Planta Medica

Journal of Medicinal Plant and Natural Product Research

Publishers

Georg Thieme Verlag KG
Stuttgart · New York
Rüdigerstraße 14
D-70469 Stuttgart
Postfach 30 11 20
D-70451 Stuttgart

Thieme Publishers
333 Seventh Avenue
New York, NY 10001, USA
www.thieme.com

Beneficial Impact of *Zingiber zerumbet* on Insulin Sensitivity in Fructose-Fed Rats

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Key words
- fructose
- glucose transporter subtype 4
- insulin resistance
- phosphoenolpyruvate carboxykinase
- *Zingiber zerumbet*
- Zingiberaceae

received October 8, 2011
revised Nov. 28, 2011
accepted Dec. 6, 2011

Bibliography
DOI http://dx.doi.org/10.1055/s-0031-1298136
Published online January 10, 2012
Planta Med 2012; 78: 317–325

ISSN 0032-0943

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Abstract

Zingiber zerumbet (L) Smith (Zingiberaceae), commonly known as the pinecone or shampoo ginger, is distributed in many parts of Asia. It has been demonstrated that the aqueous extract of *Z. zerumbet* exerted a potential blood glucose lowering effect in normoglycemic and streptozotocin-induced hyperglycemic rats. The present study was undertaken to clarify whether the ethanol extract of *Zingiber zerumbet* (EEZZ) is effective in improving insulin resistance. Insulin resistance was induced in rats by feeding a high-fructose diet for six weeks. Thereafter, rats were maintained on the same diet and treated with oral EEZZ or pioglitazone once daily for eight weeks. At the end of treatment, the degree of basal insulin resistance was measured by homeostasis model assessment (HOMA-IR). Insulin sensitivity was calculated using the composite whole body insulin sensitivity index (ISIcomp). Protein expression was evaluated by immunoblotting. Phytochemicals in EEZZ were determined through liquid chromatography-tandem mass. Not only curcumin but also quercetin and kaempferol were abundant in EEZZ. EEZZ (300 mg/kg/day) displayed similar characteristics to pioglitazone (20 mg/kg/day) in reducing HOMA-IR and elevating ISIcomp as well as enhancing hepatic glycogen accumulation. Elevated glycosylated hemoglobin levels and hyperinsulinemia were ameliorated by EEZZ. Further, EEZZ enhanced the action of insulin on muscle glucose transporter subtype 4 translocation and attenuated hepatic phosphoenolpyruvate carboxykinase expression. This study suggests that EEZZ may be an ethnomedicine for improving insulin sensitivity.

Introduction

Insulin resistance is a metabolic disorder, whose prevalence is increasing alarmingly in populations worldwide. Insulin resistance occurs when the body tissues become increasingly resistant to insulin, leading to a marked decrease in glucose metabolism. Findings from recent studies suggest that insulin resistance results from complex interactions between genetic and environmental factors and is associated with common diseases such as type 2 diabetes, hypertension, obesity, and coronary heart disease [1]. Thiazolidinediones (TZD), agonists of the peroxisome proliferator-activated receptor (PPAR)γ, are a class of oral antidiabetic agents that enhance insulin sensitivity and improve metabolic control in patients with type 2 diabetes [2]. Despite their proven efficacy, a number of deleterious side effects, such as weight gain and an increased risk of advanced heart failure due to fluid retention have been associated with TZD use [3]. It seems, therefore, that developing new agents without side effects would definitely be helpful in the therapy of diabetic patients with insulin resistance.

Zingiber zerumbet (L) Smith (Zingiberaceae family), commonly known as the pinecone or shampoo ginger, is a perennial, tuberous root herb plant that has gained much interest from scientists all over the world because of its high medicinal values [4]. The juice of boiled *Z. zerumbet* rhizomes has been used for the treatment of worm infestation in children. *Z. zerumbet* has been shown to inhibit prostaglandin-induced paw edema, a commonly used acute inflammatory reaction, and the efficacy is equivalent to the nonsteroidal anti-inflammatory drug mafenamic acid [5]. The ethanol extracts of *Z. zerumbet* displayed significant antipyretic activity in Brewer's yeast-induced pyrexia in rats [6]. *Z. zerumbet* is also used in herbal medicinal practice for the treatment of rheumatological conditions and muscular dis-

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comfort [7]. Furthermore, the methanol extract of *Z. zerumbet* possesses inhibitory effects on platelet-activating factor and against the Den2 virus NS2B/NS3 protease activity [8].

That the aqueous extract of *Z. zerumbet* exerted a potential blood glucose lowering effect in normoglycaemic and streptozotocin-induced hyperglycemic rats has been demonstrated [9]. It is not known whether *Z. zerumbet* ameliorates insulin resistance. The aim of the present study was to assess insulin resistance in rats treated with *Z. zerumbet*. Fructose has been used to induce insulin resistance in experimental animals [10]. We induced insulin resistance by feeding rats a diet containing 60% fructose.

