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EVALUATION OF ANTI-HYPERGLYCEMIC ACTIVITIES OF PHLORIDZIN IN DIABETIC MICE

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Abstract

Background: The aim of the study was to investigate the hypoglycemic effects of Phloridzin.

Methods and Materials: High fat diet induced diabetic KKAy mice were administered with phloridzin at an oral dose (60 mg/kg/day, ig.) for 10 weeks. A range of parameters, including blood glucose and lipid, serum insulin, glucose tolerance, were tested to evaluate its anti-hyperglycemic effects.

Results: Phloridzin decreased water-intake, body weight, FBG, FINS, HOME-IR, Serum leptin, and CRP levels, increased serum adiponectin level in diabetic mice. Phloridzin also improved the oral glucose tolerance test (OGTT) to a certain degree. In addition, phloridzin decreased liver index, and epididymal, perirenal white adipose tissue indexs, increased pancreas index in diabetic mice. At last, phloridzin increased hepatic GK activity and hepatic glycogen level, decreased hepatic PEPCK, G-6-Pase activities in diabetic mice.

Conclusions: Phloridzin possessed anti-hyperglycemic activities.

Key words: Diabetes, Phloridzin, FBG, Insulin resistance, Area under curve

Introduction

As a complex metabolic condition, diabetes mellitus is a risk factor for coronary artery diseases that affect both individuals and society as a whole. It is estimated that in 2030, 522 million adults will be suffering from diabetes worldwide (García-Toro et al, 2016). Type-2 diabetes is generally identified by reduced insulin secretion (Hattori et al., 1985) and increased insulin resistance, which are associated with inflammation (DeFeudis, 1991). Insulin resistance, which is associated with insulin response, causes problems in the liver and adipose tissue and continues throughout the chronic inflammatory state.

Physiologically active substances in food have lesser adverse side effects, lower costs and the better health benefits of these therapies, as well as their acceptability among people. They have been developed as functional foods to prevent diabetes in conjunction with traditional antidiabetic drugs (i.e., sulfonylurea, thiazolidinedione, and glucagon-like peptide 1 analogs) (Haines et al., 2000). Phloridzin is found in apples and apple products (Gosch et al., 2010; Perez-Jimenez et al., 2010). Wojdylo et al. showed that the dihydrochalcones phloridzin and phloretin 2′-xyloglucose account for 0.5–5% of the polyphenols in apple varieties (Wojdylo et al., 2008). According to the Phenol-Explorer database (http://www.phenol-explorer.eu), phloridzin exists in apples with a mean content of 2.75 mg/100 g of fresh weight. In recent years, studies of phloridzin in diabetes have been performed in many countries (Brouwers et al., 2013; Randhawa et al., 2013). It is suggested that phloridzin contributes to the high antioxidant activity of apples (Boyer et al., 2004; Lee et al., 2003; Wojdylo et al., 2008). Phloridzin is of significant interest to mammalian physiologists because of its ability to block sodium-linked glucose transport and block renal re-absorption of glucose in the kidney (Ehrenkranz et al., 2005). Thus, phloridzin is a promising food component that may prevent diabetes mellitus and other lifestyle-related diseases by its antioxidant ability or its ability to inhibit glucose absorption.

In this study, we isolated phloridzin from apple polyphenols. We investigated the hypoglycemic effects of phloridzin orally administered to diet induced obese hyperglycemic mice *in vivo*.

Materials and Methods

Preparation of Apple Polyphenols Extract and Preparative HPLC Isolation for Phloridzin

Extraction was performed on a weighed amount of dried apple peels (100 g) with 2000 ml of EtOAc at room temperature for 2 h under magnetic stirring. At the end of extraction, the polyphenols extract was paper filtered and concentrated to dryness under vacuum at 40 °C. Apple polyphenols extract was washed with dichloromethane to remove lipids and non-polar constituents. To remove polar non-phenolic compounds such as sugars and organic acids, a solid phase extraction was carried out using C18 cartridges. The methanolic fraction was concentrated under reduced pressure at 40 °C and used directly for the preparative HPLC isolation. The peaks of interest were isolated using a Varian-218 preparative-HPLC system connected to a Pursuit XRS 5 diphenyl preparative column (250 mm × 10 mm). The mobile phase was composed of (A) water/formic acid (1000:0.05 v/v) and (B) acetonitrile. Solvents were delivered at a total flow rate of 3 ml/min. The gradient profile was from 10% B to 70% B linearly in 50 min followed by 10 min isocratic and a return to 10% B at 90 min and 10 min isocratic to re-equilibrate. The UV chromatograms were recorded at 254, 280 and 320 nm (λmax). The injection volume was 400 µl. Isolation method was repeated several times to obtain enough amounts of the individual compound (phloridzin). The isolated compound (phloridzin) was analyzed by HPLC–HRMS and HPLC–MSn for its identification and characterization with the help of authentic standard.

