 2]. Diabetic cardiomyopathy is the leading cause of diabetes-related morbidity and mortality [3]; this condition shares many of the characteristics of other types of cardiomyopathy, including ventricular hypertrophy, decreased ventricular diastolic relaxation and reduced peak filling rate; however, it is distinct from other types of cardiomyopathy because it often occurs in the absence of coronary artery disease and hypertension [4, 5]. Ventricular hypertrophy, also known as diabetic cardiac hypertrophy, is the major cardiovascular abnormality in DM patients, and is associated with increased risk for developing congestive heart failure and sudden death [6, 7].

Rhizoma coptidis has been used to treat DM in China for centuries. Berberine ($[C_{20}H_{18}NO_4]^+$, Figure 1), one of the main ingredients of *Rhizoma coptidis* and *Cortex Phellodendri*, is an isoquinoline alkaloid with multiple pharmacological applications, including its use as an anti-inflammatory, antibacterial, antidiarrheal, and anticancer drug [8–10]. Multiple clinical trials and animal experiments have shown that berberine can improve insulin resistance, correct disorders of lipid metabolism, lower hyperglycemia, and reduce body weight [11]. In the context of the present work, some studies

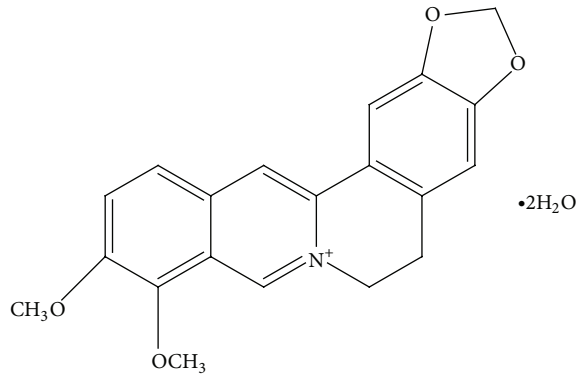

Figure 1: Chemical structure of berberine.

have also demonstrated that berberine has therapeutic potentials in rats with experimental cardiac hypertrophy [12], yet relatively little is known about the potential blunting effect of berberine on diabetic cardiomyopathy, especially on cardiac hypertrophy under diabetic condition.

Recent studies suggested that the abnormal regulation of peroxisome proliferator-activated receptors (PPARs) was related to the metabolic syndrome, particularly in the advanced stages of DM [13]. PPARs are ligand-dependent transcription factors belonging to the nuclear receptor superfamily. There are three known PPAR isoforms, α, β/δ, and γ, which exhibit tissue-specific distribution and ligand-specific effects. In particular, PPARα is abundant in tissues with oxidative energy demands that depend on mitochondrial fatty acid oxidation as a primary energy source, such as the heart [14]. The activation of PPARα may improve diabetic cardiomyocyte hypertrophy, but the downstream molecular mechanisms have not been fully understood. One possible mechanism is through nitric oxide (NO) signaling. *In vitro* and *in vivo* studies have shown that NO could inhibit cardiac hypertrophy induced under various conditions such as hypertension, valvular disease, volume overload, and endothelin-1 challenge [15–17]. Studies that focused on the relaxation effect of PPARα on aortic vessel walls and the cerebral microvasculature have explored the potential therapeutic role of PPARα agonists. This work demonstrated that a PPARα agonist could induce vasodilation, which was mediated through modulating endothelial NO synthase (eNOS) and inducing the release of NO [18, 19]. Our previous experiments have shown that berberine could specifically bind to and activate PPARα [20]; as such the goal of the current study was to determine whether berberine can inhibit high glucose- and insulin-induced cardiomyocyte hypertrophy through the activation of the PPARα/NO signaling pathway.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) except berberine (Division of Chinese Material Medical and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health, Beijing, China) and *L*-arginine and *L*-NAME (Alexis, Lausen, Switzerland).

2.2. Primary Neonatal Rat Cardiomyocyte Isolation and Culture. The experimental procedures were approved by the Animal Laboratory Administration Center and Ethics Committee of Chongqing Medical University (SYXK (Chongqing) 2007-0001). Ventricular myocytes from 1- to 3-day-old Sprague-Dawley rats (Animal Laboratory Center of Chongqing Medical University, Chongqing, China) were prepared and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 0.1 mmol/L 5′-bromodeoxyuridine [21]. The seeding density was from 0.5×10^5 to 1×10^5 cells/mL for measuring cell surface area, or from 1.5×10^6 to 3×10^6 cells/mL for mRNA extraction, evaluating cellular total protein content, or determining NOS activity and NO concentration in the media. The medium was replaced by serum-free DMEM for a further 48 h before pharmacological treatment. High glucose and insulin (25.5 mmol/L glucose and 0.1 μmol/L insulin, HGI) was used to stimulate the cardiomyocytes. The antihypertrophic effects of berberine (with 99% purity and final concentrations from 0.1 μmol/L to 100 μmol/L), fenofibrate (0.3 μmol/L), and *L*-arginine (100 μmol/L) were studied. MK886 (0.3 μmol/L) or N^G-nitro-*L*-arginine methyl ester (*L*-NAME, 100 μmol/L) were used to investigate the relationship between the antihypertrophic effects of berberine and the PPARα/NO pathway.

2.3. Morphometric Analysis. Cellular hypertrophy was evaluated by measuring cardiomyocyte cell surface using a digital image analysis system (Leica QwinV3, Leica Microsystems Ltd., Cambridge, UK). Five random fields (with approximately 10 to 15 cells per field) from every sample were averaged and expressed as μm^2/cell. All experiments were repeated three times.

2.4. Measurement of Cardiomyocyte Protein Content. Collected cardiomyocytes were separated by trypsin and counted; they were then washed three times with ice-cold phosphate-buffered solution (PBS), then homogenized with RIPA lysis buffer (Beyotime, Jiangsu, China) and finally centrifuged at 12 000 g for 20 min at 4°C. The protein concentration in the supernatant was determined with a BCA protein assay kit (Beyotime, Jiangsu, China), and then the protein concentration per 10^6 cells was calculated.

2.5. Real-Time RT-PCR Analysis of mRNA. Total RNA was extracted from cardiomyocytes with Trizol reagent (Takara Biotech Co., Dalian, China), quantified by ultraviolet spectrometric detection (Eppendorf, Germany), and reverse transcribed into cDNA using PrimeScript RT reagent kit (Takara Biotech Co., Dalian, China), according to the manufacturer's instructions. Real-time RT-PCR was performed according to the standard protocol of SYBR *Premix Ex Taq* II (Takara Biotech Co., Dalian, China) on the IQ5 real-time RT-PCR

Table 1: Oligonucleotide sequences for real-time RT-PCR.

Gene	Forward (5′-3′ orientation)	Reverse (5′-3′ orientation)
ANF	TGACAGGATTGGAGCCCAGAG	TCGAGCAGATTTGCTGTTATCTTC
eNOS	TGCAACAAACCGAGGCAATC	CACCAGCTGGCTGTTCCAGA
PPARα	CTGACATTTGTGACTGGTCAAGCTC	TTTCCAGGTCATCTGCTTCAAGTG
β-actin	GGCCAACCGTGAAAAGATGA	CAGCCTGGATGGCTACGTACA

ANF: atrial natriuretic factor; eNOS: endothelial nitric oxide synthase; PPARα: peroxisome proliferator-activated receptor-α.

Table 2: Effect of berberine on cardiomyocyte hypertrophy induced by high glucose and insulin (HGI).

Group (μmol/L)	Cell surface area (μm^2/cell; $n = 3$)	Protein level ($\mu g/10^6$ cell; $n = 6$)	ANF mRNA ($n = 3$)
Control	459.7 ± 64.1	23.1 ± 5.3	30.6 ± 6.4
HGI	1229.6 ± 99.5*	47.3 ± 3.6*	179.4 ± 25.5*
HGI + berberine (3)	711.2 ± 43.0$^\#$	37.4 ± 2.0$^\#$	81.8 ± 7.5$^\#$
HGI + fenofibrate (0.3)	678.9 ± 21.3$^\#$	33.8 ± 6.0$^\#$	37.9 ± 9.5$^\#$
HGI + MK886 (0.3)	1208.8 ± 27.1	49.9 ± 5.9	184.7 ± 22.3
HGI + fenofibrate (0.3) + MK886 (0.3)	1212.4 ± 82.7$^\bullet$	50.2 ± 5.1$^\bullet$	161.5 ± 36.5$^\bullet$
HGI + berberine (3) + MK886 (0.3)	1257.4 ± 29.6$^\blacktriangle$	47.2 ± 3.3$^\blacktriangle$	154.0 ± 17.6$^\blacktriangle$
HGI + *L*-arginine (100)	768.1 ± 49.0$^\#$	38.6 ± 4.1$^\#$	80.7 ± 8.7$^\#$
HGI + *L*-NAME (100)	1213.1 ± 84.0	48.1 ± 3.5	147.3 ± 20.4
HGI + *L*-arginine (100) + *L*-NAME (100)	1199.9 ± 75.7$^\triangle$	48.8 ± 4.9$^\triangle$	163.3 ± 20.8$^\triangle$
HGI + berberine (3) + *L*-NAME (100)	972.4 ± 41.1$^\blacktriangle$	43.9 ± 2.1$^\blacktriangle$	110.0 ± 9.5$^\blacktriangle$

ANF: atrial natriuretic factor; *L*-NAME: N^G-nitro-*L*-arginine-methyl ester. Results are mean ± SEM of n independent experiments. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus HGI; $^\bullet P < 0.05$ versus HGI + fenofibrate (0.3 μmol/L); $^\blacktriangle P < 0.05$ versus HGI + berberine (3 μmol/L); $^\triangle P < 0.05$ versus HGI + *L*-arginine (100 μmol/L).

system (Bio-Rad, USA). The standard cycling conditions were 95°C for 8 min, followed by 40 cycles of 95°C for 15 s, annealing for 1 min (atrial natriuretic factor: 61.8°C; PPARα: 60.9°C; eNOS: 59.1°C; β-actin: 59.1°C), and 72°C for 40 s. The primers used for SYBR green real-time RT-PCR were synthesized by Takara Biotech Co. (Dalian, China; Table 1). The amount of target gene mRNA relative to the internal control gene, β-actin, was calculated using the ΔCt (Ct = cycle threshold) method as follows: the relative expression = $2^{-\Delta Ct}$, ΔCt = Ct (target gene) − Ct (β-actin). Results of three independent experiments were used for statistical analysis.

2.6. Western Blotting Analysis of Protein. The isolated protein (25 μg) from cardiomyocytes was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride nylon membranes. The blots were probed with mouse anti-rat PPARα (1 : 700 dilution) or rabbit anti-rat eNOS primary antibodies (1 : 900 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then with horseradish peroxidase-conjugated secondary antibodies (1 : 2000 dilution), and visualized using an ECL detection kit (Amersham Biosciences, Piscataway, NJ, USA). The optical densities of the bands were quantified by densitometric analysis performed with a quantitative imaging system (Bio-Rad, USA). All western blot experiments were repeated three times.

2.7. NOS Activity Assay. NOS activity in the conditioned medium of cardiomyocytes was measured using the NOS detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The optical density values of the samples were measured at 530 nm with a spectrophotometer. The enzyme activity was expressed as units per mg of protein. Results of six independent experiments were used for statistical analysis.

2.8. Nitrite Production Assay. Levels of the NO derivative nitrite were determined in the conditioned medium of cardiomyocytes with the Griess reaction. A nitrite detection kit (Beyotime, Jiangsu, China) was used according to the manufacturer's instructions, and a standard curve using $NaNO_2$ was generated for quantification. Briefly, 100 μL of medium or standard $NaNO_2$ was mixed with 100 μL of Griess reagent in a 96-well plate. After 15 min, optical density was read in a microplate reader (Tecan Austria Ges.m.b.H) at 540 nm. Results of six independent experiments were used for statistical analysis.

2.9. Statistical Analysis. All data in this study were expressed as mean ± SEM. Results were analyzed by one-way ANOVA or *SNK-q* test using the SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences in the mean were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of Berberine on HGI-Induced Cardiomyocyte Hypertrophy. HGI stimulation caused significant cardiomyocyte

hypertrophy following a 48 h incubation period, as determined by cell morphometric analysis (Figure 2). The data in Table 2 shows that HGI-stimulation caused a 2.7-fold increase in cell surface and a 2-fold increase in total protein content, compared with those of the corresponding control cells ($P < 0.05$). Treatment with berberine (from 0.1 μmol/L to 100 μmol/L) significantly relieved the changes induced by HGI in a concentration-dependent manner ($P < 0.05$). The IC_{50} (concentration producing a 50% maximal inhibition) for total protein content were 5.5 μmol/L.

Fenofibrate (0.3 μmol/L), a selective PPARα agonist, had effects similar to berberine ($P < 0.05$). MK886 (0.3 μmol/L), a selective PPARα antagonist, could completely abolish the effects of 3 μmol/L berberine or 0.3 μmol/L fenofibrate ($P < 0.05$). The NO precursor, *L*-arginine (100 μmol/L), also had effects similar to berberine ($P < 0.05$). *L*-NAME (100 μmol/L), a NOS inhibitor, could completely block the effects of *L*-arginine ($P < 0.05$), but only partially block the effect of berberine (3 μmol/L; $P < 0.05$; Figure 2; Table 2).

HGI-stimulated cardiomyocyte hypertrophy also led to an approximately 4.1-fold induction in the mRNA levels of atrial natriuretic factor ($P < 0.05$), which could be significantly antagonized by berberine, fenofibrate, or *L*-arginine ($P < 0.05$). MK886 abrogated the effects of berberine and fenofibrate on atrial natriuretic factor expression completely ($P < 0.05$), whereas *L*-NAME completely blocked the effect of *L*-arginine, but only partially blocked the effect of berberine ($P < 0.05$; Table 2).

3.2. Effects of Berberine on NOS Activity and NO Concentration in the Conditioned Medium of Cardiomyocytes. The levels of NOS activity and NO concentration were significantly decreased to 61% and 46% of control levels in HGI-stimulated cardiomyocytes ($P < 0.05$), an effect which was counteracted by berberine in a concentration-dependent manner (0.1 μmol/L to 100 μmol/L; $P < 0.05$). Fenofibrate (0.3 μmol/L) and *L*-arginine (100 μmol/L) had effects similar to berberine ($P < 0.05$), both of which being able to rescue the HGI-induced decrease in NOS activity and NO concentration. MK886 (0.3 μmol/L) was able to abolish the effects of berberine (3 μmol/L) and fenofibrate ($P < 0.05$). Similarly, *L*-NAME (100 μmol/L) could also abolish the effects of berberine (3 μmol/L) and *L*-arginine ($P < 0.05$; Figure 3).

3.3. Effect of Berberine on the Expression of PPARα and eNOS mRNA and Protein in HGI-Stimulated Cardiomyocytes. In HGI-conditioned cardiomyocytes, the expression level of PPARα and eNOS decreased by 54% and 26% at the mRNA level, and by 76% and 62% at the protein level, respectively, compared with control ($P < 0.05$). Berberine treatment (1, 3, or 10 μmol/L) markedly elevated the mRNA and protein expression of both PPARα and eNOS in a concentration-dependent manner ($P < 0.05$); the effects of fenofibrate (0.3 μmol/L) similar to berberine were also observed ($P < 0.05$). The rescue effects of berberine and fenofibrate on PPARα and eNOS expression were completely abolished by MK886 (0.3 μmol/L; $P < 0.05$; Figures 4 and 5).

4. Discussion

It is well known that DM is characterized by hyperglycemia. This acts as a stimulus for pancreatic beta cells to augment insulin secretion to maintain normal glucose homeostasis; however, long-term hyperglycemia impairs the insulin signaling pathway and depresses the sensitivity to insulin, leading to glucose intolerance and insulin resistance [1]. Therefore, hyperglycemia coupled with hyperinsulinemia develops over time [22], a process which is intimately involved in the pathophysiological process of diabetic cardiomyocyte hypertrophy [23, 24]. In the current study, we used an *in vitro* model to recapitulate diabetic cardiomyocyte hypertrophy in a laboratory setting. The ability of HGI to increase cell surface area, total protein content, and atrial natriuretic factor mRNA expression in rat primary cardiomyocytes suggested that we had induced cardiomyocyte hypertrophy, indicating that high glucose and insulin could mimic the human diabetic condition.

Berberine has been used as a therapeutic agent in treating many human diseases in Korea, China, and other Asian countries. Although it is one of the most important elements in traditional formulae for the treatment of diabetes in China [25] and has therapeutic effects on chronic heart failure [12], there are few reports on its potential role in the treatment of diabetic cardiomyocyte hypertrophy. In the present investigation, the effect of berberine on HGI-induced cardiomyocyte hypertrophy, for the first time, was evaluated. We observed that berberine effectively inhibited cardiomyocyte hypertrophy caused by HGI in a concentration-dependent manner, suggesting that berberine can effectively inhibit the progression of cardiomyocyte hypertrophy in DM. Notably, our previous experiments showed that berberine could specifically activate PPARα with an EC_{50} of 5.8 μmol/L [20], which was similar to the IC_{50} of berberine (5.5 μmol/L) that inhibited HGI-induced increases in total protein content of cardiomyocytes. We interpreted these similar values to indicate that the antihypertrophic effect of berberine may be related with the activation of the PPARα signaling pathway.

PPARα plays an important role in the regulation of lipid synthesis and degradation by virtue of its ability to control key transport proteins and enzymes involved in triglyceride metabolism; therefore, the PPARα signaling pathway may be impaired in diabetes [1]. It is known that transgenic mice with PPARα deletion develop a cardiac hypertrophy mimicking what is observed in the human diabetic condition [26]. Similarly, our results indicated that cardiomyocyte PPARα expression, at both the mRNA and protein levels, was suppressed by HGI stimulation. It is noteworthy that berberine could not only reverse HGI-induced cardiomyocyte hypertrophy, but also markedly upregulate PPARα expression. Meanwhile, MK886 abolished these effects of berberine. In accordance with these findings, treatment of HGI-induced hypertrophic cardiomyocytes with fenofibrate, a PPARα activator, could activate PPARα and improve experimental measures of cardiomyocyte hypertrophy *in vitro*; an effect which was also abolished by MK886. These observations confirm the hypothesis that PPARα is a major intermediate in facilitating the beneficial effects of berberine; however, the downstream

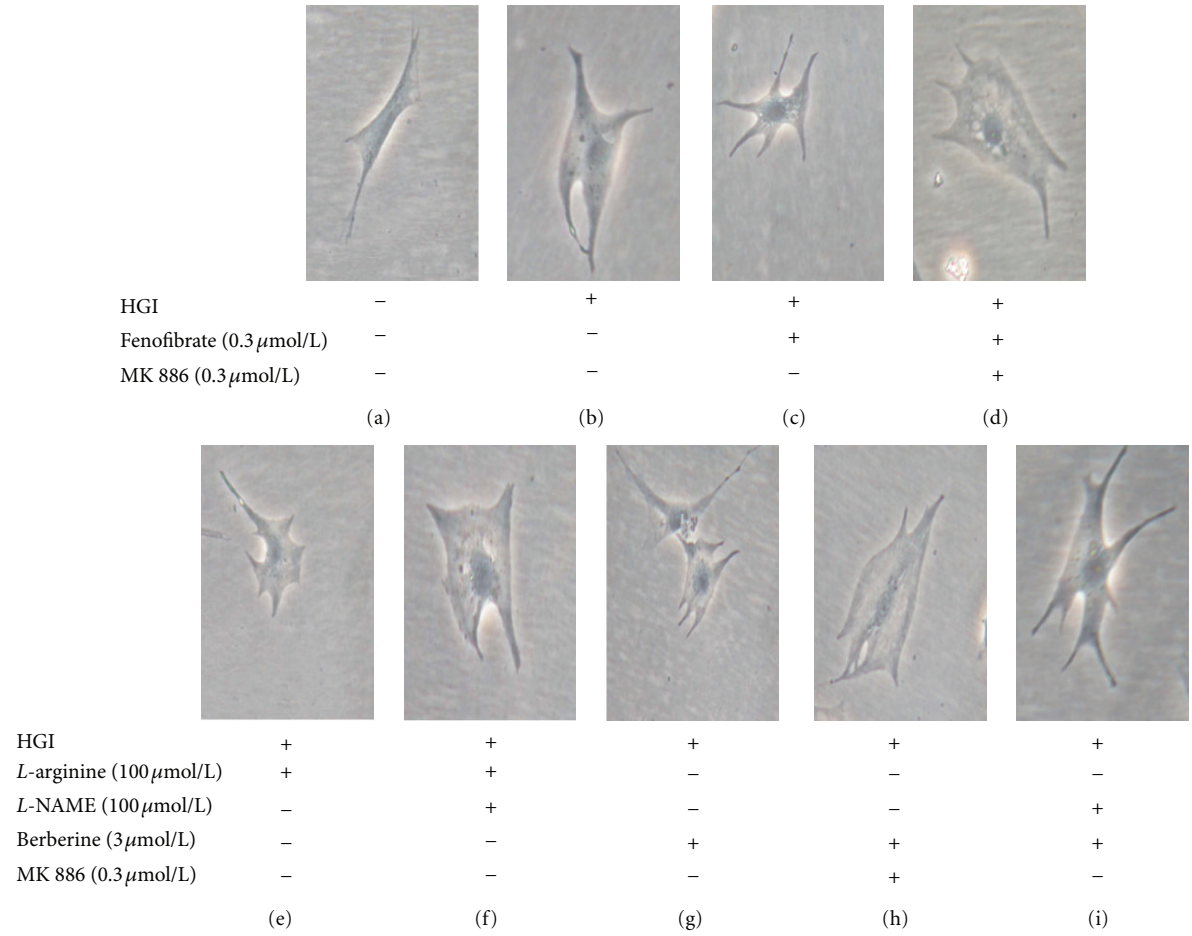

Figure 2: Representative photomicrographs of primary neonatal cardiomyocytes. Stimulation with HGI (25.5 mmol/L glucose and 0.1 μmol/L insulin) for 48 h caused significant hypertrophy (b), compared with the control group (a). Treatment with fenofibrate (0.3 μmol/L) (c), *L*-arginine (100 μmol/L) (e), or berberine (3 μmol/L) (g) inhibited cardiomyocyte hypertrophy induced by HGI. The inhibitory effects of fenofibrate and berberine were completely blocked by cotreatment with MK886, a selective PPARα antagonist (d, h) and the inhibitory effect of *L*-arginine was completely blocked by cotreatment with *L*-NAME, a NOS inhibitor (f). However, the inhibitory effect of berberine was only partially blocked by *L*-NAME (i). "+" or "−": treatment with or without relevant reagent.

molecular mechanisms of PPARα signaling pathway were unclear.

NO is synthesized from *L*-arginine by the catalytic reaction of different isoforms of NOS, including neuronal NOS, inducible NOS, and eNOS. Of interest in the current study, is the fact that eNOS is constitutively expressed in cardiomyocytes [27]. In recent years, NO has been emerged as an important regulator of cardiac remodeling and as a potent antihypertrophic mediator [15–17]. Moreover, other studies have revealed that DM impairs eNOS-induced NO production and causes endothelial dysfunction in humans and animals [28–30]. Consistent with these observations, our study also found that eNOS expression in cardiomyocytes, as well as culture medium NOS activity and NO concentration, was significantly decreased by HGI-induced cardiomyocyte hypertrophy, suggesting that this model is related to the reduction of eNOS-modulated NO production. Recently, Yakubu et al. found that the activation of PPARα could increase eNOS expression at the transcriptional and translational levels and further enhance NO production in cerebral microvascular endothelial cells [19]. Similarly, Goya et al. also demonstrated that PPARα activation enhanced NOS expression and activity in isolated endothelial cells [18]. Therefore, we examined the potential crosstalk between PPARα signaling pathway and the eNOS-NO transduction pathway in hypertrophic cardiomyocytes in order to explore the restorative mechanisms of berberine treatment against HGI-induced cardiomyocyte hypertrophy.

In this regard, our results indicated that both mRNA and protein expression levels of eNOS, as well as culture medium NOS activity and NO concentration, were restored by berberine or fenofibrate treatment in HGI-induced hypertrophic cardiomyocytes. An effect which was correlated with decreased measures of cardiomyocyte hypertrophy and evidence of PPARα activation. Moreover, coadministration of MK886 abolished the stimulatory effects of berberine and fenofibrate. We interpret these results to suggest that NO plays an important role in the antihypertrophic effect of

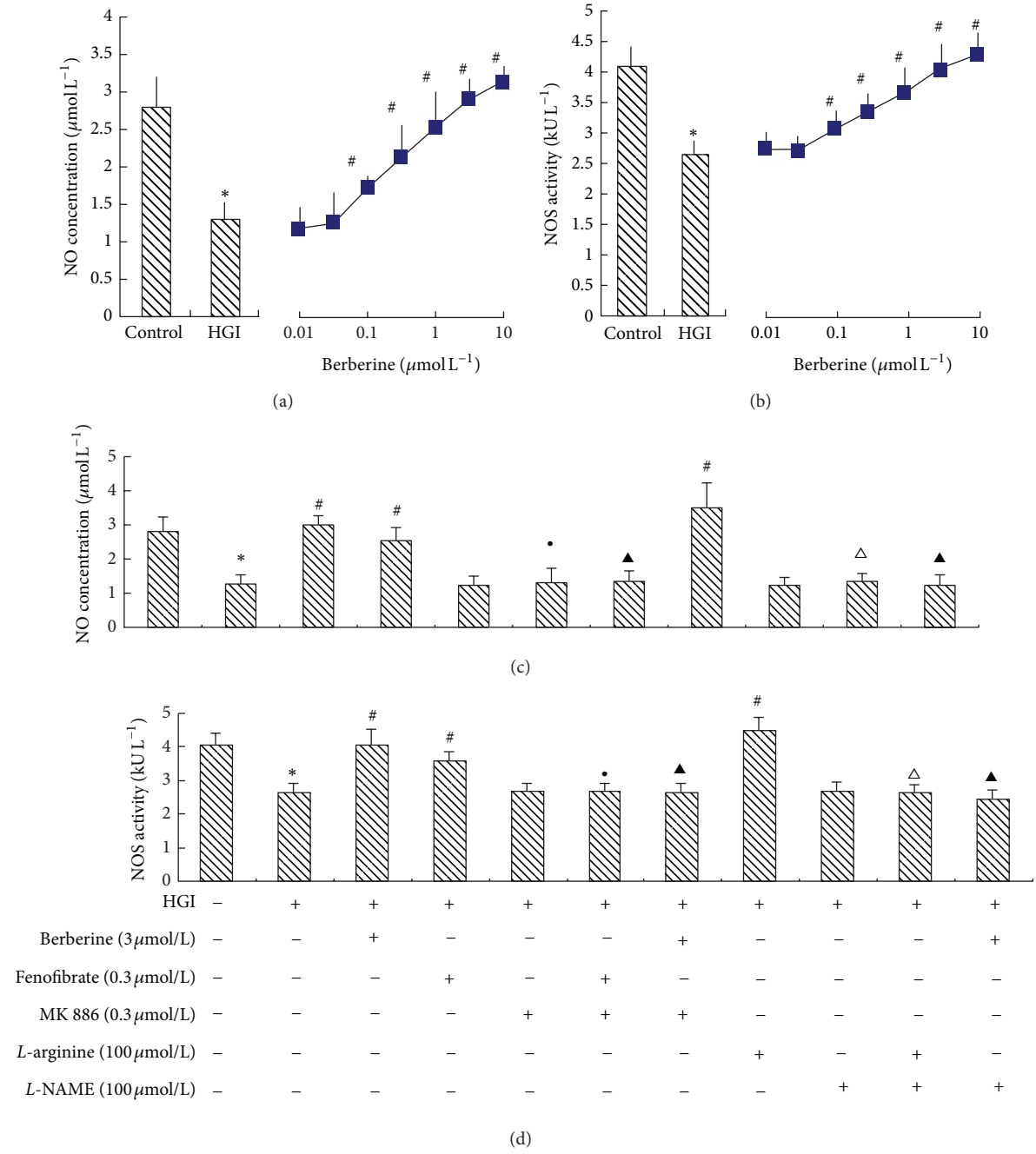

Figure 3: The effect of berberine on NO concentration and NOS activity in hypertrophic cardiomyocytes induced by HGI (25.5 mmol/L glucose and 0.1 μmol/L insulin). Cardiomyocytes were pretreated with various agents for 30 min and then stimulated by HGI for 48 h. The media were then collected for measurement of NO concentration and NOS activity. Levels of NO concentration and NOS activity decreased in the HGI group, and berberine was able to reverse the decrease in NO concentration (a) and NOS activity (b) in a concentration-dependent manner. The effect of various agents, alone or in combination, on NO concentration and NOS activity is shown in (c) and (d). Fenofibrate (0.3 μmol/L), a selective PPARα agonist, or *L*-arginine (100 μmol/L) had effects similar to berberine (3 μmol/L). Moreover, MK886 (0.3 μmol/L), a selective PPARα antagonist, could abolish the effects of both berberine and fenofibrate. *L*-NAME (100 μmol/L), a NOS inhibitor, could abolish the effects of both berberine and *L*-arginine. Results are represented by mean ± SEM of 6 experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HGI; $^{\bullet}P < 0.05$ versus HGI + fenofibrate (0.3 μmol/L); $^{\blacktriangle}P < 0.05$ versus HGI + berberine (3 μmol/L); $^{\triangle}P < 0.05$ versus HGI + *L*-arginine (100 μmol/L). "+" or "−": treatment with or without relevant reagent.

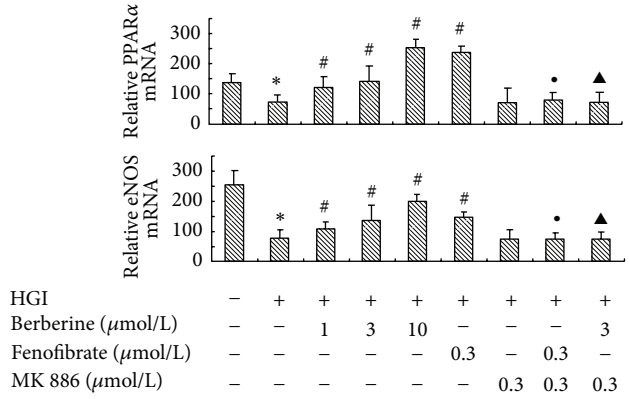

Figure 4: Concentration-dependent effects of berberine on mRNA expression of PPARα and eNOS. Cardiomyocytes were pretreated with various agents for 30 min and then stimulated by HGI (25.5 mmol/L glucose and 0.1 μmol/L insulin) for 48 h. Berberine (1, 3, or 10 μmol/L) markedly restored the decreased PPARα and eNOS mRNA expression level in a concentration-dependent manner. Fenofibrate (0.3 μmol/L) had an effect similar to berberine ($P < 0.05$). MK886 (0.3 μmol/L) abrogated the effects of both berberine and fenofibrate. Results are represented by mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HGI; $^{\bullet}P < 0.05$ versus HGI + fenofibrate (0.3 μmol/L); $^{\blacktriangle}P < 0.05$ versus HGI + berberine (3 μmol/L). "+" or "−": treatment with or without relevant reagent.

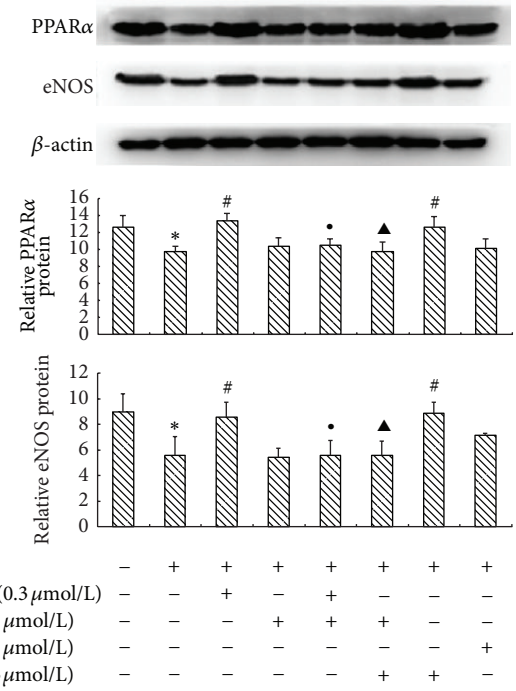

Figure 5: The effect of berberine on the protein expression level of PPARα and eNOS. Cardiomyocytes were pretreated with various agents for 30 min and then stimulated by HGI (25.5 mmol/L glucose and 0.1 μmol/L insulin) for 48 h, and protein expression level was analyzed by Western blot. Berberine (1 and 3 μmol/L) could reverse the HGI-induced decrease in PPARα and eNOS protein expression level. Fenofibrate (0.3 μmol/L) had effects similar to berberine (3 μmol/L). All of the effects of berberine and fenofibrate could be completely abolished by MK886 (0.3 μmol/L). Results are represented by mean ± SEM of 3 independent experiments. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus HGI; $^{\bullet}P < 0.05$ versus HGI + fenofibrate (0.3 μmol/L); $^{\blacktriangle}P < 0.05$ versus HGI + berberine (3 μmol/L). "+" or "−": treatment with or without relevant reagent.

berberine-modulated PPARα activation. Furthermore, the increased NO level was accompanied with enhancement of eNOS mRNA expression, indicating that the activation of PPARα could directly modulate the expression of eNOS. It is intriguing to compare the effects of berberine with NO donors, such as *L*-arginine. Both berberine and *L*-arginine attenuated HGI-induced cardiomyocyte hypertrophy, as well as increased eNOS mRNA expression, NOS activity, and NO concentration. Furthermore, the effects of berberine and *L*-arginine could be abolished by the NOS inhibitor, *L*-NAME; however, it is noteworthy that *L*-NAME did not completely block berberine-mediated attenuation of markers of HGI-induced cardiomyocyte hypertrophy. Therefore, the actions of berberine may not be totally dependent on the NO synthetic pathway; as such the relationship between the effects of berberine and other transduction pathways needs further investigation.

In conclusion, berberine can inhibit HGI-induced cardiomyocyte hypertrophy, which we consider an *in vitro* model of diabetic cardiomyocyte hypertrophy. Our mechanistic studies reveal that berberine acts via the activation of the PPARα signaling pathway which may, at least in part, promote the expression of eNOS, enhance eNOS activity, and result in a beneficial increase in the production of NO. We believe that our findings should stimulate further interest in berberine as potential therapeutic drug against diabetes-associated heart disease, especially on cardiac hypertrophy under diabetic condition. However, many of the aforementioned effects of berberine require further confirmation in appropriate diabetes models *in vivo*, and validation in patients.

Abbreviations

DM: Diabetes mellitus
PPARα: Peroxisome proliferator-activated receptor-α
NO: Nitric oxide
HGI: High glucose and insulin
NOS: Nitric oxide synthase
PPARs: Peroxisome proliferator-activated receptors
eNOS: Endothelial nitric oxide synthase
DMEM: Dulbecco's modified Eagle's medium
L-NAME: N^{G}-nitro-*L*-arginine methyl ester
PBS: Phosphate-buffered solution.

Conflict of Interests

None of the authors has any conflict of interests related to this study.

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Research Article

The Extract of Herbal Medicines Activates AMP-Activated Protein Kinase in Diet-Induced Obese Rats

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Our study investigated whether the extract of six herbal medicines (OB-1) has an inhibitory effect on obesity. High-fat diet- (HFD-) induced rats and controls were treated with 40 mg/100 g body weight of OB-1 or saline once a day for 5 weeks. After significant changes in body weight were induced, OB-1 and saline were administered to each subgroup of HFD and control groups for additional 5 weeks. No statistically significant decrease of body weight in OB-1-treated rats was found compared to controls. However, OB-1-treated rats were found to be more active in an open-field test and have a reduction in the size of adipocytes compared to controls. We observed no changes in the mRNA expressions of leptin and adiponectin from adipocytes between OB-1- and saline-treated rats with HFD-induced obesity group. However, OB-1 treatments were shown to be inversely correlated with accumulation of lipid droplets in liver tissue, suggesting that OB-1 could inhibit a lipid accumulation by blocking the pathway related to lipid metabolism. Moreover, the phosphorylation of AMP-activated protein kinase (AMPK) was significantly increased in OB-1-treated rats with HFD compared to controls. These results suggest that OB-1 has no direct antiobesity effect and, however, could be a regulator of cellular metabolism.

1. Introduction

Obesity due to disequilibrium of energy intake and expenditure has reached epidemic proportions in some parts of the world. Besides higher fat mass and body weight [1], obesity is associated with a higher risk for health problems such as cardiovascular disease, insulin resistance and diabetes mellitus, hyperlipidemia, arthrosis, many forms of cancer, and psychological stress [2, 3].

OB-1 consists of *Benincasae semen*, *Laminaria japonica* Areschon., *Pini Folium*, *Moli Folium*, *Citrus aurantium* Linn., and *Ephedra herb* (Materia medica, ISBN: 8985897373). *Benincasae semen* is a diuretic that has been used to eliminate toxins and edema from the body since early times. *Laminaria japonica Areschon* was reported to have an effect of anti-obesity [4]. It is known that *Pini Folium* increases serum lipid metabolism, and *Moli Folium* suppresses obesity. It was also reported that *Citrus aurantium Linn* increases the basal metabolic rate, acts as a diuretic, and reduces the activity of lipase [5]. The *Ephedra herb* is a well-known anti-obesity medicine that reduces body weights [6].