Materials and Methods

Plant material and extraction

Z. zerumbet rhizomes were purchased from a local market in Dongshan, Dongshan Dist. (Tainan City, Taiwan) during August 2010. Macroscopic and microscopic examinations, as well as thin-layer chromatography and high-performance liquid chromatography, were used to confirm the authenticity of the plant material provided (this analysis was performed by Dr. T. Y. Hong, Department of Biotechnology, Collage of Pharmacy and Health Care, Tajen University). Random amplified polymorphic DNA analysis of the *Z. zerumbet* rhizomes supplied was also performed to identify DNA polymorphisms. A voucher specimen (Lot No. 20100821) has been deposited in our laboratory. Extraction was performed by maceration and air drying, and 5 kg of pulverized *Z. zerumbet* rhizomes was added to 10 L of 95% ethanol at room temperature for 7 days, and the mixture was occasionally shaken. The ethanol extract of *Z. zerumbet* rhizomes (EEZZ) was evaporated to dryness under reduced pressure for the total elimination of alcohol, followed by lyophilization, yielding approximately 583 g of dry residue (w/w yield: 11.6%). EEZZ was kept at − 20 °C until use and suspended in distilled water. The samples were then analyzed using the LC/MS/MS protocol described below.

LC/MS/MS system

Chromatographic separation was performed using an HPLC apparatus equipped with two Micropumps Series 200 (PerkinElmer), a UV/VIS series 200 detector (PerkinElmer) at a wavelength of 280 nm, and a Prodigy ODS3 100A column (250 mm × 4.6 mm, particle size 5 µm) (Phenomenex). The eluents were (A) 0.2% formic acid in water, and (B) acetonitrile/methanol (60 : 40, v/v). The following gradient program was used: 20–30% B (6 min), 30–40% B (10 min), 40–50% B (8 min), 50–90% B (8 min), 90–90% B (3 min), 90–20% B (3 min) at a constant flow of 0.8 mL/min. The LC flow was split, and 0.2 mL/min was sent to the mass spectrometer. Three 20-µL injections were performed for each sample. MS and MS/MS analyses of EEZZ were performed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems), equipped with a TurboIonSpray source and working in the negative ion mode. The analyses were performed using the following settings: drying gas (air), 400 °C; capillary voltage (IS), 4000 V; nebulizer gas (air), 12 (arbitrary units); curtain gas (N_2), 14 (arbitrary units); and collision gas (N_2), 4 (arbitrary units). To optimize the declustering potential, focus potential, and collision energy for each compound, standard solutions (10 µg/mL) were infused directly into the mass spectrometer at a constant flow rate of 5 µL/min using a model 11 syringe pump (Harvard Apparatus). Kaempferol (purity ≥ 97.0%; Sigma-Aldrich), quercetin (purity ≥ 98.0%; Sigma-Aldrich), or curcumin (purity ≥ 98.0%; Sigma-Aldrich) at concentrations of 12.5 to 400 µg/mL were used to construct the standard curve. The retention times of the main compounds were 7.20, 6.89, and 7.73 min for kaempferol, quercetin, and curcumin, respectively. The linearity of the peak area (y) vs. concentration (x, µg/mL) curve for kaempferol, quercetin, and curcumin was used to calculate the contents of the main components in EEZZ. The standard curves for kaempferol, quercetin, and curcumin are $y = 270.48\,x - 303.26$ ($R^2 = 0.99$), $y = 906.31\,x - 1.67$ ($R^2 = 0.99$), and $y = 333.14\,x - 5.68$ ($R^2 = 0.99$), respectively.

Animal models

Eight-week-old male Wistar rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The rats were housed in a temperature-controlled room (25 ± 1 °C) and maintained on a 12-h light/12-h dark cycle (lights on at 06 : 00 h) in our animal center. Food and water were available *ad libitum*. The rats were divided into two experimental groups. One group of rats was randomly assigned to receive fructose-rich rat chow containing 60% fructose, 5% fat, and 18% protein (Harlan Teklad) for 6 weeks to induce insulin resistance [11]. The homeostasis model assessment of basal insulin resistance (HOMA-IR) was used to quantify insulin resistance [12]. The other group of rats (designated as the control group) received standard rat chow containing 60% vegetable starch, 5% fat, and 18% protein (Harlan Teklad) during the 6-week period. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act (approval number: IACUC 99–16; approval date: September 9, 2010).

Treatment protocols

Insulin-resistant rats were dosed by oral gavage once per day with EEZZ (100, 200, and 300 mg/kg) in a volume of 2 mL/kg distilled water. The selection of dosage regime for the present studies was according to the previous report which demonstrated that the aqueous extract of *Z. zerumbet* at 50 to 150 mg/kg exerted potential blood glucose lowering effect in normoglycaemic and streptozotocin-induced hyperglycemic rats with some modification [9]. Another group of fructose chow-fed rats was treated with vehicle (distilled water) during the 8-week treatment period. A further group of fructose-fed rats was treated by oral gavage with pioglitazone hydrochloride (Takeda Pharmaceutical Co. Ltd.: 20 mg/kg/day) for 8 weeks [13]. Rats were maintained on the fructose-rich chow diet for the 8 week treatment period, during which time water was available *ad libitum*.

Eight weeks after treatment was commenced, rats were anesthetized with intraperitoneal sodium pentobarbital (30 mg/kg), weighed, and blood samples were collected from the lateral tail vein. Blood samples were centrifuged at 2000 *g* for 10 minutes at 4 °C, and the resultant plasma was aliquoted for later analysis. After blood sampling, individual rats were placed in metabolic cages (Shineteh Instruments Co.) for 24 h urine collection.

Oral glucose tolerance test

After 8 consecutive weeks of EEZZ or pioglitazone treatment, rats were food-restricted and provided with water throughout the night prior to the oral glucose tolerance test (OGTT). The OGTT involved the administration of an oral glucose load of 1 g/kg. Plasma glucose and insulin concentrations were measured before and 30, 60, 90, and 120 min after the glucose load. Insulin sensi-

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tivity during the OGTT was calculated using the composite whole body insulin sensitivity index (ISIcomp) [14].

