**Gynura procumbens** extract improves insulin sensitivity and suppresses hepatic gluconeogenesis in C57BL/KsJ-db/db mice

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**BACKGROUND/OBJECTIVES:** This study was designed to investigate whether *Gynura procumbens* extract (GPE) can improve insulin sensitivity and suppress hepatic glucose production in an animal model of type 2 diabetes.

**MATERIALS/METHODS:** C57BL/KsJ-db/db mice were divided into 3 groups, a regular diet (control), GPE, and rosiglitazone groups (0.005 g/100 g diet) and fed for 6 weeks.

**RESULTS:** Mice supplemented with GPE showed significantly lower blood levels of glucose and glycosylated hemoglobin than diabetic control mice. Glucose and insulin tolerance test also showed the positive effect of GPE on increasing insulin sensitivity. The homeostatic index of insulin resistance was significantly lower in mice supplemented with GPE than in the diabetic control mice. In the skeletal muscle, the expression of phosphorylated AMP-activated protein kinase, pAkt substrate of 160 kDa, and PM-glucose transporter type 4 increased in mice supplemented with GPE when compared to that of the diabetic control mice. GPE also decreased the expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in the liver.

**CONCLUSIONS:** These findings demonstrate that GPE might improve insulin sensitivity and inhibit gluconeogenesis in the liver.

Keywords: Gluconeogenesis, db/db mice, *Gynura procumbens*, hyperglycemia, insulin resistance

**INTRODUCTION**

Diabetes mellitus and its complications are a cause for concern worldwide. The current global prevalence of diabetes mellitus is estimated to be 2.8 percent and is expected to reach about 7.7 percent by 2030 [1]. Thus, finding a treatment for this epidemic disease is crucial. Diabetes mellitus is an endocrine system disease that occurs metabolic disorders that induce to multiple organ damages. Causes of type 2 diabetes outbreak are complex and include insulin resistance in target tissues such as the skeletal muscle and adipose tissue [2], excessive hepatic glucose production, and decreased insulin secretion [3]. Although many oral antidiabetic drugs for type 2 diabetes such as metformin and thiazolidinediones [4,5] are available, they may have undesirable side effects [6]. Therefore, studies have been designed to identify natural products to treat diabetes.

Insulin regulates the metabolism of glucose by promoting glucose uptake from the blood to the skeletal muscles and adipose tissue and inhibits the production of glucose by the liver. Glucose uptake in the skeletal muscle is stimulated through activation of the AMPK-AS160-GLUT4 pathway. AMP-activated protein kinase (AMPK) is an important enhancer of insulin sensitivity. Phosphorylated AMPK activates AS160, thereby promoting glucose transporter 4 (GLUT4) translocation to the plasma membrane and glucose uptake in the cells [7,8]. The activation of this pathway enhances insulin sensitivity and is effective for the treatment of type 2 diabetes mellitus. Recent studies reported that some natural compounds such as flavonoids promote the activation of AMPK and glucose uptake in skeletal muscle [9,10]. The liver is also responsible for maintaining normal concentrations of blood glucose. In type 2 diabetes mellitus, increased gluconeogenesis was identified as the main cause of elevated glucose production and fasting hyperglycemia [11]. Therefore, regulation of gluconeogenic enzymes such as glucose-6-phosphatase (G6pase) and phosphoenolpyruvate carboxykinase (PEPCK) is important in hepatic glucose metabolism.

*Gynura procumbens* (*G. procumbens*) belongs to the family of Asteraceae. It recently received attention for its antidiabetic function as a folk remedy [12]. To date, studies showed that *G. procumbens* extract (GPE) presents anti-inflammatory, antioxidant, antibacterial, and antihypertensive effects [13-16]. The antidiabetic effect of *G. procumbens* was reported using streptozotocin-induced type 1 diabetic mice [17]. However, its effect on insulin secretion has been conflicting due to the differing response of various cell lines when treated with *G. procumbens* [18]. Especially, there is no report regarding the improvement of hyperglycemia and insulin sensitivity in type 2 diabetic mice.

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Therefore, we investigated the effect of GPE on hyperglycemia and insulin sensitivity in C57BL/KsJ-db/db mice, which show the characteristics of type 2 diabetes mellitus.