Animals Grouping and Treating

Twelve-week-old male KKAy mice and age-matched C57BL/6J mice (C57BL) (The Experimental Animal Center, Beijing Huafukang biology science technology Ltd, Beijing) were housed in individual cages under controlled temperature (23 ± 1 °C) and humidity (55 ± 5%) on a 12:12 h light–dark cycle and were given standard rodent chow and free access to water, unless otherwise noted. They were acclimatized for 1 week with ad libitum access to tap water and a normal chow diet. After acclimation, the mice were divided into the following groups:

Group 1 (n = 15): C57BL/6J mice administered distilled water: normal control.

Group 2 (n = 15): KKAy mice administered distilled water: vehicle-treated KKAy.

Group 3 (n = 15): KKAy mice administered rosiglitazone (15 mg/kg body weight) by oral gavage once a day for 10 weeks: KKAy + rosiglitazone.

Group 4 (n = 15): KKAy mice administered phloridzin (60 mg/kg body weight) by oral gavage once a day for 10 weeks: KKAy + phloridzin.

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Taizhou University. Their care was in accordance with institution guidelines, and the ethical committee of our institution approved of the study. The KKAy mice were fed a purified high-fat diet consisting (as a percentage of total kcal) of 41% fat, 41% carbohydrates, and 18% protein. The C57BL/6J mice were fed a normal chow diet consisting of 12% fat, 60% carbohydrates, and 28% protein.

Food and water consumptions and body weight were recorded twice weekly. When the mice had been fed experimental diets for 10 weeks, they were weighed and then sacrificed after having been anesthetized with diethyl ether from 8:00 to 11:00. Blood was drawn from the descending part of the abdominal aorta without anti-coagulants, and it was then centrifuged at 2000 × g for 15 min for the separation of serum. The liver, kidney, pancreas, and epididymal, perirenal white adipose tissue (WAT) were removed rapidly, weighed, washed with cold saline, and then frozen using liquid nitrogen, followed by storage at −80 °C until analysis.

Oral Glucose Tolerance Test (OGTT) and Fasting Blood Glucose (FBG)

Oral glucose tolerance testing was performed during the last week of treatment. After a 12-hour fasting, the animals were orally gavaged with 2 g/kg body weight of glucose dissolved in water (40%, wt/vol). Blood glucose levels were measured at 0 and 120 min by glucose oxidase method according to the instruction.

Assay of FBG, FINS, Serum Leptin, Adiponectin, and CRP

Blood glucose concentration was determined with a handheld glucometer (Ascensia Contour glucometer, Bayer) (Woods et al., 2003). Plasma insulin concentration was measured by enzyme-linked immunosorbent assay kit (Seikagaku Corp, Kanagawa, Japan) (Liversy et al., 1980).

The HOMA-IR was calculated as described by Matthews et al (1985) as follows: HOMA-IR = (fasting insulin [μU/mL] × fasting glucose [mmol/L])/22.5. This method is routinely used for assessment of insulin resistance in rodents and is strongly correlated with glucose clamping (the criterion standard for determination of insulin sensitivity).

Serum leptin, adiponectin, and CRP were detected by ELISA kits.

Statistical Analysis

All experiments and analyses were performed at least in triplicate. Results are expressed as means ± SE. Statistical analyses were performed using the Student′s t-test. Values of $P < 0.05$ were considered to be statistically significant.