Obesity-induced alterations in adipocyte tissue result in altered expression or function of important endocrine hormones like leptin and adiponectin. Fasting leptin levels are remarkably elevated in adipocyte from obese individuals, and its gene expression is significantly increased in rats with diet-induced obesity [1, 7]. Unlike leptin, adiponectin is reduced in adipocyte tissue from obese individuals [8].

AMPK is known as a key molecule that regulates energy balance, body weight, food intake, and metabolic balance of lipid and glucose. The activation of AMPK switches cells from ATP consumption to active ATP-producing processes like

fatty acid and glucose oxidation. From these reasons, AMPK has become the focus of many recent studies as a therapeutic target of metabolic disease [9–11].

2. Methods and Materials

2.1. Preparation of OB-1. Six herbs, *Benincasae semen, Laminaria japonica Areschon, Pini Folium, Moli Folium, Citrus aurantium Linn,* and *Ephedra herb,* were purchased from Omniherb (Gyeong Buk, Korea) and immersed in 1 L of 80% ethanol and then sonicated for 30 min. The resulting extract was filtered through a glass filter using a vacuum pump. A rotary vacuum evaporator (Eyela, Japan) was used to concentrate the liquid extract at 45°C. The concentrated extract was then lyophilized and reconstituted in saline at the working concentration. OB-1 is prepared from these six herbs extracts in the ratio of 1 : 1 : 1 : 1 : 1 : 1.

2.2. Experimental Design. Four-week-old male Wistar rats weighing 140–160 g were purchased from Central Laboratory Animal, Inc. (Seoul, Republic of Korea). The animals were examined in compliance with Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. Four rats were housed per cage under a 12 : 12 hour light-dark cycle, 50% humidity, and 23 ± 1°C. The nutrient component and composition ratio of the control and high-fat diets are indicated in Table 1 [1, 12]. The rats were fed with a standard laboratory pellet chow (Purina Co.; Republic of Korea) and acclimatized to their environment for 7 days before commencing the experiment. After acclimatization, the control group ($n = 8$) received a standard laboratory chow diet (control diet) and the high-fat diet group ($n = 10$) received the diet described in Table 1. The nutrient component of the control diet (3.665 kcal/g) was 65% carbohydrate, 20% protein, and 4.5% lipid. The high-fat diet (4.058 kcal/g) was a mix containing highly palatable human foods (cookies, cheese, sausage, chips, chocolate, and almonds) in a proportion of 2 : 2 : 2 : 2 : 1 : 1 and an equal amount (in grams) of the control laboratory chow diet. This high-fat diet contained 32%, 12%, and 31% of its energy as carbohydrate, protein, and fat, respectively. The animals were weighed at the start of the experiment and every week thereafter. After 5 weeks of feeding the rats either control or high-fat diets, each group was randomly divided into saline-treated or OB-1-treated groups. Rats were fed the indicated diet treated with saline or 40 mg/100 g of OB-1 daily for 5 weeks. Rats were sacrificed by administration of anesthesia 10 weeks after the start of the dietary treatment.

2.3. Organ Samples. Epididymal adipose tissue and liver samples were enucleated from the rats and washed in cold saline solution. Epididymal adipocyte samples were immediately stored in a −70°C deep freezer for subsequent mRNA isolation. Liver samples were fixed overnight in 10% neutral buffer formalin (NBF) in preparation for histological staining. Fixed liver samples were then soaked in 30% sucrose (Sigma; St. Louis, MO, USA) until the liver samples sank to the bottom of bottle. After removing excess fluid from samples, they were stored at −70°C.

2.4. RT-PCR Analysis. Total RNA was isolated using TRIzol Reagent according to the manufacturer's instructions (Invitrogen; Grand Island, NY, USA). Total RNA was treated with 2 units of RNase-free DNase (Promega, Madison, WI, USA) at 37°C for 30 min, extracted with phenol/chloroform/isopropanol (25 : 24 : 1; Fluka; Milwaukee, Wi, USA), and precipitated with ethanol. For reverse transcriptase (RT) reactions, 2 μg of total RNA was used as a template to synthesize cDNA, as follows: total RNA was combined with 4 μg random hexamer (Amersham Biosciences; Buckinghamshire, UK), incubated at 65°C for 10 min, and cooled on ice for 2 min. The RT reaction was carried out in a 30-μL total volume with 2 units of M-MLV reverse transcriptase (Invitrogen; Carlsbad, CA, USA) at 42°C for 1 h, followed by heating at 95°C for 5 min to stop the reaction. Subsequent PCR was carried out in a 25-μL reaction mixture consisting of the cDNA template, 10 pmole of each gene-specific primer, 5X first strand buffer, 2.5 mM dNTP mixture, and 1 unit of *Taq* DNA polymerase (Takara Korea; Seoul, Republic of Korea). PCR was performed using the following primers for leptin (5′ ATG TGC TGG AGA CCC CTG T 3′; 5′ ATT CAG GGC TAA GGT CCA ACT 3′) and GAPDH (5′ CAA AGT GGA CAT TGT TGC CA 3′; 5′ TTC ACC ACC TTC TTG ATG TCA 3′). The resulting PCR products were resolved in 2.0% agarose gels containing ethidium bromide.

2.5. Immunoblot Analysis. Tissues were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and protease inhibitor cocktail (104 mM AEBSF, 0.08 mM Aprotinin, 2 mM Leupeptin, 4 mM Bestatin, 1.5 mM Pepstatin A, and 1.4 mM E-64) on ice. Homogenized tissue was incubated for 30 min on ice, followed by centrifugation at 14,000 rpm for 30 min at 4°C. The supernatant was used to conduct a Bradford assay (Bio-rad) to determine protein concentration. Then, 50 μg of total protein was separated on 10% reducing polyacrylamide gels and transferred to membranes. Immunoblot analysis was performed using a phospho-AMPK antibody (Cell Signaling Technology; Beverly, MA, USA) or α-tubulin antibody, and immunoreactive proteins were detected using chemiluminescence.

2.6. Isolation of Fat Cell from Adipocyte. Fat cells were isolated by collagenase treatment, in accordance with a method previously described [13]. Briefly, epididymal adipose tissue samples were minced at room temperature and incubated with 1.5 g/L of collagenase in 10 mL Krebs-Ringer bicarbonate (KRB; 10 nM HEPES, 6 mM glucose, and 30 g/L bovine serum albumin, pH 7.4, pregassed with 95% O_2/5% CO_2) for 30 min at 37°C in a shaking water bath. Adipocytes were then visualized by microscopy and photographed.

2.7. Liver Morphology. The fixed liver samples described above were embedded in Optical Cutting Temperature (OCT) compound, and 10 μM sections were cut on a cryostat. Tissue sections were stained with Oil Red O (Sigma), to visualize neutral lipids, and nuclei were counterstained with hematoxylin (Gill No. 2; Sigma). Oil Red O was dissolved

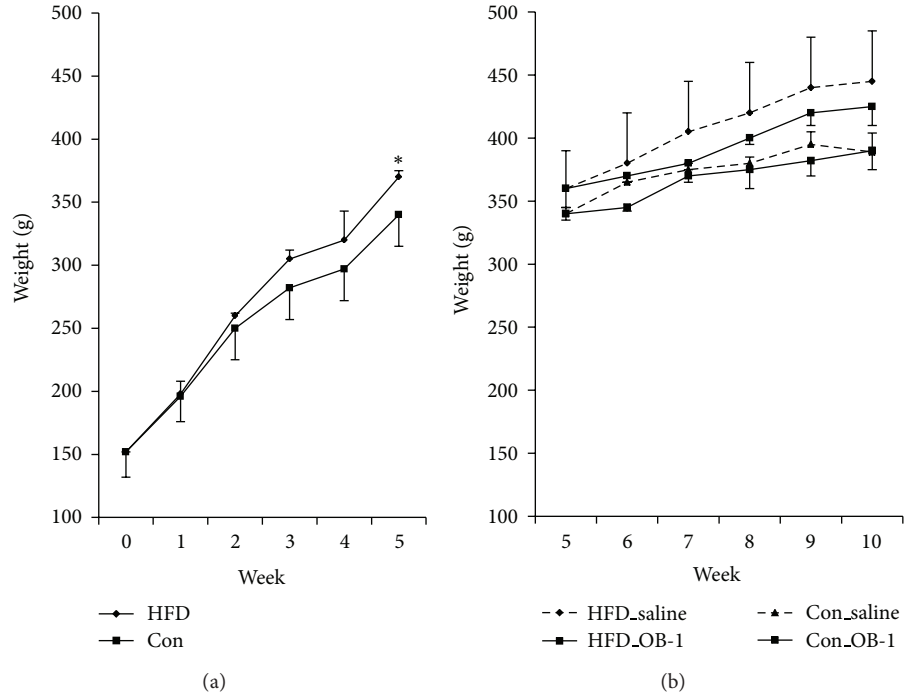


Figure 1: The change of body weight for total 10 weeks. (a) Obesity in rats ($n = 10$) was induced by high-fat diet for five weeks compared to controls ($n = 8$). (b) After subclassification of each group, herbal medicine (OB-1) and saline were treated for additional five weeks. Each data presents the mean ± S.E.M. $^*P < 0.05$ compared to controls.

in 99% isopropanol, left overnight at room temperature, and filtered with Whatman filter paper no. 2 (Whatman; UK). This stock solution was mixed with distilled water (2 : 3) and refiltered with Whatman filter paper no. 2 before use. Slides containing sectioned liver tissue were rinsed with isopropanol for 10 min and stained with the Oil Red O working solution for 15 min. The slide was then decolorized with 70% isopropanol for 3 min, rinsed with distilled water for 5 min, and stained with hematoxylin for 30 sec. Stained slides were given a final wash with distilled water, air dried, and mounted with glycerin jelly.

2.8. Open Field Test. To determine whether OB-1 treatment increased the activity level of rats, we monitored and compared visit counts, rearing, and grooming of the OB-1-treated group to the saline group in an open field arena. We used a 75 cm × 75 cm arena with 30 cm high walls, constructed of five pieces of thick paper and marked with a cross stripe across the bottom at a distance of 15 cm. A video camera was placed 250 cm above the arena, and it was used to record 10 min of activity per rat. Incidences of three kinds of activity (visit counts, rearing, and grooming) were monitored.

2.9. Statistical Analysis. Statistical analyses were performed using window SPSS (version 12.0). All of the quantitative data were analyzed by independent T-tests for the differences between two means and one-way ANOVA for the differences among four means. P values of < 0.05 indicated significant differences.

Table 1: The nutrient component and composition ratio of control and high-fat diet.

Component	Experimental diet (g/kg diet)	
	Control diet	High-fat diet
Protein	200.0	120.0
Carbohydrate	615.0	320.0
Fat	45.0	310.0
Fiber	60.0	30.0
Crude ash	70.0	35.0
Alsium	5.0	2.5
Phosphorous	5.0	2.5
Energy (kcal/g)	3.665	4.058

3. Results

3.1. Effect of OB-1 on Body Weight of High-Fat Diet-Induced Rats. High-fat diet-induced rats ($n = 10$) were significantly overweighted compared to controls of standard laboratory chow diet rats ($n = 8$) after five weeks ($P = 0.02$, Figure 1). Then, half of each HFD-induced and control groups were randomly subclassified and administered with OB-1 and saline for additional five weeks, as like HFD_OB-1, HFD_saline, Con_OB-1, and Con_saline, respectively. OB-1 treatments in HFD-induced rats showed about 3.1% reduction of body weight at 10 weeks, but not significant ($P > 0.05$). In addition, there was no difference in food intake between OB-1 and saline treatment groups. As like HFD group, there was no

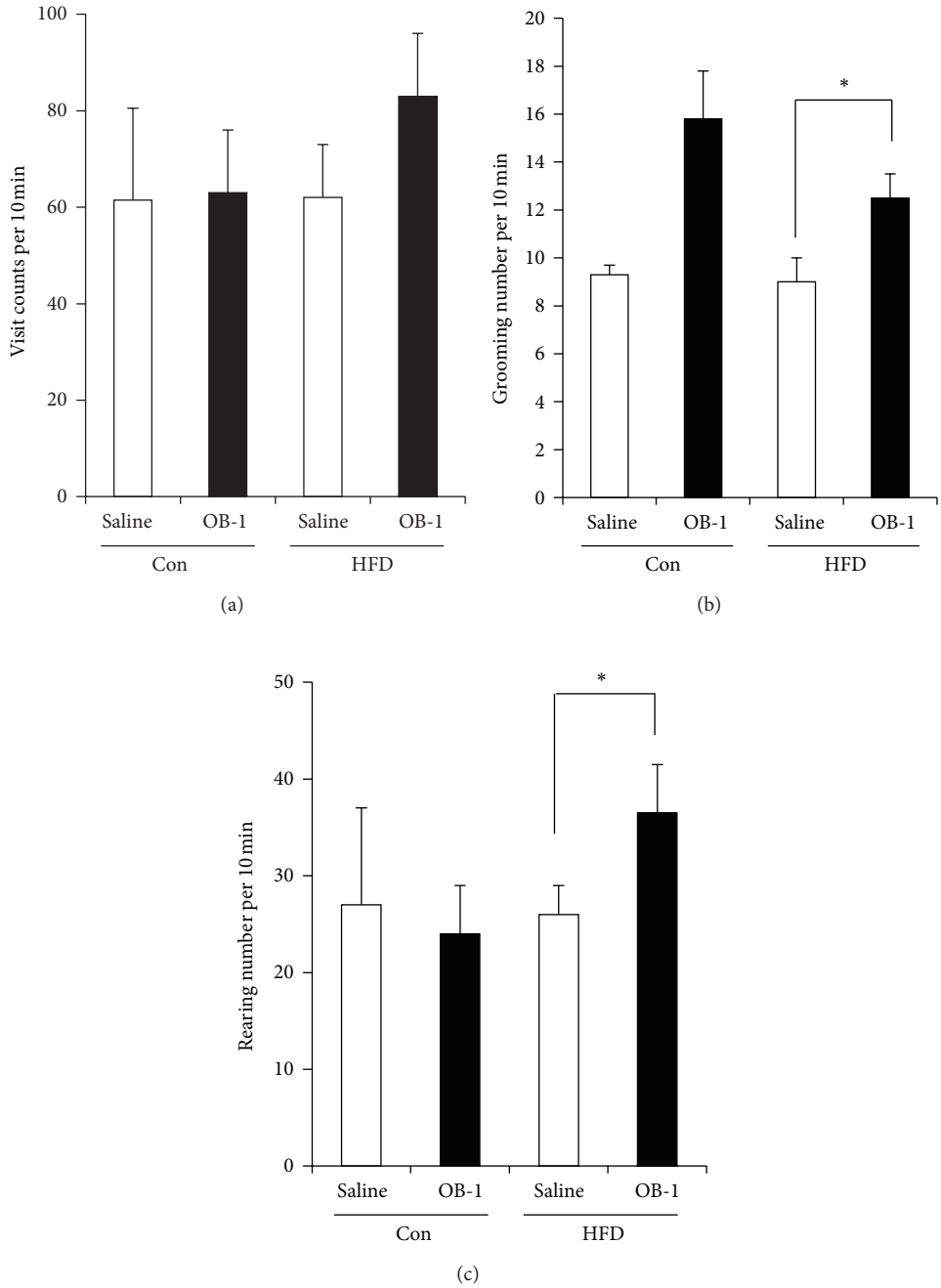
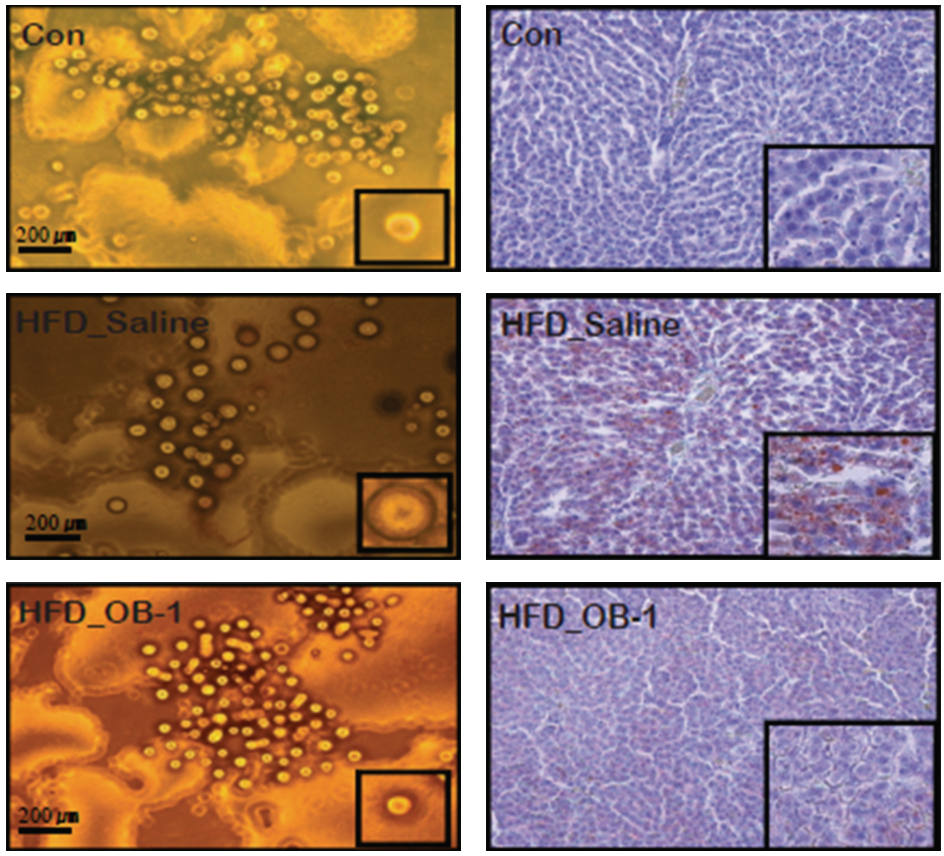


Figure 2: Increased activity of movements in open field test by OB-1. Animals were placed in open field arenas and allowed to move freely for 10 minutes. Visit counts (a), grooming (b), and rearing (c) of control_saline ($n = 4$), control_OB-1 ($n = 4$), HFD_saline ($n = 5$), HFD_OB-1 ($n = 5$) were monitored to measure activity levels. Each data presents the mean ± S.E.M. $^{*}P < 0.05$ compared to saline treatment of HFD-induced obese rats.

difference between OB-1 and saline treatment of controls in body weight.

3.2. Increased Activity of Movements in Open-Field Test by OB-1. To evaluate the effects of OB-1 on general activity levels of rats, rats of each group were subjected to an open field test to evaluate their levels of activity. During the 10 min they were allowed to roam freely, the visiting, rearing, and grooming of rats were monitored. Interestingly, OB-1 treatments in HFD-induced rats significantly increased the activity of movements compared to HFD_saline group ($P < 0.05$, Figure 2).

3.3. Effects of OB-1 on Adipocytes and Lipid Accumulation. To observe whether OB-1 affects the morphology of cells, white adipocytes cells were first isolated from rats of each group and then compared by microscopy. Epididymal white adipocytes



(a) (b)

Figure 3: Effects of OB-1 on adipocytes and lipid accumulation. (a) Photomicrographs of isolated epididymal white adipocytes from HFD-induced rats. (b) For microscopic observation (200x), liver tissues from each group were stained with Oil Red O to visualize neutral lipids and counterstained the nuclei with hematoxylin.

from HFD-induced rats were shown to be enlarged compared to the controls that were fed standard chow diet, whereas the size of adipocytes from OB-1-administered rats with HFD-induced obesity was found to be recovered (Figure 3(a)). Since obesity is related to lipid accumulation in the liver, liver tissues from each group were harvested and then stained with Oil Red O, a dye specific for lipid staining, and counterstained the nuclei with hematoxylin. Microscopic observation showed lipid droplets in the liver tissues from obesity-induced rats without administration of OB-1 (HFD_saline), whereas those from OB-1 treatment group showed no lipid droplets, similar to control samples (Figure 3(b)), indicating that OB-1 treatments could modulate the formation of lipid droplets in liver tissues.

3.4. Effects of OB-1 on the Expression of Leptin and Adiponectin mRNA. Based on the changes in adipocytes and liver tissue after OB-1 treatments, it was investigated whether the expression of leptin and adiponectin genes could be regulated by OB-1. Although the transcriptional level of leptin in epididymal white adipocytes from HFD-induced rats was significantly increased compared to controls, there was no difference between OB-1 and saline treatments (Figure 4(a)). In case of adiponectin, each group showed no significant change (Figure 4(b)). These results indicate that OB-1 has no effect to regulate the expression of both leptin and adiponectin.

3.5. Stimulatory Effect of OB-1 on the Phosphorylation of AMPK. Since AMPK plays a key role in energy metabolism within cells, it was further investigated whether OB-1 affects AMPK activity in the liver tissues from rats. There were no differences in the AMPK protein expression among controls and saline-administered group with HFD-induced obesity. However, the phosphorylation of AMPK was significantly increased in the OB-1-administered group compared to saline-treated obese group as well as controls ($P < 0.05$, Figure 5).

4. Discussion

OB-1, a mixture of six herbal remedies, has been utilized for detoxification or metabolic applications in oriental medicine. Each of the six components (*Benincasae semen*, *Laminaria japonica Areschon*, *Pini Folium*, *Moli Folium*, *Citrus aurantum Linn,* and *Ephedra herb*) has been independently reported

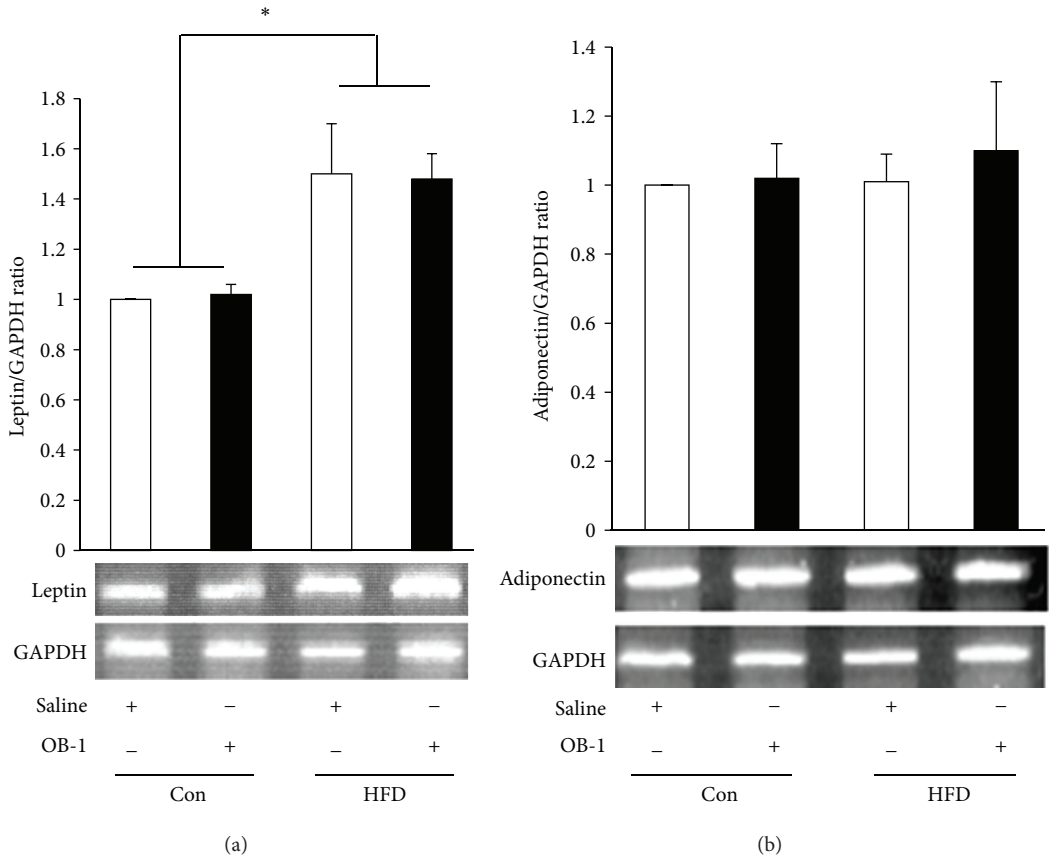


Figure 4: No change in mRNA expressions of leptin and adiponectin. Transcriptional levels of leptin (a) and adiponectin (b) were determined by RT-PCR to evaluate the effect of OB-1 on HFD-induced obese rats. Each data presents the mean ± S.E.M. from three independent experiments. $^{*}P < 0.05$ compared between HFD-induced rats and controls.

to exert its clinical effects on the human body, including regulation of lipid metabolism, reduction of body fat mass, and elimination of toxic compounds. *Citrus aurantum*, as a dietary supplement to reduce obesity, was examined to promote weight loss and metabolic rate [14, 15]. The *Ephedra herb* is a well-known anti-obesity medicine for weight loss. Multinutrient containing *ephedra* especially has been shown to have effects of weight loss and to improve metabolic risks in obese persons [16]. The *Laminaria japonica* is widely eaten as a healthy food in East Asia, and its preventive effects in streptozotocin-induced diabetic rat liver were reported recently [17]. Therefore, we hypothesized that OB-1 might have a significant effect on obesity and then established and evaluated its molecular functions using *in vitro* and *in vivo* studies. To induce an obesity (significant changes in body weight), 5-week treatment with high-fat diet was appropriate in our experimental model. Moreover, 40 mg/100 g body weight of OB-1 dose and additional 5-week treatments were appropriate to observe activity of movements in open field test, reduction in the size of adipocytes, inhibition of lipid accumulation, and phosphorylation of AMPK. Our findings revealed that OB-1 had no direct effect on anti-obesity and regulation of leptin and adiponectin; however, OB-1 increased the phosphorylation of AMPK and the activity of movement *in vivo*.

AMPK plays an important function in maintaining the energy balance within cells. Also, liver is a center for energy metabolism and glucose homeostasis. To control energy homeostasis, AMPK activation decreases gluconeogenesis and lipogenesis, whereas it increases fat oxidation and glucose uptake by switching on ATP-generating pathways and switching off ATP-consumption pathways [9, 18, 19]. In this study, OB-1 treatments were also shown to be negatively correlated with accumulation of lipid droplets in liver tissue, suggesting that OB-1 could inhibit a lipid accumulation by blocking the pathway related to lipid metabolism. In support of this idea, OB-1 positively regulated the AMPK activity in liver tissue from OB-1-treated group with HFD-induced obesity. Therefore, OB-1 might negatively regulate the lipid metabolism or gluconeogenesis by activating AMPK. However, it is needed to elucidate the underlying molecular mechanisms in detail.

According to our results of open field tests [20], OB-1-administered rats with HFD-induced obesity showed the increased grooming and rearing activities compared to saline-treated control group. Although OB-1-administered obese rats showed no significant reduction of body weight compared to saline-treated obese group, these increased movements suggest that the activation of AMPK and its related mechanisms might control not body weight or food intake but energy metabolism [10, 21]. This increased energy

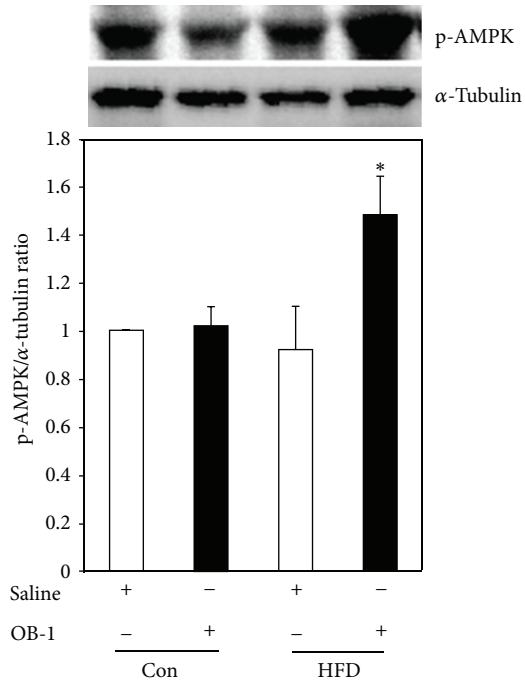


Figure 5: Stimulatory effect of OB-1 on the phosphorylation of AMPK. The phosphorylated AMPK protein levels in liver tissue were detected by western blot analysis. OB-1 treatment to HFD-induced obese rats showed a significant increase in phosphorylation of AMPK compared to saline treatment. Each data presents the mean ± S.E.M. from three independent experiments. $^{*}P < 0.05$ compared to saline treatment of HFD-induced obese rats.

metabolism by OB-1 could decrease about 3.1% reduction of body weight of rats in this study, but not significant, and might increase the activity of movements.

Leptin and adiponectin, as adipocyte-derived hormones, play key roles in obesity and energy homeostasis [22, 23]. Since leptin regulates body fat stores through its effects on food intake and energy metabolism, leptin is an important molecule in the obesity process [24]. It has been recently reported that there is a significant correlation between leptin expression and adipocyte size [25]. In addition, there is a correlation between the expression of adiponectin, which is an adipokine that is specially secreted by adipocytes, and adipocyte size in obesity [26, 27]. Based on these facts, the expression of leptin and adiponectin was evaluated, but no significant change in the mRNA expression of the genes by treatment of OB-1 was found. However, the size of adipocyte from OB-1-administered rats with HFD-induced obesity was shown to be recovered compared to the enlarged adipocytes of obesity-induced rats, suggesting that other signals might be involved in the expression and regulation of adipocytes in treatment of OB-1. It was reported that TNF is expressed in human adipocytes, and TNF level is positively correlated with obesity [28]. This suggests that TNF signaling might be involved in the expression and regulation of adipocytes in treatment of OB-1.

Physical exercise and contraction are correlated with the increased phosphorylation of AMPK in human or rat skeletal muscle [29, 30]. Therefore, our findings that administration of OB-1 increased the phosphorylation of AMPK suggest that components from the six herbals could stimulate the mechanism of physical movements and exercise through the activation of AMPK and/or its involved regulators. In conclusion, this study showed that treatments with OB-1 to HFD-induced obese rats significantly increased the phosphorylation of AMPK and reduced the enlarged size of adipocytes from HFD-induced obesity and lipid accumulation. Therefore, it is suggested that these effects of OB-1, especially related with the activation of AMPK, might alter the metabolic processes. Our findings need future studies at the levels of molecular mechanism to understand how OB-1 herbal extract or its components modulate metabolic processes.

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Research Article

Quercetin Preserves β-Cell Mass and Function in Fructose-Induced Hyperinsulinemia through Modulating Pancreatic Akt/FoxO1 Activation

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Fructose-induced hyperinsulinemia is associated with insulin compensative secretion and predicts the onset of type 2 diabetes. In this study, we investigated the preservation of dietary flavonoid quercetin on pancreatic β-cell mass and function in fructose-treated rats and INS-1 β-cells. Quercetin was confirmed to reduce serum insulin and leptin levels and blockade islet hyperplasia in fructose-fed rats. It also prevented fructose-induced β-cell proliferation and insulin hypersecretion in INS-1 β-cells. High fructose increased forkhead box protein O1 (FoxO1) expressions *in vivo* and *in vitro*, which were reversed by quercetin. Quercetin downregulated Akt and FoxO1 phosphorylation in fructose-fed rat islets and increased the nuclear FoxO1 levels in fructose-treated INS-1 β-cells. The elevated Akt phosphorylation in fructose-treated INS-1 β-cells was also restored by quercetin. Additionally, quercetin suppressed the expression of pancreatic and duodenal homeobox 1 (Pdx1) and insulin gene (Ins1 and Ins2) *in vivo* and *in vitro*. In fructose-treated INS-1 β-cells, quercetin elevated the reduced janus kinase 2/signal transducers and activators of transcription 3 (Jak2/Stat3) phosphorylation and suppressed the increased suppressor of cytokine signaling 3 (Socs3) expression. These results demonstrate that quercetin protects β-cell mass and function under high-fructose induction through improving leptin signaling and preserving pancreatic Akt/FoxO1 activation.

1. Introduction

High-fructose feeding is suggested to cause metabolic syndrome characterized by hyperinsulinemia, hyperleptinemia, and insulin resistance, exacerbating the development of type 2 diabetes in rodents [1–4]. Recent *in vitro* study shows that fructose stimulates insulin secretion in human and mouse islets by directly targeting β-cells [5]. Leptin via its signaling affects β-cell growth and function and directly suppresses insulin secretion from pancreas to prevent hyperinsulinemia occurrence [6]. Leptin (ob/ob) or leptin receptor (ObR) (db/db)-deficient mice develop islet hyperplasia, which is possibly associated with deficient leptin signaling and consequent insulin action enhancement [7, 8]. Protein kinase B/Akt/forkhead box protein O1 (FoxO1) pathway mediates leptin action in pancreas and plays an important role in controlling β-cell size and survival [7, 9, 10]. It has been observed that ObR gene mutation or deficiency in animals with compensatory β-cell growth also enhances Akt and FoxO1 phosphorylation [7, 11]. Furthermore, pancreatic FoxO1 regulates β-cell proliferation and function through inhibiting pancreatic and duodenal homeobox 1 (Pdx1, insulin promoter factor 1) [8, 12]. Mutation of FoxO1 in mice can cause insulin hypersecretion and β-cell mass, while FoxO1 haploinsufficiency partially restores β-cell proliferation in Irs2 knockout mice [13, 14]. High-fructose feeding is confirmed to cause leptin resistance and leptin downstream signaling janus kinase 2/signal transducers and activators of transcription 3 (Jak2/Stat3) pathway impairment in peripheral tissues of rats [15–17]. Thus, we propose that fructose-impaired leptin signaling in pancreas may play a critical role in the direct and/or indirect induction on β-cell mass and function. Although there is limited evidence for direct involvement of Akt/FoxO1 pathway in the etiology of fructose-induced

β-cell hyperplasia, hepatic FoxO1 dysregulation has been proved in high-fructose-fed hamsters with leptin resistance [18].

Quercetin as an important dietary flavonoid is found in a variety of plant-based foods such as red onions, apples, tea, broccoli, capers, lovage, parsley, red grapes, and berries [19]. Quercetin exhibits beneficial effects on human health with its broad pharmacological properties, including anti-inflammation and antioxidation [19–21]. Recently, quercetin is confirmed to alleviate hepatic fat accumulation and metabolic changes in western-style diet-fed animals [22, 23]. It also protects β-cell damage to ameliorate hyperglycemia in type 2 diabetic animals [24–26]. Our previous study showed that quercetin reduced serum insulin and leptin levels, improving insulin and leptin resistance with regulation of insulin and leptin signaling in the liver of fructose-fed rats [27]. However, the precise molecular mechanism of quercetin action against fructose-induced hyperinsulinemia has not been elucidated, which prompted us to evaluate the effects of quercetin on pancreatic Akt/FoxO1 pathway impairment involved in β-cell mass and function using fructose-treated rats and INS-1 β-cells.

In the present study, quercetin was found to prevent fructose-induced compensatory β-cell hyperplasia and preserve β-cell mass and function by suppressing Akt/FoxO1 phosphorylation in rats and INS-1 β-cells, which were associated with its improvement of pancreatic leptin signaling. The direct mechanism was that quercetin preserved nuclear FoxO1 transcription activation, thereby inhibiting Pdx1 and insulin gene expression in β-cells under high-fructose induction.

2. Materials and Methods

2.1. Materials. Fructose was provided for animals by Huikangyuan Biotechnology Co., Ltd. (Beijing, China) and for cells by Sigma-Aldrich (St. Louis, MO, USA). Quercetin (98%), recombinant rat leptin, Histopaque 1077, and DAPI were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diagnostic kit for serum glucose levels was obtained from Jiancheng Biotech Institution (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits for serum insulin and leptin levels were purchased from Alpco Inc. (Salem, NH; Crystal Chem, Downers Grove, IL). Insulin ELISA kit used for measurement of INS-1 β-cell insulin secretion was from Millipore Corporation (MA, USA). Primary antibodies including rabbit polyclonal antibodies against FoxO1, phospho-FoxO1 (Ser256), Pdx1, Akt, p-Akt (Ser473), Jak2, p-Jak2 (Tyr1007), Stat3, p-Stat3 (Tyr705), suppressor of cytokine signaling 3 (Socs3), and α-tubulin were provided by Cell Signaling Technology (Boston, MA, USA) and Jak2 and p-Jak2 by Abcam (Cambridge, MA, USA). ECL-Plus Western blotting detection reagents were provided by Perkin Elmer (Wellesley, MA, USA). Mouse polyclonal antibody against insulin was obtained from Santa Cruz Biotechnology (CA, USA). Alexa Fluor 555 donkey anti-mouse IgG was obtained from Invitrogen (Carlsbad, CA, USA). Other chemicals of the highest analytical grade were purchased from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China).

2.2. Animal Experiments. Male Sprague-Dawley rats, weighted 250–280 g, were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China) and housed in a temperature- and humidity-controlled environment with a 12 h light-dark cycle. The animals had access to diet and water *ad libitum*. All animal use procedures were conducted in accordance with Chinese legislation on the Use and Care of laboratory animals and were approved by the Institute for Experimental Animals of Nanjing University.