MATERIALS AND METHODS

Preparation of GPE

G. procumbens was collected from Uiwang Nae-Son province (Gyeonggi-do, Korea), washed in distilled water, freeze dried, and ground into a powder. G. procumbens powder was then extracted with water for 12 h three times at room temperature. GPE was concentrated in a rotary vacuum evaporator, freeze-dried to a powder, and then stored in a deep freezer (−80°C). The yield of GPE was 5 ± 0.2%. GPE total flavonoid content was 10.33 ± 0.88 mg catechin equivalents (CE)/g dry weight (DW). It was reported that GPE contained 464.53 ± 1.81 μg/g DW of kaempferol, 251.10 ± 3.67 μg/g DW of myricetin, 135.87 ± 0.40 μg/g DW of quercetin [19].

Animals and diets

Male C57BL/KsJ-db/db mice were purchased from Japan SLC (Hamamatsu, Japan). Five-week-old db/db mice were fed a pelletized commercial chow diet for 2 weeks after arrival. They were subsequently randomly divided into 3 groups (n = 7 per group): db/db mice in the control group (DMC) were fed a standard semi-synthetic diet (AIN-93G) for 6 weeks, while animals in the other 2 groups were fed the AIN-93G diet supplemented with either rosiglitazone (0.005% w/w) or GPE.

Plasma membrane fraction of skeletal muscles

Muscle tissue was placed in a buffer (5 mM sodium azide, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaHCO3, pH 7.0) at 4°C. Sub-fractionation of the muscle membranes was performed as previously described [21], using procedures modified from those described by Klip et al. [22,23]. Dissected skeletal muscle was homogenized and centrifuged at 1,000 g for 10 min and the supernatant was collected and stored. The resulting pellet was resuspended in the buffer and re-homogenized in a glass homogenization tube. The supernatant was collected, combined with the first supernatant, and the combination was centrifuged at 9,000 g for 10 min. The resulting supernatant was then centrifuged at 190,000 g for 60 min. The membranes were subsequently applied to a discontinuous sucrose gradient containing 25, 30, and 35% sucrose (wt/vol) solutions and were centrifuged at 190,000 g for 16 h. Membranes were collected from the top of each sucrose gradient, resuspended in the buffer, pelleted by centrifugation at 190,000 g for 60 min, and resuspended in buffer.

Western blot

Western blot analysis was performed on tissue extracts from the liver and skeletal muscles. Liver and skeletal tissues were
Results

Body weight, food intake, and water intake

Body weights as well as food and water intake of all mice were observed during the experiment. Table 1 presents the monitored body weights as well as food and water intake of the mice during the 6 weeks of the experiment. Body weights of mice in db/db-control, db/db-RG, and db/db-GPE groups did not differ significantly at the beginning of the experiment. Body weights increased gradually during the 6-week period. At the end of the experiment, mice in the db/db-RG group exhibited significantly higher body weights than mice in the db/db-control and db/db-GPE groups. However, water intake was not significantly different between the db/db-control, db/db-RG, and db/db-GPE groups. Water intake was significantly higher in the db/db-control group mice than in the db/db-RG and db/db-GPE groups, respectively.

Blood glucose and HbA1c levels

Fasting blood glucose levels in db/db-GPE group mice are shown in Fig. 1. At the beginning of the study, blood glucose levels were not significantly different between groups. However, fasting blood glucose levels of db/db-GPE mice after 4 weeks were significantly lower than those of the db/db-control mice. Blood glucose levels measured in db/db-control mice were elevated throughout the experiment, likely reflecting the progress of diabetes mellitus. As shown in Table 2, HbA1c values were 12.92 ± 0.31, 7.45 ± 0.4, and 8.87 ± 1.09 in db/db-control, db/db-RG, and db/db-GPE mice, respectively. These values were significantly lower in db/db-GPE mice than in db/db-control and db/db-RG mice. HbA1c values were significantly different among groups (P < 0.05). HbA1c: blood glycosylated hemoglobin, HOMA-IR: homeostatic index of insulin resistance, QUICKI: quantitative insulin sensitivity check index.