Table 1: Effect of phloridzin on food-intake and water-intake quantity in experiment mice

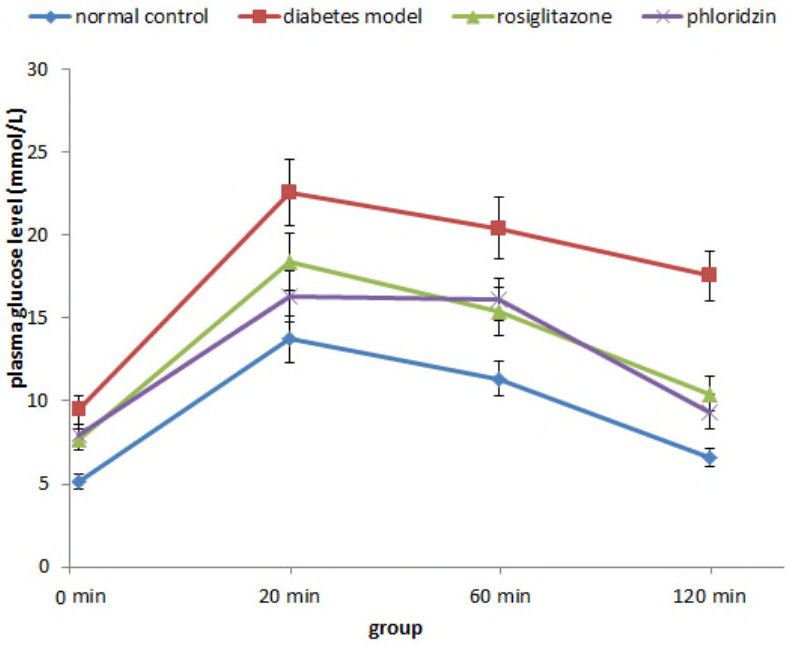
Indexs	Group	Time (week)		
		0	5	10
Food-intake (g/week)	Normal	43.27	40.24	38.13
	Diabetes model	27.36	25.06	23.14
	Rosiglitazone (15 mg/kg bw)	26.02	27.91	25.38
	Phloridzin (60 mg/kg bw)	29.21	29.83	24.28
Water-intake (ml/week)	Normal	72.13	68.38	70.02
	Diabetes model	83.18	80.61	79.38
	Rosiglitazone (15 mg/kg bw)	84.16	71.24	53.27
	Phloridzin (60 mg/kg bw)	82.63	74.29	58.14
Body weight (g)	Normal	25.16±2.74	27.06±3.83	29.78±4.06
	Diabetes model	28.79±3.51 *	34.62±4.85 *	38.21±5.91 **
	Rosiglitazone (15 mg/kg bw)	28.03±3.99	30.19±3.88 #	32.36±4.92 #
	Phloridzin (60 mg/kg bw)	27.95±3.94	29.38±3.41 #	31.27±1.81 ##

*p<0.05, **p<0.01, compared to Normal control; #p<0.05, ##p<0.01, compared to Diabetes model control

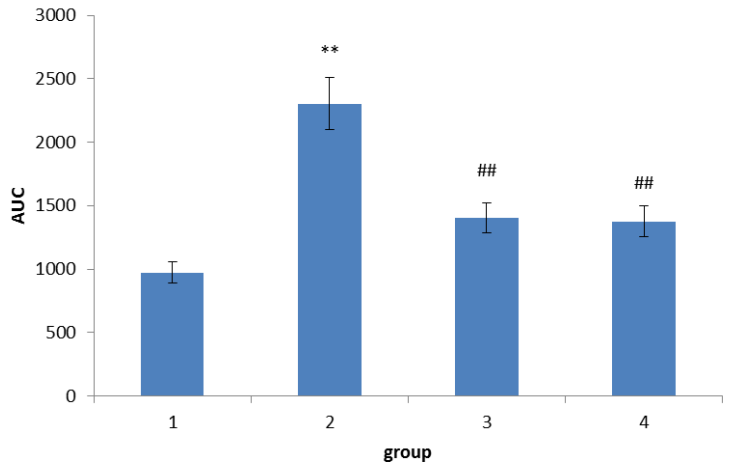
Results

Food-intake in diabetes model group was significantly lower than that in normal group. Both Rosiglitazone and Phloridzin treatment didn't obviously affect the food intake relative to diabetes model control; Water-intake in diabetes model group was significantly lower than that in normal group. Both Rosiglitazone and Phloridzin treatment obviously decreased the water intake relative to diabetes model control; Body weight in diabetes model control group was significantly increased compared to normal control group. Both Rosiglitazone and Phloridzin treatment obviously decreased the body weight of diabetes mice (Table 1).

The results of the OGTT at the end of the experiment (week 10) are shown in Fig. 1A and B. Glucose tolerances were impaired at 10 weeks in the diabetes model control, rosiglitazone and phloridzin treatment groups, while the $AUC_{glucose}$ values in the rosiglitazone and phloridzin treatment groups were effectively lower than that in the diabetes model control group ($p < 0.01$).