Rats were fed either normal drinking water (as control group) or water containing 10% (wt/vol) fructose according to the previous report [15]. After 4 weeks, fructose-fed rats were divided into 3 subgroups (10 rats per group). One group (vehicle group) received 1 mL/kg water by intragastric administration. Other two groups were treated with 50 or 100 mg/kg quercetin suspended in water by intragastric administration (1 m/kg body weight) once daily from 2:00 PM to 3:00 PM for an additional 4 weeks, respectively. Simultaneously, drinking water with 10% (wt/vol) fructose was continued for all of the fructose-fed rats. The chosen quercetin dosages were based on our and other previous experiments [19–21, 27, 28]. Body weight was detected weekly, and oral glucose tolerance test (OGTT) was performed during the last week of the feeding period. At the end of experiments, tail-vein blood samples were collected and centrifuged (3000 g) at 4°C for 10 min to get serum for the measurement of glucose and insulin and leptin levels. Pancreases from 4 rats of each group were harvested for immunohistochemical analyses and islet morphometry as described in the following.

2.3. OGTT. At the end of quercetin treatment, OGTT was performed. Rats were weighed and fasted for 14 h and then orally administered with glucose (1.5 g/kg body weight). Tail-vein blood samples were collected from the tip of the rat tails before glucose administration and at different times afterwards up to 120 min, which were centrifuged (3000 g) at 4°C for 10 min to get serum for glucose assay by the method described previously.

2.4. Isolation of Rat Islets. At the end of experiments, six rats in each group were injected i.p. with 100 μg/kg leptin 30 min before they were anesthetized to investigate the response of pancreatic islet Akt/FoxO1 pathway to leptin action in normal group, and fructose-fed groups treated with vehicle or quercetin. Rat pancreatic islets were isolated by collagenase digestion [29] and used for RNA or protein extraction, respectively. In brief, animals were sacrificed and the pancreas was infused with a cold solution of collagenase P (1 mg/mL) in HBSS from the liver through a catheter introduced into the part of the choledoco running. Then the filled pancreas was extracted and digested at 37°C for 20 min. Islets were enriched on Histopaque 1077 (500 g, 15 min, 4°C) and purified by handpicking under a microscope.

2.5. Cell Culture and Treatment. Isolated insulinoma cell line INS-1 β-cells were maintained in RPMI 1640 medium containing physiologic concentrations of glucose (5 mM) supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin,

100 mg/mL streptomycin, 1 mM sodium pyruvate, and 50 mM 2-mercaptoethanol. According to the experimental design, fructose (1 mM) and/or quercetin (5–100 μM) were added in baseline condition medium to stimulate cells, respectively. MTT colorimetric assay was used to measure cell proliferation *in vitro*. After treating, INS-1 β-cells were washed with PBS twice and fixed in 4% (vol/vol) paraformaldehyde for immunofluorescence staining according to the following method.

2.6. Insulin Secretion. When INS-1 β-cells reached approximately 70% confluence, they were treated with fructose or/and quercetin according to each experimental condition in a 24-well dish. Insulin secretion from INS-1 β-cells was measured as described previously [30]. Before stimulation, β-cells were preincubated in 1 mL of Krebs-Ringer-bicarbonate-(KRB-) buffered solution without glucose for 1 h at 37°C to increase the sensitivity of β-cells to glucose. Cells were then incubated with 0.25 mL KRB buffer at 8.3 mM glucose for an additional hour, after which the supernatant was collected, for insulin measurement using insulin ELISA kits. Cells in the same well were subsequently collected and total protein contents were measured by a BCA protein assay kit. Under each experimental condition, we independently measured insulin from four separate wells, which were then averaged to represent a single value for the group. Averages of three to eight independent cell experiments were shown in this study.

2.7. Quantitative RT-PCR. Reverse-transcribed RNA in rat islets and INS-1 β-cells was analyzed by real-time PCR using SYBR Green or TaqMan technology. The primers were used as follows: Pdx1, GTG CCA GAG TTC AGT GCT AAT CC (fwd) and ACT TCC CTG TTC CAG CGT TCC (rev); Ins1, CAA GTC CCG TCG TGA AGT G (fwd) and GCA GTA GTT CTC CAG TTG GTA G (rev); Ins2, CAG TCG GAA ACC ATC AGC AAG C (fwd) and CCA CCA AGT GAG AAC CAC AAA GG (rev). β-actin was used to normalize gene expression.

2.8. Western Blot Analysis. Whole cell protein extracts from rat islets and INS-1 β-cells were prepared as described previously [30]. Equal amounts of proteins from each group were fractionated by 10% (wt/vol) SDS PAGE, transferred to a nitrocellulose membrane, and incubated with primary antibodies of FoxO1, p-FoxO1 (Ser256), Pdx1, Akt, p-Akt (Ser473), Jak2, p-Jak2 (Tyr1007), Stat3, p-Stat3 (Tyr705), and Socs3. Proteins were detected using ECL-Plus Western blotting detection reagents and imaged using VersaDoc Imaging System (Bio-Rad). Bands were densitometrically quantified by Image Lab software (Bio-Rad).

2.9. Nuclear Extract Preparation. Nuclear protein extracts from INS-1 β-cells were prepared using a detergent lysis procedure. Cells lysed in a buffer of 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P40, 0.4 mM phenylmethanesulfonyl fluoride, 0.01 ng/mL leupeptin, and 0.02 ng/mL aprotinin were incubated on ice for 30 min. Proteins were extracted from nuclear pellets by an incubation with a high-salt buffer containing 420 mM NaCl, 1 mM EDTA, 20 mM HEPES (pH 7.9), 20% glycerol, 1 mM dithiothreitol, 0.4 mM phenylmethanesulfonyl fluoride, 0.01 ng/mL leupeptin, and 0.02 ng/mL aprotinin with vigorous shaking. The nuclear debris was pelleted by a centrifugation at 2000 g for 30 min, and the supernatant was stored at −20°C. For the determination of FoxO1 and Pdx1 localization, Western blot analysis was conducted with nuclear protein extracts using rat FoxO1 and Pdx1 primary antibodies as described previously.

2.10. Islet Morphology and Immunohistochemistry. Pancreases of four rats in each group were rapidly dissected, weighed, fixed in 4% (vol/vol) paraformaldehyde, cut into 8 μm section for hematoxylin and eosin (H&E), and immunofluorescence-stained as described previously [8]. Mouse polyclonal antibody against insulin was used to mark insulin in islets on pancreatic section. DAPI was used for nuclear staining. Alexa Fluor 555 donkey anti-mouse IgG was used for secondary antibody. Images were recorded by a Leica TCS SP5 confocal microscope (Leica, Richmond Hill, ON, Canada). β-cell mass was calculated by point counting morphometry on three to four immunofluorescence-stained sections of each pancreas as described previously [31]. Data were analyzed systematically using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, USA) covering at least 250 fields per rat.

2.11. Statistical Analysis. Statistical analyses were performed using 2-tailed Student's t-test when 2 conditions were compared and one-way or two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons variance by the computer software Prism 5 from Graph-Pad Software Inc. (San Diego, CA). Results were represented as mean ± SEM (unless stated otherwise), and P value <0.05 was considered significant.

3. Results

3.1. Quercetin Blockaded Islets Hyperplasia in Fructose-Induced Rats. Consistent with the previous studies [1, 2, 15], fructose-fed rats exhibited obesity, fasting hyperinsulinemia and hyperleptinemia, but did not develop fasting or postprandial hyperglycemia (Figures 1(a)–1(d)). Furthermore, postprandial hyperglycemia was observed in OGTT in fructose-fed rats (Figure 1(b)). These results indicate that compensatory insulin secretion occurs under insulin resistance condition to control blood glucose levels in fructose-fed rats. Treatment with quercetin at 50 and 100 mg/kg significantly decreased body weight and fasting serum insulin and leptin levels and reduced serum glucose levels to the normal in OGTT (Figures 1(a)–1(c)), suggesting its improvement of insulin and leptin resistance in this model.

Furthermore, optical and statistical results showed a 2-fold increase of islet size in high-fructose-fed rats compared with control group (Figures 1(d)–1(f)). Pancreatic β-cell mass was also significantly increased secondary to the increased numbers of β-cells but not to the increased size of β-cells in these rats (Figure 1(g)), confirming the compensatory β-cell hyperplastic response to maintain normoglycemia.

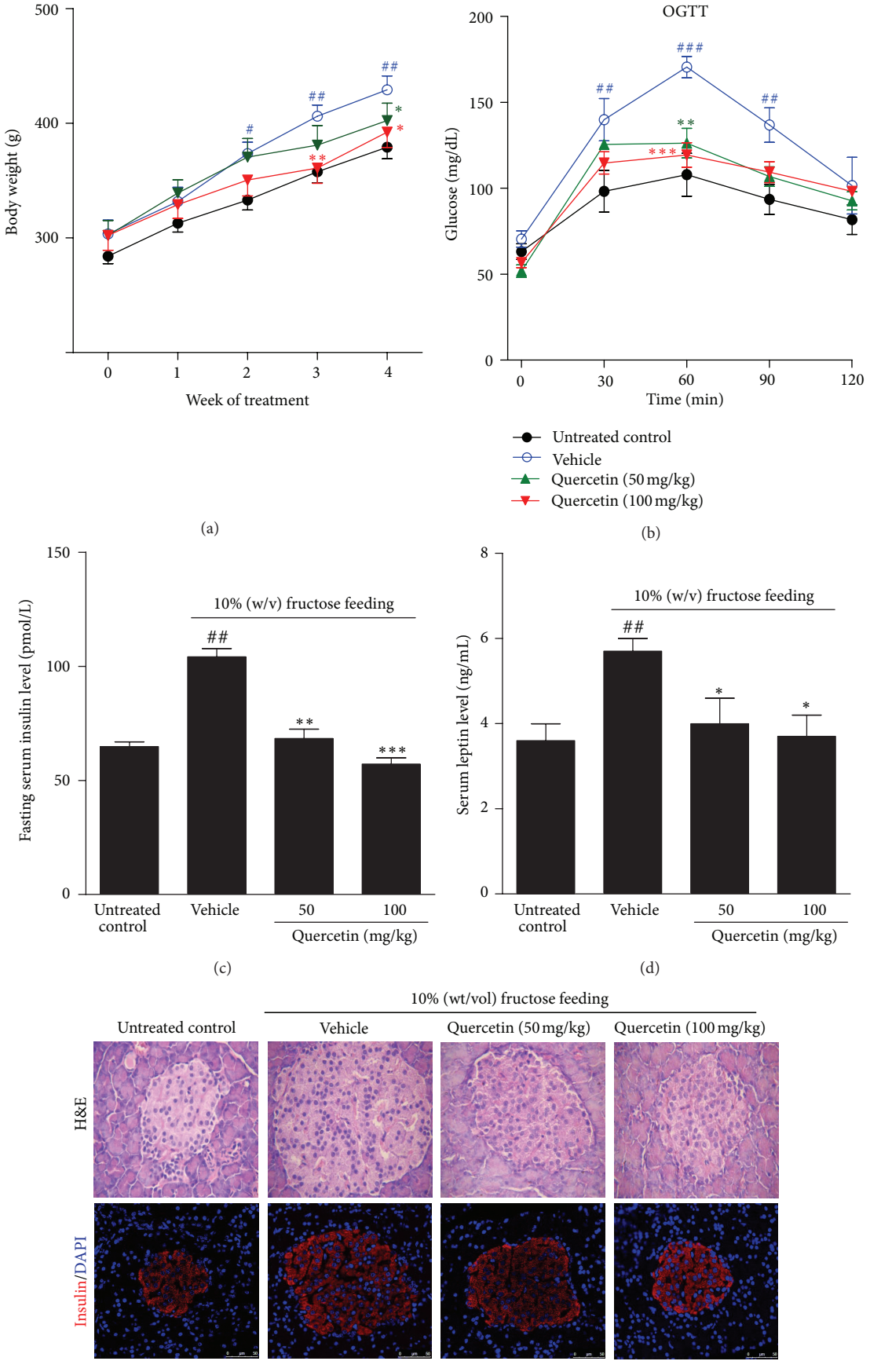

Figure 1: Continued.

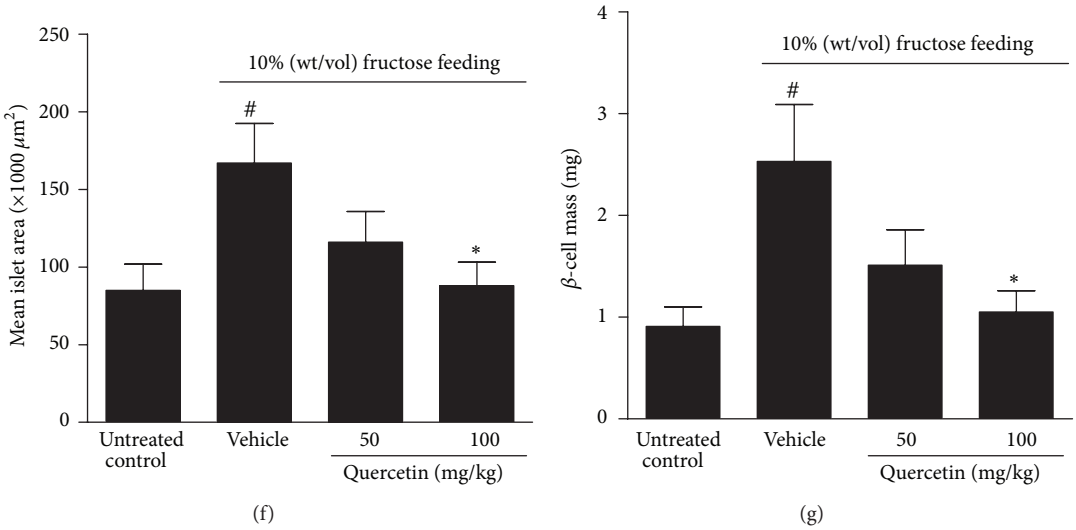

Figure 1: Protective effects of quercetin on fructose-induced insulin hypersecretion and islets hyperplasia. Rats were fed with 10% (wt/vol) fructose for 8 weeks and treated with 50 or 100 mg/kg quercetin in the last 4 weeks. (a) Body weight, (b) OGTT assay, and (c, d) fasting serum insulin and leptin levels were tested in rats ($n = 8$). (e) H&E staining (×400) and immunofluorescence staining (scale bars represent 50 μm) in pancreas sections. Representative images were showed for insulin (red) of pancreas sections. Nuclei were stained with DAPI (blue). Mean islet area (f) and β-cell mass (g) were estimated by morphometric analyses. Mean islet area was detected, at least 20 islets from 4 individual rats for each genotype. Data were presented as the mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ relative to untreated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to fructose-fed rats treated with water (vehicle).

The treatment with quercetin at 50 and 100 mg/kg blockaded fructose-induced islet hyperplasia and β-cell mass in rats (Figures 1(d)–1(g)). These observations indicate that quercetin preserved islet morphology in fructose-fed rats, possibly having prevention for the development of overt type 2 diabetes.

3.2. Quercetin Restored Fructose-Induced Akt/FoxO1 Pathway Activation in Islets of Rats. Western blotting showed significant phosphorylation enhancement of Akt at Ser473 (p-Akt) and FoxO1 at Ser256 (p-FoxO1) in islet lysate of fructose-fed rats by 1.9- and 4.3-fold, with the increased expression of FoxO1 protein levels by 3.1-fold (Figures 2(a)–2(c)) compared with control group, respectively. These data demonstrated fructose-induced islet lysate Akt/FoxO1 pathway activation in fructose-fed rats, which might contribute to an increase in islet size and mass. Accordingly, Pdx1 mRNA and protein levels were significantly elevated in islet lysate of fructose-fed rats by 2.8- and 2.2-fold, respectively (Figures 2(a), 2(c), and 2(d)). Islet Ins1 and Ins2 mRNA levels were also enriched in this model by 1.8- and 3.1-fold, respectively (Figure 2(d)). After the treatment with quercetin at 50 mg/kg, the increased expression levels of p-Akt, FoxO1, p-FoxO1, and Pdx1 were partly attenuated in islet lysate of fructose-fed rats, with a reduced tendency of Ins1 and Ins2 expression levels (Figures 2(a)–2(d)). 100 mg/kg quercetin treatment completely restored the increased protein levels of p-Akt, FoxO1, p-FoxO1, and Pdx1, as well as the increased mRNA levels of Pdx1, Ins1, and Ins2 in islet lysate of fructose-fed rats (Figures 2(a)–2(d)). Taken together, these data demonstrate that the preservation of quercetin on islet morphology and β-cell mass may be associated with its suppression of pancreatic Akt/FoxO1 activation in fructose-fed rats.

3.3. Quercetin Prevented Fructose-Induced Cell Proliferation and Insulin Secretion in INS-1 β-Cells. In vitro study showed that INS-1 β-cell proliferation was significantly increased by 45% after 24 h incubation with 1 mM fructose, evidenced by results of MTT assay (Figure 3(a)), confirming the direct stimulation of fructose on β-cell mass. 5–20 μM of quercetin treatment dose-dependently prevented fructose-stimulated INS-1 β-cell proliferation (Figure 3(a)), further suggesting its preservation of β-cell mass. Alone treatment of quercetin at 5–20 μM showed no significant effect on the proliferation of INS-1 β-cells (Figure 3(a)), but at 50–100 μM significantly decreased INS-1 β-cell proliferation (Figure 3(a)), showing potent cytotoxicity that was alleviated by 1 mM fructose.

As expected, 1 mM fructose significantly increased the ability of INS-1 β-cells to secret insulin (Figure 3(b)). Lower dosage of quercetin (5 and 10 μM) increased insulin secretion in normal INS-1 β-cells, but failed to prevent the changes of insulin secretion in fructose-treated INS-1 β-cells (Figure 3(b)). It was noted that 20 μM quercetin prevented the changes of insulin secretion in fructose-treated INS-1 β-cells, but not in normal cells (Figure 3(b)). However, 50 and 100 μM quercetin showed potent cytotoxicity to significantly decrease cell proliferation and glucose-stimulated insulin secretion in normal and fructose-treated INS-1 β-cells (Figure 3(b)).

3.4. Quercetin Blocked Fructose-Induced Nuclear FoxO1 Translocation in INS-1 β-Cells. Time-course study showed that

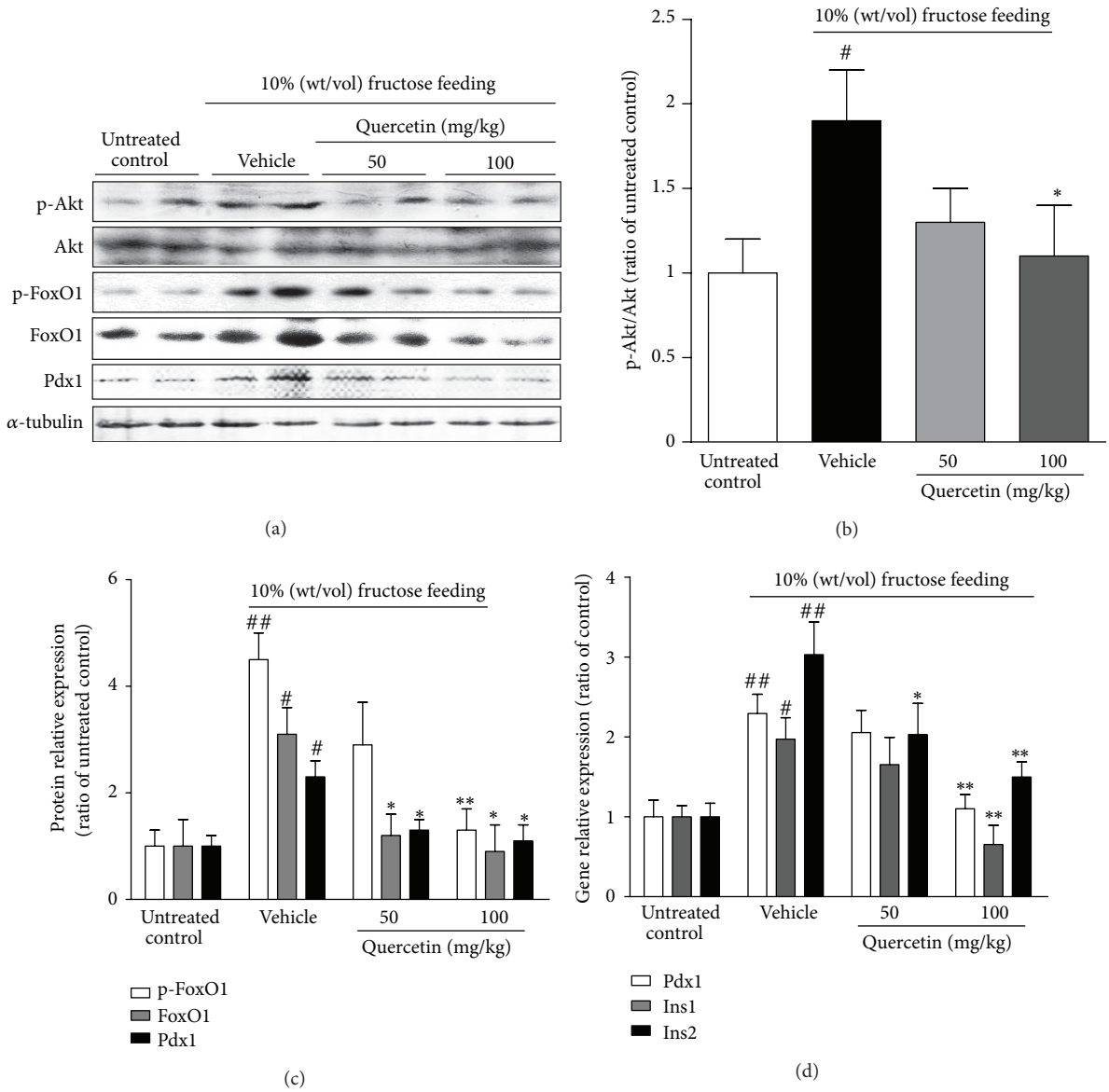

FIGURE 2: Effects of quercetin on the increased expression of FoxO1, Pdx1, and insulin gene in islets of fructose-fed rats. Rats were fed with 10% (wt/vol) fructose for 8 weeks, treated with 50 or 100 mg/kg quercetin in the last 4 weeks, and injected i.p. with 100 μg/kg leptin 30 min before they were anesthetized. (a) The bands of Western blot analyses for protein levels of Akt, p-Akt, FoxO1, p-FoxO1, and Pdx1 in islets of rats. (b, c) The intensity of bands was measured, and the ratio of treated samples to untreated control was showed. (d) Real-time PCR for gene expression levels of Pdx1 and Ins1/2 in islets of rats. Islets were from three to four individual mice in each group, and data were presented as the mean ± SEM of three independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to untreated control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to fructose-fed rats treated with water (vehicle).

total FoxO1 protein levels were rapidly increased in INS-1 β-cells induced by 1 mM fructose within 4 h, and this augment in FoxO1 expression was sustained for up to 24 h (Figure 4(a)). Conversely, the nuclear FoxO1 protein levels were simultaneously decreased in fructose-treated INS-1 β-cells (Figure 4(a)). Nuclear import of FoxO1 contributes to the suppression of Pdx1 gene expression in β-cells of pancreas [32]. We also found that nuclear Pdx1 protein levels were markedly elevated in INS-1 β-cells induced by 1 mM fructose starting from 8 h and sustaining for up to 24 h (Figure 4(a)), further confirming that fructose impairs FoxO1 transcriptional suppression on Pdx1 in β-cells. 20 μM quercetin time dependently prevented 1 mM fructose-stimulated protein alterations of total FoxO1, nuclear FoxO1, and nuclear Pdx1 in INS-1 β-cells (Figure 4(a)).

Furthermore, 24 h quercetin treatment dose-dependently suppressed the increased total FoxO1 protein levels and increased nuclear FoxO1 protein levels in 1 mM fructose-treated INS-1 β-cells and displayed the strongest effect at 20 μM (Figure 4(b)). The increased Pdx1 protein levels in

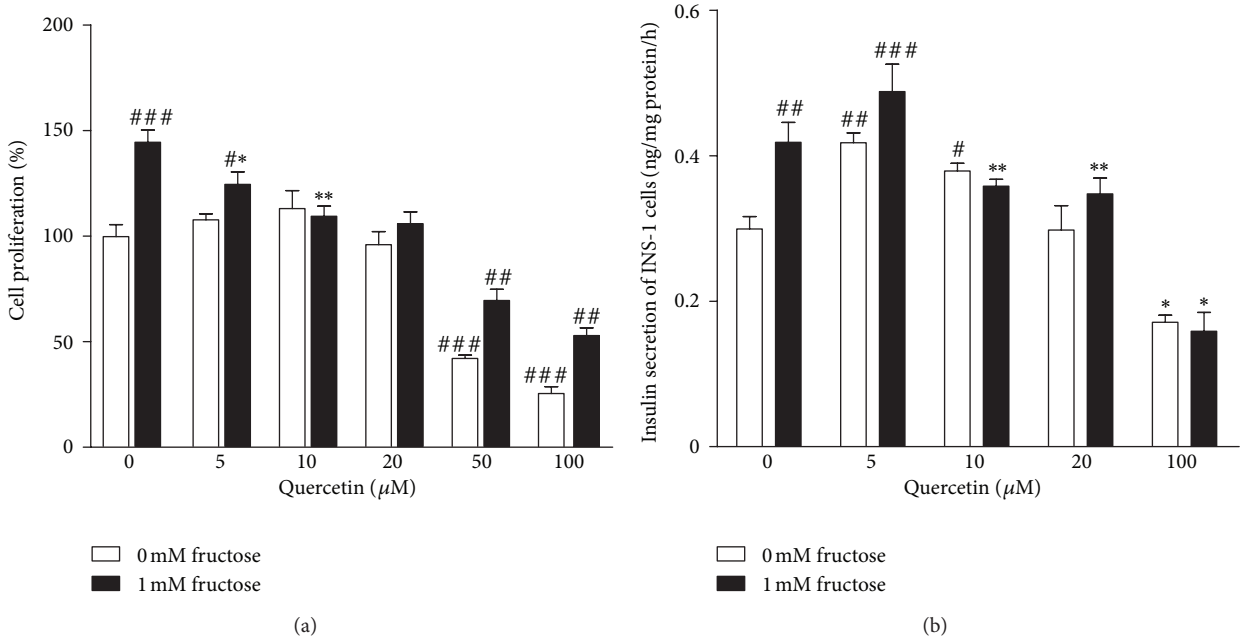

Figure 3: Effects of quercetin on cell proliferation and insulin secretion in INS-1 β-cells with or without 1 mM fructose treatment. INS-1 β-cells were treated with 5–100 μM quercetin in the absence or presence of 1 mM fructose from 24 h. Data were presented as the mean ± SEM of three to six independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ relative to control cells without fructose and quercetin treatment; $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to vehicle cells only with fructose treatment.

nuclear of INS-1 β-cells induced by 1 mM fructose were inhibited by quercetin at a dose-dependent manner and completely recovered to the normal at 10 and 20 μM quercetin (Figure 4(b)), demonstrating the protection of quercetin against fructose-impaired FoxO1 transcriptional activation in β-cells.

3.5. Quercetin Reversed the Increased Phosphorylation of Akt in Fructose-Treated INS-1 β-Cells. The elevated phosphorylation of Akt, upstream of FoxO1, was observed in INS-1 β-cells induced by 1 mM fructose starting from 4 h and sustaining for up to 24 h (Figure 5(a)). 20 μM quercetin time-dependently reversed 1 mM fructose-induced p-Akt (Ser473) elevation in INS-1 β-cells (Figure 5(a)). In addition, 24 h quercetin treatment suppressed the increased p-Akt (Ser473) in this cell model at a dose-dependent manner (Figure 5(b)). These data provide another evidence for the regulation of quercetin on Akt/FoxO1 pathway in fructose-induced β-cell impairment.

3.6. Quercetin Improved Leptin Downstream Signals in Fructose-Treated INS-1 β-Cells. We also found that fructose reduced phosphorylation levels of Jak2 (Tyr1007) and Stat3 (Tyr705) in INS-1 β-cells (Figure 6(a)). Conversely, 1 mM fructose significantly increased Socs3 expression, an inducible inhibitor that negatively regulates Stat signaling pathway, in INS-1 β-cells (Figure 6(b)). These data indicate the impairment of fructose on leptin downstream signaling in β-cells. Quercetin treatment for 24 h dose-dependently upregulated the decreased p-Jak2 and p-Stat3 (Figure 6(a)), as well as reduced Socs3 expression (Figure 6(b)) in fructose-incubated INS-1 β-cells. 20 μM quercetin completely corrected fructose-induced phosphorylation changes of Jak2 and Stat3 in this cell model (Figure 6(a)).

4. Discussion

Fructose-induced hyperinsulinemia is associated with pancreatic β-cell compensative insulin secretion and islets hyperplasia in humans and animals, predicting the onset of type 2 diabetes and metabolic diseases [4, 33, 34]. Here, we demonstrated that quercetin improved leptin signaling impairment and preserved islets morphology and β-cell function under high-fructose induction by regulating Akt/FoxO1 pathway, as well as Pdx1 and insulin gene expression in β-cells.

Akt/FoxO1 pathway links leptin signaling to Pdx1 regulation of pancreatic β-cell function and growth [12, 32, 35]. Our results demonstrated that fructose induced activation of pancreatic Akt/FoxO1 pathway in rats and INS-1 β-cells, which contributed to the increased β-cell mass and insulin secretion *in vivo* and *in vitro*. Quercetin as an antioxidant and anti-inflammatory agent possesses various potential effects. It can prevent the reduction of glucose or STZ-stimulated insulin secretion in rat islets [36] and protect β-cells against cytokine and STZ-induced damage [37, 38]. Moreover, quercetin is confirmed to effectively control postprandial blood glucose levels in STZ-induced diabetic rats and db/db mice, suggesting that it is a leading potential candidate for the prevention and treatment of diabetes [39, 40]. Our previous study found that quercetin normalized

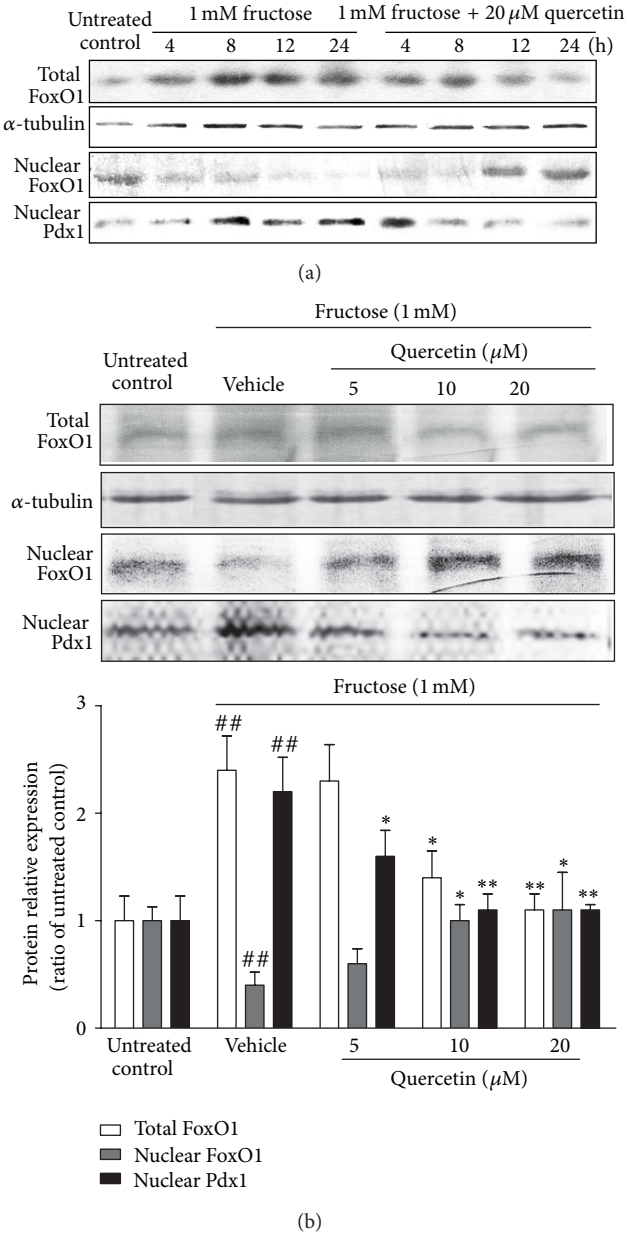

Figure 4: Time-course (a) and dose-dependent (b) effects of quercetin on protein levels of total FoxO1 and nuclear FoxO1 and Pdx1 in fructose-treated INS-1 β-cells. INS-1 β-cells were treated with 1 mM fructose in the absence or presence of 5, 10, and 20 μM quercetin. Data were presented as the mean ± SEM of three to six independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to untreated control cells; $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to fructose-treated vehicle cells.

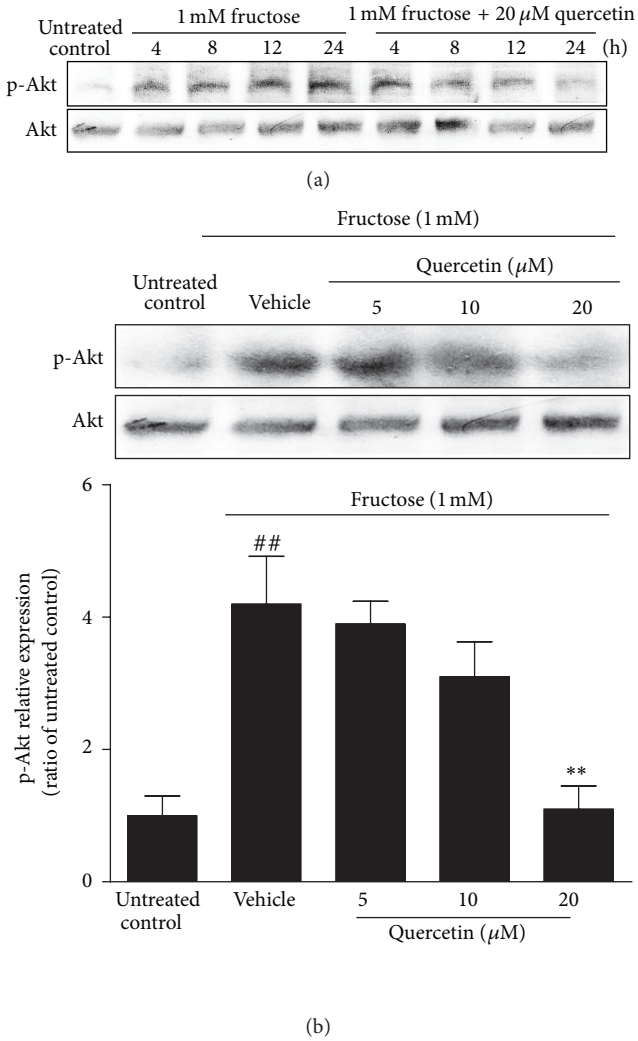

Figure 5: Time-course (a) and dose-dependent (b) effects of quercetin on the elevated phosphorylation of Akt in fructose-treated INS-1 β-cells. INS-1 β-cells were treated with 1 mM fructose in the absence or presence of 5, 10, and 20 μM quercetin. Data were presented as the mean ± SEM of three to six independent experiments. $^{\#\#}P < 0.01$ relative to untreated control cells; $^{**}P < 0.01$ relative to fructose-treated vehicle cells.

cyclical insulin and leptin levels and improved insulin and leptin signaling in liver and kidney of high-fructose-fed rats, showing beneficial effects on insulin and leptin resistance [27]. In the present study, quercetin was found to restore fructose-induced compensatory hyperplasia in rats, further confirming its protection of β-cells. These observations indicate that quercetin possibly prevents the onset of prediabetes driven by excess fructose. Indeed, direct phosphorylation by Akt inhibits transcriptional activation of FoxO1, causing its translocation from the nucleus into the cytoplasm [41]. Interestingly, quercetin was found to reduce phosphorylation levels of Akt and FoxO1 in fructose-fed rat islets and increase the nuclear FoxO1 levels in fructose-treated INS-1 β-cells. In parallel, the elevated phosphorylation levels of Akt in fructose-treated INS-1 β-cells were also restored by quercetin. Moreover, quercetin suppressed Pdx1, Ins1, and Ins2 protein or gene expressions in fructose-treated rat islets and INS-1 β-cells, implying that quercetin preserves fructose-induced nuclear FoxO1 activation by regulating insulin signaling. Therefore, the protective effect of quercetin on β-cells from high-fructose-induced insulin secretion enhancement and islet hyperplasia seems to occur through the modulation of pancreatic Akt/FoxO1 activation.