Plasma insulin levels, HOMA-IR, and QUICKI

Plasma insulin levels measured in experimental animals are shown in Table 2. Plasma insulin levels in C57BL/KsJ-db/db mice fed AIN-93G diet supplemented with G. procumbens extract (GPE) were significantly lower than those in the C57BL/KsJ-db/db mice fed AIN-93G diet with rosiglitazone (0.005 g/100 g diet). Plasma insulin levels were markedly lowered in the mice of the db/db-RG group (139.06 ± 21.96 pmol/L) compared to those of the mice in the db/db-control group (251.04 ± 25.24 pmol/L). Similarly, db/db-GPE mice exhibited

| Table 1. Effects of GPE supplementation on water and food intakes as well as body weights of C57BL/KsJ-db/db mice |
|-----------------------------------------------|
| db/db-control | db/db-RG | db/db-GPE |
| Water intake (mL/day) | 28.57 ± 3.02 | 4.74 ± 1.74 | 18.62 ± 1.97 |
| Food intake (g/day) | 5.51 ± 0.77 | 4.81 ± 1.55 | 5.03 ± 0.96 |
| Body weights (g) | | | |
| Initial | 31.10 ± 2.49 | 31.54 ± 1.02 | 31.20 ± 1.90 |
| Final | 46.18 ± 4.89 | 57.00 ± 2.12 | 45.80 ± 4.90 |

**Note:** Values are presented as means ± SD, n = 7 per group. a-c Mean values designated by different letters are significantly different among groups (P < 0.05).

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Fig. 2. Effect of G. procumbens extract (GPE) supplementation on intraperitoneal glucose tolerance tests (A) and insulin tolerance tests (B) in C57BL/KsJ-db/db mice. Blood glucose concentrations were measured at the indicated times and presented as percentages of glucose measured at the time of injection (t=0) of glucose (0.5 g/kg of BW) or insulin (2 units/kg of BW). db/db (diabetes mellitus control): C57BL/KsJ-db/db mice fed AIN-93G diet; db/db-RG: C57BL/KsJ-db/db mice fed AIN-93G diet supplemented with rosiglitazone (0.005 g/100 g diet); db/db-GPE: C57BL/KsJ-db/db mice fed AIN-93G diet supplemented with GPE (0.5 g/100 g diet). Values are presented as means ± SD, n = 7 per group. Mean values designated by different letters are significantly different between groups (P<0.05).

Fig. 3. Effect of G. procumbens extract (GPE) supplementation on PM-GLUT4, pAMPK, and pAS160 expression in the skeletal muscle. Western blotting and signal intensities were determined by densitometric analysis using Multi Gauge V3.1 software. Representative blots of PM-GLUT4, pAMPK, and pAS160 protein expression are shown with protein expression levels quantified relative to the expression level observed in samples from db/db-control mice. Each value is expressed as mean ± SD of experiments performed in triplicate. Values denoted by different letters are significantly different (P<0.05), as analyzed by Duncan’s multiple range test. PM-GLUT4: plasma membrane- glucose transporter type 4; pAMPK: phosphorylated AMP-activated protein kinase; pAS160: phosphorylated Akt substrate of 160 kDa.
Fig. 4. Effect of G. procumbens extract (GPE) supplementation on G6Pase and PEPCK expression and hepatic glycogen in the liver of C57BL/KsJ-db/db mice. Representative blots of G6Pase and PEPCK protein expression are shown with protein expression levels quantified relative to the expression level observed in samples from db/db-control mice. Each value is expressed as mean ± SD of experiments performed in triplicate. Values denoted by different letters are significantly different (P < 0.05), as analyzed by Duncan’s multiple range test. PEPCK: Phosphoenolpyruvate carboxykinase, G6Pase: Glucose-6-phosphatase.

significantly lowered plasma insulin levels than mice in the db/db-control group. HOMA-IR values were significantly lower in the db/db-GPE mice than in the db/db-control mice (P < 0.05). Additionally, QUICKI values were significantly higher in the db/db-GPE mice than in the db/db control mice.

Intraperitoneal glucose and insulin tolerance tests

We performed IPGTT in db/db mice receiving GPE to evaluate GPE’s ability to improve glucose tolerance. The results are presented as percentages of the measurement performed at the time of glucose injection (t = 0) and significant differences among db/db-control, db/db-RG, and db/db-GPE group are presented. As shown in Fig. 2 (A), blood glucose levels in the db/db-GPE mice peaked at 60 minutes after injection and recovered to levels close to the basal value at 120 min. A similar pattern was observed in mice from the db/db-RG group. IPITT was also performed to determine the effects of GPE supplementation on insulin tolerance. The results expressed as percentages of the measurement at the time of insulin injection (t = 0) and significant differences among db/db-control, db/db-RG, and db/db-GPE group are presented. As shown in Fig. 2 (B), db/db-GPE and db/db-RG mice exhibited a rapid reduction in blood glucose levels compared with the db/db-control mice, with blood glucose levels decreasing within 120 min of the insulin injection.