Biochemical parameter analysis

Kits for determining plasma glucose (Cat. No. 10009582) concentration were purchased from Cayman Chemical Company. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to quantify plasma insulin concentration (LINCO Research, Inc.) and glycosylated hemoglobin (HbA_{1c}) levels (Intergrated Bio Ltd.). All samples were analyzed in triplicate.

Estimation of hepatic glycogen content

Liver tissue (200 mg) was homogenized with 20 mL of 5% trichloroacetic acid, and the resultant protein precipitate was filtered. Clear filtrate (2 mL) was aliquoted into a 20-mL calibrated test tube, and 10 N KOH (2 mL) was added. The tube was then placed in a boiling water bath for 1 h. After cooling, 1 mL of glacial acetic acid was added to neutralize the excess alkali, and the tube was filled with distilled water. Two ml of the solution were then added to a test tube containing 4 mL of anthrone reagent, which was placed in cold water to prevent excessive heating. After thorough mixing, the tube was placed in a boiling water bath for 10 min for color development and then cooled with running tap water. The optical density of the solution was read within 2 h using a spectrophotometer (SPECORD 200; Analyjetika) set at 650 nm [15].

In vivo insulin receptor activation

To assess the effect of EEZZ on insulin receptor activation *in vivo*, rats in the fed state were anesthetized with sodium pentobarbital at the end of the 8-week treatment period. A bolus of insulin (Novo Nordisk; 10 units/kg) was then injected into the portal vein as previously described [16]. Approximately 120 s after insulin injection, rats were sacrificed and the soleus muscle and liver were immediately removed, washed with cold phosphate buffer and cut into 200–300 mg portions for separate storage at − 70 °C and subsequent immunoblot analysis.

Western immunoblotting

After homogenization of tissue samples using a glass/Teflon homogenizer, protein concentrations were determined using the BioRad protein dye binding assay. Cytosol and membrane fractions of the soleus muscle were prepared according to a previously described method [17]. The homogenates (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis were performed using either an anti-rat antibody to bind glucose transporter subtype 4 (GLUT 4) (1 : 1000) (R&D Systems, Inc.) in soleus muscle samples or an anti-rat antibody (1 : 1000) to bind cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) (Cayman Chemical Company) in liver samples. Blots were incubated with the appropriate peroxidase-conjugated secondary antibody. The membranes were then washed three times in TBST (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20) and visualized on X-ray film using the enhanced chemiluminescence detection system (Amersham). Band densities were determined using ATTO Densitograph Software (ATTO Corporation) and quantified as the ratio to β-actin. The primary antibody to β-actin was obtained from Santa Cruz Biotechnology, Inc. The mean value for samples from the vehicle-treated standard chow-fed group on each immunoblot, expressed in densitometry units, was adjusted to a value of 1.0. All experimental sample values were then expressed relative to this adjusted mean value.

Statistical analysis

Data are expressed as the mean ± SEM for each group of animals at the number indicated in tables. Statistical analysis was performed with one-way analysis of variance (ANOVA). The Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. A p value < 0.05 was considered statistically significant.

Results

The chromatogram of the sample solution is shown in **Fig. 1**. The contents of kaempferol, quercetin, and curcumin in EEZZ were 266.32 ± 0.21, 82.20 ± 0.14, and 75.32 ± 0.18 µg/g, respectively.

Among the fructose chow-fed rats, body weight gain was lower in EEZZ- (300 mg/kg/day) and pioglitazone- (20 mg/kg/day) treated rats than in vehicle-treated rats (**Table 1**). No significant differences were observed in daily food intake between the groups during the experimental period, despite the fact that water intake was slightly higher in the vehicle-treated, fructose chow-fed group as compared to the other groups (**Table 1**).

Twenty-four-hour urinary volume was not significantly different between vehicle-treated, standard chow-fed rats, and EEZZ- (300 mg/kg/day) and pioglitazone- (20 mg/kg/day) treated, fructose chow-fed rats, but was slightly higher in vehicle-treated, fructose chow-fed rats (**Table 1**).

Plasma glucose and insulin concentrations, HbA_{1c}, and HOMA-IR were markedly higher in vehicle-treated, fructose chow-fed rats than in the vehicle-treated, standard chow-fed rats (**Table 2**). After 8 weeks of treatment with EEZZ (300 mg/kg/day), plasma glucose concentrations were approximately 14% lower in fructose chow-fed rats compared to their vehicle counterparts. Corresponding HbA_{1c}, plasma insulin, and HOMA-IR values were 26%, 8%, and 20% lower. After 8 weeks of treatment with pioglitazone (20 mg/kg/day), plasma glucose, HbA_{1c}, and plasma insulin, HOMA-IR values were 24%, 37%, 50%, and 60% lower, respectively, in fructose chow-fed rats compared to their vehicle counterparts. Glycogen synthesis in vehicle-treated, fructose chow-fed rats was 65% lower than in vehicle-treated, standard chow-fed rats (**Table 2**). Fructose chow-fed rats treated with EEZZ or pioglitazone (20 mg/kg/day) had elevated hepatic glycogen levels compared with the standard chow-fed group.

The OGTT findings at the end of the 8-week treatment period are summarized in **Fig. 2**. Plasma glucose levels were significantly higher in fructose chow-fed rats compared with standard chow-fed rats at all time points (**Fig. 2a**). In the vehicle-treated, fructose chow-fed group, plasma glucose concentrations increased from fasting levels of 142.7 ± 5.1 mg/dL to 225.5 ± 5.4 mg/dL by 30 min and remained greatly elevated above baseline levels 2 h after the oral glucose challenge. The fructose chow-fed rats treated with EEZZ (300 mg/kg/day) showed a significant elevation in plasma glucose concentrations at 30 min; however, concentrations returned to baseline by 2 h. Similar results were seen in rats treated with pioglitazone (20 mg/kg/day) (**Fig. 2a**).