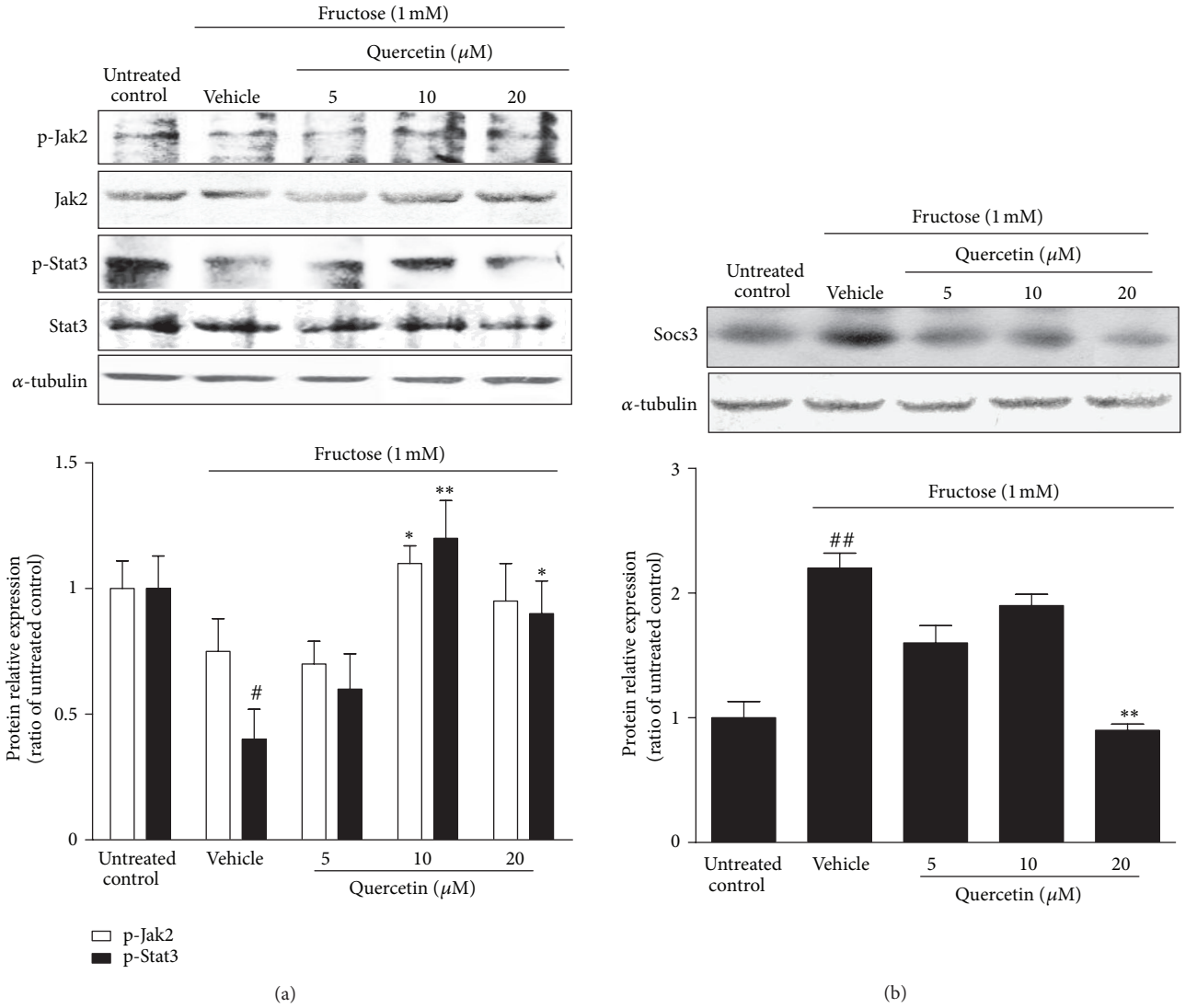

Figure 6: Effects of quercetin on the reduced phosphorylation of Jak2-Stat3 pathway (a) and the increased expression of Socs3 (b) in fructose-treated INS-1 β-cells. INS-1 β-cells were treated with 1 mM fructose in the absence or presence of 5, 10, and 20 μM quercetin for 24 h. Data were presented as the mean ± SEM of three to six independent experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ relative to untreated control cells; $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to fructose-treated vehicle cells.

Leptin signaling suppresses insulin secretion in physiological condition [42, 43]. Leptin resistance in pancreatic β-cells is suggested to contribute to hyperinsulinemia, β-cell failure, and consequent glucose intolerance in the obese state [7, 44]. The absence of leptin signaling significantly enhances phosphorylation of Akt and FoxO1, possibly resulting in an increase of β-cell size and islet mass in MIN6 β-cells and pancreas-ObR-KO mice [7]. FoxO1 in turn binds to Stat3 and inhibit in Stat3-mediated leptin actions *in vivo* and *in vitro* studies [45, 46]. It was noted that the increased activation of Akt/FoxO1 pathway was observed in islet of fructose-fed rats under leptin stimulation in this study, indicating that impairment of fructose on leptin signaling and its action contributed to the increased FoxO1 expression. The reduction of Jak2/Stat3 phosphorylation levels in fructose-treated INS-1 β-cells provided the direct evidence for this impairment. More studies demonstrate that Jak2/Stat3 pathway may be a molecular target for quercetin's antioxidant and anti-inflammatory effects [20, 47]. In our previous study, quercetin improved leptin resistance and repaired renal Jak2-Stat3 pathway in fructose-fed rats [27]. In this study, quercetin treatment elevated phosphorylation levels of Jak2 and Stat3 in fructose-treated INS-1 β-cells, suggesting that quercetin repairs leptin signaling disruption. Therefore, quercetin-mediated FoxO1 expression reduction may be related to its upregulation of p-Stat3 in fructose-treated INS-1 β-cells. The increased Socs3, a negative regulator of leptin signaling, is suggested to be responsible for leptin resistance in peripheral tissues of fructose-fed rats [15, 16]. Quercetin treatment suppressed Socs3 expression in fructose-incubated INS-1 β-cells. Thus, improvement of leptin signaling with suppression of pancreatic Akt/FoxO1 activation by quercetin is considered

to be one of the molecular mechanisms of its protection of fructose-induced compensative β-cells and hyperinsulinemia.

Hyperinsulinemia is associated with cardiovascular diseases and obesity [48]. Quercetin is suggested to be a candidate for reducing cardiovascular risk factors in humans [49] and preventing human obesity-related diseases [21]. It has been reported that dried grapes rich in quercetin reduce postprandial insulin response, modulate glucose absorption, and enhance leptin and ghrelin-mediated satiety in humans [50], suggesting that quercetin may be used as a nutritional and available supplement to improve health status in patients with diabetes [51]. Thus, the full spectrum of quercetin benefits needs to be evaluated appropriately in the treated and placebo subjects with or without excess fructose consumption.

In conclusion, our experimental results demonstrated that quercetin prevented compensatory β-cell hyperplasia in fructose-treated rats and INS-1 β-cells by decreasing pancreatic Akt/FoxO1 activation and affecting FoxO1 nuclear translocation. This protective effect of quercetin may be associated with the improvement of leptin resistance in whole body and leptin signaling in β-cells. This study provides more evidence for quercetin to be considered as a nutritional agent with potential for the prevention and treatment of hyperinsulinemia and its related metabolic diseases caused by high-fructose intake.

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Review Article

Metabolic Syndrome and Inflammation: A Critical Review of *In Vitro* and Clinical Approaches for Benefit Assessment of Plant Food Supplements

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Metabolic syndrome is defined as the clustering in an individual of several metabolic abnormalities associated with insulin resistance, type 2 diabetes, and obesity, in which low-grade chronic inflammatory activity is commonly observed. Part of the European Project PlantLIBRA is concerned with methods to assess the benefits of plant food supplements (PFSs) in countering inflammatory activity and metabolic syndrome. This paper summarizes the current methods used for benefit assessment of PFS, taking into consideration only *in vitro*, in silico, and clinical methodologies used to investigate the anti-inflammatory properties of plants. No in silico studies (using computer simulation) related to metabolic syndrome were found; these methods appear to be used exclusively for identifying or testing potentially effective compounds in drug development. Most *in vitro* methods for the assessment of beneficial effects of botanicals or plant food supplements in diabetes were based on a quantitative polymerase chain reaction (PCR), whereas the preferred kind of clinical study was the double-blind randomized controlled clinical trial. Only two parameters were observed to change after treatment with botanicals in both *in vitro* and *in vivo* studies: interleukin-6 and tumour necrosis factor-α, and these biomarkers should be carefully considered in future studies for PFS benefit assessment.

1. Introduction

Metabolic syndrome (MS) defines the clustering in an individual of multiple metabolic abnormalities [1]. World Health Organization and programs including the National Cholesterol Education Program (NCEP) and Adult Treatment Program III (ATP III) have now agreed [2, 3] to consider MS as a disease characterized by five traits: (1) increased abdominal girth, (2) low levels of high-density lipoprotein cholesterol (HDL-C), (3) hypertriglyceridemia, (4) hypertension, and (5) fasting hyperglycemia. A low-grade chronic inflammatory activity is commonly observed in metabolic diseases such as obesity and type 2 diabetes (T2D).

A major shortcoming of current definitions of MS is the lack of inclusion of measures of a proinflammatory state and oxidative stress [4, 5]. The multicenter Insulin Resistance Atherosclerosis Study had shown a linear relation between the inflammatory marker C-reactive protein (CRP) and a number of metabolic disorders [6]. Other proinflammatory markers known to increase in patients with MS include fibrinogen [2], cytokines such as interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α).

Type 2 diabetes (T2D) is considered an MS-related disease and an inflammatory disease. As with MS, patients with T2D show higher levels of circulating CRP, fibrinogen, plasminogen activator inhibitor (PAI), and proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6. Circulating levels of IL-18 have been reported to be elevated in subjects with the metabolic syndrome and closely associated with the biomarkers of the syndrome to predict cardiovascular events and mortality in populations affected by MS-related diseases [7]. In patients with T2D, metabolic stress promotes insulin resistance and activation of IkB kinase-β (IKKβ) and JUN N-terminal kinase (JNK), which suggests that these kinases

have key roles in the pathogenesis of this disease [8]. IKKβ activates nuclear factor-κB (NF-κB) which induces expression of NF-κB-dependent genes such as proinflammatory cytokines (e.g., TNFα and IL-1β), so that the suppression of this transcription factor could reduce metabolic disorders and the complications occurring in diabetes (retinopathy, nephropathy, and neuropathy) [8].

In the last 10 years, the link between inflammation and nutrition has become increasingly apparent [8–10]. It has been shown that excessive macronutrient intake can contribute to the inflammatory response occurring in MS [11], whereas some dietary polyphenols are able to reduce the incidence of MS, including diabetes [12]. Many of the metabolites occurring in plants are now recognized as useful for the maintenance of human health, hence the recommended use of plant food supplements (PFSs). A methodology for the safety assessment of botanicals has been promoted by EFSA (European Food Safety Authority), but major bottlenecks remain in its implementation.

The European Project PlantLIBRA (acronym for plant food supplements: levels of intake, benefit, and risk assessments) aims to promote the safe use of PFS or botanicals and the measurement of the risk/benefit ratio related to their consumption. Part of the project is devoted to the discovery of methods for the evaluation of the benefits of PFS and their application and validation. The first step was to review the evidence for PFS benefit in epidemiological, clinical, and intervention studies, in particular the value of PFS as anti-inflammatory agents [13, 14].

The aim of the present paper was to identify the *in vitro* and *in vivo* methods that can detect a decrease of the inflammatory biomarkers that play a key role in metabolic syndrome and diabetes.

2. Methods

2.1. Source and Search Strategy. The following databases were searched electronically to identify relevant articles published up to September 2011: PubMed/Medline, SciFinder Scholar, and Cochrane Library. Search limits were *in vitro*, *in silico*, *clinical* methodologies, and the European languages, without limits of year of publication.

A search strategy was developed for each electronic database using specific medical subject heading (MeSH) terms (e.g., inflammation mediators, C reactive protein CRP, and metabolic X syndrome) in addition to relevant text keywords (plant extract, plant preparation, methods, and analytical approaches).

The same MeSH terms were used in the T2D area and in the MS area to search for inflammatory biomarkers and plant extracts, and the specific terms diabetes mellitus, noninsulin-dependent OR diabetes mellitus, and type II were combined with relevant keywords (plant extract, plant preparation, methods, and analytical approaches).

Titles and abstracts of retrieved citations were first screened to identify publications reporting *in vivo* methods developed in humans, *in vitro* and *in silico* methods used in inflammation conditions related to MS or diabetes. Animal studies were not considered since PlantLIBRA neither uses nor promotes *in vivo* experiments on animals. Other exclusion criteria were the use of plant ingredients for homeopathy, topical use, aerosol/inhalation, and hygiene products. Reviews, commentaries, and patents were also discarded.

3. Results and Discussion

The search by title and abstract retrieved 46 papers for MS and 68 for diabetes. After removal of duplicates and application of the inclusion/exclusion criteria, the total number of papers was 43. Papers were also rejected if they were not in a European language. *In silico* methods for assessing PFS benefit in MS-related diseases were not found. All the studies selected for diabetes were related to T2D.

3.1. Metabolic Syndrome Studies. Neither *in silico* nor *in vitro* studies for assessing inflammation in metabolic syndrome were found. Although diabetes is one of the features of MS, the methods related to diabetes have been considered separately from those relating to MS, because of the complexity of the metabolic syndrome, which includes several alterations of metabolic conditions not specifically associated with diabetes.

3.1.1. In Vivo Methods. Table 1 reports the *in vivo* methods used in clinical trials. The preferred type of clinical study to evaluate the anti-inflammatory effect of PFS in humans was the double-blind randomized controlled clinical trial.

Several publications reported in the present review used randomization in clinical trials, but in a few cases, randomization was not described in detail or incompletely applied (e.g., no randomization for age or gender). Positive aspects of randomization include the elimination of biases, balanced arms, and the capacity to form the basis for statistical tests.

A method of randomization should be considered appropriate if it allows each study participant to have the same chance of receiving the intervention [22]. Methods of allocation using the date of birth or of admission, hospital numbers, or alternation are not appropriate. Suitable methods for randomization include using a table of random numbers or computer generation. In most studies, the subjects enrolled included people of both sexes. Studies of only male or female subjects do not reflect the whole population and the results are not reliable. Almost all studies reported the number of dropouts, but it was not always clear if this was due to lack of efficacy or due to adverse effects. The analytical method used to quantify inflammatory markers such as CRP, cytokines, malondialdehyde (MDA), and adhesion molecules was reported in most studies. In several cases, methods were well described, and manufacturers' protocols were appropriate. The methods most frequently applied were the ELISA test, immunoturbidimetry, and real-time PCR.

3.1.2. Inflammatory Biomarkers Affected by PFS in Metabolic Syndrome. The inflammatory parameters decreasing after PFS treatment were also reviewed. Although changes after

Table 1: Inflammatory parameters measured in metabolic syndrome.

Method[1]	Botanical or botanical derivatives used	No. of participants and length of treatment	Parameters measured	Results	Reference
Double-blind randomized controlled cross-over trial	Olive oil (*Olea europaea* L.)	20 (6-week washout period plus 4-h study session)	Expression of inflammatory genes (CCL3, CXCL1, CXCL3, CXCR4, IL-1β, IL-6, and OSM	Expression of inflammatory genes was reduced	Camargo et al., 2010 [15]
Randomized double-blind controlled trials	ProAlgaZyme (freshwater algae infusion)	60 (10 weeks)	hs-CRP, IL-6, and TNF-α	Significant reduction of all parameters	Oben et al., 2007 [16]
Single-blind randomized controlled trial	Plant sterol margarines (30 g/day)	53 (5 weeks)	CRP, IL-6, CD 40 ligand (CD40L), and E-selectin	No changes	Gagliardi et al., 2010 [17]
Randomized open controlled cross-over trials	Soy nut/soy proteins	482 (8 weeks)	Serum endothelin-1, sICAM-1, sVCAM-1, E-selectin, IL-2, IL-6, IL-18, TNF-α, SAA, and CRP	Reduction of CRP for soy protein, E-selectin, TNF-α, IL-18, and CRP for soy nuts	Azadbakht et al., 2007 [18]
Randomized open controlled clinical trials	Berries and derivatives*	61 (20 weeks)	TNF-α, ICAM, VCAM, E-selectin, and hs-CRP	No significant differences	Lehtonen et al., 2010 [19]
Open nonrandomized noncontrolled clinical trials	Freeze-dried strawberry extract	35 (4 weeks)	Malondialdehyde (MDA), hs-CRP, and adiponectin	No significant differences	Basu et al., 2009 [20]
Cross-sectional studies	Fruit food group** Vegetable fruit group***	486 (1 year)	CRP	Reduction of CRP for both groups	Esmaillzadeh et al., 2006 [21]

*Participants consumed lingonberry, bilberry, black currant, sea buckthorn as such or as derivatives (juice, oil, and powder).

**Pears, apricots, cherries, apples, raisins or grapes, bananas, cantaloupe, watermelon, oranges, grapefruit, kiwi, strawberries, peaches, nectarines, tangerines, mulberry, plums, persimmons, pomegranates, lemons, pineapples, fresh figs, and date.

***Vegetable fruit group: cabbage, cauliflower, Brussels sprouts, kale, carrots, tomatoes, spinach, lettuce, cucumber, mixed vegetables, eggplant, celery, green peas, green beans, green pepper, turnip, corn, squash, mushrooms, and onions.

[1]Clinical studies used to assess inflammatory parameters are listed from the best to the worst method applied.

PFS treatment depend on the plant used and on the bioavailability of the active compounds and the method conditions, parameters that change in a short period of time can be selected as useful biomarkers of the anti-inflammatory properties of PFS. In patients affected by MS (Table 1), CRP levels decreased in three of the seven studies measuring it: a randomized open controlled cross-over study involving 482 patients for eight weeks [18], a 10-week randomized double-blind controlled clinical trial involving 60 patients [16], and a 1-year cross-sectional study involving 486 patients [21].

On the contrary, the other four of these studies [17, 19, 20, 23] reported no changes in CRP values. This discrepancy could be due to the nature of the botanicals used, the bioavailability of active compounds, the number of patients enrolled in the study, the type of study, and/or the laboratory methods for biomarker measurement. A larger number of studies is necessary to assess if CRP is a suitable parameter to evaluate the decrease of inflammatory status in MS after treatment with PFS.

E-selectin levels were evaluated in four studies [17–19, 23], but a decrease was recorded in only one [18], which rules out this biomarker as suitable for the purpose. TNF-α levels were evaluated in three studies [16, 18, 19], and two of them [16, 18] reported a significant reduction after treatment with soy proteins (8 weeks) and fresh algae infusion (10 weeks). No significant differences before and after treatment with berry derivatives were found [19]. IL-18 was evaluated in only one study [18], and no conclusions can be drawn. IL-6 levels: two studies [17, 23] reported no significant differences after 5 and 9 weeks; another three studies [16, 18, 20] reported a significant reduction in both gene expression and serum after 8, 10, and 20 weeks.

We may conclude that TNF-α and IL-6 may be suitable biomarkers to evaluate in a rather short time (6–10 weeks) the improvement of the inflammatory status in humans after PFS treatment, but careful consideration of the botanical formulation used in the study (i.e., kind of extract, occurrence of active principles, and their bioavailability) and the amount of PFS taken before should be given before ruling a proinflammatory biomarker in or out. Additional studies are needed before considering CRP and IL-18 as biomarkers modulated by PFS treatment, since the data occurring in the literature do not allow us to draw clear conclusions. The importance of IL-6, TNF-α, and CRP as proinflammatory biomarkers is well documented. TNF-α is released by adipose tissue and is overexpressed in obesity, and TNF-α can modulate insulin resistance in a variety of clinical trials related to obesity [6, 15, 24]. CRP and IL-6 are peripheral inflammatory markers, and their measurement improves the prediction of the risk of cardiovascular events [24].

3.2. Diabetes Studies

3.2.1. In Vitro Methods. Table 2 reports the *in vitro* methods applied to investigate inflammatory markers in diabetes. The method mostly used for the assessment of beneficial effects of botanicals/plant food supplements on diabetes were based on quantitative PCR (qPCR), a real-time PCR coupled with reverse transcriptase. This is a very sensitive and specific method for analyzing mRNA levels as a marker of gene expression. Uemura et al. [25] reported the use of SYBR Green assay, while Chuang et al. [26, 27] and Cao et al. [28] reported the use of the highly specific and sensitiveTaQman assay. Other frequently used methods are ELISA assay, immunoblotting, and transfections, in a variety of cell cultures, with a plasmid containing the luciferase reporter gene under the control of the NF-κB responsive element(pNF-κB-luc); the latter method is widely used for *in vitro* assays because it assesses events upstream of the inflammatory cascade which activates the NF-κB pathway.

In the luciferase transfection assay only, Chuang et al. [26, 27] used primary cultures (from humans or animals) in their experiments; they are considered the most predictive, as primary cells retain the characteristics of the starting tissue. However, the isolation of appropriate cells from primary cultures can be difficult as the cell population is heterogeneous. Moreover, primary cultures have a limited life. Considering the challenges associated with modelling a chronic disease such as diabetes, it is encouraging to see that several *in vitro* methods for investigating inflammation have been developed, but few of them have been applied to the evaluation of PFS benefit assessment. In this sense, it would be interesting to develop methods to evaluate the following inflammatory events:

(1) nuclear translocation of NF-κB factor in the nucleus (usually evaluated by ELISA test) and the consequent transcription of inflammatory genes and the expression of adhesion molecules on endothelium, leading to the vascular complications of diabetes;

(2) expression of adhesion molecules (i.e., sICAM-1, sICAM-2 and sVCAM-1, and E-selectin), which are typically overexpressed in diabetes and in cardiovascular disease [38]. Adhesion molecule expression is usually measured by RT-PCR (mRNA levels) and ELISA (protein expression on the cell surface);

(3) evaluation of metalloproteinase-9 (MMP-9) secretion and gene expression. MMP-9 is induced by hyperglycemia and accelerates some diabetic complications such as retinopathy [39]. MMP-9 gene expression is usually measured by RT-PCR, secretion and enzymatic activities by zymography or western blotting;

(4) evaluation of monocyte-macrophage chemotaxis in the endothelium. Indeed, monocytes and macrophages play a role in accelerating diabetes and in the development of atherosclerosis. They express specific receptors for advanced glycation end products (AGEs), which are proteins or lipids that become nonenzymatically glycated and oxidized after contact with aldose sugars.

After the binding of AGEs and intracellular processing, monocytes/macrophages synthesize and secrete growth-promoting cytokines such as TNF-α, interleukin 1, and insulin-like growth factor, responsible for vascular complications in diabetes [40]. Furthermore, circulating AGEs may interact with endothelial receptors, which leads to

Table 2: *In vitro* methods and inflammatory parameters measured in diabetes.

Method	Botanical used	Parameters measured	Results	Reference
Flow cytometry	Peanut oil (*Arachis hypogaea*)	Apoptosis induced by TNF-α in rat β-pancreatic cell line (INS-I)	Reduction of apoptosis	Vassiliou et al., 2009 [29]
Luciferase assay	*Sinocrassula indica* Berger (Shilianhua) extract	Transcriptional activity of NF-kB induced by LPS in RAW264.7 macrophages	Reduction of transcriptional activity of NF-kB	Yin et al., 2009 [30]
Quantitative polymerase chain reaction (qRT-PCR)	*Zizyphus lotus* L. Desf. extract	mRNA levels of IL-2 in human (Jurkat) T cells stimulated with anti-CD3 antibodies	Reduction of mRNA and IL-2 levels	Uemura et al., 2010 [25]
	Lyophilized grape powder (*V. vinifera*)	Gene expression of IL-6, IL-8, IL-1β, MCP-1, COX-2, and TLR-2 in primary human adipocytes stimulated with TNF-α	Attenuation of all parameters measured	Chuang et al., 2011 [26]
	White grape seed extract (*V. vinifer*a)	Gene expression of CYP, PPARγ, LEP, APM1, IL-6, and MCP-1 in THP-1 cells and human adipocytes stimulated with LPS and TNF-α, respectively	Reduction of IL-6 and MCP-1 expressions; modulation of APM1 and LEP (adipokine) gene expressions	Kar et al., 2009 [31]
	Lyophilized grape powder (*V. vinifera*)	Gene expression of IL-6, IL-8, IL-1β, and TNF-α in human macrophages and adipocytes stimulated with LPS	Reduction of IL-6, IL-8, IL-1β, and TNF-α gene expressions	Chuang et al., 2010 [27]
	Cinnamomum burmannii extract	Expression of tristetraprolin (TTP) in mouse RAW264.7 macrophages treated with LPS	Induction of TTP	Cao et al., 2008 [28]
	Crataegus pinnatifida Bunge var. *typica* Schneider and *C. pinnatifida* Bunge	Gene expression of iNOS and COX-2 in murine RAW264.7 macrophages treated with LPS	mRNA levels of iNOS and COX2 were inhibited by *C. pinnatifida* Bunge	Li et al., 2010 [32]
	Fructus Xanthii	Protection from IL-1β and IFN-γ and NF-kB induced in pancreatic cell line RINM5F	Complete protection	Song et al., 2009 [33]
	Lyophilized grape powder (*V. vinifera*) obtained from red, green, and blue-purple seeded and seedless California table grapes	Concentration of IFN-γ inducible protein-10 (IP-10) in human adipocytes and macrophages after treatment with LPS	Reduction of IP-10	Chuang et al., 2010 [27]
	Palmitic acid, oleic acid, or DHA	Production of TNF-α and IL-10 in 3T3-L1 murine adipocytes	Increase of IL-10, no effect on TNF-α	Bradley et al., 2008 [34]
	Lyophilized grape powder, (*V. vinifera*)	Activation of NF-kB factor and Ikα degradation in primary cultures of human adipocytes treated with TNF-α	Reduction of NF-kB activity and mediation of Ikα degradation	Chuang et al., 2011 [26]
	Lyophilized grape powder, (*V. vinifera*)	Activation of NF-κB factor in primary cultures of human adipocytes and macrophages treated with LPS	Inhibition of NF-kB activation	Chuang et al., 2010 [27]
	Cinnamomum burmanii	Cytokine (TNF-α, IL-6, and COX-2) production by mouse RAW 264.7 macrophages treated with LPS	Reduction of cytokine (TNF-α, IL-6, and COX-2) production	Cao et al., 2008 [28]

Table 2: Continued.

Method	Botanical used	Parameters measured	Results	Reference
Chromogenic assay (TMPD)	Extract of rhizome of *Zingiber officinale* Roscoe	Enzymatic activity of COX in C2C12 cells	Reduction of enzymatic activity	Priya Rani et al., 2011 [35]
ELISA	Diosgenin from seeds of *Trigonella foenum-graecum*	Levels of adiponectin and MCP-1 in 3T3-L1 preadipocytes	Increase of adiponectin levels and decrease of MCP-1	Uemura et al., 2010 [25]
Caspase-3 activity luminometric assay	*Hypericum perforatum* L.	Determination of apoptosis in rat insulinoma cell line (INS-1E) stimulated with cytokines	Inhibition of apoptosis	Menegazzi et al., 2008 [36]
	White grape seed extract (*V. vinifera*)	P65 translocation and PIKBα protein in human monocyte cell line THP-1 and human adipocyte treated with LPS and TNF-α	Partial inhibition	Chacón et al., 2009 [37]
No production colorimetric assay	*Crataegus pinnatifida* Bunge var. *typica* Schneider and *C. pinnatifida* Bunge	Cytotoxicity and no inhibitory activity in murine RAW 264.7 macrophages stimulated with LPS	Inhibition of cytotoxicity and no activity only by *C. pinnatifida* Bunge	Li et al., 2010 [32]
Nitrite measurement colorimetric assay	Fructus Xanthii	No production in RINm5F cells treated with IL-1β and IFN-γ	Inhibition of no production	Song et al., 2009 [33]
NF-kB binding assay	Dietary fatty acids	Binding activity of the P65 subunit of NF-kB in nuclear extracts from 3T3-L1 murine adipocytes	Decrease of binding activity	Bradley et al., 2008 [34]

Table 3: Inflammatory parameters assessed in clinical trials of botanicals in diabetes.

Method[1]	Botanical or botanical derivatives used	No. of participants and length of treatment	Parameters measured	Results	Reference
Double-blind randomized controlled cross-over trial	Grape seed extract (*V.vinifera*)	32 (4 weeks)	hs-CRP	Decreased hs-CRP	Kar et al., 2009 [31]
Double-blind randomized controlled multicenter trial	Pycnogenol (extract of bark from the French Maritime Pine, *Pinus pinaster*)	77 (12 weeks)	Endothelin-1 and ketoprostaglandin F1-α	Decreased endothelin-1 and increased ketoprostaglandin F1-α	Liu et al., 2004 [45]
Randomized double-blind controlled trial	Pomegranate (*Punica granatum* L.), green tea (*C. sinensis* L.) extract	114 (12 weeks)	Plasma MDA	Decrease of MDA	Kutan Fenercioglu et al., 2010 [46]
	Blueberry (*Vaccinium arctostaphylos* L.) leaves water extract	42 (4 weeks)	Serum CRP	Decreased hs- CRP	Abidov et al., 2006 [47]
Single-blind randomized controlled trial	Coffee	47 (1-month washout, 1 month 4 cups/day, and 1 month 8 cups/day)	CRP, IL-6, IL-1, IL-18, 8-isoprostane, MIF, adiponectin, leptin, and SSA	Decreased IL-18, 8-isoprostane and increased adiponectin and other markers unchanged	Kempf et al., 2010 [48]
Randomized controlled cross-over trial	Green tea (*C. sinensis* L.) aqueous extract	55 (4 weeks)	hs-CRP and major cytokine mediator (IL-6)	Both mediators unchanged	Ryu et al., 2006 [49]
Randomized open controlled clinical trial	Black tea (*C. sinensis*)	46 (4 weeks: 150 mL week 1, 300 mL week 2, 450 mL week 3, and 600 mL week 4)	Serum CRP, MDA, and fibrinogen	Decreased serum CRP after consumption of 600 mL and MDA after consumption of 300 mL; fibrinogen unchanged	Neyestani et al., 2010 [50]
Open noncontrolled nonrandomized clinical trial	*Ginkgo biloba* extract (24% ginkgo flavone glycosides and 6% terpenes	47 (12 weeks)	Urinary metabolites of thromboxane B2 (TXB_2) and prostacyclin (PGI2)	Decreased urinary TXB_2 and PGI2	Kudolo et al., 2003 [51]
Prospective cohort studies	Coffee	2040 (6 years)	Adiponectin	Higher in drinkers of >4 cups/day	Williams et al., 2008 [52]

Macrophage migration inhibitory factor (MIF) and malondialdehyde (MDA).
[1]Clinical studies used to assess inflammatory parameters are listed from the best to the worst method applied.

perturbation of cellular properties, such as upregulation of the transcription and the translocation of nuclear factor NF-κB [41]. Chemotaxis is generally measured under agarose [42, 43] or by chemotaxis assay, based on evaluation of cell migration through apposite filters after inflammatory stimuli have been placed in chambers (Boyden Chamber Assay) [44].

3.2.2. In Vivo Methods. Table 3 reports the *in vivo* methods developed for human trials.

The majority of clinical trials evaluating the effect of botanicals on inflammatory biomarkers in diabetic patients were randomized, double-blind randomized controlled trials and randomized, open and controlled studies. The lack of blinding is particularly critical when the treatment is applied versus placebo. In fact, the placebo effect is a major cause of bias due to patient or doctor awareness [22]. Several studies have indicated that nonrandomized trials are more likely to yield a positive result for a new treatment than for an established conventional one. In some of the clinical trials considered, randomization was not described in detail or was unsuitable. In two papers [53, 54], the number of subjects was limited. In some studies [49, 51, 55], the dropout rate and the reasons for it were not critically discussed. The most often used methods for biomarker quantification were ELISA assay and HPLC analysis.

3.2.3. Inflammatory Biomarkers Affected by PFS in Diabetes. The parameters most frequently measured in clinical trials were as follows: CRP = MDA > endothelin-1 = urinary thromboxane B_2 metabolites > IL-6, IL-18, TNF-α, and PGI_2, 8-isoprostane. Six studies [31, 47–50, 55] examined CRP levels; among them, three [31, 47, 50] reported a significant reduction in serum CRP levels after 4 weeks of treatment, two studies reported no significant changes [48, 49], and one reported increased levels of CRP [55]. As for MS, further studies are mandatory before conclusions on the efficacy of *in vivo* CRP measurement in diabetes after PFS treatment can be drawn.

Serum levels of malondialdehyde (MDA) were measured in three studies [46, 50, 56], and all reported a significant reduction after 4, 8, and 12 weeks of PFS consumption. MDA is an important biomarker of oxidative stress in diabetes as a consequence of persistent hyperglycemia and lipid peroxidation. These trials establish serum MDA measurement as appropriate for the purpose.

IL-6 levels were measured in three studies [48, 49, 56] and one reported a significant reduction after PFS treatment [56]. IL-18 [48] and TNF-α [56] each were measured in only one study, and in both cases, a significant decrease was found. Nevertheless, further studies are needed to evaluate IL-6 and IL-18 as suitable inflammatory parameters. Endothelin-1, generally overexpressed in diabetes models [57], was found to decrease in two studies [45, 56], after 12 and 8 weeks of treatment, respectively.

Two studies showed a significant reduction of thromboxane B_2 (TXB_2) urinary metabolites after 4 and 12 weeks of treatment, respectively [51, 54]. PGI_2 [51] and 8-isoprostane [48] were each evaluated only in one study; both decreased after PFS treatment.TXB_2, PGI_2, and 8-isoprostane are key proinflammatory biomarkers in diabetes. They are massively released in the wake of hypercoagulation and the vascular modifications that are typical for this disease. The paucity of studies in which these mediators were assayed in response to PFS treatment does not allow us to establish their usefulness in investigating the benefit of PFS treatment.

4. Conclusions

The aim of the present paper was to collect and critically discuss the existing experimental approaches used *in vitro, in silico*, and *in vivo* for benefit assessment of botanicals or plant food supplements (PFSs) in decreasing inflammation in MS-related diseases. PFS often consist of a complex mixture of compounds, which makes benefit assessment difficult and subject to interferences and false positives. The development of reliable methods for evaluating benefit assessment and their application and validation is therefore crucial.

No *in silico* methods were found in MS-related diseases. qRT-PCR was the *in vitro* method most widely applied for measuring the expression of inflammatory cytokines in diabetes, but no *in vitro* methods were found for testing PFS benefits in MS. Data from the *in vivo* studies (clinical trials) show that the inflammatory markers CRP, MDA, and PGI_2 are likely to be useful in judging the efficacy of PFS treatment, although more studies are needed to validate this conclusion. In addition, two proinflammatory biomarkers, IL-6 and TNF-α, were the only two parameters to change in both *in vitro* and *in vivo* systems, and these biomarkers should be carefully considered in future studies.

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Research Article

Managing the Combination of Nonalcoholic Fatty Liver Disease and Metabolic Syndrome with Chinese Herbal Extracts in High-Fat-Diet Fed Rats

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Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome (MetS). The aim of the study was to evaluate the effects of Chinese herbal extracts from *Salvia miltiorrhiza* and *Gardenia jasminoides* (SGE) on the combination of NAFLD and MetS induced by a high-fat diet (HFD) in rats. After 6 weeks of HFD feeding, rats ($n = 10$ each group) were treated with saline, rosiglitazone (RSG), and SGE for 4 weeks. HFD rats were obese, hyperinsulinemic, hyperlipidemic and increased hepatic enzymes with the histological images of NAFLD. Treatment with SGE significantly reduced serum triglycerides (TG), nonesterified fatty acids and enhanced insulin sensitivity, and ameliorated the elevated serum hepatic enzymes compared with HFD-saline group. SGE treatment also attenuated hepatic TG by 18.5% ($P < 0.05$). Histological stains showed SGE decreased lipids droplets in hepatocytes ($P < 0.05$) and normalized macrovesicular steatosis in HFD rats. Significant reduction of TNF-α and IL6 in adipose tissue was detected in SGE treated rats. The anti-inflammatory action may be, at least in part, the mechanism of SGE on MetS associated with NAFLD. This study discovered that SGE is capable of managing metabolic and histological abnormalities of NAFLD and MetS. SGE may be an optimal treatment for the combination of NAFLD and MetS.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a pathologic entity, including a spectrum of liver damage ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), advanced fibrosis, and progression to cirrhosis [1]. Pathophysiology of NAFLD still has not been completely clarified but a large body of clinical and experimental evidence suggests that ectopic fat deposition in the liver plays a fundamental role in the development and progression of NAFLD [2–4]. The increased hepatocellular lipids are correlated to central obesity, insulin resistance, dyslipidaemia, and impaired glucose tolerance, a cluster of metabolic syndrome (MetS) [5, 6]. The prevalence of NAFLD has reached epidemic proportions in recent years, in parallel with the increasing prevalence of obesity and MetS worldwide. NAFLD is currently conceptualised as the hepatic manifestation of MetS, and it is an early warning sign of future risk for type 2 diabetes and cardiovascular disease [5–8].

Given the rising coincidence of MetS and NAFLD, development of an effective treatment for obesity-related NAFLD to prevent future disease-related morbidity and mortality is a priority. To date, intervention for MetS and NAFLD remains mainly lifestyle modifications and no pharmacological treatment has been proven to be effective for NAFLD associated with MetS. The pathogenesis of the combination of NAFLD and MetS is multifactorial, including hepatic insulin resistance, increased ectopic fat deposition in the liver and other non-adipose tissues, and adipocytokines-trigged inflammation [9]. Thus, novel therapy for the disease should be capable of managing insulin resistance, lowing hyperlipidaemia and anti-inflammation

as well rendering benefits on liver histological outcomes [10].