Expression of PM-GLUT4, GLUT4, pAMPK, AMPK, pAS160, and AS160 in the skeletal muscle

In this study, we studied the expression of PM-GLUT4, GLUT4, pAMPK, AMPK, pAS160, and AS160 in the skeletal muscle. RG supplementation, positive control, showed the highest PM-GLUT4, pAMPK, and pAS160 levels in the skeletal muscle. GPE supplementation also increased PM-GLUT4, pAMPK, and pAS160 levels in the skeletal muscle. As shown in Fig. 3, the expression of GLUT4 in the plasma membrane of the skeletal muscle significantly increased, reaching levels that were 2.2 and 3.3-fold
higher in db/db-GPE and db/db-RG mice than those measured in db/db-control mice, respectively. No significant difference was observed in AMPK expression among db/db-RG, db/db-GPE and db/db-control mice. However, the phosphorylation levels of AMPK and AS160, two upstream regulators of GLUT4 translocation to the plasma membrane, were significantly increased by GPE supplementation. This means that GPE has the ability to stimulate glucose uptake through activation of the AMPK-AS160-GLUT4 pathway in the skeletal muscle.

Expression of pAMPK and gluconeogenic enzymes, G6Pase and PEPCK, and glycogen levels in the liver

As shown in Fig. 4, pAMPK activation in the liver of db/db-GPE mice was significantly higher than that in the db/db-control mouse. The expression of G6Pase in the liver significantly decreased in db/db-GPE mice, with 0.5-fold lower levels than those observed in the db/db-control mice. Hepatic glycogen levels in the db/db-RG mice were the highest with values of 119.33 ± 4.04 mg/g of liver. db/db-GPE mice were significantly higher than the levels in the db/db-control mice, with values of 109.67 ± 4.16 and 90.25 ± 4.75 mg/g of liver measured in db/db-GPE and db/db-control mice, respectively (Fig. 4).

DISCUSSION

Common characteristics of type 2 diabetes are chronic hyperglycemia caused by insulin resistance and increased gluconeogenesis in the liver [24]. Treatment for type 2 diabetes, aim at increasing insulin sensitivity and decreasing hyperglycemia. However, pharmacological treatments for type 2 diabetes exhibit a number of limitations, including side effects. Thus, in this study, we investigated the effect of a medicinal plant extract, GPE on insulin sensitivity and glucose metabolism using an animal model of type 2 diabetes, C57BL/KsJ- db/db mice.

The daily food intake was not significantly different among db/db-control, db/db-RG and db/db-GPE groups. The body weight was also not significantly different between db/db-control and db/db-GPE groups. But it was significantly more increased in db/db-RG group than those measured in the db/db-control and db/db-GPE groups. Weight gain of db/db-RG mice was attributable to an increase in the adipose tissue mass. Some studies attributed weight gain to rosiglitazone treatment. RG may affect the differentiation of preadipocytes in humans [25]. This effect is markedly enhanced in subcutaneous fat [26].

Water intake was significantly lower in db/db-GPE mice than in db/db-control mice. The increase in water intake shows the state of polydipsia, one of the typical characteristics of diabetes [27]. In this study, GPE supplementation in db/db mice ameliorated the symptoms of diabetes, reducing water intake.

After 4 weeks of experimental diet, fasting blood glucose levels in the db/db-GPE mice were significantly lowered when compared with those of the db/db-control mice. Hyperglycemia is associated with the development of type 2 diabetes. Therefore, controlling blood glucose levels is important for preventing diabetes mellitus and diabetic complications [28]. In addition, GPE treatment significantly lowered HbA1c levels. HbA1c is generally used as an accurate index of long-term blood glucose regulation and is a simple way to investigate the glycemic control in diabetes [29]. Lowering HbA1c levels in diabetes mellitus can effectively prevent the risk of complications such as retinopathy and nephropathy [30]. The flavonoids present in GPE may be responsible for the reduction of hyperglycemia. Components of GPE such as quercetin, kaempferol, and myricetin have antidiabetic effects by lowering blood glucose levels [31,32]. This suggests that GPE supplementation has positive effects in controlling hyperglycemia in type 2 diabetes.