Fasting plasma insulin concentrations were significantly higher in fructose chow-fed rats compared with standard chow-fed rats and remained higher at all time points throughout the OGTT study period (**Fig. 2b**). Plasma insulin concentrations in both

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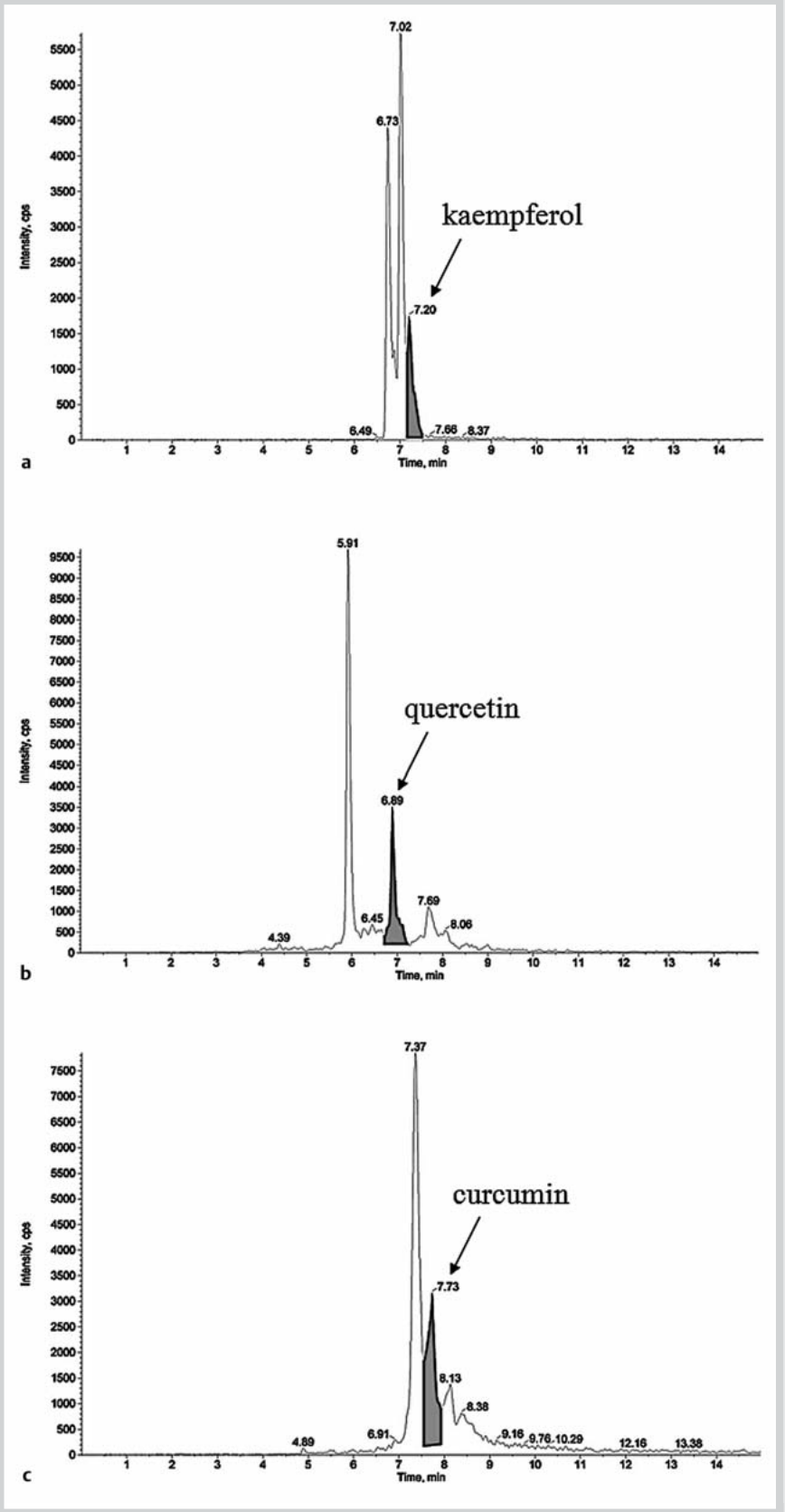

Fig. 1 Chromatogram for kaempferol (**a**), quercetin (**b**), and curcumin (**c**) in the EEZZ sample.

EEZZ- and pioglitazone-treated, fructose chow-fed rats were increased after 30 min, but returned to fasting levels by 2 h (**Fig. 2b**).

The ISIcomp was significantly lower in the fructose chow-fed group compared with the standard chow-fed group (**Fig. 2c**). EEZZ (300 mg/kg/day) treatment for 8 weeks increased the ISIcomp in fructose chow-fed rats by 2.6-fold relative to the value

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Table 1 General characteristics of fructose chow-fed rats receiving 8-week administration with EEZZ or pioglitazone.