A



B

Figure 1: Varying levels of blood glucose (mmol/L) (A) and $AUC_{glucose}$ value (B) in mice as revealed by oral glucose tolerance tests.

1. Normal; 2. Diabetes model; 3. Rosiglitazone (15 mg/kg bw); 4. Phloridzin (60 mg/kg bw)

**p<0.01, compared to Normal control; #p<0.05, ##p<0.01, compared to Diabetes model control

In the diabetic control group, FBG, FINS, HOME-IR, serum leptin, and CRP levels significantly (p<0.01) increased within 12 weeks compared to normal control. However, serum adiponectin in the diabetic control mice decreased significantly within 12 weeks (p<0.01, Table 2). Phloridzin (60 mg/kg bw) treatment significantly (p<0.01) decreased FBG, FINS, HOME-IR, serum leptin, and CRP levels, increased serum adiponectin in Phloridzin treatment group compared to diabetic control mice (Table 2). In the positive control group, rosiglitazone (15 mg/kg bw) led to a significant decrease in FBG, FINS, HOME-IR, serum leptin, and CRP levels and increase in serum adiponectin within 12 weeks compared to the diabetic control group (p<0.05, p<0.01).

Table 2: Effect of phloridzin on Fast blood glucose, Fast insulin, HOME-IR, Serum leptin, adiponectin, and CRP levels in experiment mice

Group	Fast blood glucose (mmol/L)
Normal	6.07±0.54
Diabetes model	19.35±1.28 **
Rosiglitazone (15 mg/kg bw)	12.15±1.16 ##
Phloridzin (60 mg/kg bw)	8.93±0.75 ##
	Fast insulin (mU/ml)
Normal	14.05±1.36
Diabetes model	21.81±2.09 **
Rosiglitazone (15 mg/kg bw)	16.53±1.63 ##
Phloridzin (60 mg/kg bw)	18.36±1.84 #
	HOME-IR
Normal	3.79±0.28
Diabetes model	18.75±1.55 **
Rosiglitazone (15 mg/kg bw)	8.93±0.84 ##
Phloridzin (60 mg/kg bw)	7.29±0.62 ##
	Leptin (mmol/L)
Normal	8.73±1.82
Diabetes model	17.39±4.28 **
Rosiglitazone (15 mg/kg bw)	9.32±3.11 ##
Phloridzin (60 mg/kg bw)	10.11±1.74 ##
	adiponectin (mmol/L)
Normal	29.64±2.84
Diabetes model	9.06±0.78 **
Rosiglitazone (15 mg/kg bw)	22.63±1.93 ##
Phloridzin (60 mg/kg bw)	13.18±2.44 #
	CRP (mmol/L)
Normal	74.39±5.92
Diabetes model	336.14±29.48 **
Rosiglitazone (15 mg/kg bw)	128.41±13.04 ##
Phloridzin (60 mg/kg bw)	140.52±11.52 ##

**p<0.01, compared to Normal control; #p<0.05, ##p<0.01, compared to Diabetes model control

In the diabetic control group, the liver index, and epididymal, perirenal white adipose tissue indexs increased significantly during the experiment acompared to the normal control group ($p<0.01$). Pancreas index decreased significantly ($p<0.01$) during the experiment in the diabetic control group. Phloridzin (60 mg/kg bw) significantly inhibited the tendency of liver index, and epididymal, perirenal white adipose tissue indexs to increase and of pancreas index to decrease in diabetic mice during the entire experiment ($p<0.01$). In the positive control groups, rosiglitazone (15 mg/kg bw) also significantly inhibited the tendency of liver index, and epididymal, perirenal white adipose tissue indexs to increase and of pancreas index to decrease in diabetic mice during the entire experiment ($p<0.01$). At the end of the experiment, there wasn't significantly statistical difference in kidney index between groups. (Table 3)

Table 3: Effect of phloridzin on liver index, kidney index, pancreas index, and epididymal, perirenal white adipose tissue indexs