This study showed that GPE supplementation could improve plasma insulin levels. The levels of plasma insulin in db/db-control mice were higher than those in db/db-GPE mice, suggesting that db/db-control mice still exhibited hyperinsulinemia. db/db mice generally show an initial phase of hyperinsulinemia, while insulinopenia could develop with age [33]. It is a common feature exhibited in the late stage of type 2 diabetes. In this study, the plasma insulin levels in db/db-GPE mice were significantly lower than those measured in db/db-control mice. The mice in the db/db-control group still displayed hyperinsulinemia. On the other hand, db/db-RG and db/db-GPE mice showed reduced hyperinsulinemia. Hyperinsulinemia is caused by insulin resistance, which is a characteristic of the early stage of type 2 diabetes [34]. When the insulin reaction does not function properly in target tissues, more insulin is needed, resulting in hyperinsulinemia. Some studies reported that flavonoids have the ability to improve insulin resistance, thereby, reducing hyperinsulinemia and preventing metabolic symptom [9,32]. HOMA-IR and QUICKI calculated from fasting insulin and glucose levels are the commonly used measures of insulin sensitivity. HOMA-IR is an index of insulin resistance, which increases with increasing insulin resistance [35]. QUICKI is an index of insulin sensitivity, with values that decrease with increasing insulin resistance [36]. In this study, HOMA-IR was significantly lowered in mice of the db/db-GPE group compared with the values measured in db/db-control mice. These results suggest that GPE supplementation might improve insulin sensitivity.

In type 2 diabetes mellitus, hyperglycemia presents as a reduction in the insulin-mediated blood glucose disposal. Our results showed that GPE supplementation may improve both glucose and insulin tolerance. The results of IPGTT showed improved glucose levels in db/db-GPE and db/db-RG mice by lowering blood glucose levels to the basal level, compared to the levels measured in db/db-control mice. IPITT was also improved in mice supplemented with GPE and RG compared to that of mice in the db/db-control group. These results suggest that GPE and RG might act as insulin sensitizers. RG is an antidiabetic drug and was used as the positive control for GPE in this study. RG works as an insulin sensitizer by binding to the PPAR receptor in fat cells making the cells more responsive to insulin [26]. db/db-GPE mice also showed improved insulin sensitivity, which is important for lowering blood glucose levels, even if the effect of GPE was lower than that of RG.

Enhancing insulin sensitivity is very important in the treatment of diabetes. We investigated the expression of genes related to insulin sensitivity using western blotting. Glucose
uptake is regulated by insulin binding to the insulin receptor on the cell surface via GLUT4 [37]. GLUT4 is the key glucose transporter in response to insulin in the skeletal muscle [38]. The action of insulin on glucose transport in the skeletal muscle is also affected by AMPK, suggesting that AMPK can be a therapeutic target for the treatment of type 2 diabetes, especially with insulin resistance [39]. Through an insulin-dependent mechanism, increased activation of AMPK increases GLUT4 translocation to the plasma membrane [40]. Dietary GPE supplementation in db/db mice significantly increased AMPK activation. This resulted in the increased expression of GLUT4 in the plasma membrane, which contributed to the improvement in insulin sensitivity. GLUT4 expression in the plasma membrane was 2.2 and 3.3-fold higher in db/db-GPE and db/db-RG mice than those measured in db/db-control mice, respectively. Although GLUT4 expression in the plasma membrane of db/db-GPE mice was lower than that in db/db-RG mice, GLUT4 expression in the plasma membrane in db/db-GPE mice was significantly higher than that in db/db-control mice. GPE supplementation also increased the expression of phosphorylated AS160, suggesting that it may stimulate glucose uptake in the skeletal muscles and reduce hyperglycemia. AS160 is a major mediator of GLUT4 translocation from the intracellular pool to the plasma membrane is promoted [6]. Through the upregulation of the AMPK-AS160-GLUT4 pathway, glucose uptake and utilization in the skeletal muscle are enhanced. GPE contains flavonoids such as quercetin and 3-O-glycosides of kaempferol. These flavonoids stimulate AMPK activation and GLUT4 translocation in the skeletal muscle, thereby, increasing basal glucose uptake in the skeletal muscle [41].

The liver plays a significant role in regulating glucose production from gluconeogenesis or glycogenolysis. In the postprandial state, insulin stimulates the liver to take up more glucose from the blood and synthesize glycogen. In the starving state, the liver produces more glucose to maintain blood glucose levels. However, under the diabetic state, hepatic gluconeogenesis is abnormally enhanced and glycogen synthesis is inhibited [42]. In this study, the expressions of G6Pase and PEPCK, the key gluconeogenic enzymes, was significantly decreased by GPE supplementation, while hepatic glycogen levels were significantly increased. These results show that GPE might be beneficial in type 2 diabetes and useful as an antidiabetic supplement.

**CONFLICT OF INTEREST**

The authors declare no potential conflicts of interests.

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