Variable (s)	Standard chow-fed	Fructose chow-fed				
	Vehicle	Vehicle	EEZZ (mg/kg/day)			Pioglitazone
			100	200	300	(20 mg/kg/day)
Initial body weight (g/rat)	183.8 ± 7.3[b]	219.2 ± 8.2[a]	217.7 ± 9.4[a]	217.0 ± 7.5[a]	216.1 ± 9.4[a]	218.9 ± 8.3[a]
Body weight gain (g/rat)	18.4 ± 6.3	25.3 ± 7.7	24.3 ± 5.5	21.4 ± 6.6	20.2 ± 7.0	19.6 ± 6.1
Food intake (g/rat/day)	18.8 ± 5.4	22.3 ± 6.3	21.8 ± 6.1	20.6 ± 6.7	20.7 ± 7.1	20.5 ± 6.5
Water intake (mL/rat/day)	73.9 ± 8.5[b]	97.5 ± 7.6[a]	91.1 ± 7.2[a]	89.4 ± 7.8[a]	87.8 ± 8.2[a]	86.7 ± 6.9[a]
Urine volume (mL/rat/day)	33.4 ± 6.7[b]	50.3 ± 5.5[a]	49.6 ± 7.3[a]	47.5 ± 6.3[a]	40.2 ± 5.2	38.6 ± 7.1

EEZZ or pioglitazone was dissolved in distilled water for oral administration at the desired doses in a volume of 10 mL/kg once a day in fructose chow-fed rats. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SEM) were obtained from each group of 8 animals in each group during and/or after 8 weeks of the experimental period. [a] $P < 0.05$ compared to the values of vehicle-treated standard chow-fed rats in each group, respectively. [b] $P < 0.05$ compared to the values of vehicle-treated fructose chow-fed rats in each group, respectively

Table 2 Effects on plasma glucose, HbA_{1c}, plasma insulin, insulin resistance index, and hepatic glycogen content in fructose chow-fed rats receiving 8-week administration of EEZZ or pioglitazone.

Variable (s)	Standard chow-fed	Fructose chow-fed				
	Vehicle	Vehicle	EEZZ (mg/kg/day)			Pioglitazone
			100	200	300	(20 mg/kg/day)
Plasma glucose (mg/dL)	93.4 ± 5.1[d]	150.3 ± 5.9[b]	141.4 ± 5.7[b]	137.8 ± 5.2[b]	129.8 ± 4.7[a,c]	114.8 ± 4.9[a, c]
HbA_{1c} (%)	4.4 ± 0.8[d]	7.6 ± 0.7[b]	7.1 ± 0.8[b]	6.4 ± 0.6[a,c]	5.6 ± 0.8[c]	4.8 ± 0.7[d]
Plasma insulin (μU/mL)	19.8 ± 5.8[d]	60.5 ± 7.3[b]	56.7 ± 8.1[b]	56.3 ± 6.7[b]	55.9 ± 6.2[b]	30.1 ± 5.1[a,d]
HOMA-IR score	4.6 ± 0.7[d]	22.6 ± 1.5[b]	19.9 ± 1.6[b]	19.1 ± 1.2[b]	18.1 ± 0.9[b,c]	8.6 ± 1.2[a]
Hepatic glycogen (mg/g of liver tissue)	10.3 ± 2.0	6.4 ± 1.8	7.3 ± 1.9	8.0 ± 1.6	8.6 ± 2.1	9.4 ± 2.2

EEZZ or pioglitazone was dissolved in distilled water for oral administration at the desired doses in a volume of 10 mL/kg once a day in fructose chow-fed rats. The vehicle (distilled water) used to dissolve the tested medications was given at the same volume. Values (mean ± SEM) were obtained from each group of 8 animals. [a] $P < 0.05$ and [b] $p < 0.01$ compared to the values of vehicle-treated standard chow-fed rats in each group, respectively. [c] $P < 0.05$ and [d] $p < 0.01$ compared to the values of vehicle-treated fructose chow-fed rats in each group, respectively

in fructose chow-fed rats who received the vehicle (**Fig. 2c**). Pioglitazone (20 mg/kg/day) treatment for 8 weeks increased the ISIcomp in fructose chow-fed rats by nearly 3.3-fold relative to the value in fructose chow-fed rats who received the vehicle (**Fig. 2c**).

Following insulin stimulation, GLUT 4 soleus muscle membrane protein expression levels in fructose chow-fed rats were 43% of those in standard chow-fed rats, while protein expression levels in the cytosolic fraction of the same rats were approximately 171% of those in standard chow-fed rats (**Fig. 3**). Pioglitazone-treated (20 mg/kg/day), fructose chow-fed rats had both membrane and cytosolic fraction soleus muscle GLUT 4 protein expression levels that were similar to those in standard chow-fed rats. GLUT 4 protein expression levels in the soleus muscle cytosolic fraction from rats treated with pioglitazone were 60% of those in vehicle-treated, fructose chow-fed rats (**Fig. 3**). Rats treated with EEZZ (300 mg/kg/day) for 8 weeks had membrane fraction GLUT 4 protein expression levels that were 83% of those in standard chow-fed rats; while, cytosolic fraction GLUT 4 protein expression levels were decreased by 76% relative to expression levels in vehicle-treated, fructose chow-fed rats (**Fig. 3**).

PEPCK protein expression levels were increased by approximately 3-fold in the livers of fructose chow-fed rats compared with standard chow-fed rats. After 8 weeks, hepatic PEPCK protein expression levels in pioglitazone-treated (20 mg/kg/day), fructose chow-fed rats were similar to those in standard chow-fed rats. After 8 weeks, hepatic PEPCK protein expression levels in EEZZ-treated (300 mg/kg per day), fructose chow-fed rats were 58% of those in vehicle-treated fructose chow-fed rats (**Fig. 4**).