Group	Pancreas index
Normal	0.674±0.049
Diabetes model	0.438±0.028 **
Rosiglitazone (15 mg/kg bw)	0.599±0.035 ##
Phloridzin (60 mg/kg bw)	0.624±0.043 ##
	Liver index
Normal	5.032±0.337
Diabetes model	6.241±0.292 **
Rosiglitazone (15 mg/kg bw)	5.484±0.344 #
Phloridzin (60 mg/kg bw)	5.217±0.481 #
	Kidney index
Normal	1.326±0.122
Diabetes model	1.298±0.109
Rosiglitazone (15 mg/kg bw)	1.304±0.131
Phloridzin (60 mg/kg bw)	1.322±0.109
	perirenal white adipose tissue index
Normal	0.362±0.042
Diabetes model	1.042±0.092 **
Rosiglitazone (15 mg/kg bw)	0.649±0.042 ##
Phloridzin (60 mg/kg bw)	0.584±0.039 ##
	epididyma white adipose tissue index
Normal	1.217±0.098
Diabetes model	2.632±0.174 **
Rosiglitazone (15 mg/kg bw)	1.957±0.109 ##
Phloridzin (60 mg/kg bw)	1.693±0.121 ##

**$p<0.01$, compared to Normal control; #$p<0.05$, ##$p<0.01$, compared to Diabetes model control

In the diabetic control group, the hepatic PEPCK, G-6-Pase activities increased significantly during the experiment compared to the normal control group ($p<0.01$). Hepatic GK activity and glycogen level decreased significantly during the experiment in the diabetic control group ($p<0.01$). Phloridzin (60 mg/kg bw) significantly inhibited the tendency of hepatic PEPCK, G-6-Pase activities to increase and of hepatic GK activity and glycogen level to decrease in diabetic mice during the entire experiment ($p<0.01$). In the positive control groups, rosiglitazone (15 mg/kg bw) also significantly inhibited the tendency of hepatic PEPCK,

G-6-Pase activities to increase and of hepatic GK activity and glycogen level to decrease in diabetic mice during the entire experiment ($p<0.01$) (Table 4).

Table 4: Effect of phloridzin on hepatic GK, PEPCK, G-6-Pase activities and hepatic glycogen level

Group	GK (mU/g)
Normal	18.47±1.21
Diabetes model	11.19±1.07 **
Rosiglitazone (15 mg/kg bw)	17.28±1.58 ##
Phloridzin (60 mg/kg bw)	14.11±0.72 ##
	PEPCK (mIU/mg)
Normal	17.63±1.09
Diabetes model	35.49±2.22 **
Rosiglitazone (15 mg/kg bw)	21.37±1.73 ##
Phloridzin (60 mg/kg bw)	29.84±1.18 #
	G-6-Pase (U/g)
Normal	6.93±0.54
Diabetes model	10.42±1.11 **
Rosiglitazone (15 mg/kg bw)	7.87±0.68 ##
Phloridzin (60 mg/kg bw)	9.11±0.61 #
	hepatic glycogen (mg/g)
Normal	21.08±2.31
Diabetes model	8.37±0.93 **
Rosiglitazone (15 mg/kg bw)	17.35±1.81 ##
Phloridzin (60 mg/kg bw)	18.94±1.74 ##

**$p<0.01$, compared to Normal control; #$p<0.05$, ##$p<0.01$, compared to Diabetes model control

Discussion

We found that treating mice with high-fat diet-induced T2D with 60 mg/kg/day of phloridzin significantly reduced their water-intake and body weight, but didn't affect food-intake. Additional oral glucose tolerance data indicated that phloridzin significantly increased plasma glucose tolerance, including the altered pattern and the reduced averaged glucose levels of the AUC for diabetic mice at the end of our study. Impaired OGTT is an important standard for type 2 diabetes, which has often been used to derive estimates of the relative roles of insulin secretion and insulin resistance in population studies of glucose tolerance (Guo et al., 2015). A diet containing 0.5% or 1% phloridzin significantly reduced the blood glucose levels in BALB/c mice after 7 days of feeding. A 0.5% phloridzin diet significantly suppressed the blood glucose levels in STZ-induced diabetic mice most likely by inhibiting the absorption of glucose through SGLT1 in the small intestine (Masumoto et al., 2009). Kobori et al (2012) reported that dietary phloridzin reduces blood glucose levels in healthy normal mice as well as STZ-induced diabetic mice by inhibiting glucose absorption in the small intestine. Administration of phloridzin reduced water intake and body weight in a dose-dependent manner (Najafian et al., 2012; Shen et al., 2012). These have been well confirmed by our current results. These results indicated that phloridzin could efficiently improve glucose tolerance and prevent the development of hyperglycemia in type 2 diabetic mice as they grew over time.