Chinese Herbal Medicine (CHM) has been used in China and other parts of Asian counties for thousands of years. A special feature of Chinese medicine is the use of a formula containing several herbs (like a cocktail) to ameliorate a set of abnormalities related to a disease. Herbal extracts contain multiple naturally occurring compounds that can target different pathological pathways involved in the disease, providing therapeutic effects via a spectrum of actions. In our previous study, we demonstrated that a Chinese herbal formula, containing a high amount of *Salvia miltiorrhiza* Bge extract, effectively reversed metabolic syndrome in a high-fatdiet (HFD) [11]. The dried root of *Salvia miltiorrhiza (S. miltiorrhiza)* is a Chinese herb commonly included in prescriptions to ischemic heart disease, hyperlipidaemia [12, 13], and chronic liver disease [14]. The fruit of *Gardenia jasminoides (G. jasminoides)* is a Chinese herb for cleaning away toxicity in TCM. Recent study showed that the active ingredient of *G. jasminoides* (geniposide) has an alleviating effect on fatty liver in obese diabetic mice [15]. Based on our previous study and other's findings, we combined the extracts from *S. miltiorrhiza* and *G. jasminoides* and evaluated their effects on the coexisting NAFLD and MetS induced by HFD feeding in rats.

2. Materials and Methods

2.1. Preparation of Chinese Herbal Extracts. Chinese herbal extracts of *S. miltiorrhiza* and *G. jasminoides* were prepared by Kanion Pharmaceuticals (Lianyungang, Jiangsu, China). In general, each dried herb was authenticated using microscopic examination to ascertain the species' authenticity. Ground herbs were first extracted for 2 h with 65% aqueous ethanol at room temperature followed by 2 h decoction at 120°C. The result was then vacuum-filtered through a filter paper and concentrated in a rotating vacuum evaporator (Yahya Rong Biochemical Instrument, Shanghai, China) at 40°C. The highly concentrated solution was freeze-dried to obtain a solid powder with a yield of 8% and 10% (powder versus raw herb, w/w) for Salvia root and Gardenia fruit, respectively. The quality control for the herbal extracts was performed using an Agilent 1200 series liquid chromatography/mass selective detector equipped with QTOF 6510 mass spectrometer (Agilent Technologies Inc., CA, USA) with botanical markers of tanshinone IIA for *S. miltiorrhiza* and gypenoside for *G. jasminoides* obtained from the Beijing Institute of Materia Medica. The content of tanshinone IIA and gypenoside in 1 g of herbal extract was 120 mg and 30 mg, respectively. The mixture of *S. miltiorrhiza* and *G. jasminoides* extracts, coded as SGE, was used for animal study.

2.2. Animals and Treatment. Male Sprague-Dawley (SD) rats at 6 weeks of age were supplied by the Animal Resources Centre (Perth, Australia). All experimental procedures were approved by the University of Technology Sydney Animal Ethics Committee, following guidelines issued by the National Health and Medical Research Council of Australia. SD rats were maintained on a 12 h light/dark cycle (lights on 0600) under constant temperature (22°C) with *ad libitum* access to standard chow diet or a high-fat diet (HFD, 59% fat, 21% protein, and 20% carbohydrate by energy composition) for 6 weeks to induce MetS and NAFLD. After 6 weeks of HFD feeding, HFD fed rats were randomly divided into three groups ($n = 10$ each group) to receive saline 5 mL/kg of body weight (HF-Con), rosiglitazone (HFD-RSG, 3 mg/kg of body weight), and SGE (HFD-SGE, 2 g/kg of body weight) by daily oral gavage for 4 weeks. HFD feeding continued during the 4-week treatment period. Meanwhile, saline (5 mL/kg of body weight) was administrated to chow fed rats (chow, $n = 10$) as the model control.

2.3. Determination of Metabolic Parameters, Liver Enzymes, and Insulin Sensitivity. At the end of treatment, blood samples were collected from the tail of rats after an overnight fast (12 hours). Fasting serum total cholesterols (TCs), high-density lipoprotein cholesterol (HDL-C), triglycerides (TGs) and nonesterified fatty acids (NEFAs) were analysed using enzymatic colorimetric kits obtained from Roche Diagnostic (Mannheim, Germany) and Wako Pure Chemical Industries (Japan), respectively. Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated by Friedewald's formula: LDL-C (mmol/L) = TC − HDL-C − TG/2.2 [16]. Fasting serum insulin concentration was measured using a RIA kit (Linco Diagnostic Services). Fasting serum glucose, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined by spectrophotometric analysis using commercial kits (Dialab, Vienna, Austria). All experimental assays were carried out according to the manufacturer's instruction.

Whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: [fasting serum glucose (mmol) times fasting serum insulin (mU/mL)]/22.5 [17]. Because HOMA is negatively correlated with insulin sensitivity, low HOMA-IR values indicate high insulin sensitivity, whereas high HOMA-IR values indicate insulin resistance.

2.4. Tissue Collection and Measurement of Liver Triglycerides. At the end of the experiment, animals were anesthetized using inhalant aesthetic gas (isoflurane and nitrous oxide) after 12 h of fasting. Liver and visceral fat (epididymal and perirental adipose tissues) were quickly excised, washed by ice-cold PBS, then stored at −80°C for subsequent histological and molecular assays.

Approximately 50 mg of liver tissues was homogenized at 4°C in RIPA lysis buffer (Sigma-Aldrich, St. Luis, MO, USA). Lipids from total liver and muscle homogenate were extracted using chloroform/methanol method (2 : 1), evaporated, and dissolved in 1 mL ethanol (Sigma-Aldrich). TG concentration was assayed using kits from *Roche Diagnostic (Mannheim Germany)* following the manufacturers' instructions.

2.5. Measurement of Adipokines. Frozen epididymal fats (200 mg) were homogenized in 1 mL lysis buffer containing 150 mM NaCl, 1 mM PMSF, 10% Glycerol, and the complete

Table 1: Summary of metabolic parameters in chow and HFD fed rats with variety of treatments.

	Chow	HFD-Con.	HFD-RSG	HFD-SGE
Initial body weight, g	372 ± 16	414 ± 14†	409 ± 20	417 ± 16
Final body weight, g	412 ± 22	468 ± 19†	483 ± 32	438 ± 27*
Visceral fat mass, g	7.8 ± 1.2	14.60 ± 1.20‡	17.0 ± 1.4	8.9 ± 1.0**
Liver wt, g	11.7 ± 0.2	12.50 ± 0.90	13.3 ± 0.6	11.8 ± 0.6
Serum glucose, mM	6.75 ± 0.14	8.87 ± 0.24‡	7.65 ± 0.14*	8.15 ± 0.18
Serum insulin, mU	26.60 ± 0.22	46.25 ± 0.35‡	24.01 ± 0.18**	27.03 ± 0.31**
HOMA-IR	7.98 ± 0.09	18.23 ± 0.28‡	8.16 ± 0.17**	9.76 ± 0.29**
Serum TC, mM	3.98 ± 0.21	5.67 ± 0.24†	5.13 ± 0.32	4.35 ± 0.18*
Serum TG, mM	0.41 ± 0.03	1.03 ± 0.06†	0.59 ± 0.03*	0.51 ± 0.02**
Serum HDL-C, mM	1.20 ± 0. 22	0.86 ± 0.13‡	0.90 ± 0.16	0.96 ± 0.15
Serum LDL-C, mM	2.59 ± 0.52	4.34 ± 0.59‡	3.96 ± 0.67	3.16 ± 0.56*
Serum NEFA, mM	0.50 ± 0.10	0.77 ± 0.21†	0.57 ± 0.12*	0.58 ± 0.12*

Data are means ± SEM; $n = 10$ rats/group. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus chow control; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HFD-Con. HOMA is defined as (fasting insulin × fasting glucose)/22.5. Visceral fat included epididymal fat pad and inguinal fat. TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; NFFA: nonfree fatty acids.

protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The homogenates were incubated on ice for 30 min and spun at 10,000 g at 4°C for 10 min. The supernatant was collected for analysis of leptin, necrosis factor-α (TNF-α), interleukin 6 (IL6) in fat tissue using multiplex rat adipocyte Linoplex kits obtained from Linco Research (Millipore, St. Charles, MO) for the simultaneous quantification of leptin, IL-6, and TNF-α according to the manufacturer's instruction. Resulting leptin, TNF-α, and IL-6 contents were determined using a multiplex reader (BioRad Bio-Plex 200 System; Bio-rad Laboratories, Hercules, CA, USA) and expressed as nM in mg of fat tissue.

2.6. Histological Analysis. A small portion of frozen liver tissue was cut and embedded with precooled optimal cutting compound (Torrance, CA, USA) for cryostat sectioning at 6 μm. The sections were mounted on microscope slides then fixed with 10% formaldehyde solution for 48 h. The samples were then stained with Haematoxylin and Eosin (H&E) or Oil Red O (Sigma-Aldrich) to investigate architecture of the liver and hepatic lipid droplets. Stained Oil Red O (ORO) slides were visualized with the Olympus microscope, and images were captured with an Olympus digital camera (DP70, Tokyo, Japan) using Image-Pro6.2 software (Media Cybernetics, Inc., MD, USA). Lipid droplets was quantified at least 5 different high-power fields in a blinded way. Masson's Trichome (MT) stain was used for evaluation of liver fibrosis. For each group, liver samples from 6 to 8 rats were prepared and stained and six fragments from each liver were analysed. All slides were scanned at an absolute magnification of 200x using Image-Pro6.2 software (Media Cybernetics) under a light microscopy (Olympus, BX51 microscope, Tokyo, Japan).

2.7. Statistical Analysis. All values are expressed as the means ± SEM. Comparisons across the four groups were done using one-way ANOVA followed by post-hoc analysis of Tukey's test to determine significant differences between the two groups using Prism version 4 (GraphPad Inc., San Diego, CA, USA). P-value <0.05 was considered statistically significant.

3. Results

3.1. Effects of SGE on Metabolic Profiles and Insulin Sensitivity. HFD feeding significantly increased body weight and visceral fat mass compared with chow fed rats (Table 1). Weight gain in SGE group was significantly lower than that in HFD control rats and RGS treated rats ($P < 0.01$). SGE treatment also markedly reduced visceral fat mass by 46% ($P < 0.01$). The liver weights were not significantly different amongst three groups of HFD rats, though SGE slightly reduced liver weight. After a total of 10 weeks of HFD feeding, SD rats became mildly hyperglycaemic, and SGE treatment reduced fasting serum glucose levels by 8%, but the reduction did not achieve a statistical significance (Table 1). SGE reduced serum TC, TG, and NEFA levels by 23.2% ($P < 0.05$), 51.4% ($P < 0.01$), and 22.3% ($P < 0.05$), respectively, when compared with the HFD control. LDL-C concentrations were also decreased in SGE-treated HFD-rats ($P < 0.05$). SGE also raised HDL-C levels by 11.6% compared to HFD control, but the statistical significance was not achieved. HFD rat was insulin resistant reflected by hyperinsulinaemia as well as significantly increased value of Home-IR (Table 1). SGE treatment produced a similar effect to RGS on insulin resistance, evidenced by reduction of fasting serum insulin levels and improved HOME-IR (Table 1).

3.2. SGE Reduced Hepatic Triglycerides and Ameliorated the Elevated Liver Enzymes of HFD Rats. Figure 1(a) showed that HFD feeding increased hepatic TG by 2.2-fold compared to chow fed rats (15.34 ± 0.23 μM/g versus 7.11 ± 1.10 μM/g). SGE treatment significantly attenuated the elevated hepatic TG levels by 18.5% ($P < 0.05$, compared with HFD-Con.), and there was no significant reduction of hepatic TG in RSG treated rats (Figure 1(a)).

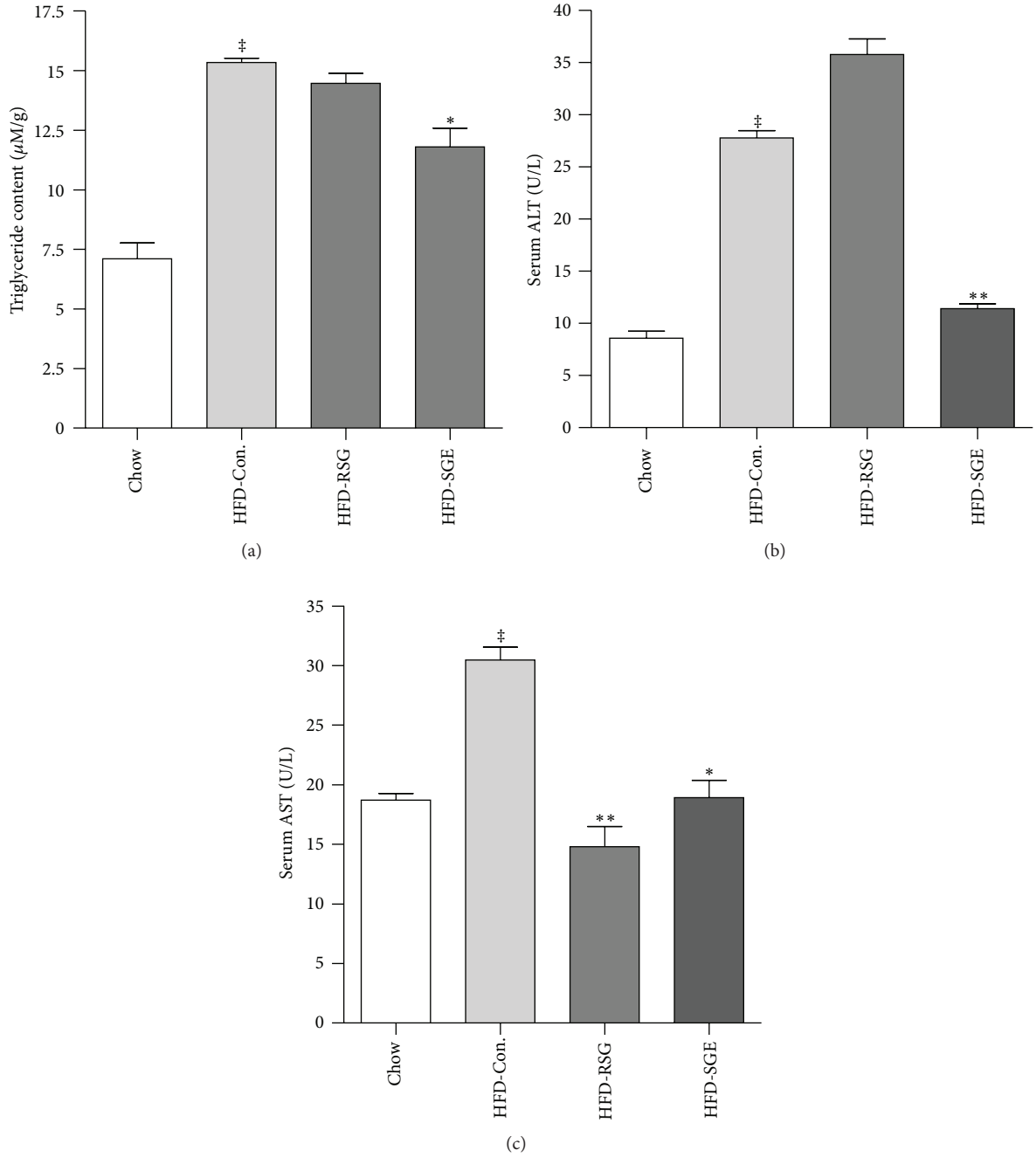

Figure 1: Hepatic triglyceride contents (a), serum alanine aminotransferase (b), and aspartate aminotransferase (c) values in chow fed rats and HFD control rats treated with saline (5 mL/kg of body weight), and HFD rats treated with rosiglitazone (HFD-RSG, 3 mg/kg of body weight) or SGE (HFD-SGE, 2 g/kg of body weight). Data are means ± SEM; $n = 10$ rats/group. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus chow control; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HFD-Con.

Figures 1(b) and 1(c) showed that serum ALT and AST concentrations in HFD fed rats were significantly higher than those in chow fed rats (27.2 ± 1.3 and 31.18 ± 1.66 U/L versus 8.3 ± 0.6 and 16.92 ± 0.79 U/L, resp.). The RSG treatment had further increased ALT levels (36.22 ± 1.30 U/L). In contrast to RSG, SGE treatment significantly attenuated the elevated ALT and AST values to 11.01 ± 0.61 U/L ($P < 0.01$ versus HFD-Con.) and 17.3 ± 2.1 U/L ($P < 0.05$ versus HFD-Con.).

3.3. Effects of SGE on Adipokines. Leptin, TNF-α, and IL-6 were determined in adipose tissue of chow fed rats and HFD rats treated with saline, RGS, or SGE, respectively. As shown in Figure 2, HFD feeding significantly increased TNF-α by 26.5% and IL-6 by 49.8% and decreased leptin levels by 81.6% when compared with chow fed rats. Both SGE and RGS were capable of significantly inhibiting HFD-induced TNF-α and IL-6 (Figures 2(b) and 2(c), $P < 0.05$ and $P < 0.01$

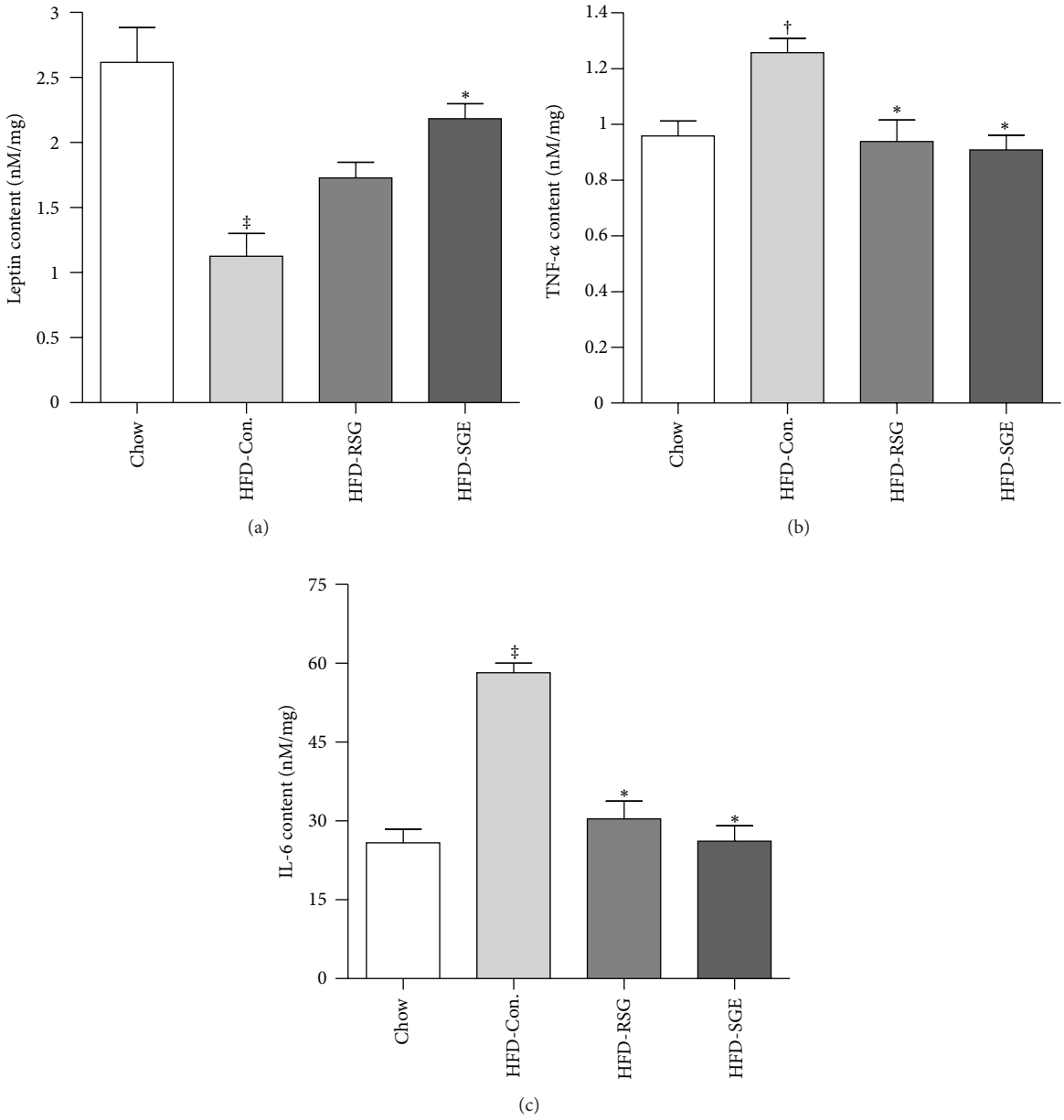

Figure 2: Effects of SME on leptin (a), TNF-α (b), and IL-6 (c) expression in adipose tissue of chow fed rats and HFD control rats treated with saline (5 mL/kg of body weight), and HFD rats treated with rosiglitazone (HFD-RSG, 3 mg/kg of body weight) or SGE (HFD-SGE, 2 g/kg of body weight). Adipose leptin, TNF-α, and IL-6 were determined using a multiplex rat adipocyte Linoplex kit stated in method section. Data are means ± SEM; $n = 10$ rats/group. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus chow control; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HFD-Con.

versus HFD control, resp.). SGE treatment also significantly increased leptin expression ($P < 0.05$ versus HFD control) in adipose tissue of HFD rats.

3.4. SGE Improved Fatty Liver, Liver Steatosis, and Fibrosis. The photomicrographs of the H&E stain showed that HFD feeding increased hepatic fat deposits, evidenced by the majority of the hepatocytes of HFD rats that were distended by fat in comparison to the chow group (Figures 3(a) and 3(b)). The images of H&E stain also displayed macrovesicular steatosis, as many single large droplets had displaced the nucleus and ballooning degeneration causing conspicuous swelling of the cell and cytoplasmic vacuolation as shown in Figure 3(b). The treatment of HFD rats with RGS and SGE reduced fat liver depots (Figures 3(c) and 3(d)). The SGE groups showed histological features similar to the chow group with no macrovesicular steatosis as revealed in the HFD group as shown in Figure 3(b).

ORO staining on frozen liver sections exhibited many lipid droplets in liver sections of HFD fed rats (Figure 4(b)), whereas a few lipid droplets were seen in the liver sections from the chow (Figure 4(a)) and SGE treated HFD rats

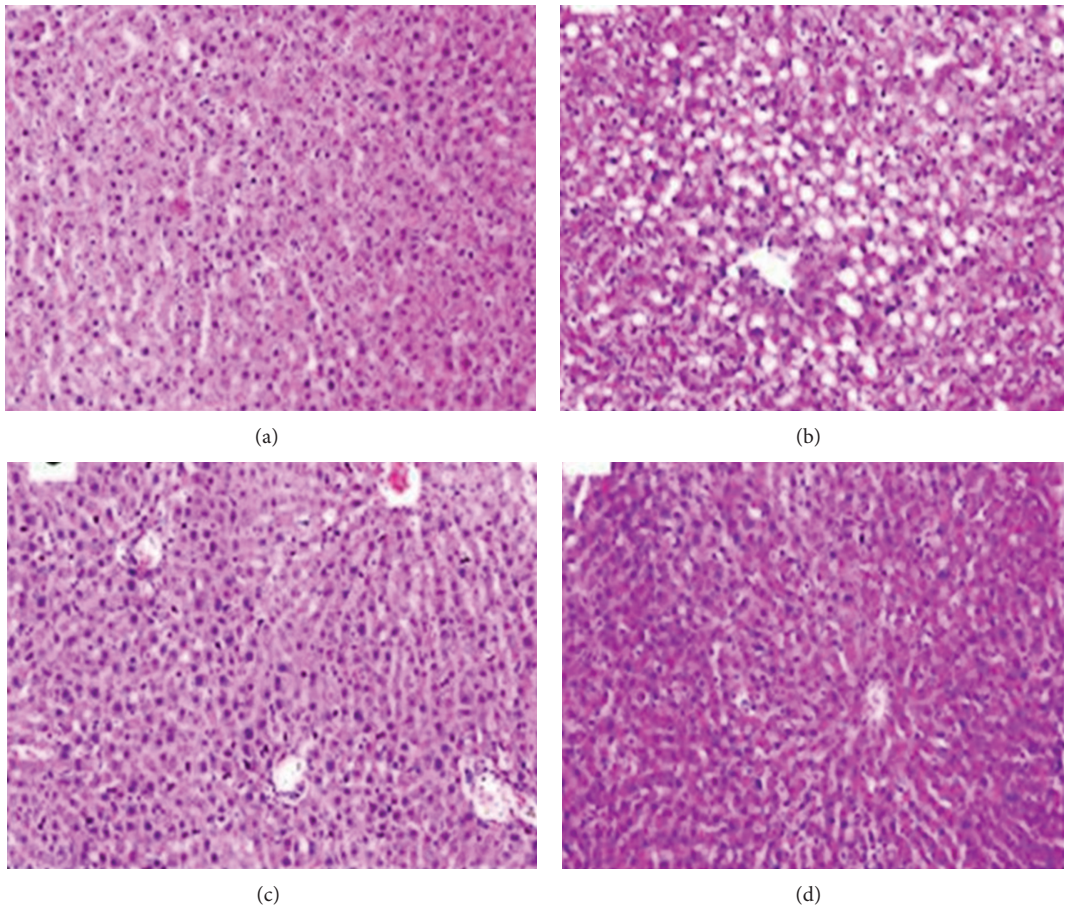

Figure 3: Representative images of hematoxylin and eosin (H&E) staining to visualize architecture of the liver and hepatocytes on sections of the liver section (6 μm thick) from chow fed rats (a) and HFD control rats (b) treated with saline (5 mL/kg of body weight), and HFD rats treated with rosiglitazone (RSG, 3 mg/kg of body weight, (c)) or SGE (2 g/kg of body weight, (d)). All photomicrographs were taken at a magnification of 200x. The scale bar represents 50 μM. Data are means ± SME, $n = 6–8$.

(Figure 4(d)). Strikingly, there was an increase in the lipid content of liver tissue in HFD-RSG rats (Figure 5(c)). Analysis of blindly scored ORO-stained sections showed a statistically significant increase in the lipid content of liver tissue of HFD rats (Figure 4(e)). Levels of lipids were higher in the liver of HFD rats (10.77 ± 0.45%) compared with those in chow group (8.80 ± 0.34%). The RSG group had elevated levels of lipids (9.78 ± 0.21%), similar to the HFD fed group. The SGE treatment significantly lowered lipids values (9.10 ± 0.34%, $P < 0.05$ versus HFD control).

In the photomicropictographs of Masson's Trichrome stain taken in the chow group displayed no fibrotic changes in the hepatocytes (Figure 5(a)). In the HFD group, intracytoplasmic fat was seen as clear vacuoles around the vessels and more distinct and slight fibrotic tissues were stained blue as shown in Figure 5B. In RSG treated HFD rats, fibrosis were observed around the vessels by the purplish green colour in comparison to chow fed rats (Figures 5(c) and 5(a)). SGE treated rats showed similar histological features in MT staining as chow fed rats (Figures 5(d) and 5(a)).

4. Discussion

As with our previous study, feeding a high-fat diet to SD rats led to visceral obesity associated with hyperlipidaemia, hyperinsulinaemia, and slight hyperglycaemia, which are characteristics of metabolic syndrome [11]. In the present study, we have demonstrated those ten weeks of high-fat diet feeding induced fatty liver disease in SD rats. The HFD fed rats assembled key biochemical features of MetS and NAFLD, including hyperinsulinaemia and increased HOMRE-IR value, marked elevation of hepatic enzymes, and hyperlipidaemia associated with increased TG accumulation in the liver. Histological evaluation remains the most important method of identifying NAFLD lesions, including steatosis, lobular and portal inflammation, hepatocyte injury as ballooning, and fibrosis [18]. In the present study, H&E, MT, and ORO stains on the liver samples revealed distended hepatocytes with increased lipid droplets, macrovesicular steatosis, indicating the simple steatohepatitis and mild fibrosis with no cirrhotic displacements. The histological abnormalities in the HFD rats of this study were consistent

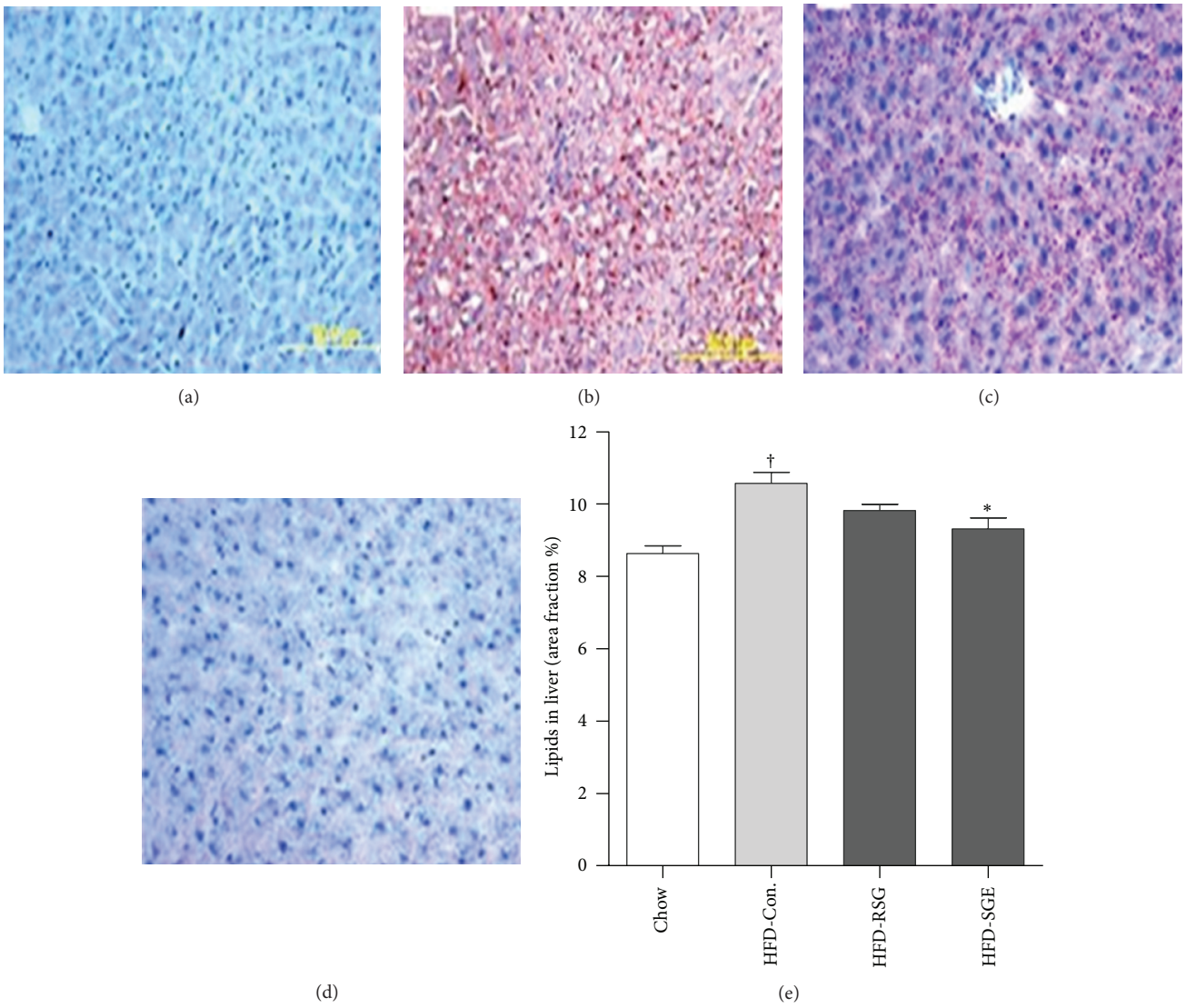

Figure 4: Representative images of Oil Red O (ORO) staining to visualize lipid droplets on liver sections (6 μm thick) from chow fed rats (a) and HFD control rats (b) treated with saline (5 mL/kg of body weight), and HFD rats treated with rosiglitazone (RSG, 3 mg/kg of body weight, (c)) or SGE (2 g/kg of body weight, (d)). Images from the stained liver were taken at a magnification of 200x. The scale bar represents 50 μM. Lipid droplets quantified at least 5 different high-power fields, being blind by two independent assessors. The intensity of staining of liver tissue with ORO provides a qualitative measure of the lipid contents (e). Data are means ± SEM, n = 6–8, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus chow control; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HFD-Con.

with the findings of the previous literatures [19, 20]. High fat diet induced animal model of NAFLD has been widely used to identify the pathogenesis and evaluate new treatment for NAFLD [20, 21].

The elevated liver aminotransferase is a nonspecific clinical feature of NAFLD, which positively correlated to 90% patients with NASH [22]. Treatment with SGE for 4 weeks has proven to significantly normalise the liver hepatic aminotransferase (ALT and AST) to the level as normal chow control. Specifically, SGE treatment was effective in impeding fat infiltration (evidenced by decreased hepatic TG contents and lipid droplets) and preventing hepatic fibrosis, as shown in similar histological appearances as the chow control group. The results from biochemistry and histology assays demonstrated that SGE was protective against HFD-induced liver lesion and prevented NAFLD in HFD rats.

NAELD's pathogenic mechanisms are still under investigation; however, fat accumulation, mainly triglycerides filtration within hepatocytes, is considered the first step in the development of NAFLD [23]. The clinical and animal studies demonstrated that levels of hepatic triglycerides are positively correlated to visceral obesity and insulin resistance [4, 24]. Under insulin resistant status, FFAs from lipolysis of visceral tissue are increased with decreased oxidative capacity. The elevated FFAs in the blood stream will directly circulate into the portal vein where the liver deposits FFAs as triglyceride in the hepatocytes and contribute to liver fibrosis. In addition to over-accumulation of triglycerides, *de no* synthesis of

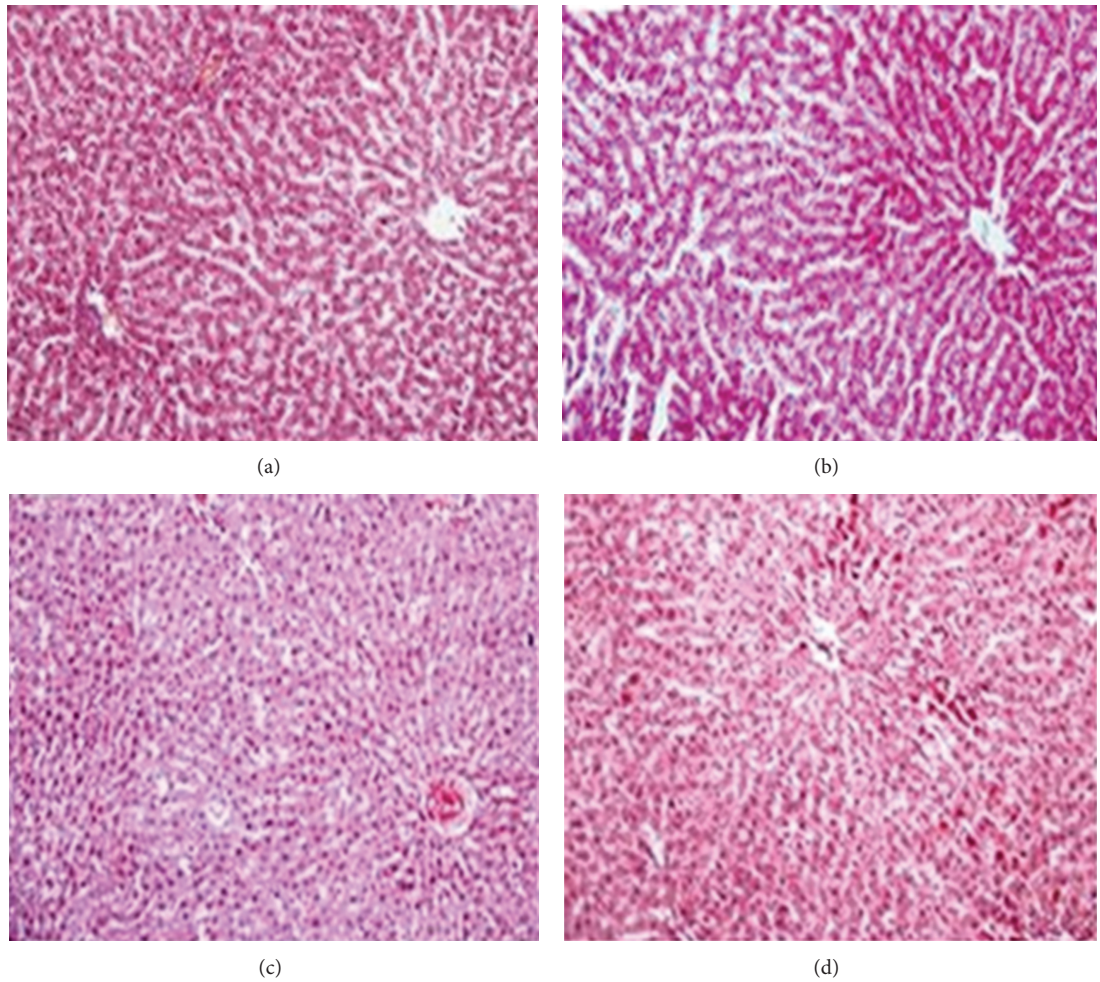

Figure 5: The photomicropictographs of Masson's Trichrome staining for assessment of the degree of fibrosis in the liver section from chow fed rats (a) and HFD control rats (b) treated with saline (5 mL/kg of body weight), and HFD rats treated with rosiglitazone (HFD-RSG, 3 mg/kg of body weight, (c)) or SGE (HFD-SGE, 2 g/kg of body weight, (d)). All photomicrographs were taken at 200x magnification.

unoxidised fatty acids (ceramides and diglycerides) in the liver also increases, which trigger apoptosis of lipid-laden hepatocytes and impair insulin signal pathway. Therefore, aggressive treatment of hyperlipidaemia and inhibition of adipose lipolysis play a critical role in the overall management of patients with NAFLD and MetS. Statins are the first-line agents to treat hyperlipidaemia but there is a risk for liver injury in patients with NAFLD [25]. Ten weeks of HFD feeding in rats caused atherogenic dyslipidaemia with high levels of TG, low levels of HDL-C, and marked elevations in LDL-C concentrations. In this study, we showed that SGE had effects on hypertriglyceridemia and attenuated the elevated serum NEFAs, indicating that SGE may inhibit adipose lipolysis or enhanced FFAs availability. SGE also presented similar effects to RSG (insulin sensitizer) on hyperinsulinaemia and improved HOME-IR, but its potency was much less than RSG as the higher dose (2 g/kg of body weight) than RSG (3 mg/kg of body weight) was needed. SGE also raised HDL-C levels; however, the statistical significance was not achieved. Increasing the proportion of *S. miltiorrhiza* may be necessary if the formula would target at atherogenic dyslipidemia because the previous study has shown that *S. miltiorrhiza* markedly raised HDL-C in patients with ischemic cerebrovascular disease [12]. Specifically, we demonstrated that SGE had a beneficial effect on body weight and significantly reduced visceral fat mass but RSG treated rats gained more weight than HFD control. Thus, present study has proven that SGE had preventive effect on NAFLD as well as improved several metabolic abnormalities in HFD rats.