Discussion

In the present study, we found that water intake and urinary volumes as well as HOMA-IR were significantly increased in fructose chow-fed rats, but not in fructose chow-fed rats treated with EEZZ or pioglitazone. EEZZ treatment was also found to increase whole-body insulin sensitivity in fructose chow-fed rats (as did pioglitazone treatment) as evidenced by the marked elevation of ISIcomp. This finding suggests that EEZZ can ameliorate insulin resistance associated with high fructose feeding.

The level of glycosylated hemoglobin is a widely used marker of glucose homeostasis due to its stability over the red blood cell lifetime [18]. Similar to pioglitazone treatment, we found that an 8-week EEZZ (300 mg/kg/day) administration regimen significantly inhibited the glycosylation of hemoglobin by promoting normoglycemia in fructose chow-fed rats, thus maintaining HbA_{1c} levels near to normal. Clearly, these results indicate that EEZZ can reverse impaired insulin responsiveness in insulin-resistant rats to ameliorate hyperglycemia, demonstrating the insulin-sensitizing effect of this medicinal herb.

The increased responsiveness of skeletal muscle to insulin is likely to be related to increased protein expression and/or the functional activity of several key components of the insulin signal transduction cascade. Considering that the regulation of glucose

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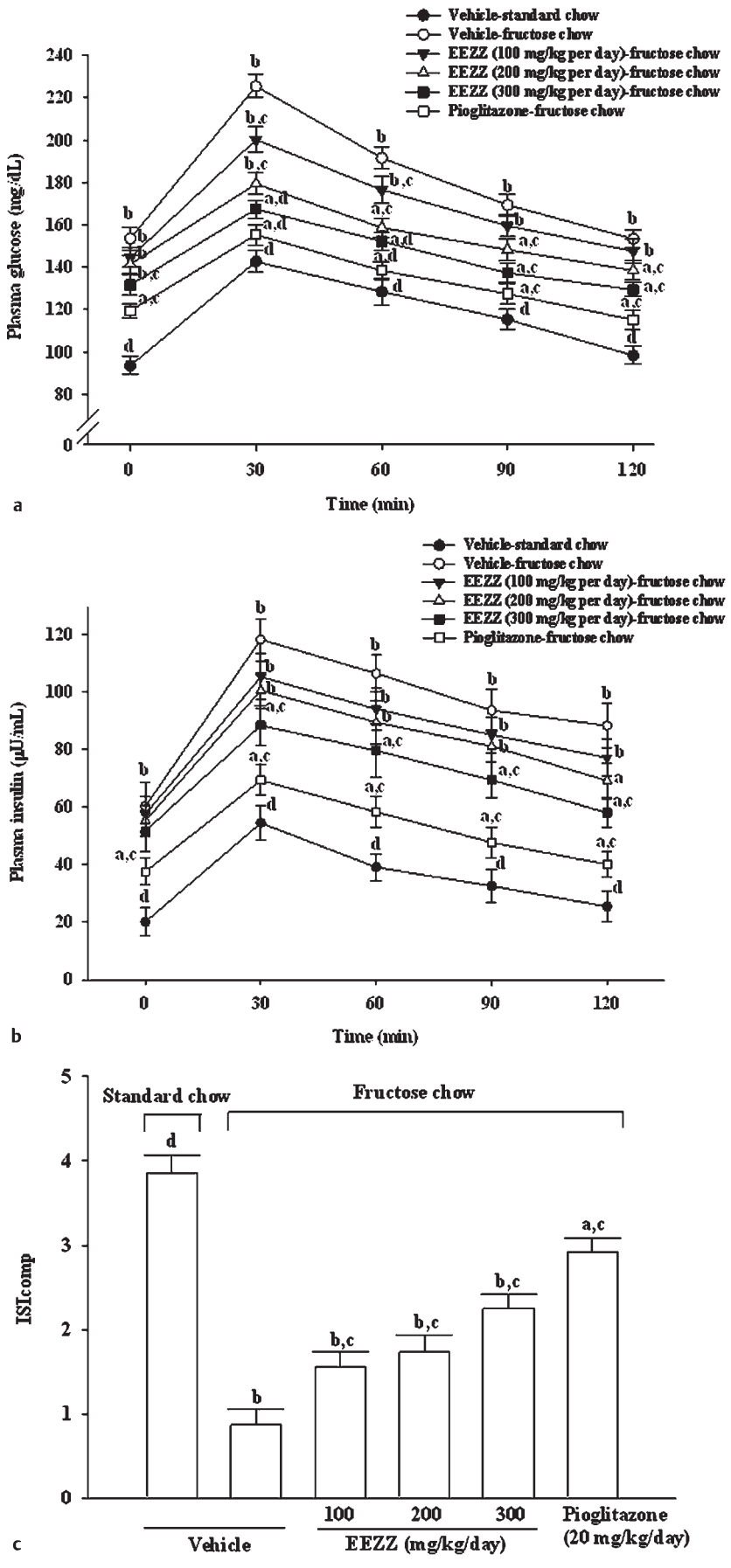

Fig. 2 Effects of treatment on insulin sensitivity. **a** Plasma glucose concentrations during an oral glucose (1 g/kg) tolerance test (OGTT) in fructose chow-fed rats treated once daily with EEZZ (100 mg/kg [▼], 200 mg/kg [△], or 300 mg/kg [■]) for 8 weeks. **b** Plasma insulin concentrations during the OGTT in the same rats. **c** Insulin sensitivity was calculated using the composite whole body insulin sensitivity index after the 2 h OGTT. Pioglitazone was given by oral gavage (20 mg/kg/day) for 8 weeks to a separate group of the fructose chow-fed rats (□). Rats not receiving any treatment were given the same volume of vehicle (distilled water) used to dissolve the test medications. Values (mean ± SEM) were determined from each group of 8 animals. [a] $P < 0.05$ and [b] $p < 0.01$ compared with the values of vehicle-treated, standard chow-fed rats (●) at the indicated times. [c] $P < 0.05$ and [d] $p < 0.01$ compared with the values of vehicle-treated, fructose chow-fed rats (○) at the indicated times.