High concentrations of plasma glucose and insulin levels were maintained by feeding high-fat diet without phloridzin to KKAy mice for 10 weeks. Hyperglycemia is associated with the consequences of hyperinsulinemia, insulin resistance, and glucose

intolerance in diabetes (Lamming et al., 2013). During the feeding period, we confirmed that hyperinsulinemia and insulin resistance worsened in mice fed the high-fat diet. However, the elevation of FBG and fasting insulin levels in diabetic mice was markedly suppressed by the administration of phloridzin. One possible reason for this effect is that phloridzin prevents chronic elevation of postprandial blood glucose and insulin levels.

Leptin, another adipokine of 16 kDa, is located on 7q31.3 chromosomal region and is a product of obesity gene (Zhang et al., 1994). Hyperleptinemia, represents a condition of elevated leptin levels and is commonly accompanied by components of metabolic syndrome, that is: hyperlipidemia, hypertension, increased adipose tissue, and insulin resistance (Leyva et al., 1998). Lemieux et al (2001) reported relationships between blood levels of hs-CRP and adiponectin with obesity indices and metabolic markers in populations consisting of both obese and lean subjects involving a wide range of weight and body mass index. In this study, phloridzin (60 mg/kg bw) treatment significantly decreased serum leptin, and CRP levels, increased serum adiponectin in phloridzin treatment group compared to diabetic control mice.

Our data show that compared to the normal control group, the diabetes control group developed noticeably greater lipid accumulation in the liver tissue and that body and liver weights in the diabetes control group were also dramatically increased compared to those of the normal control group. However, supplementation with phloridzin significantly lowered lipid accumulation in the liver and suppressed body and liver weight increases compared to those of the diabetes control group. Our data also suggests that phloridzin suppresses high-fat-diet-induced adipose tissue mass and body gain, and may inhibit lipid accumulation in adipose tissue in particular.

The liver is mainly responsible for maintaining normal concentrations of blood glucose by regulating glycolysis and gluconeogenesis (Roden & Bernroider, 2003). In glycolysis, GK is key rate-limiting enzymes mediating glucose oxidation, which catalyzes the conversion of glucose to pyruvate and results in ATP generation (Palsamy & Subramanian, 2009; Zhang et al., 2009). Many researchers have reported that GK is the most sensitive indicators of the glycolytic pathway in diabetic animals. Insufficiency of the enzyme in the diabetic state can cause decreased utilization of glucose for energy production (Roden & Bernroider, 2003; Zhang et al., 2009). In the current study, we found remarkable increase of the hepatic glycolysis enzyme (GK) in diabetic mice treated with phloridzin (60 mg/kg) for 12 weeks. Taken together, these results indicate that phloridzin accelerates hepatic glucose metabolism. Increasing number of studies have shown that excessive hepatic glucose production via the gluconeogenesis pathway is crucial for the elevated glucose levels observed in patients with T2DM (He et al., 2009; Li et al., 2012). The rate of gluconeogenesis is regulated by the activity of rate-limiting gluconeogenic enzymes, namely, PEPCK and G-6-Pase (Xia et al., 2011). Inhibition of hepatic gluconeogenesis contributes to glycemic control in diabetic patients (Foretz et al., 2010). In the present study, significant decrease in activitiess of PEPCK and G-6-Pase, which suggests attenuation of gluconeogenesis and consequent decrease in glucose-6-phosphate dephosphorylation to free glucose, were detected in DPM-treated diabetic mice. Glycogen is the primary intracellular storable form of glucose, and its levels in various tissues, especially in the liver, kidney, and skeletal muscles, directly reflect insulin activity, which in turn regulates glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase (Palsamy & Subramanian, 2009). Reinstatement of glycogen levels in diabetic mice with phloridzin treatment suggests that phloridzin facilitates conversion of glucose-6-phosphate to glycogen, which then results in attenuation of hepatic glucose output.

In conclusion, our results showed that phloridzin supplementation reduced blood glucose, serum leptin, CRP levels, increased serum adiponectin level, improved insulin resistance in type 2 diabetic patients. In addition, phloridzin supplementation increased hepatic GK, decreased hepatic PEPCK, G-6-Pase activities. This suggested that phloridzin promoted glycolytic pathway, decreased gluconeogenic pathway and increased hepatic glycogen synthase in type 2 diabetic patients.

Conflict of interest: There is no conflict of interest.

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