Accumulating data demonstrate that obesity and insulin resistance lead to NAFLD and hepatic fibrosis through not only fatty infiltration but also adipocytokines-induced inflammation [26]. Adipocytokines are cytokines secreted primarily by adipose tissue, including adiponectin, leptin, resistin, TNF-α, interleukins, and others. Decreased secretion of adiponectin and leptin from adipose tissue is involved in central obesity and insulin resistance [27]. Leptin is a key fat-derived hormone produced by adipose tissue, acting on the brain to increase satiety, hence inhibiting food intake and

controlling weight gain [28]. After a high-fat meal, leptin secretion usually increased in rodents [29]. Our study showed that chronic HFD feeding was associated with reduced leptin expression in adipose tissue. This reduced leptin levels may contribute to the subsequent weight gain that we observed in the control HFD rats. We propose two mechanisms that may explain why a high dietary fat intake was associated with decreased leptin expression. Firstly, a long-term high-fat exposure (10 weeks) may overstimulate leptin secretion, hence depleting leptin contents in the adipose tissue. Secondly, increases in lipolysis have also been associated with decreased leptin synthesis in the previous study [30]. Decreased leptin synthesis may explain reduced leptin levels in adipose tissue after fat feeding because increased lipolysis has been documented after high-fat diets [31]. Our study has demonstrated that SGE treatment enhanced leptin expression, which is consistent with a recent study that circulating leptin was reversely associated with weight loss and VLDL-TG secretion in obese subjects [32]. Furthermore, the marked hepatic steatosis has been observed in leptin-deficient *ob/ob* mice [33]. Enhanced leptin expression may be one of mechanisms by which SGE ameliorated NAFLD and hepatic fibrosis in HFD rats.

Overexpression of TNF-α and IL6 has been identified in the adipose tissue of obese patients [34]. TNF-α together with IL6 can mediate macrophage infiltration locally and at distant sites, such as liver. Hepatic inflammation resulting from adipose proinflammatory cytokines plays an important role in the development of NAFLD and progression of the fibrogenic process [35]. In the present study, HFD feeding significantly increased the weight of visceral fat and insulin resistance. In particular, decreased leptin and increased TNF-α and IL6 expression in visceral fat were found in HFD rats compared with standard chow fed rats. Treatment with both SGE and RGS significantly inhibited adipose TNF-α and IL6 expression but only SGE increased leptin expression in adipose tissue. Anti-inflammatory action may be one of the mechanisms that SGE attenuate HFD-induced liver pathology.

The coexisting MetS and NAFLD provide a rationale for using a formula with multiple naturally occurring compounds that can target different pathological pathways involved in this complicated metabolic disorder, providing therapeutic effects via a spectrum of actions. SGE contains active compounds existing in *S. miltiorrhiza* such as 3, 4-dihydroxyphenyl lactic acid (named as Danshensu) and diterpenoid quinines (tanshinone IIA) from *S. miltiorrhiza,* and active compounds such as geniposide and genipin from *G. jasminoides*. The previous studies have demonstrated that the active compounds in *S. miltiorrhiz* ahave beneficial effects on the cardiovascular system through reducing oxidant stress, inhibiting inflammatory cytokine (plasminogen activator inhibitor-1), and improving lipid profiles [12, 13, 36]. The results in this study may also account for the pharmacological action of ingredients from *G. jasminoides*. Geniposide is one of the major iridoid glucosides in the fruit of *G. jasminoides*, which has been reported to possess anti-inflammatory activity [37]. A recent animal study has demonstrated that geniposide has an antiobesity effect, and the metabolite genipin shows a direct effect on the fatty liver through inducing expression of a lipid metabolism-related gene [15]. We have performed screening tests on many herbal extracts from single herb, but their actions were mild and lack of the capability to control most components of metabolic syndrome (data not published). The SGE used in this study have substantially exemplified that the effects of natural compounds together may provide synergistic effects.

In summary, this study demonstrated that SGE improved circulating lipid profiles, reduced visceral fat, and improved insulin resistance, as well as effectively impeded the accumulation of triglycerides in the liver and fat infiltration in the hepatocytes with normal histological appearances and hepatic enzymes in HFD fed rats. SGE prevented the development of HFD-induced NAFLD maybe through attenuating release of inflammatory cytokine from adipose tissue. Because two herbs have been used in TCM for thousand years, it has been considered for its safety and tolerability. Large scale of clinical trials is worth being performed to prove that SGE is an optimal approach to the combination of non-alcoholic fatty liver disease and metabolic syndrome.

Abbreviations

NAFLD: Nonalcoholic fatty liver disease
HFD: High-fat diet
RSG: Rosiglitazone
SD: Sprague Dawley
SGE: *Salvia miltiorrhiza*, *Gardenia jasminoides* extracts.

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Research Article

Artemisia iwayomogi Extract Attenuates High-Fat Diet-Induced Obesity by Decreasing the Expression of Genes Associated with Adipogenesis in Mice

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The objective of the present study was to determine whether *Artemisia iwayomogi* (AI) extract reduces visceral fat accumulation and obesity-related biomarkers in mice fed a high-fat diet (HFD), and if so, whether these effects are exerted by modulation of the expression of genes associated with adipogenesis and inflammation. AI extract supplementation for 11 weeks significantly prevented HFD-induced increments in body weight, visceral adiposity, adipocyte hypertrophy, and plasma levels of lipids and leptin. Additionally, AI extract supplementation resulted in downregulation of adipogenic transcription factors (PPARγ2 and C/EBPα) and their target genes (CD36, aP2, and FAS) in epididymal adipose tissue compared to the HFD alone. The AI extract effectively reversed the HFD-induced elevations in plasma glucose and insulin levels and the homeostasis model assessment of insulin resistance index. Furthermore, the extract significantly decreased gene expression of proinflammatory cytokines (TNFα, MCP1, IL-6, IFNα, and INFβ) in epididymal adipose tissue and reduced plasma levels of TNFα and MCP1 as compared to HFD alone. In conclusion, these results suggest that AI extract may prevent HFD-induced obesity and metabolic disorders, probably by downregulating the expression of genes related to adipogenesis and inflammation in visceral adipose tissue.

1. Introduction

Adipogenesis is the process by which mesenchymal precursor cells differentiate into adipocytes [1]. Although its presence is necessary in many ways, excess adipose tissue is associated with serious health problems such as obesity, cardiovascular disease, and type 2 diabetes [2, 3]. Presently, CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ2 (PPARγ2) are considered the 2 primary transcription factors that mediate adipogenesis. It has been reported that inactivation of PPARγ2 and C/EBPα in adipose tissue protects against obesity in rodent models [4–6]. Thus, potential therapeutic agents that have the ability to inhibit adipogenesis could have a profound impact as a strategy for preventing obesity and related metabolic disorders.

Adipose tissue not only serves as an organ for energy storage, but also as an endocrine organ by releasing various inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin- (IL-)6 [7–9]. Proinflammatory molecules produced by adipose tissue have been implicated as active participants in the development of inflammation and the increased risk of obesity-related insulin resistance [9–11]. Increased production of monocyte chemoattractant protein 1 (MCP1), interferon (IFN) α, IFNβ, TNFα, and IL-6 in adipose tissue has been reported in animal models of obesity [8–10, 12]. Therefore, therapeutic agents that attenuate proinflammatory cytokines may prove useful in the medical management of obesity-induced inflammation.

Artemisia iwayomogi (AI), a member of *Compositae*, is a perennial herb easily found throughout Korea. It has been used as a traditional medicine and is known to have

antiallergic, antiapoptotic, and antioxidant effects [13–16]. It has been reported that a carbohydrate fraction from AI suppresses spontaneous or 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced apoptotic death of mouse thymocytes [14, 15]. *Artemisia iwayomogi* extract displays scavenging activity of peroxynitrite, a potent cytotoxic oxidant formed by the reaction between nitric oxide and superoxide radicals [16]. In addition, to our knowledge, few studies have described the beneficial effects of AI in high-fat diet-(HFD-) fed obese rodents. One study reported that oral administration of AI extract significantly reduced serum lipid levels in HFD-fed rats [17]. A recent study by Cho et al. reported that oral administration of AI extract to HFD-fed mice provoked upregulation of PPARβ and its target genes involved in fatty acid oxidation in the skeletal muscle [18]. To date, however, no study has assessed the protective effects of AI extract on adipose tissue dysfunction in diet-induced obese animal models. Therefore, the present study aimed to investigate whether AI extract could reduce visceral fat accumulation and improve obesity-related biomarkers in HFD-fed mice, and if so, whether these effects were exerted by modulation of the expression of genes associated with adipogenesis and inflammation.

2. Material and Methods

2.1. Preparation of AI Extract. *Artemisia iwayomogi* was collected from Korean standard products in March 2008, and was identified by Professor Wan Kyun Whang of the Pharmaceutical Botany Laboratory at Chung-Ang University in Seoul, Republic of Korea, where a voucher specimen has been deposited. Dried AI (500 g) was washed in water, oven dried at 40°C, mechanically fragmented, and then was powdered in an electric mill. The powder was extracted 5 times with ethanol (powder : solvent = 1 : 5) at room temperature. After filtration, the extraction was vacuum concentrated to yield 6.25% ethanol extract (31.3 g), which was stored at −4°C until use.

2.2. HPLC Analysis. Chromatography was performed using a Water HPLC system (Water Corporation, Milford, MA, USA) with an autosampler. HPLC separation was conducted using a Kromasil C18 column (4.6 mm × 250 mm, 5-μm inner diameter) at 30°C with a flow rate of 1.0 mL/min using a gradient mobile phase composed of water (A) and acetonitrile (B). The mobile phase comprised an 80 : 20 mixture of component A to B as the initial condition of the chromatographic run, and component B was increased to 80% in a linear gradient in 30 min. The sample injection volume was 10 μL. The identities of the compounds in the AI extract were confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS, with individual purities of not less than 95%.

2.3. Animals and Experimental Protocol. Male C57BL/6J mice (5 weeks old) were purchased from Orient Bio (Gyeonggi-do, Republic of Korea) and were maintained in 12 h light/dark with *ad libitum* access to food and water. After a 1-week acclimatization period, the mice were divided into 3 groups (n = 10 per group): normal diet (ND), HFD, and 0.5% AI extract-supplemented diet (AED). The ND was a purified diet based on the AIN-76 rodent diet composition. The HFD was identical to the ND, but to which 200 g fat/kg (170 g lard plus 30 g corn oil) and 1% cholesterol had been added. The AED was identical to the HFD but contained 0.5% (w/w) AI extract. The mice were fed the experimental diets for 11 weeks. Diet consumption was monitored daily, and body weight was monitored weekly. At the end of the feeding period, mice were anesthetized with diethyl ether after an overnight fasting for 16 h, and their blood samples were collected in EDTA-coated tubes. Plasma samples were isolated by centrifugation at 4000 ×g for 20 min and stored at −80°C for subsequent analysis. Adipose and liver tissues were collected, washed with phosphate-buffered saline, and frozen at −80°C. All animal experiments were performed in accordance with the Korean Food and Drug Administration guidelines. The Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center reviewed and approved the protocols.

2.4. Biochemical Analysis. Plasma concentrations of triglycerides (TGs), free fatty acids (FFAs), glucose, total cholesterol, and HDL cholesterol were measured using commercial kits (Bio-Clinical System, Gyeonggi-do, Republic of Korea). LDL + VLDL cholesterol levels were calculated by subtracting HDL cholesterol from total cholesterol. Plasma insulin levels were analyzed using a mouse insulin ELISA kit (ALPCO Diagnostics, Windham, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as fasting plasma glucose concentration (mmol/L) multiplied by fasting insulin level (pmol/L) divided by 22.5. Plasma levels for leptin, TNFα, and MCP1 were measured using a mouse ELISA kit (ID Labs, Cambridge, MA, USA). Hepatic lipids were extracted as described by Folch et al. [19], using a chloroform-methanol mixture (2 : 1 v/v), and the dried lipid residues were dissolved in 2 mL ethanol. Concentrations of cholesterol, triglyceride, and free fatty acids in the hepatic lipid extracts were measured using the same enzymatic kits that were used for the plasma analysis.

2.5. Histological Analysis. White adipose tissues (WATs) were fixed in neutral buffered formalin, embedded in paraffin, and sectioned into 5 μm sections onto slides. For histology, sections were stained with hematoxylin and eosin (H&E). The sectional areas of WAT were analyzed to quantify the size of the adipocytes.

2.6. RNA Extraction and Semiquantitative RT-PCR. Total RNA was isolated from the epididymal adipose tissue of each mouse with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Isolated RNA was quantified using a spectrophotometer, and cDNA was synthesized using reverse transcriptase (Invitrogen). The PCR was programmed as follows: 10 min at 94°C; 30–33 cycles at 94°C for 30 s, 55°C for 30 s; 72°C for 1 min; 10 min incubation at 72°C. Four microliters of each PCR reaction were mixed with 1 μL of 6-fold concentrated loading buffer and loaded onto 2% agarose gel containing ethidium bromide. The GenBank accession numbers of the relevant

Table 1: Primer sequences and RT-PCR conditions.

Gene description	Primers	Sequences ($5' \rightarrow 3'$)	T_m (°C)	Size (bp)
Peroxisome proliferator-activated receptor γ2 (PPARγ2)	F	TTCGGAATCAGCTCTGTGGA	55	148
	R	CCATTGGGTCAGCTCTTGTG		
CCAAT/enhancer binding protein α (C/EBPα)	F	AAGGCCAAGAAGTCGGTGGA	55	189
	R	CCATAGTGGAAGCCTGATGC		
Adipocyte protein 2 (aP2)	F	ACATGAAAGTGGGAGTG	55	128
	R	AAGTACTCTCTGACCGGATG		
Cluster of differentiation 36 (CD36)	F	ATGACGTGGCAAAGAACAGC	55	160
	R	GAAGGCTCAAAGATGCCTCC		
Fatty acid synthase (FAS)	F	TTGCCCGAGTCAGAGAACC	55	171
	R	CGTCCACAATAGCTTCATAGC		
Monocyte chemoattractant protein 1 (MCP1)	F	CCAGCAAGATGATCCCAATG	55	450
	R	CTTCTTGGGGTCAGCACAGA		
Interferon α (IFNα)	F	ATGGCTAG(G/A)CTCTGTGCTTTCCT	60.2	500
	R	GGGCTCTCCAGA(T/C)TTCTGCTCTG		
Interferon β (IFNβ)	F	TGGAGCAGCTGAATGGAAAG	55	122
	R	GAGCATCTCTTGGATGGCAA		
Tumor necrosis factor α (TNFα)	F	TGTCTCAGCCTCTTCTCATT	55	156
	R	AGATGATCTGAGTGTGAGGG		
Intereukin 6 (IL-6)	F	TTGCCTTCTTGGGACTGATG	55	162
	R	CCACGATTTCCCAGAGAACA		
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F	AGAACATCATCCCTGCATCC	60	321
	R	TCCACCACCCTGTTGCTGTA		

templates and forward (F) and reverse (R) primer sequences are shown in Table 1. The measured mRNA levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

2.7. Statistical Analysis. The data on body weight gain, plasma biochemistries, and adipocyte diameter are presented as the mean ± SEM of 10 mice. RT-PCR results are presented as the mean ± SEM of at least 3 separate experiments. All analyses were performed using SPSS (version 12.0). Data were analyzed by 1-way ANOVA, followed by Duncan's multiple range tests. P values <0.05 were considered significant.

3. Results

3.1. Chromatographic Analysis of Artemisia iwayomogi Extract. The HPLC chromatogram revealed that scopolin (AI-I, 1.21% w/w), acetophenone glycoside (AI-II, 0.26% w/w), and scopoletin (AI-III, 0.38% w/w) were the major components among the organic molecules of the AI extract, which exhibited maximum absorbance at 280 nm (Figure 1 and Table 2).

3.2. Body and Visceral Fat Pad Weights. AED-fed mice exhibited significantly decreased body weight gain (−52%) and final body weight (−19%) compared to HFD-fed mice without their food intake being affected (Figures 2(a)–2(c)).

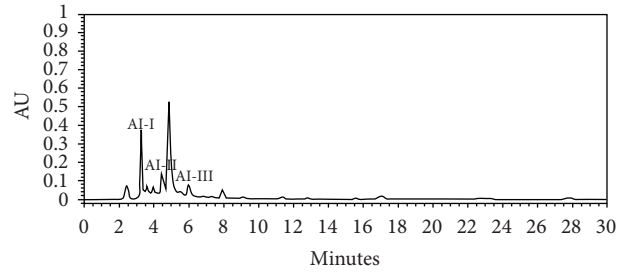
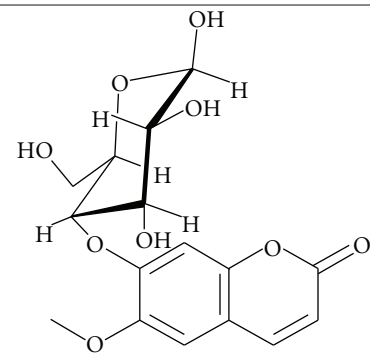
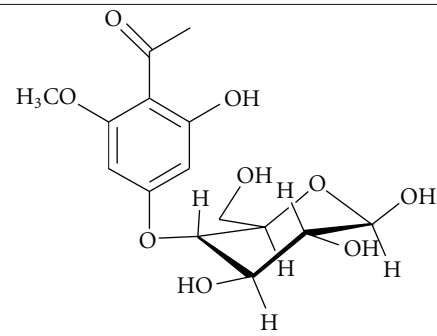
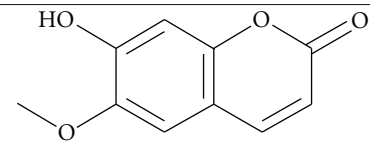


Figure 1: HPLC chromatogram of the *Artemisia iwayomogi* extract. The peaks were assigned based on the isolation of each compound. AI-I: scopolin; AI-II: acetophenone glycoside; AI-III: scopoletin (see Table 2).

The food efficiency ratio (FER) was significantly lower in AED-fed mice (−54%) compared to HFD-fed mice (Figure 2(d)). The AI extract supplementation led to a significant decrease in total visceral fat pad weight (−64%) compared to the HFD alone. This was attributable to weight decreases in the epididymal (−54%), retroperitoneal (−58%), perirenal (−83%), and mesenteric (−69%) adipose depots ($P < 0.05$ for all depots; Figures 2(e) and 2(f)). The H&E sections of epididymal adipose tissues revealed that adipocyte diameter (−20%) was significantly decreased in AED-fed mice than in HFD-fed mice (Figure 2(g)).

Table 2: Profile of compounds in the *Artemisia iwayomogi* extract.

Compound	IUPAC name	Structure	Contents (%)
(1) Scopolin	6-methoxy-2-oxo-2*H*-chromen-7-yl β-D-glucopyranoside		1.21 ± 0.07
(2) Acetophenone glycoside	2,4-dihydroxy-6-methoxyacetophenone 4-O-β-D-glucopyranoside		0.26 ± 0.01
(3) Scopoletin	7-hydroxy-6-methoxychromen-2-one		0.38 ± 0.04

3.3. Plasma Biochemistries. Plasma concentrations of TG (−47%), FFA (−47%), total cholesterol (−47%), HDL cholesterol (−34%), and LDL + VLDL cholesterol (−59%) were all significantly lower in AED-fed mice than in HFD-fed mice ($P < 0.05$, Figures 3(a)–3(e)). AED-fed mice had significantly lower plasma concentrations of leptin (−71%) than HFD-fed mice (Figure 3(f)). Likewise, the AI extract significantly attenuated the elevation in plasma concentrations of glucose (−42%) and insulin (−21%) in HFD-fed mice (Figures 3(g) and 3(h)). The HOMA-IR calculations revealed that the AI extract significantly decreased the HOMA-IR index (−49%) compared to HFD alone (Figure 3(i)). AED-fed mice had significantly lower plasma concentrations of MCP1 (−60%) and TNFα (−46%) than HFD-fed mice (Figures 3(j) and 3(k)).

3.4. Hepatic Lipid Accumulation. AED-fed mice had significantly lower liver weight (−30%) than in HFD-fed mice (Figure 4(a)). Hepatic triglyceride (−66%), cholesterol (−51%), and free fatty acid (−75%) concentrations were markedly lower in AED-fed mice than in HFD-fed mice (Figures 4(b)–4(d)).

3.5. Expression of Genes Related to Adipogenesis. Examination of adipogenic gene expression in epididymal adipose tissue showed that mRNA levels of PPARγ2 (−44%) and C/EBPα (−18%), regulators of adipogenic molecules, were significantly lower in AED-fed mice than in HFD-fed mice. The expressions of PPARγ2 target genes, including cluster of differentiation 36 (CD36) (−29%), adipocyte fatty acid binding protein (aP2) (−35%), and fatty acid synthase (FAS) (−58%), were all significantly decreased in AED-fed mice than in HFD-fed mice (Figure 5).

3.6. Expression of Genes Related to Inflammation. Based on the active roles of proinflammatory cytokines in obesity-related inflammation, we examined the effect of AI extract on proinflammatory cytokine expressions in epididymal adipose tissue. Compared to HFD-fed mice, the epididymal adipose tissue in AED-fed mice contained significantly decreased mRNA levels of several proinflammatory cytokines, including TNFα (−41%), IL-6 (−28%), MCP1 (−32%), IFNα (−41%), and IFNβ (−38%) (Figure 6).

4. Discussion

This feeding study was designed to assess whether AI extract supplementation for 11 weeks could improve diet-induced obesity and obesity-related biomarkers in mice. Based on a preliminary study involving different AI extract dosages (0.5%, 1%, and 2%), we determined that 0.5% AI extract was the minimal effective dose for preventing weight gain

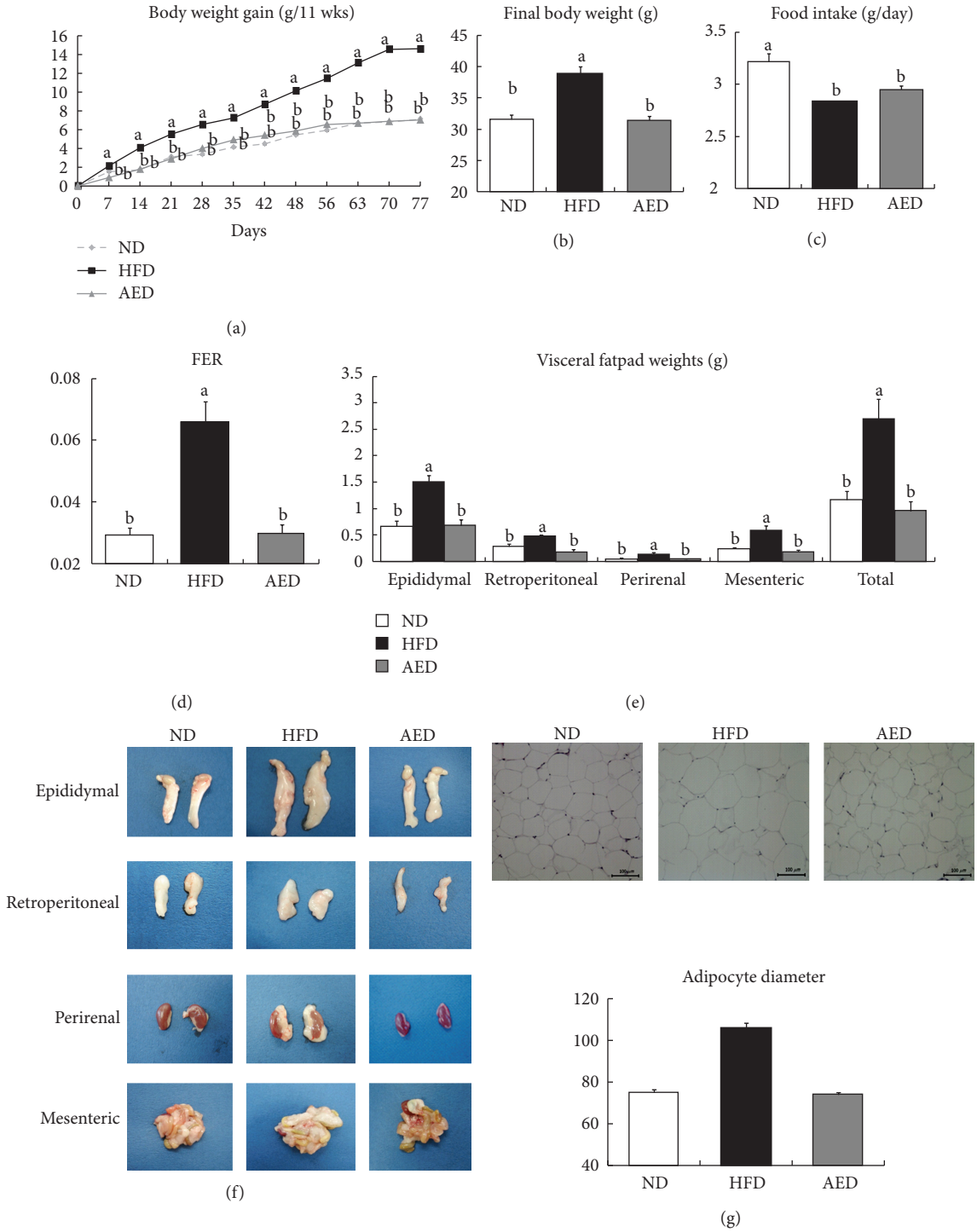


FIGURE 2: Effects of *Artemisia iwayomogi* extract supplementation on body weight gain, food efficiency ratio, and visceral fat pad weights of mice fed with high-fat diet. Mice were fed ND, HFD, or AED for 11 weeks. Changes in (a) body weight gain, (b) final body weight, (c) food intake, (d) FER, (e), (f) visceral fat pad weights, (g) representative pictures of H&E-stained fat cells from mice epididymal adipose tissue (×100), and densitometric analysis of adipocyte diameter in epididymal tissue. Data represent mean ± SEM, $n = 10$. Means without a common letter differ, $P < 0.05$. FER = (body weight gain for experimental period (g))/(food intake for experimental period (g)).

in HFD-fed mice (data not shown). Hence, the 0.5% AI extract was considered for this study. In the present study, the AI extract significantly decreased not only body weight gain, but also visceral adiposity and adipocyte hypertrophy in HFD-fed mice. Since visceral adiposity is positively correlated to plasma leptin concentration, the circulating leptin level is an ideal indicator for assessing obesity in both experimental animals and humans [20, 21]. In this context,

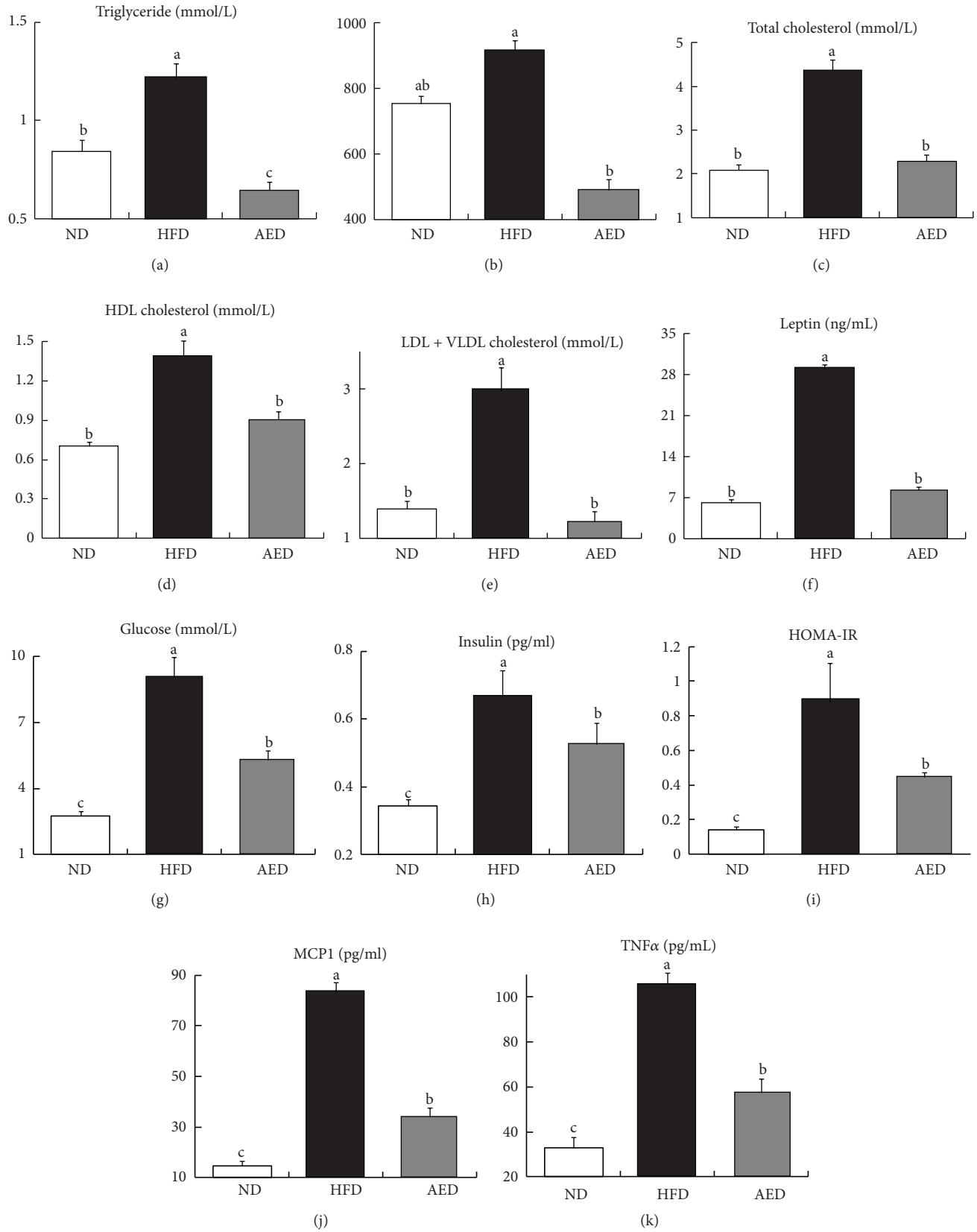


Figure 3: Effects of *Artemisia iwayomogi* extract supplementation on plasma levels of lipids, leptin, glucose, insulin, and proinflammatory cytokines in mice fed with high-fat diet. (a) Triglyceride, (b) free fatty acid, (c) total cholesterol, (d) HDL cholesterol, (e) LDL + VLDL cholesterol, (f) leptin, (g) glucose, (h) insulin, (i) HOMA-IR, (j) MCP1, and (k) TNFα. Bars represent mean ± SEM, $n = 10$. Means without a common letter differ, $P < 0.05$.

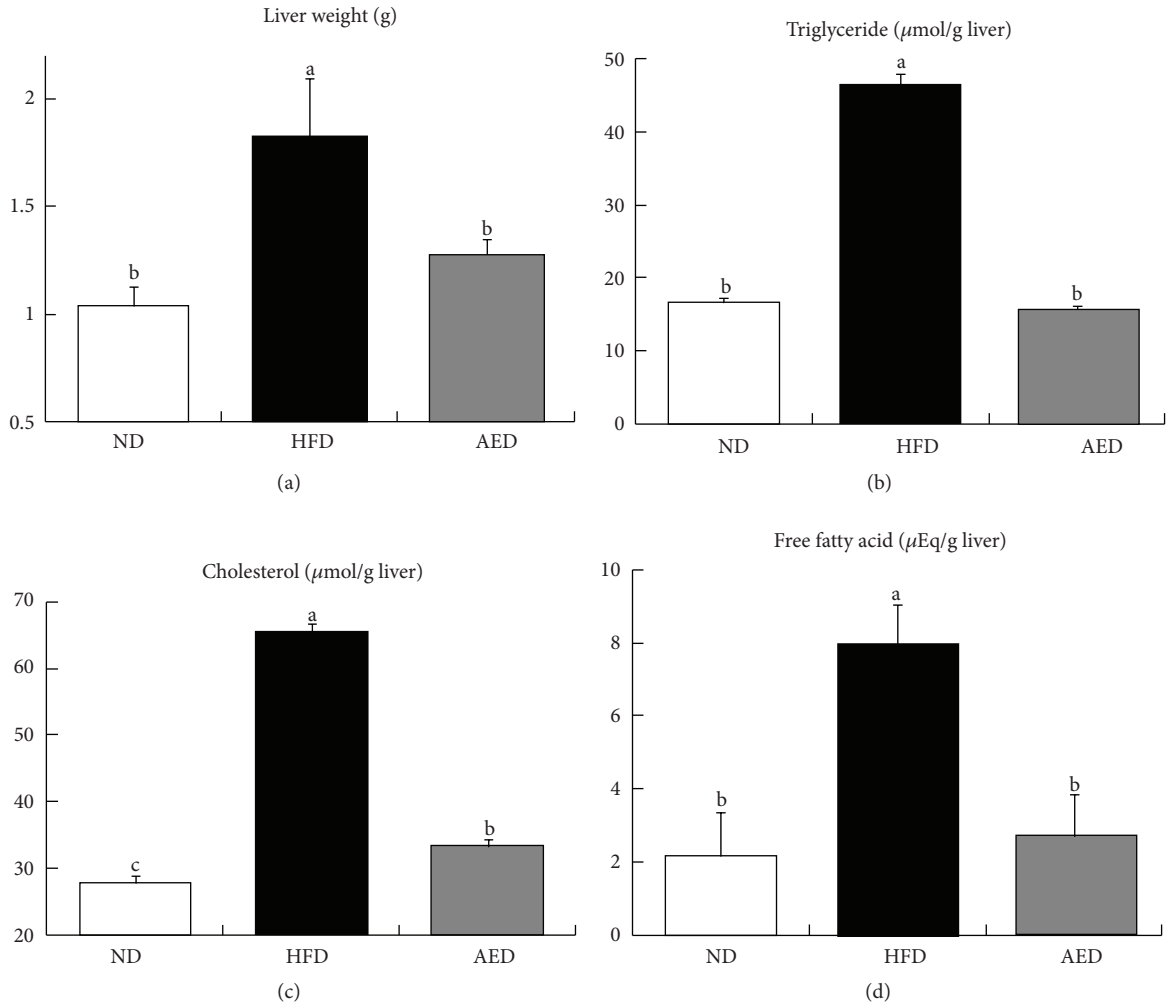


Figure 4: Effects of *Artemisia iwayomogi* extract supplementation on liver weights and hepatic lipid levels in mice fed with high-fat diet. (a) Liver weights and concentrations of hepatic (b) triglyceride, (c) cholesterol, and (d) free fatty acids. Means without a common letter differ, $P < 0.05$.

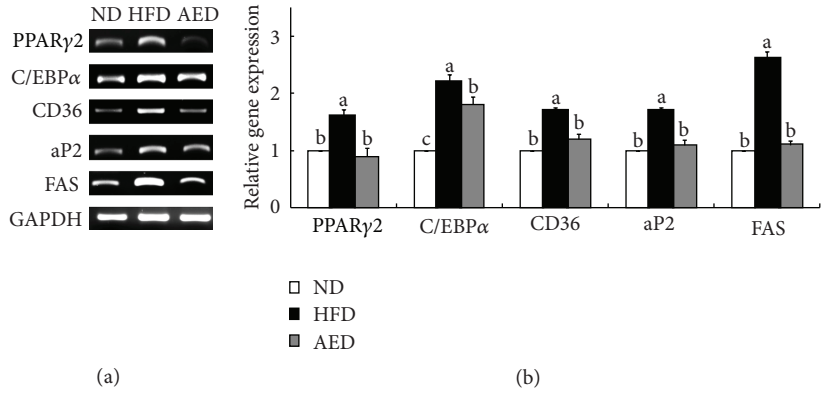


Figure 5: Effects of *Artemisia iwayomogi* extract supplementation on genes regulating adipogenesis in epididymal adipose tissue of mice fed with high-fat diet. (a) Representative example of semiquantitative RT-PCR revealing the expression levels of adipogenic genes in epididymal adipose tissue and their quantitative analysis. The data represent relative density normalized to GAPDH. Data represent the results of 3 independent experiments. Bars represent mean ± SEM. Means without a common letter differ, $P < 0.05$.