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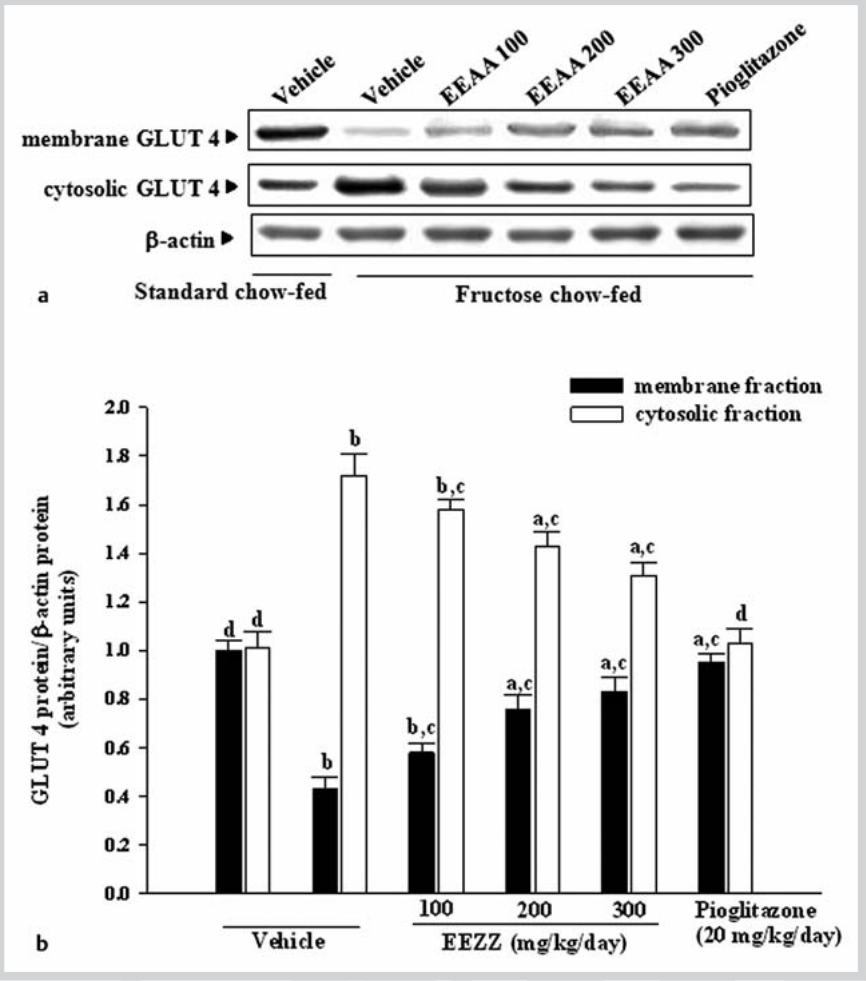

Fig. 3 Effect of treatment on GLUT 4 in soleus muscle. **a** Soleus muscle membrane (■) and cytosolic fraction (□) glucose transporter subtype 4 (GLUT 4) protein expression after insulin stimulation in fructose chow-fed rats treated once daily with EEZZ (100 mg/kg [EEZZ 100], 200 mg/kg [EEZZ 200], or 300 mg/kg [EEZZ 300]) for 8 weeks. Pioglitazone was given by oral gavage (20 mg/kg/day) for 8 weeks to a separate group of the fructose chow-fed rats. Rats not receiving any treatment were given the same volume of vehicle (distilled water) used to dissolve the test medications. Similar results were found in four additional replications. **b** Protein levels are expressed as the mean with SEM (n = 5 per group) in each column. [a] $P < 0.05$ and [b] $p < 0.01$ compared with the values of vehicle-treated standard chow-fed rats. [c] $P < 0.05$ and [d] $p < 0.01$ compared with the values of vehicle-treated fructose chow-fed rats.

uptake into muscle cells via GLUT 4 is fundamental to the action of insulin, and that this process is impaired in type 2 diabetes [19], we examined the cytosolic and membrane expression of GLUT 4 in soleus muscle. We observed a definite improvement in the translocation of GLUT 4 from intracellular vesicles to the plasma membrane of the soleus when rats fed a high fructose diet were treated with EEZZ. EEZZ reversed the abnormal responsiveness to insulin caused by consumption of a high fructose diet by enhancing GLUT 4 translocation and promoting glucose homeostasis.

In classical forms of diabetes, elevated blood glucose concentrations are thought to be due to increased hepatic glucose output in concert with reduced peripheral glucose utilization [20]. PEPCK is a catalyst of the first step in gluconeogenesis, and gene transcription of the cytosolic form of PEPCK (PEPCK-C) is highly regulated by the glucagon/insulin axis [21]. Induction of PEPCK-C correlates with increased rates of gluconeogenesis in the liver and kidney [21], indicating that PEPCK-C plays a key role in the control of hepatic energy metabolism and thus is a potential target for treatment intervention. In fructose chow-fed rats, we found that EEZZ treatment was associated with attenuated hepatic PEPCK-C expression. Further, an increase in glycogen accumulation may contribute to enhance glucose utilization in EEZZ-treated, fructose chow-fed rats. The results indicate that EEZZ ameliorates the disturbance in hepatic glucose metabolism in rats fed a high fructose diet. This effect may due to impaired gluconeogenesis as a result of enhanced glycogen accumulation and decreased hepatic PEPCK expression.

A considerable content of flavonoids such as quercetin, curcumin, and kaempferol has been found in EEZZ. The antidiabetic activity of quercetin is responsible for the stimulation of the insulin-independent AMP-activated protein kinase pathway [22]. The actions of curcumin on the insulin sensitivity improvement are attributed at least in part to its anti-inflammatory properties as evident by the attenuating tumor necrosis factor-α level in high-fat diet fed rats [23]. Kaempferol improves chronic hyperglycemia-impaired pancreatic beta-cell viability, and insulin secretory function has been demonstrated [24]. The pharmacological effects of EEZZ on the amelioration of insulin resistance could be attributed to the presence of these valuable constituents. Although the effects of EEZZ on the insulin sensitivity improvement are not as effective as those produced by the standard drug pioglitazone, EEZZ may be a suitable therapeutic adjunct for the patients who are particularly sensitive to the TZD-induced side effects [3,25].

In conclusion, the study shows that EEZZ exhibits a potent inhibitory effect on the progression of high fructose diet-induced insulin resistance in rats. EEZZ resulted in apparent improvement in overall insulin sensitivity by ameliorating hyperinsulinemia and gluconeogenesis. Further, treatment with EEZZ was associated with improved GLUT 4 translocation and decreased PEPCK expression. Our findings are encouraging, and suggest that, with

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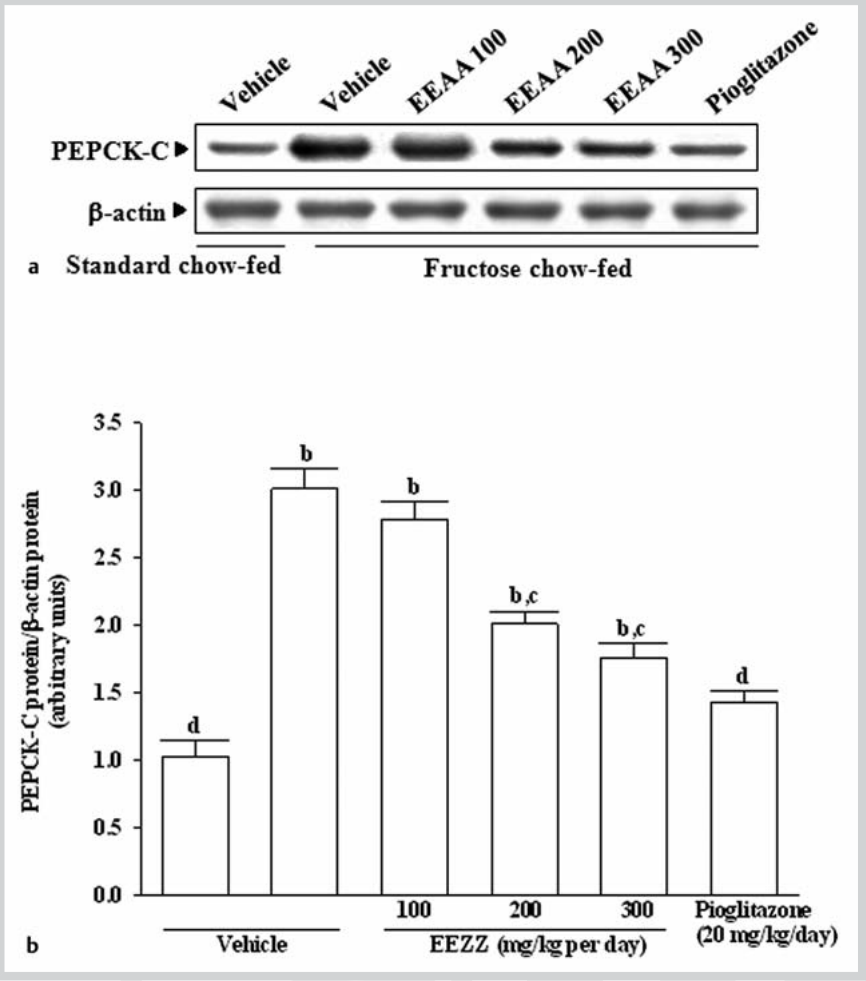

Fig. 4 Effect of treatment on expression of hepatic PEPCK protein. **a** Hepatic cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) protein expression levels in fructose chow-fed rats treated once daily with EEZZ (100 mg/kg [EEZZ 100], 200 mg/kg [EEZZ 200], or 300 mg/kg [EEZZ 300]) for 8 weeks. Pioglitazone was given by oral gavage (20 mg/kg/day) for 8 weeks to a separate group of the fructose chow-fed rats. Rats not receiving any treatment were given the same volume of vehicle (distilled water) used to dissolve the test medications. Similar results were found in four additional replications. **b** Protein levels are expressed as the mean with SEM (n = 5 per group) in each column. [b] $P < 0.01$ compared with the values of vehicle-treated standard chow-fed rats. [c] $P < 0.05$ and [d] $p < 0.01$ compared with the values of vehicle-treated fructose chow-fed rats.

further research and development, EEZZ may prove to be a useful treatment for diabetes and related symptoms.

Acknowledgements

The present study was supported by a grant from the National Science Council (NSC 100–2324-B-127-001-CC2 of Taiwan, the Republic of China.

Conflict of Interest

No competing financial interests exist.

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