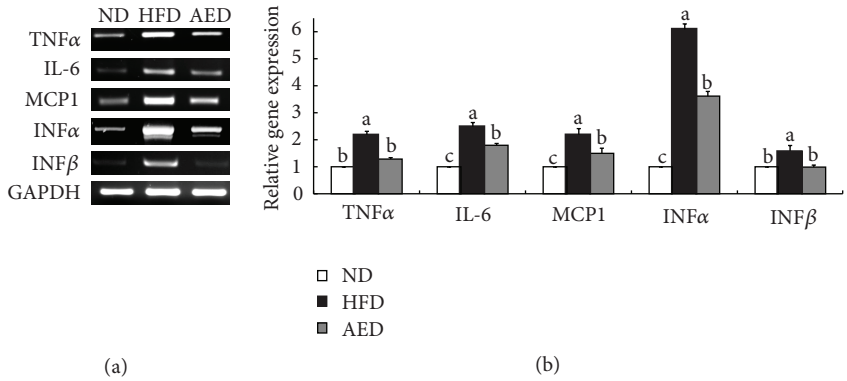


Figure 6: Effects of *Artemisia iwayomogi* extract supplementation on expression of proinflammatory cytokine genes in epididymal adipose tissue of mice fed with high-fat diet. (a) Representative example of semi-quantitative RT-PCR revealing the expression levels of proinflammatory cytokines in epididymal adipose tissue and their quantitative analysis. Data represent the results of 3 independent experiments. Bars represent mean ± SEM. Means without a common letter differ, $P < 0.05$.

the lower plasma leptin level recorded in the AED-fed mice may be attributable to the prevention of visceral adipocyte hypertrophy.

As there was no significant difference in food consumption between HFD- and AED-fed mice, the beneficial effects of the AI extract on body weight gain and visceral fat accumulation evidently did not depend on decreased energy intake. Thus, we hypothesized that the AI extract reduced HFD-induced body weight gain and visceral fat accumulation by mediating the inhibition of adipogenesis. That the expression of PPARγ2 and C/EBPα in WAT is upregulated in HFD-induced obese animals is well known [4, 22]. Increased PPARγ2 and C/EBPα function cooperatively to transactivate adipocyte genes, including FAS, CD36, and aP2 [23–26]. FAS catalyzes the reactions for the synthesis of long-chain fatty acids [27], whereas CD36 and aP2 facilitate the uptake of long-chain fatty acids in adipocytes [23, 28], thereby increasing adipocyte size and fat accumulation. In the current study, the AI extract significantly reversed the HFD-induced upregulation of adipogenic transcription factors (PPARγ2 and C/EBPα) and their target genes (FAS, CD36, and aP2) in the epididymal adipose tissue of mice. These decreased expressions of adipogenic genes by the AI extract may have contributed to the lower visceral adiposity and body weight gain. In addition, the AI extract significantly decreased plasma FFA levels in HFD-fed mice. As increased plasma concentrations of FFA form a vicious relationship with PPARγ2 activation in diet-induced obese animals [29, 30], the decreased FFA level in the AED-fed mice might also be considered associated with PPARγ2 inactivation.

Increased adipose tissue accumulation in obese individuals correlates with the overproduction of proinflammatory cytokines that play crucial roles in the development of obesity-induced inflammation [8, 11]. In obese animals, excess adipose tissue increases the expression and secretion of TNFα, a prototypical inflammatory cytokine [4, 9, 12]. In turn, increased TNFα activates adipocytes, thereby further enhancing the expression of various proinflammatory genes such as MCP1, IL-6, IFNα, and IFNβ [31, 32]. IL-6 induces a hepatic acute-phase reaction with upregulated acute-phase proteins, including C-reactive protein and fibrinogen [33, 34], whereas MCP1 contributes to macrophage infiltration into adipose tissue, which leads to chronic inflammation [35]. IFNα/β directly stimulates IFNγ production, a regulator of innate immune response, in T cells [36]. Our results suggested that the decrease in visceral adiposity by the AI extract might have contributed, in part, to the decreased expression of proinflammatory cytokines (TNFα, MCP1, IL-6, IFNα, and IFNβ) and reduced plasma levels of TNFα and MCP1. Consequently, the decreased gene expression and secretion of proinflammatory cytokines in the AED-fed mice may have contributed to the prevention of obesity-induced inflammation.

Several studies have demonstrated that cytokines play crucial roles in the development of insulin resistance [35, 37–39]. In diet-induced obese animals, increased plasma levels of TNFα, IL-6, MCP1, and leptin have been shown to impair the ability of insulin to activate signal transduction and stimulate glucose uptake into skeletal muscle and adipose tissue [35, 37–41]. Thus, in the present study, improvement of insulin resistance by the AI extract might be associated with decreased gene expression and/or production of cytokines such as TNFα, IL-6, MCP1, and leptin.

Separation and determination of chemical constituents are generally recommended for standardization and quality control of herbal products and herb-related investigations [42]. Several studies have demonstrated that AI contains flavonoids such as genkwanin and jaceosidin [43], essential oils, including eugenol and 1,8-cineole [44], and coumarin compounds such as scopoletin and scopolin [43, 45]. In addition, scopoletin has been used as a standard compound for the verification and identification of AI [46, 47]. In the present study, we isolated not only scopoletin, but also scopolin and acetophenone glycoside from the AI extract (Figure 1 and Table 2). Of these, scopolin (1.21%) was the most abundant, followed by scopoletin (0.38%) and acetophenone glycoside (0.26%). In addition, the scopolin content of the AI extract was higher in our study than that

observed by Kim et al. (0.49% w/w) and Ding et al. (0.2% w/w) [43, 45]. This difference may be due to the differing extraction and isolation methods as well as differences in plant material [43, 45]. Previous studies have shown that coumarin compounds from *Fraxinus rhynchophylla* [48], *Angelica gigas* [49], and *Ionidium suffruticosum* [50] inhibit adipocyte differentiation in 3T3-L1 cells and/or reduce body weight gain and plasma lipid levels in mice fed a high-fat diet. Thus, the antiobesity activity of AI extract observed in the present study may be attributable to the presence of high amounts of coumarin compounds in the plant. Further studies are needed to determine the major active compounds in AI extract that are responsible for decreased visceral adiposity and other obesity-related biomarkers.

In summary, the present study showed that AI extract reduced visceral fat accumulation in HFD-fed mice and improved the risk factors related to the metabolic syndrome, such as inflammation and insulin resistance. The evidence obtained in this study suggests that the beneficial effects of AI extract may be due to, at least in part, downregulation of the genes related to adipogenesis and inflammation in the visceral adipose tissue of mice. Therefore, dietary supplementation with this extract, if validated in human studies, may provide an adjunctive therapy for the prevention and/or treatment of obesity and metabolic syndrome.

Abbreviations

AI:	*Artemisia iwayomogi*
AED:	*Artemisia iwayomogi* extract-supplemented diet
aP2:	Adipocyte protein 2
C/EBPα:	CCAAT/enhancer binding protein α
CD36:	Cluster of differentiation 36
FAS:	Fatty acid synthase
FFA:	Free fatty acids
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HFD:	High-fat diet
HOMA-IR:	Homeostasis model assessment of insulin resistance
IFNα:	Interferon α
IFNβ:	Interferon β
IFNγ:	Interferon γ
IL-6:	Interleukin-6
MCP1:	Monocyte chemoattractant protein 1
ND:	Normal diet
PPARγ2:	Peroxisome proliferator-activated receptor γ2
TG:	Triglyceride
TNFα:	Tumor necrosis factor α.

Acknowledgments

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Research Article

Oat Attenuation of Hyperglycemia-Induced Retinal Oxidative Stress and NF-κB Activation in Streptozotocin-Induced Diabetic Rats

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The overproduction of reactive oxygen species (ROS) plays a central role in the pathogenesis of endothelial damage in diabetes. To assess the effect of oat on experimental diabetic retinopathy, five groups of Albino rats were studied: nondiabetic control, untreated diabetic, and diabetic rats treated with 5%, 10%, and 20% (W/W) oat of the diet for 12 weeks. Novel data were obtained in this study indicating a protective role of oat against oxidative stress and diabetic retinopathy. The effects of oat on parameters of oxidative stress, AGE, and nuclear factor kappa B (NF-κB) were assessed by ELISA and NF-κB activation by electrophoretic mobility shift assay. Tumor necrosis factor alpha (TNFα) and vascular endothelial growth factor (VEGF) were also determined. After 12 weeks of diabetes, oat treatment reduced blood glucose levels, HbA1c, all oxidative stress markers, CML, normalized NF-κB activation and TNFα expression. Furthermore it reduced VEGF in the diabetic retina by 43% ($P < 0.001$). In conclusion, oat modulates microvascular damage through normalized pathways downstream of ROS overproduction and reduction of NF-κB and its controlled genes activation, which may provide additional endothelial protection.

1. Introduction

Diabetes is a wide distributed disease characterized by high concentrations of the circulating glucose. It is a lifelong progressive disease and results from body's inability to either produce insulin or use insulin to its full potential. Diabetes is the fourth leading cause of death by disease globally, every 10 seconds a person dies because of the late diabetic complications. Diabetes is a disease which can be controlled but it does not go away. In diabetes, the chronic hyperglycaemia attacks both macrovessels and microvessels throughout the body. In many of the developed countries, diabetes is the leading cause of noninjury amputation, blindness and visual impairment, and end-stage renal disease in adults. It can threaten vision; patients with diabetes develop cataracts at an earlier age. The risk to get glaucoma is nearly twice in diabetic patients compared to nondiabetics [1]. In addition, wound healing impairments is a direct cause of diabetes, and diabetic patients are two to four times more likely to develop cardiovascular disease than people without diabetes.

Diabetic retinopathy, a disease of the retina, is the leading cause of acquired blindness in adults. The microvasculature of the retina is damaged, the blood vessels swell and leak fluid, and, if not prevented, new vessels start to grow and ultimately lead to the detachment of the retina [2, 3]. It is a duration-dependent disease that develops in stages; the incidence of retinopathy is rarely detected in the first few years of diabetes, but the incidence increases to 50% by 10 years, and to 90% by 25 years of diabetes. The prevalence of diabetic retinopathy is increasing due to the prolonged survival of diabetic patients.

Continued high concentrations of the circulating glucose in this life-long disease can damage retina via many acute (and/or repeated) and also cumulative long-term changes. Although the capillaries of the retina are lined with endothelial cells that maintain the blood retinal barrier and are supported with an equal number of pericytes that provide

tone to the vessels, the ratio of endothelial cells to pericytes in diabetes is altered to be about 4 : 1 [4]. All the blood vessels of the retina have tight junctions that help to protect them against leaking, but prolonged high concentrations of glucose damage these tight junctions and the vessels become leaky allowing the fluid or/and blood to seep into the retina, which results in the swelling of the retina [5]. Due to progressive dysfunction, the capillaries die prematurely resulting in ischemia that can be followed by neovascularization and finally retinal detachment and blindness [6, 7].

In the development of diabetic retinopathy, the basement membrane thickens and the blood pressure is altered. In addition pericytes and endothelial cells undergo accelerated apoptosis leading to pericyte ghosts and acellular capillaries [8]. The leukocytes become less deformable, and retinal leukostasis is increased affecting the endothelial function [9, 10].

The linkage between the development and progression of diabetic retinopathy and any pathway is still largely speculative. Several studies suggest that glucose-induced production of reactive oxygen species (ROS) stimulates several of the biochemical mechanisms that can be involved in hyperglycaemia-mediated complications of diabetes, including retinopathy. Cumulative studies suggest that many related hyperglycaemia-affected pathways play a major role in the pathogenesis of diabetic retinopathy. The most actively studied pathways are the oxidative stress pathway [11], polyol pathway activity [12, 13], formation of advanced glycation end product (AGEs) [14, 15], activation of protein kinase C (PKC) isoforms [16, 17], and increase in augmentation of the hexosamine pathway flux [18]. The linkage between the development of diabetic retinopathy and any pathway is still largely speculative. Previous studies used STZ to induce diabetes in experimental animals [6, 8, 14, 15, 18].

Cumulative studies demonstrated that dietary fiber can significantly reduce the risk of cardiovascular disease and type 2 diabetes mellitus [19]. This is due in part to the ability of fiber to reduce postprandial glycemia and improve long-term glycemic control [20, 21]. It was postulated that the rheological properties of soluble dietary fibers are highly related to their effects on control of the glucose concentration [22]. For instance, the ability of oat-derived β-glucan to reduce postprandial glycaemia has been strongly correlated with its viscosity [23], demonstrating an inverse linear relationship between the logarithm of viscosity measures and peak postprandial plasma glucose and insulin responses after consuming various doses of purified oat β-glucan with a 50 g oral glucose load. Despite these findings, the levels of viscosity required to achieve specific glucose-lowering effects are poorly understood. Still, the majority of trials investigating dietary fiber have not accounted for the principles of polysaccharide solubility and viscosity as the main determinants of its physiological outcome. While a small number of studies have shown the effect of oat on diabetes [24, 25], none examine its effect on the development and progression of diabetic retinopathy.

The aim of this study is to evaluate the effect of oat on the hyperglycemia-induced oxidative stress and if this can attenuate the development of diabetic retinopathy. Because oat is natural and widely used, the results of this study may provide an alternative for enhancing nutrition and diabetic control during diabetic retinopathy.

2. Materials and Methods

2.1. Induction of Diabetic Retinopathy Model and Study Design. Nine-week-old 200 ± 20 g male Albino rats were housed in cages and received normal rat chow diet and tap water ad libitum in a constant environment (room temperature 28 ± 2°C, room humidity 60 ± 5%) with a 12 h light, 12 h dark cycle. The animals were kept under observation for one week prior to the start of the experiments. All procedures were done according to the Animal Ethics Committee. 10 rats were randomly selected as control group (group 1, n = 10), which received a single tail vein injection of 0.1 mol/L citrate buffer only. The other 45 rats received a single dose of STZ (Sigma S-0130) in citrate buffer pH 4.5 through the indwelling catheters over 2 min, at a fixed dose of 60 mg/kg [16]. Only rats with blood glucose higher than 250 mg/dL after two days were considered as being diabetic in the fasting state. Blood glucose was measured by using *one touch select* Sensor Analyzer (Life Science, UK). Rats with blood glucose levels lower than 200 mg/dL were excluded from the study. All studies were carried out two days after STZ injection. Diabetic rats were classified to four groups each contains ten rats: group 2, untreated diabetic untreated rats (n = 10) and groups 3–5 (n = 10 rats each), oat treated diabetic rats. Rats of these groups were supplemented with oat 5, 10, and 20%, respectively, on the diet (W/W). Treatment was continued for 12 weeks starting from day two after STZ administration. At the end of the experiments, the final body weight of the various groups was recorded. Then, animals were fasted overnight (18 h) and then anesthetized [26]. Blood was collected directly from the heart of each animal. Serum was used for the determination of glucose, total protein, and albumin using a Cobas integra 800 automatic analyzer of Roche Diagnostic (USA) according to the instructions of the manufacturer. Rat eyes from each group were removed, washed with cold normal saline, and used for preparation of the eye homogenate and histopathological examinations.

2.2. Eye Homogenate Preparation. Retinal protein was extracted from freshly enucleated eyes (n = 9 from diabetic groups and n = 6 from the control group) and processed as described [26]. The isolated individual retinae were rinsed thoroughly with ice-cold phosphate-buffered saline to remove blood components and homogenized in a lysis buffer (containing 63 mmol/L Tris-HCl, pH 6.8; 1% Nonidet P-40; 0.25% SDS; 150 mmol/L NaCl; 1 mmoL/L EDTA; 5 mmol/L EGTA; 1 mmol/L phenyl methyl sulphonyl fluoride; 1 μg/mL of aprotinin and leupeptin; 2 mmol/L benzamidine; 1 mmol/L NaF; 10 nM okadaic acid; and 0.1% SDS). The supernatant was aliquoted and stored at −80°C and assayed for protein concentration using BCA kit (Pierce, Rockford, USA) using albumin diluted in lysis buffer as standard.

Part of the retina was homogenized with 100 μL TOTEX buffer (100 mM HEPES-KOH, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM

EGTA, 10 μg/mL leupeptin, 0.5 mM DTT, 0.2 mM PMSF) for 30 seconds, incubated in ice bath for 30 minutes, vortexed, and centrifuged at 13000 rpm for 5 minutes. The supernatant which contained the total retinal extract was transferred to a fresh tube and kept at −80°C for electrophoretic mobility shift assay (EMSA) [27].

2.3. Determination of Reduced GSH. Glutathione (GSH) plays a central role in protecting mammalian cells against damage incurred by free radicals, oxidants, and electrophiles. Reduced GSH was measured by colorimetric end-point assay using dithionitrobenzoic acid method as described by Moron et al. and Mekheimer et al. [28, 29]. GSH concentration was expressed as μmol/mg protein using GSH Kit from Roche (Mannheim, Germany) according to the instructions of the manufacturer.

2.4. Determination of Lipid Peroxidation. The concentration of TBARS was determined as MDA according to Okhawa et al. and Sayed [30, 31]. The degree of lipid peroxidation in the retina was determined using the Bioxytech LPO-586 kit. The concentration of MDA was expressed in terms of nmol/mg protein.

2.5. Determination of SOD Activity. SOD activity was determined as the volume of homogenate that is required to scavenge 50% of the superoxide anion generated from the photo illumination of riboflavin in the presence of EDTA (1 unit of SOD activity) [32, 33]. The activity was determined using the SOD available kit from BioVision Research Products (Linda Vista Avenue, USA) according to the instructions of the manufacturer.

2.6. Assessment of Retinal CML. The supernatant of the retinal homogenate was tested for CML using the anti-CML rat autoantibody ELISA kit which employs the semiquantitative enzyme immunoassay technique. The absorbance of the resulting yellow product is measured at 450 nm [34–36]. The levels of CML of the retinal extract were determined using the ELISA kit from Roche Diagnostics (Mannheim, Germany) according to the instructions of the manufacturer.

2.7. Assay of TNFα and VEGF. The levels of TNFα and VEGF in the isolated retinal proteins were determined as previously described [37], using a specific rat ELISA kit. The ELISA kits were obtained from BD Biosciences, Pharmingen (San Diego, CA, USA). Determination of TNFα and VEGF were performed according to the manufacturer's instructions. The reaction was stopped and absorbance was read immediately on an ELISA reader (Model 3550, BIO-RAD Laboratories, Ca, USA). The levels of TNFα and VEGF were expressed as pg/mg protein.

2.8. Electrophoretic Mobility Shift Assay (EMSA). The retinal extract was assayed for the transcription factor binding activity using the NF-κB-p65 consensus sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides as previously described [38, 39]. EMSA experiments were performed at least three times.

2.9. Histopathological Examinations. After 12 weeks of the experiment, rats were scarified and eyes were enucleated and fixed in formalin. Sections of the entire globe were prepared, stained with haematoxylin and eosin (H&E), and examined by light microscope.

2.10. Statistical Analysis. Statistical analysis was performed using the SPSS software. The effect of each parameter was assessed using the one way analysis of variance. Individual differences between groups were examined using Dunnett's test and those at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Blood Biochemical and Physiological Parameters. The weights of the rats at the beginning of the study were similar in all groups. At the end of the experiment, diabetic animals presented a significant weight loss. The initial and final body weights were not significantly different in groups 1, 3, 4, and 5 (Table 1). Treatment of rats with STZ resulted in a significant increase in blood glucose levels in the diabetic untreated group compared with the control group ($P = 0.01$), while treatment with 5, 10, and 20% oat resulted in a significant decrease in blood glucose compared with the untreated diabetic animals ($P < 0.05$, Table 1). As a result of diabetes, HbA1c and total protein were significantly increased ($P < 0.05$) in the diabetic untreated group. Treatment of animals with oat improves the two parameters in a dose-dependent manner (Table 1). The concentration of serum albumin was not affected by STZ treatment.

3.2. Oxidant/Antioxidant Parameters. The antioxidant enzyme activities like catalase, glutathione reductase, and glutathione peroxidase and the concentration of reduced GSH in the blood of diabetic animals were significantly reduced as a result of STZ treatment. Supplementation of oat resulted in a significant increase of the activity of these enzymes and in the level of GSH (Table 1). This increase in the activity of the antioxidant enzymes was found to be dose-dependent. In addition, the oxidative stress biomarkers in the retina were assayed. The activities of catalase, SOD, glutathione-S-transferase, and glutathione peroxidase were significantly reduced as a result of STZ administration. As a result of oat supplementation, the reduced activities of the antioxidant enzymes were increased in a dose-dependent manner (Table 2). The degree of lipid peroxidation in the retina was significantly elevated as a result of diabetes. Administration of oat resulted in a dose-dependent decrease of MDA levels (Table 2).

3.3. Effects of Oat on the Retinal CML. As a result of diabetes the production of AGEs increases. In the present study we measure the retinal levels of CML. CML was significantly

Table 1: Initial and final body weights, blood biochemical, and physiological parameters of the rats.

	Group 1	Group 2	Group 3	Group 4	Group 5
Initial body weight, g	195.13 ± 9.5	196.44 ± 7	194.19 ± 6	197 ± 7.2	196.33 ± 6.5
Final body weight, g	265.5 ± 8.2	161.8 ± 7.2[a]	203.13 ± 16.5[a,b]	215.52 ± 8.9[a,b]	234 ± 12.3[a,b]
Glucose, mg/dL	92.78 ± 0.45	265.35 ± 1.45[a]	115.92 ± 1.4[a,b]	106.71 ± 1.23[b]	96.21 ± 0.57[a,b]
HbA1c, %	5.24 ± 0.41	9.42 ± 0.34[a]	7.88 ± 0.54[b]	6.85 ± 0.38[b]	6.88 ± 0.52[b]
Albumin, g/L	38.4 ± 5.7	38.2 ± 7.7	38.5 ± 6.5	38.7 ± 7.5	38.9 ± 8.1
Total protein, g/L	76 ± 7	62 ± 6.5[a]	63.2 ± 6.5[a]	65.6 ± 7.1	69.91 ± 7.5[b]
GSH, mmol/L	0.281 ± 0.015	0.105 ± 0.03[a]	0.116 ± 0.02[a]	0.145 ± 0.021[a,b]	0.172 ± 0.02[a,b]
Catalase, U/gHb	94.54 ± 14	50.98 ± 15[a]	62.13 ± 13[a,b]	70.11 ± 11[a,b]	86.55 ± 12[b]
Glutathione reductase, U/gHb	4.25 ± 0.09	2.25 ± 0.62[a]	2.31 ± 0.71[a,b]	2.54 ± 0.54[a,b]	3.21 ± 0.94[b]
Glutathione peroxidase, U/gHb	57.1 ± 11	145.5 ± 45[a]	129 ± 11.1[a,b]	89.54 ± 10.2[b]	69.9 ± 9.55[b]

Data are expressed as the means ± SD. Group 1: normal control; group 2: diabetic untreated and groups 3–5: diabetic rats treated with 5, 10, and 20% (W/W) oat in the diet, respectively. Each group consisted of 10 animals.

[a]$P < 0.05$ versus normal control group, [b]$P < 0.05$ versus diabetic untreated group.

Table 2: Oxidant/antioxidant parameters as well as concentration of CML in the rat retina.

	Group 1	Group 2	Group 3	Group 4	Group 5
MDA, nmol/mg protein	2.45 ± 0.16	4.75 ± 0.17[a]	3.98 ± 0.52[a,b]	3.15 ± 0.25[a]	2.95 ± 0.15[b]
GST, nmol substrat·mg protein^{-1}·min^{-1}	176 ± 31	82 ± 13[a]	105 ± 17[a,b]	125 ± 11[a,b]	151 ± 9[a,b]
GSH-Px, nmol substrat·mg protein^{-1}·min^{-1}	0.92 ± 0.17	0.34 ± 0.09[a]	0.55 ± 0.1[a,b]	0.67 ± 0.15[a,b]	0.82 ± 0.165[b]
Catalase, IU·mg protein^{-1}	2.61 ± 0.032	0.52 ± 0.03[a]	0.56 ± 0.02[a]	0.96 ± 0.04[a,b]	1.45 ± 0.21[a,b]
SOD, nmol substrat·mg protein^{-1}·min^{-1}	3.53 ± 0.45	1.82 ± 0.35[a]	1.92 ± 0.5[a]	2.3 ± 0.6[a]	2.95 ± 0.75[b]
GSH, nmol/mg protein	16 ± 3	16.11 ± 2	16.1 ± 2.1	15.95 ± 2	16 ± 2.3
CML, pg/mg protein	3.54 ± 0.22	8.81 ± 0.34[a]	5.42 ± 0.35[a,b]	3.85 ± 0.55[b]	3.2 ± 0.6[b]

Data are expressed as the means ± SD. Group 1: normal control; group 2: diabetic untreated group; groups 3–5: diabetic groups treated with 5, 10, and 20% (W/W) oat in the diet, respectively. Each group consisted of 10 animals.

[a]$P < 0.05$ versus normal control group, [b]$P < 0.05$ versus diabetic untreated group.

elevated in the STZ-diabetic rats compared with the normal control rats. Treatment of rats with 5, 10, and 20% (W/W) oat in the diet for 12 weeks resulted in lowering of these elevated levels. The reduction of CML formation was found to be significant and dose-dependent when compared with the diabetic untreated rats (Table 2).

3.4. Effect on the Levels of TNFα and VEGF. Both TNFα and VEGF play an important role in the pathogenesis of diabetic retinopathy. The levels of TNFα and VEGF were measured in the retina of the rats. As a result of STZ injection the retinal levels of TNFα and VEGF were significantly elevated indicating a considerable level of inflammation compared with the normal control rats. Administration of 5, 10 and 20% (W/W) oat in the diet for 12 weeks resulted in a significant and a dose-dependent reduction of these elevated levels. This reduction was shown in Figures 1 and 2.

3.5. Histopathological Findings. On microscopic examination, the layers of retinae were intact in all animal groups (Figure 3). There was no significant difference in the cellular content of the ganglion cell layer. A previous study showed that the observation of retinopathy in normal H&E staining can be seen only 30 weeks after induction of diabetes [40, 41].

3.6. Effects of Oat on the Activation of NF-κB. As a result of diabetes and enhanced formation of AGEs, NF-κB-p65 was markedly activated in the diabetic untreated group compared with the normal control group. The activation of NF-κB-p65 was due to the high concentration of AGEs. These elevated AGEs interact with their receptor RAGE resulting in the obtained activation of NF-κB-p65. Oat supplement resulted in a significant reduction of the activated NF-κB-p65 in a dose-dependent manner as indicated in Figure 4. As mentioned in Table 2, oat attenuated AGE (in particular CML) production which resulted in the reduction of NF-κB-p65 in Figure 4.

4. Discussion

The present study demonstrated that the interference with the overproduction of ROS by oat in the diabetic rats normalizes parameters of oxidative stress in the diabetic retina and prevents the activation of major pathways involved in hyperglycaemia-induced vascular damage. Oat reduced or

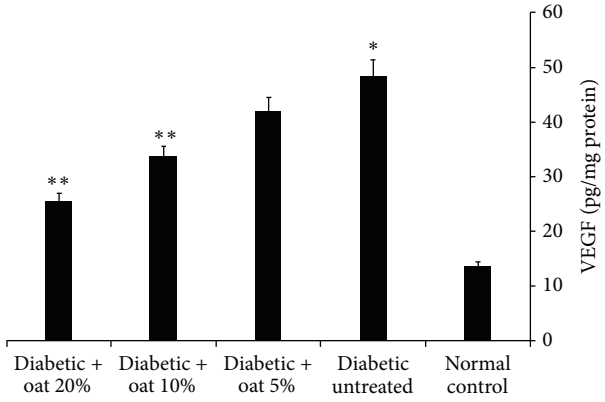

Figure 1: Retinal VEGF levels assessed at 12 weeks of oat treatment. VEGF was significantly higher in the diabetic untreated group than in the normal control group. Oat treatment resulted in a dose-dependent reduction of the elevated VEGF levels. Data are mean ± SE ($n = 10$ retinas/group), $^{*}P < 0.01$ versus normal control, and $^{**}P < 0.01$ versus diabetic untreated group.

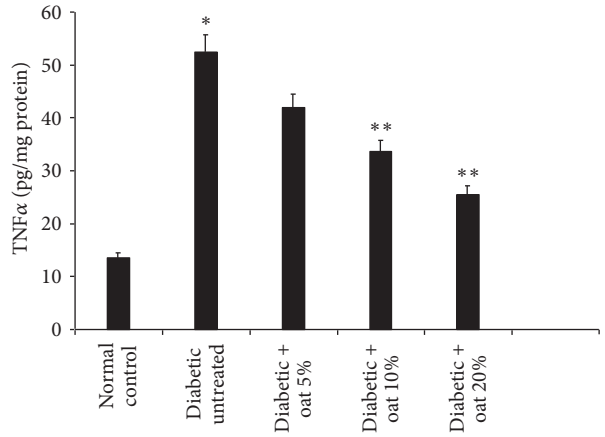

Figure 2: Retinal TNFα levels assessed at 12 weeks of oat treatment. TNFα was markedly elevated in the diabetic untreated group than in the normal control group. Tretment of rats with oat resulted in a dose-dependent reduction of the elevated TNFα levels. Data are mean ± SE ($n = 10$ retinas/group), $^{*}P < 0.01$ versus normal control, and $^{**}P < 0.03$ versus diabetic untreated group.

even normalized downstream effectors of vascular response to injury. These *in vivo* data show an evidence of oxidative protein modification. Earlier short-term studies using antioxidants showed inhibition of retinal lipid peroxidation products and superoxide dismutase, but no change in enzymes of glutathione metabolism [42]. In addition, the reduction of ROS overproduction and the associated reduction of intracellular CML suggest an indirect AGE-inhibiting effect of oat.

RAGE is an important receptor for mediating AGE effects [43]. RAGE is normally expressed in the inner vascularised part of the diabetic retina, both in neuroglial and in vascular cells [44]. Binding of AGE such as CML to RAGE enhances oxidative stress in retinal tissue [44]. RAGE signals via NF-κB activate target genes which have a harmful potential for the diabetic retina [45]. Oat reduced NF-κB-p65 antigen and NF-κB binding activity. Thus, our data suggest that part of the beneficial effects of oat includes the disruption of the detrimental AGE-RAGE-NF-κB pathways.

Both preclinical and clinical studies have shown the significance of VEGF in the pathogenesis of proliferative diabetic retinopathy [46, 47]. Similarly, in the present study expression of retinal VEGF has been found to be significantly increased in diabetic untreated rats compared to the normal control rats, while oat treatment attenuated the expression of VEGF. Various studies have shown that antioxidants inhibit VEGF-mediated angiogenesis [48]. Eventually, antioxidants have been found to inhibit angiogenesis by abrogating VEGF signaling through interfering with the formation of VEGF receptor 2 complex which may have a physiological significance in the management of diabetic retinopathy [49].

TNF-α contributes to the pathogenesis of diabetic retinopathy [50], and significantly higher levels of TNF-α are found in the plasma of patients affected by either type 1 or type 2 diabetes versus age-matched healthy control subjects [51]. Earlier, the role of inflammatory cytokine TNF-α in the apoptotic cell death of retinal endothelial cells during early and late stages of diabetic retinopathy in a rat model of streptozotocin-induced diabetes has been investigated [52]. Similarly, in the present study TNF-α expression was significantly increased in the diabetic untreated group compared with the normal control group, and the oat-treated groups showed significantly lower levels of TNF-α than the diabetic untreated group. Other studies also reported that antioxidants dose-dependently inhibit inflammatory markers like TNF-α, IL-6, and IL1β gene expression in the cells of chronic diseases and their release from a human cancer cell line [53–58].

In summary, the present study clearly demonstrates that both of controlling of hyperglycaemia and catalytic scavenging of reactive oxygen species are effective approaches for the prevention of diabetic retinopathy. Oat is a paradigm natural food supplement with a broad spectrum of beneficial biochemical and cell biological effects, based on its ability to reduce the hyperglycaemia-induced ROS overproduction. Since oat also has beneficial effects on other target tissues of diabetic angiopathy and shows beneficial effects of mediators of large vessel damage, this concept appears attractive for the prevention or delay of diabetic angiopathy. In conclusion, it can be postulated that oat could have potential benefits in the prevention of the onset and progression of retinopathy in diabetic patients.

Abbreviations

CML: Carboxymethyllysine
MDA: Malondialdehyde
TBARS: Thiobarbituric acid reactive substance
SOD: Superoxide dismutase
HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
NP-40: Nonylphenoxy polyethoxy ethanol
EGTA: Ethylene glycol-bis(β-aminoethyl ether)
DTT: Dl-dithiothreitol
PMSF: Phenylmethylsulfonyl fluoride.

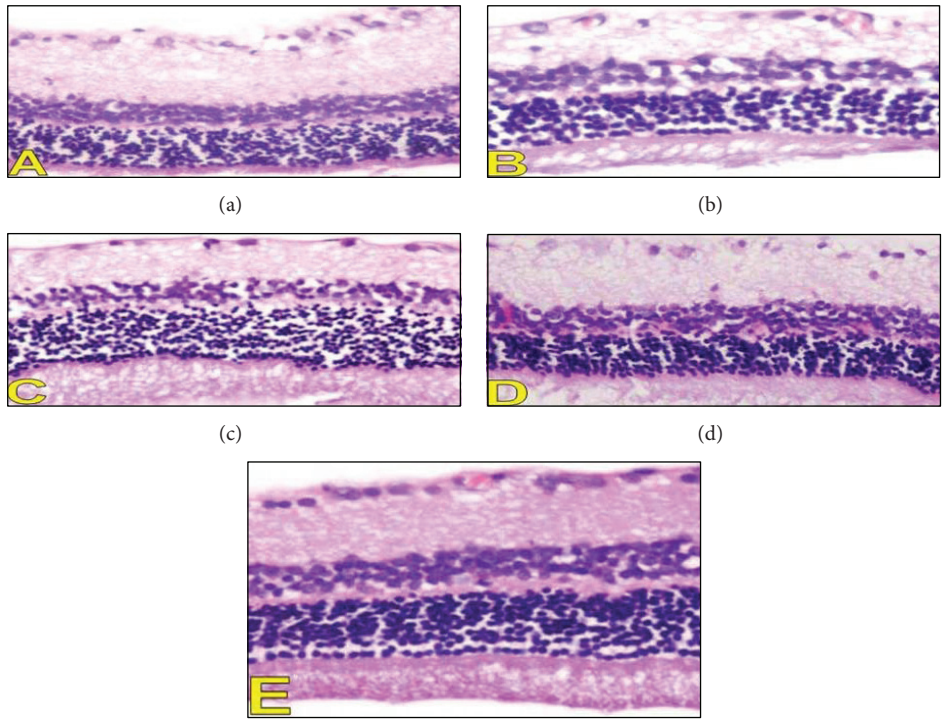

Figure 3: Histopathological findings in the retina. A photomicrography representing H&E sections from different studies groups (A: diabetic group, B–D: diabetic rats treated with 5, 10 and 20% oat in the diet (W/W) respectively, and E: Control group) (200x).

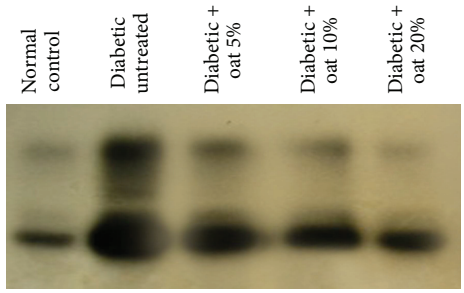

Figure 4: EMSA for NF-κB for total retinal extract. Compared with the normal group, the diabetic untreated group shows markedly increased NF-κB expression and the oat-treated groups show a dose-dependent decreased expression.

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Research Article

Fisetin Inhibits Hyperglycemia-Induced Proinflammatory Cytokine Production by Epigenetic Mechanisms

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Diabetes is characterized by a proinflammatory state, and several inflammatory processes have been associated with both type 1 and type 2 diabetes and the resulting complications. High glucose levels induce the release of proinflammatory cytokines. Fisetin, a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggygria*), and is also widely distributed in fruits and vegetables. Fisetin is known to exert anti-inflammatory effects via inhibition of the NF-κB signaling pathway. In this study, we analyzed the effects of fisetin on proinflammatory cytokine secretion and epigenetic regulation, in human monocytes cultured under hyperglycemic conditions. Human monocytic (THP-1) cells were cultured under control (14.5 mmol/L mannitol), normoglycemic (NG, 5.5 mmol/L glucose), or hyperglycemic (HG, 20 mmol/L glucose) conditions, in the absence or presence of fisetin. Fisetin was added (3–10 μM) for 48 h. While the HG condition significantly induced histone acetylation, NF-κB activation, and proinflammatory cytokine (IL-6 and TNF-α) release from THP-1 cells, fisetin suppressed NF-κB activity and cytokine release. Fisetin treatment also significantly reduced CBP/p300 gene expression, as well as the levels of acetylation and HAT activity of the CBP/p300 protein, which is a known NF-κB coactivator. These results suggest that fisetin inhibits HG-induced cytokine production in monocytes, through epigenetic changes involving NF-κB. We therefore propose that fisetin supplementation be considered for diabetes prevention.

1. Introduction

Diabetes and its complications are known for their chronic inflammatory properties. Inflammation is a defense reaction of host tissue against diverse insults. Although normally a beneficial process, when it occurs over prolonged periods, inflammation can be deleterious to cells [1]. Hyperglycemia has been implicated in diabetes-induced inflammatory disease and several diabetes-related complications [2, 3]. It has been reported to induce oxidative stress [4, 5]. In addition, high levels of glucose can activate the proinflammatory transcription factor nuclear κB (NF-κB), resulting in increased inflammatory chemokine and cytokine release [6, 7].

We and other researchers have recently shown that diabetic conditions activate inflammatory gene expression and monocyte activation by inducing epigenetic changes and chromatin remodeling [8, 9]. However, the exact molecular mechanisms induced by hyperglycemia are not fully resolved.

NF-κB is required for the expression of many inflammatory genes. Several of these genes have been associated with inflammatory diseases, including atherosclerosis, insulin resistance, metabolic syndrome, and diabetes and its complications [10].

In mammals, the NF-κB family contains 5 members: RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52. The classical NF-κB transcription factor is a p50-RelA/p65 heterodimer [11], which regulates transcription of a number of inflammatory genes. NF-κB activity is regulated by RelA/p65 acetylation and deacetylation, which are mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively [12, 13]. In addition to regulating

NF-κB activity, HATs and HDACs regulate inflammatory processes by acetylation/deacetylation of histones [14, 15].

The term "epigenetic" is used to describe heritable changes in phenotype or gene expression caused by mechanisms that do not involve modulation of DNA sequence [16]. Recently, epigenetic modifications have also been implicated in disease-associated changes in gene expression [17].

The best understood types of epigenetic regulation are DNA methylation and histone posttranslational modification. Histones can be subject to a number of different posttranslational modifications, among which acetylation and deacetylation have been extensively studied. In general, addition of acetyl groups to histones by HATs results in an "open" chromatin conformation, facilitating gene expression by allowing transcription factors to access DNA. In contrast, removal of acetyl groups by HDACs results in a "closed" chromatin environment, which has a repressive effect on transcription [18]. Thus, it is obvious that a tightly regulated balance between the activities of these 2 antagonistic enzymes is required for proper gene expression, while disruption of this balance can result in pathological conditions [19].

Altered HAT and HDAC activities can lead to several diseases, including cancer, diabetes, cardiac hypertrophy, and asthma [14, 15, 20, 21]. Recently, the *in vivo* relevance of histone acetylation in diabetes and inflammation was supported by a study demonstrating increased levels of histone lysine acetylation on inflammatory gene promoters in monocytes isolated from both type 1 and type 2 diabetes patients, relative to healthy control volunteers [9]. Other studies have addressed targeting of epigenetic mechanisms as a putative therapeutic means in the treatment of diabetes, primarily focusing on the use of small molecule HDAC modulators of natural origin [6, 7, 20–22]. However, the mechanisms of action of such molecules remain largely unknown.

Flavonoids are natural molecules examined as putative anti-inflammatory agents. They are low-molecular-weight polyphenolic compounds abundantly found in seeds, citrus fruits, red wine, tea, and olive oil. Flavonoids have diverse biological properties: in addition to their anti-inflammatory function, they have been described to exert antioxidant, antiplatelet, antithrombotic, cytoprotective, antiallergic, antiviral, and anticarcinogenic effects [23–26]. Due to their abundance in dietary products and their potential beneficial pharmacological and nutritional effects, flavonoids are of considerable interest both as drugs as well as health food supplements. Fisetin (3,7,3′,4′-tetrahydroxyflavone) is a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggygria*) and is also widely distributed in fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber. It exhibits various activities, including neurotrophic, antioxidant, anti-inflammatory, and antiangiogenic effects [23, 27–29]. Fisetin has also been reported to downregulate glycogenolysis, gluconeogenesis, and formation of glycated hemoglobin *in vitro* [30, 31]. However, to date, the molecular mechanism of fisetin action remains unknown.

In the current study, we sought to address the use of fisetin as an anti-inflammatory agent, analyzing its molecular mechanism of action under diabetic conditions. We hypothesized that fisetin suppresses proinflammatory cytokine secretion through the NF-κB signaling pathway, by altering the balance between histone acetylation and deacetylation. To test our hypothesis, we used human monocytes cultured under high-glucose conditions and analyzed the effect of fisetin on HAT and HDAC activity, NF-κB acetylation, and inflammatory gene expression.

2. Materials and Methods

2.1. Reagents. Fisetin was purchased from Sigma Aldrich (St Louis, MI, USA). Fisetin was kept as a stock solution in Dimethyl Sulfoxide (DMSO) and was diluted with culture medium. We used 0.1% (v/v) DMSO as a vehicle control in all experiments. Real-time PCR primers were purchased from Bioneer (Daejeon, Korea). Antibodies against NF-κBp65, phosphorylated NF-κBp65, acetylated p65, p300, and acetylated CBP/p300 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against HDAC-1, HDAC-2, and HDAC-3 were purchased from Abcam (Cambridge, MA, USA). Tumor necrosis factor α (TNF-α) and IL-6 ELISA assay kits were also purchased from Abcam (Cambridge, MA, USA). HAT and HDAC assay kits were purchased from Biovision (Mountain View, CA). The BCA protein assay kit was purchased from Pierce. Novex precast Tris-Glycine gels were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell Culture. The human monocytic THP-1 cell line was obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI medium containing 10% fetal bovine serum and 1% antibiotics at 37°C in a 5% CO_2 atmosphere. Fisetin dissolved in DMSO was used for treatment of the cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. THP-1 cells (1×10^5 cells/mL) were cultured in the presence of osmolar control (14.5 mmol/L mannitol), under normal glycemic (NG, 5.5 mmol/L glucose), or hyperglycemic (HG, 20 mmol/L glucose) conditions, in the absence or presence of fisetin (0, 3, 6, 10 μM) for 48 h. Following this period, the medium was collected for measurement of cytokine release; cells were washed with phosphate-buffered saline (PBS) and then harvested.

2.3. Cell Viability Assay. The toxic effects of fisetin on cultured THP-1 cells was measured by Cell Counting Kit-8 (Dojindo Molecular Technologies, ML, USA), according to the manufacturers protocol. THP-1 cells were seeded at 4×10^3 cells/well in a 96-well plate and subsequently treated with fisetin for 48 h. Absorbance was measured using a Wallac EnVision microplate reader (PerkinElmer, Finland). The inhibitory effect of fisetin on growth was assessed as the percentage of cell growth reduction compared to vehicle-treated cells, which were defined as 100% viable.

2.4. Cytokine Release Measurement. Cytokine levels were measured using ELISA assay kits (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Values were calculated based on a standard curve constructed for the assay.

2.5. Evaluation of mRNA Levels. Primers were designed using an online program. Total RNA was isolated using TRIzol reagent (Life Technologies, MD), according to the manufacturers' protocol. The concentration and purity of total RNA were assessed by measuring absorbance at 260 and 280 nm. First-strand cDNA was synthesized starting from 2 μg of total RNA, using 1 μM of oligo-dT_{18} primer and Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR was performed with the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA), using 3 μL of first-strand cDNA diluted 1 : 50 as a template, and 10 pmoles of primers, according to the manufacturer's protocols. The PCR reaction consisted of three segments: the first segment (95°C for 10 min) activated the polymerase; the second segment included 35 cycles, each consisting of 40 s of denaturation at 94°C, followed by 40 s of annealing at 60°C, and 1 min of extension at 72°C; the third segment was performed to generate temperature dissociation curves of the products, by incubation at 95°C for 1 min, followed by incubation for 30 s at 55°C and 30 s at 95°C. All reactions were run in triplicate, and data were analyzed by the $2^{-\Delta\Delta CT}$ method [32]. GAPDH was used as a normalization control gene. Significance was determined by comparison with GAPDH-normalized $2^{-\Delta\Delta CT}$ values.

2.6. Measurement of HDAC and HAT Activity Using ELISA. Following treatment with various concentrations of fisetin for 48 h, cells were harvested and nuclear lysates were prepared. For determination of HAT and HDAC activity, nuclear lysate containing 50 μg of protein was taken from each group. The experiment was performed according to the manufacturer's instructions. Absorbance was measured at 405 nm and 440 nm.

2.7. Preparation of Nuclear and Cytoplasmic Lysates. After treatment with fisetin, the medium was aspirated and cells were washed twice in PBS (10 mM, pH 7.4). Nuclear lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, IL, USA). Lysates were collected and cleared by centrifugation, and the supernatant was aliquoted and stored at −80°C. The protein concentration of the lysates was measured by BCA protein assay (Pierce, IL, USA), as per the manufacturer's protocol.

2.8. Western Blot Analysis. For western blot analysis, cells were homogenized in buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF, and one protease inhibitor cocktail tablet (Roche, Germany) at 4°C, and then centrifuged at 10,000 ×g for 15 min. The supernatant was used as the cytoplasmic protein fraction, and nuclear proteins were extracted from the pellet using the NucBuster Protein Extraction kit (Novagen, Germany). Protein concentration was determined using the BCA protein assay kit (Pierce, IL). Samples (20 μg of total protein) were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue (pH 7.6), incubated at 95°C for 15 min, and loaded on 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell system (Bio-Rad, CA). The resolved proteins were transferred on a nitrocellulose membrane (Scheicher & Schnell BioScience, Germany). To visually assess the amount of protein loaded and the efficiency of the transfer, membranes were stained with Ponceau S staining solution. For immunoblotting, membranes were washed and incubated in blocking buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 3% nonfat dry milk), and then incubated with diluted primary antibodies (1 : 1000) for 2 h at room temperature. Following incubation with the primary antibody, membranes were washed 3 times with blocking buffer and then probed with diluted secondary antibodies (1 : 2000) for 1 h. The membranes were washed 3 times (15 min each) and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, IL, USA), using a LAS-3000 luminescent image analyzer (Fuji Photo Film Co. Ltd., Japan).

2.9. Immunoprecipitation Assays. For immunoprecipitation, we isolated the nuclear fraction of cells, as per the manufacturer's protocol. Samples (300 μg of total protein) were precleared with protein A/G plus agarose (Santacruz Biotechnology Inc., CA USA) for 1 h at 4°C. After 1 h, the supernatant fraction of each sample was transferred to a fresh tube and incubated with the respective antibody (2 μg/mL) overnight at 4°C. Following incubation with the antibody, samples were mixed with 40 μL of protein A/G plus agarose for 2 h at 4°C. The samples were subject to microcentrifugation and then washed 3 times with PBS. Following the washing steps, samples were mixed with 15 μL of 2x SDS sample buffer and then subjected to western blot analysis.

2.10. Chromatin Immunoprecipitation Assays. ChIP assays were performed using MAGnify ChIP according to the manufacturer's instructions. After treatment of cells with fisetin, the cells were centrifuged and medium was aspirated. Cells were washed twice in PBS (10 mM, pH 7.4) and fixed with fresh Fixation solution (37% formaldehyde) for 10 min at room temperature, followed by glycine stop-fix solution. Cells were washed twice with cold PBS, PBS was poured off and discarded and cells were scraped, pelleted by centrifugation for 10 min at 7,000 rpm at 4°C. Cells were resuspended in 100 μL of ice-cold lysis buffer followed by 5 min incubation on ice. Pellets were spun down for 10 min at 5000 rpm. Chromatin was sheared using high power sonication using our optimized condition (16 cycle; 30 pulses of 30 s each with a 30-s rest on ice between pulses) to an average DNA size of 500 bp and lysates were cleared by centrifugation at 13,000 for 10 min at 4°C. For each ChIP,

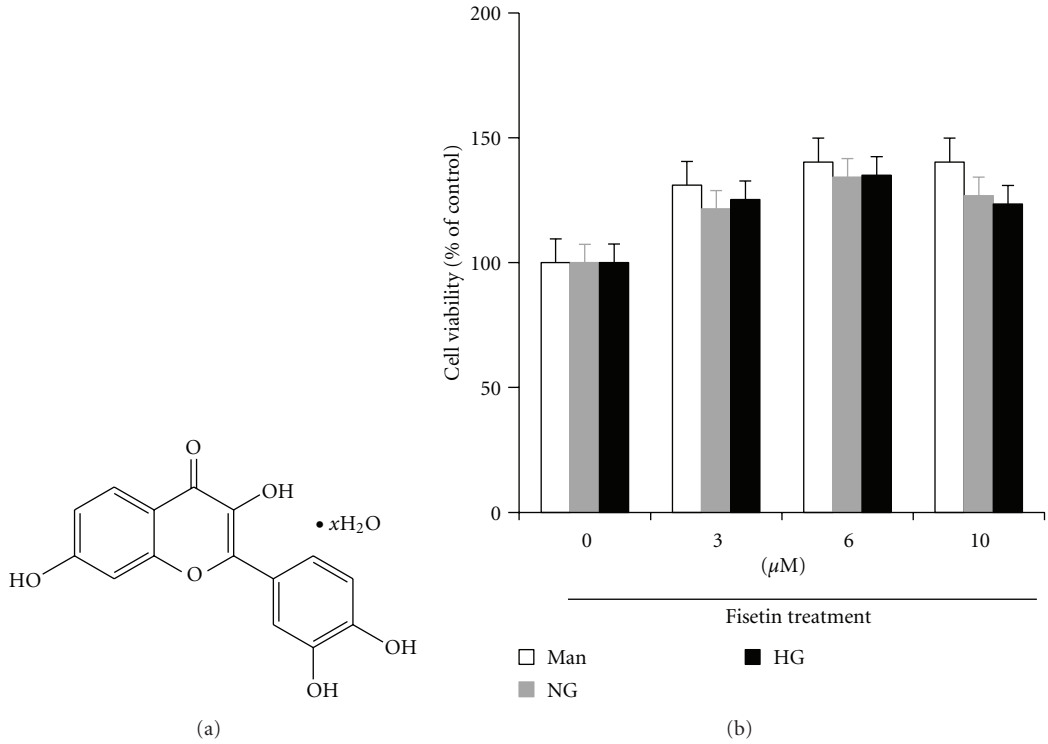

Figure 1: The cytotoxicity of fisetin to cultured high glucose-induced THP-1 cells. (a) Chemical structure of fisetin. (b) Effect of fisetin on cell viability after 48 h was evaluated by the CCK-8 assay, as described in the methods. Human monocytic (THP-1) cells (1×10^5 cells/mL) were cultured in presence of osmolar control (14.5 mmol/L mannitol) or normal glycemic (NG, 5.5 mmol/L glucose) or hyperglycemic (HG, 20 mmol/L) conditions in absence or presence of fisetin (0, 3, 6, 10 μM) for 48 h as described in the methods and the media was collected. Results are shown as mean ± SD of five different experiments.

one-tenth of the total sonicated chromatin volume (100 μL) was used. Immunoprecipitations were performed overnight at 4°C with 5 μg of the p300 antibody. Chromatin-antibody complexes were captured to magnetic beads (20 μL), and chromatin was eluted as described in manufacturer's instructions. The cross-links were reversed and DNA was purified by proteinase K. DNA was analyzed by PCR.

2.11. PCR. DNA concentration was measured spectrophotometrically at 260 nm. DNA was subjected to PCR. The antibodies against p300 were purchased from Santa Cruz Biotechnology. Primer sequences for the amplification of tumor necrosis factor α (TNF-α) were: forward: 5′-CCTCCCAGTTCTAGTTCTATC-3′ and reverse: 5′-GGGGAAAGAATCATTCAACCAG-3′. PCR was performed after a 5-minute denaturation at 94°C, and repeating the cycles of 94°C, 55°C for each 30 s and 72°C for 45 s; the 35 of cycles were specific for primer set. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide.

2.12. Statistical Analysis. Each experiment was performed at least 3 times. Results are expressed as the mean value ± standard deviation (SD). Statistical analysis was performed using Student's t-test, and statistical significance was set at $P < 0.05$ for some analyses and $P < 0.01$ for others. They have been separately indicated in the figures.

3. Results

3.1. Toxic Effects of Fisetin on Monocytes under Hyperglycemia. The chemical structure of fisetin is shown in Figure 1(a). We investigated the cytotoxic effect of fisetin on high glucose-induced THP-1 cells, using CCK-8 assay (Figure 1(b)). No toxicity was observed at concentrations of fisetin between 3 and 10 μM, for 48 h of treatment. All our experiments were performed in the latter, nontoxic concentration range of fisetin (Figure 1(b)).

3.2. Effects of Fisetin on Proinflammatory Cytokine Secretion in Monocytes under Hyperglycemic Conditions. We examined whether fisetin could inhibit proinflammatory cytokine genes, such as TNF-α and IL-6, in high-glucose-treated THP-1 cells. Under hyperglycemic conditions, inflammatory cytokine release was significantly increased compared to under normal glycemic conditions. Mannitol was used as a hyperosmolar control and did not affect cytokine release. As shown in Figure 2(a), treatment of fisetin significantly inhibited high glucose-induced mRNA expression levels of TNF-α and IL-6. To confirm the effect of fisetin on the expression of proinflammatory cytokines, culture media

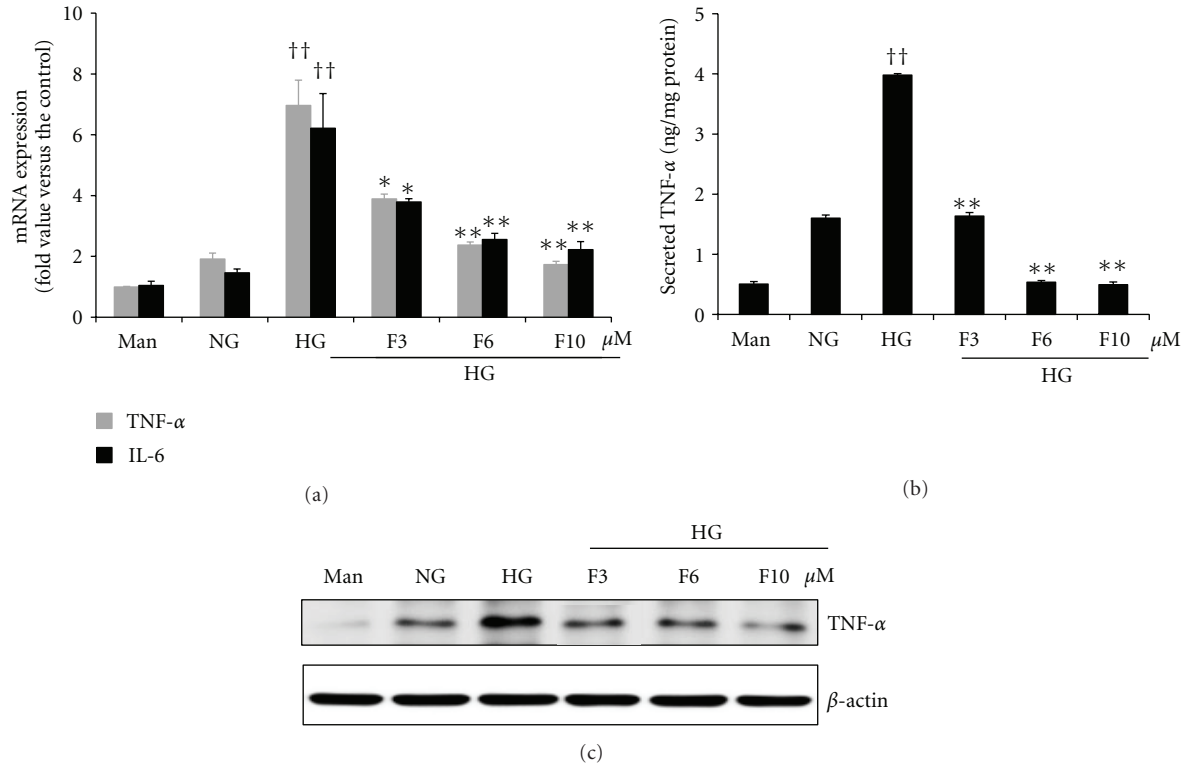

Figure 2: Fisetin-mediated inhibition of cytokine release in HG-treated THP-1 cells. (a) Cells (1×10^5 cells/mL) were treated with fisetin for 48 h and then mRNA levels were evaluated by quantitative real-time PCR. (b) Cell media were collected for TNF-α measurement by ELISA assay kit. Cytokine levels in the media were measured with ELISA assay kit according to the manufacturer's instructions. Values were calculated based on a standard curve constructed for the assay. Results are shown as mean ± SD of five different experiments. $^{\dagger\dagger}P < 0.01$ compared to NG; $^{*}P < 0.05$; $^{**}P < 0.01$ compared to HG. (c) Cell lysates were prepared and TNF-α level was evaluated by western blot analysis as described in the methods. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-actin protein. The immunoblots shown here are representative of 3 independent experiments.

were assayed for TNF-α levels by ELISA, and nuclear lysates were subjected to western blot assay. As shown in Figures 2(b) and 2(c), fisetin significantly decreased the secretion of cytokine, TNF-α, under hyperglycemic conditions in human monocytes.

3.3. Modulatory Effect of Fisetin on HAT and HDAC Activity in Monocytes under Hyperglycemic Conditions. We next addressed the mechanism by which fisetin inhibits cytokine gene expression in monocytes. To obtain further insights into the mechanisms of fisetin-induced downregulation of inflammatory cytokines, we first examined whether fisetin treatment modulates HAT and HDAC activity, using ELISA. As shown in Figure 3, under hyperglycemic conditions, there was a significant increase in HAT activity and decrease in HDAC activity compared to normal glucose conditions ($P < 0.01$). Interestingly, fisetin treatment results in a significant downregulation of HAT and upregulation of HDAC activity ($P < 0.01$). High glucose levels activate transcription factors, such as NF-κB, by recruitment of transcriptional coactivator molecules CBP/p300, which possess intrinsic HAT activity. The resulting increase in histone acetylation and DNA unwinding allow RNA polymerase to access DNA, leading to proinflammatory gene expression. As shown in Figure 3(c), THP-1 cells cultured under hyperglycemic conditions showed marked upregulation of p300 as well as its acetylation levels, compared with cells cultured under normal glycemic conditions. D-mannitol had no effect on p300. As shown Figure 3(c), p300 activation was abolished by fisetin (10 μM) treatment. Fisetin also decreased the levels of acetylated CBP/p300 in high-glucose conditions, to levels comparable to those observed under normal glucose conditions. No effects were observed with DMSO (0.1%) vehicle control treatment.

3.4. Effect of Fisetin on NF-κB p65 Activation in Monocytes under Hyperglycemic Conditions. Histone acetylation is associated with increased NF-κB activation that leads to increased acetylation of the RelA/p65 subunit of NF-κB [3]. Therefore, we studied the effect of fisetin on acetylation of p65 and subsequent NF-κB activation, under high-glucose conditions. We observed that fisetin resulted in significantly decreased acetylation and phosphorylation of NF-κBp65 in the nuclear faction (Figure 4).

3.5. Effect of Fisetin on the Interaction of p300 with Inflammation-Associated Genes. To further understand the

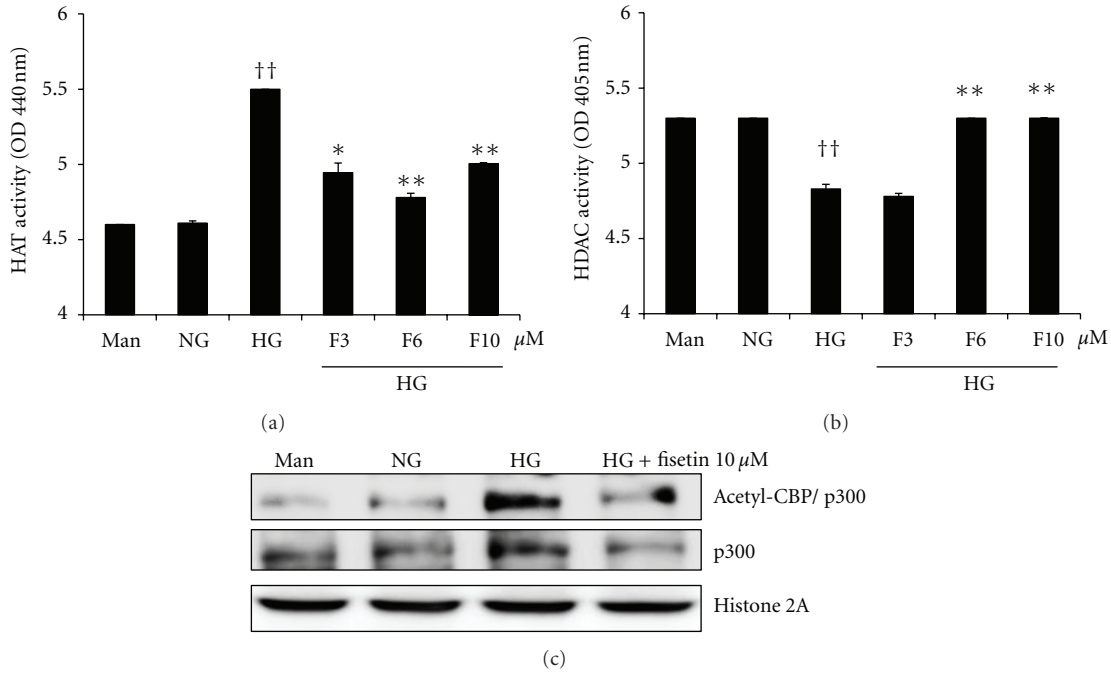

Figure 3: Effect of fisetin on HAT and HDAC activity as well as p300 and acetylated CBP/p300 levels in HG-treated THP-1 cells. Cells were harvested after 48 h of fisetin treatment and nuclear lysates were prepared. Samples were analyzed for determination of HAT (a) and HDAC activity (b). Results are shown as mean ± SD for 3 different experiments. $^{\dagger\dagger}P < 0.01$ compared to NG; $^{*}P < 0.05$; $^{**}P < 0.01$ compared to HG. (c) After nuclear protein extraction, p300 and acetylated CBP/p300 levels were evaluated by western blot. The immunoblots shown here are representative of 3 independent experiments.

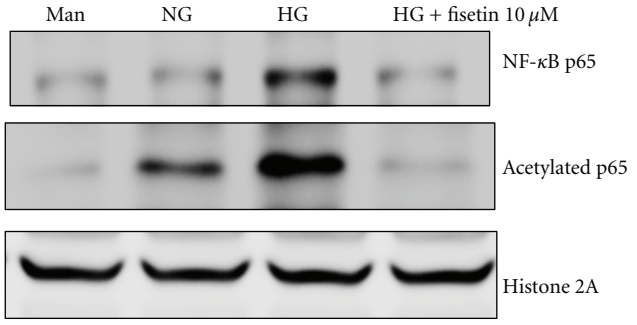

Figure 4: Fisetin-induced suppression of NF-κB activation in HG-treated THP-1 cells. Protein levels were evaluated by western blot for NF-κB p65 and acetylated p65. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for histone 2A protein. The immunoblots shown here are representative of 3 independent experiments.

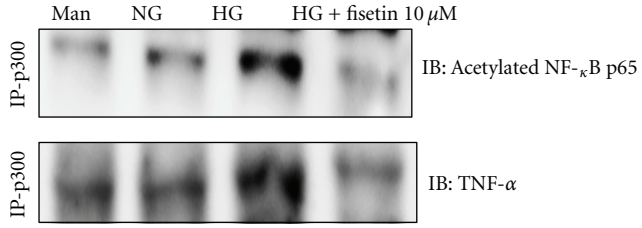

Figure 5: Effect of fisetin on the interaction of p300 with acetylated p65 and TNF-α. Cells were treated with fisetin for 48 h and then nuclear lysates were prepared. p300 was immunoprecipitated, and interaction with acetylated p65 and TNF-α was assessed by western blotting. The immunoblots shown here are representative of 3 independent experiments.

mechanism of fisetin-mediated inhibition of inflammation, we investigated its effect on the interaction between p300 and NF-κB. As shown in Figure 5, fisetin reduced the interaction of p300 with both the acetylated form of NF-κB and with TNF-α. This was associated with decreased TNF-α gene transcription in monocytes under HG conditions.

3.6. Effect of Fisetin on Chromatin Events at the Promoters of Inflammatory Genes. To confirm the epigenetic regulation of fisetin on inflammation, we next used ChIP assays to further investigate whether p300 can be bound to the promoters of NF-κB-related inflammatory cytokine genes under HG conditions. ChIP assays showed that HG increased the recruitment of p300 to the TNF-α promoter. As shown in Figure 6, Fisetin reduced the binding of p300 to the promoter region of TNF-α. This was associated with decreased TNF transcription in monocytes under HG conditions.

4. Discussion

Diabetes is a proinflammatory condition and chronic inflammation plays an important role in the progression of diabetic

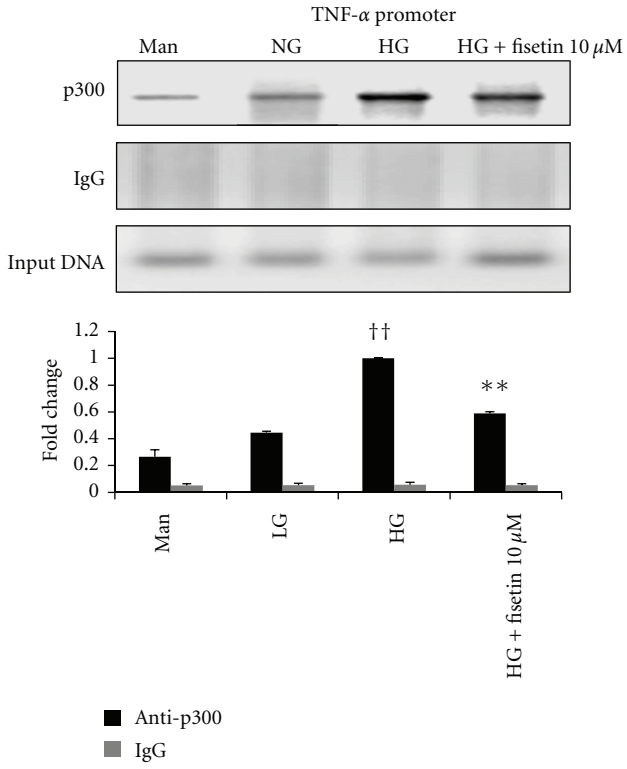

Figure 6: Effect of fisetin on chromatin event at the promoter of inflammatory gene. ChIP assays were performed using MAGnify ChIP according to the manufacturer's instructions. Immunoprecipitations were performed overnight at 4°C with 5 μg of p300 antibody. DNA was subjected to PCR. ChIP assays showed the recruitment of p300 to the TNF-α promoters. Results of 1 typical experiment of 3 are shown. Values from ChIP with anti-p300 antibody represent the fold difference relative to those from IgG control antibody. $^{\dagger\dagger}P < 0.01$ compared to NG; $^{*}P < 0.05$; $^{**}P < 0.01$ compared to HG. Results are shown as mean ± SD for 3 different experiments.

complications. Hyperglycemia has been implicated as a major contributor in several diabetes complications [2, 3].

THP-1 monocytes or human peripheral blood monocytes cultured under high-glucose conditions are a relevant cell culture model for the study of hyperglycemia. High glucose levels are known to induce expression of the inflammatory cytokine TNF-α, chemokines, and monocyte chemoattractant protein 1 (MCP-1), in these cells, in an oxidative stress-, NF-κB-, and AP-1 transcription factor-dependent manner [6, 7]. We have recently shown that hyperglycemia induces proinflammatory cytokine release (IL-1, IL-6, and TNF-α) in monocytes via an NF-κB-dependent pathway [6–8, 33, 34]. Systemic levels of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, are elevated in patients with both type 1 and type 2 diabetes [9, 34, 35]. In recent years, several clinical and animal studies have indicated that inflammatory cytokines play an important role in the development and progression of diabetic complications [36].

NF-κB plays a crucial role in the expression of cytokines, including TNF-α and IL-6 [9, 35–39]. Schmid et al. have reported that NF-κB plays a critical role in diabetes complications as it regulates transcription of a number of genes involved in inflammatory response [40]. Histone acetylation of NF-κB target genes is generally associated with increased binding of the transcription factor to its response elements and active transcription [14]. HATs and HDACs play an important role in regulating proinflammatory response.

The antioxidant and/or anti-inflammatory effects of dietary polyphenols have been shown to play a role in either controlling NF-κB activation or chromatin remodeling through modulation of HDAC and HAT activity, consequently affecting inflammatory gene expression [8, 18, 41–43].

Fisetin is a major flavonoid with a wide range of pharmacological effects, such as inhibition of angiogenesis, as well as anticancer, antiallergenic, and antithyroid activities [44–47]. Fisetin has been reported to downregulate both glycogenolysis and gluconeogenesis *in vitro* [30]. Furthermore, recent studies revealed hypoglycemic activity of fisetin in streptozotocin-induced experimental diabetes in rats [48, 49]. However, its specific regulation mechanisms at the chromatin level are not known in diabetic conditions.

The goal of this study was to determine whether fisetin can be used as a therapeutic agent for treatment of inflammation, which contributes to diabetes-related complications. We investigated the role of fisetin in regulation of high glucose-mediated proinflammatory cytokines (IL-6 and TNF-α release), HAT and HDAC modulation, and posttranslational modification of the transcription factor NF-κB, in high-glucose-treated THP-1 cells.

Production of reactive oxygen species has been implicated as a causative factor for hyperglycemic damage [50–52]. Reactive oxygen species alter nuclear histone acetylation and deacetylation balance, leading to increased NF-κB-dependent gene expression of proinflammatory mediators [51–53]. NF-κB plays an important role in the regulation of proinflammatory genes that are associated with several inflammatory diseases, including atherosclerosis, insulin resistance, metabolic syndrome, and diabetes and its complications [9]. The p65 protein is a key component of NF-κB activation and its transactivation potential is enhanced by several coactivators, including CREB-binding protein/p300, p/CAF and SRC1, which possess HAT activity [54–57]. In addition, acetylation of p300 at Lys1499 has been demonstrated to enhance its HAT activity and affect a wide variety of signaling events [58]. CBP/p300-mediated hyperacetylation of RelA is critical for NF-κB activation. Five main acetylation sites have been identified on p65. Acetylation at Lys221 enhances DNA binding by p65 and inhibits its interaction with IκBα, whereas acetylation of Lys316 is required for full transcriptional activity of p65 [57]. Accordingly, the attenuation of p65 acetylation is a potential molecular target for the prevention of chronic inflammation [9].

We found that fisetin treatment inhibited the expression of NF-κB target genes, including IL-6 and TNF-α, in high-glucose-treated THP-1 cells. We also show novel data

supporting fisetin-mediated inhibition of hyperglycemia-induced p65 acetylation, resulting in suppressed NF-κB transcription activity. We also observed that fisetin can inhibit inflammation through upregulation of HDAC activity in HG-treated THP-1 cells.

In contrast, fisetin inhibited HAT activity, preventing NF-κB-mediated chromatin acetylation and subsequent transcription of cytokines in hyperglycemic conditions. Fisetin also reduced p300 expression as well as its interaction with NF-κB. Thus, fisetin appears to suppress inflammatory cytokines through at least in part the NF-κB signaling pathway via inducing HDAC activity and suppressing HAT activity under HG conditions.

In summary, high glucose levels activate HAT (p300) and reduce HDAC activity, leading to increased acetylation of p65. Acetylation of p65, in turn, induces NF-κB activation and transcription of IL-6 and TNF-α in monocytes. Fisetin treatment was cytotoxic at 30 μM on monocytes under hyperglycemia (data not shown). We observed that 3–10 μM is nontoxic and we used very effective concentration (0, 3, 6, 10 μM) in this study. Administration of fisetin to cells cultured under hyperglycemic conditions may activate HDACs and suppress HATs, particularly p300, leading to deacetylation of the p65 subunit of NF-κB. Thus, fisetin administration suppresses proinflammatory cytokine release. It may therefore be considered for use in diabetes preventions. Future studies are needed to uncover the specific chromatin events and molecular mechanisms induced by fisetin in hyperglycemic conditions.

5. Conclusion

In the current study, we hypothesized that fisetin suppresses proinflammatory cytokine secretion through the NF-κB signaling pathway, by altering the balance between histone acetylation and deacetylation. Administration of fisetin to cells cultured under hyperglycemic conditions may activate HDACs and suppress HATs, particularly p300, leading to deacetylation of the p65 subunit of NF-κB. Thus, fisetin administration suppresses proinflammatory cytokine release. To our knowledge, this is the first report for analyzing its molecular mechanism of action under diabetic conditions. Understanding these mechanisms will be critical towards establishment of fisetin as a natural therapeutic agent for the treatment of chronic inflammation associated with diabetes and its complications.

Acknowledgments

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