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Pandanus amaryllifolius leaf extract increases insulin sensitivity in high-fat diet-induced obese mice

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ABSTRACT

Objective: To examine the effect of *Pandanus amaryllifolius* (*P. amaryllifolius*) leaf extract on the insulin resistance state in obese ICR mice.
Methods: Obesity was induced in mice fed with high-fat diet (45% fat) for 12 weeks. After the first six weeks on the diet, the obese mice were administered with the water extract of *P. amaryllifolius* leaf at 125 and 250 mg/kg/day, respectively for another six weeks. At the 5th week of treatment, oral glucose tolerance test was conducted. After six weeks of treatment, the levels of blood glucose, serum insulin, leptin, adiponectin, and lipid profiles were determined. The liver, muscle and epididymal fat tissues were removed for measuring the biochemical parameters and protein expression, as well as histological examination.
Results: Six weeks of treatment with *P. amaryllifolius* led to a significant reduction in the blood glucose level as well as improvement in the insulin resistance. *P. amaryllifolius* also increased the liver glycogen storage and serum adiponectin and decreased the serum leptin levels. A reduction in the serum and hepatic triglyceride, and non-esterified fatty acid levels was also observed. The histological examination showed that the obese mice treated with *P. amaryllifolius* reduced the lipid droplet in liver tissue and adipocyte size in epididymal fat tissue. The treatment also increased the protein expression of glucose transporter 4 in the muscle and fat tissues.
Conclusions: The treatment with *P. amaryllifolius* could decrease several parameters of impaired glucose and lipid metabolism. To the best of our knowledge, this is the first report on the role of *P. amaryllifolius* leaf extract in alleviating the insulin dysfunction in obesity state.

1. Introduction

Obesity is one of the major risk factors for diabetes, hypertension, dyslipidemia, and atherosclerosis [1,2]. The development of obesity is the etiology for inducing the status of insulin resistance, which is a primary risk factor for type 2 diabetes mellitus. The impaired insulin action in obesity can inhibit glucose output from the liver and glucose uptake into the fat and muscle cells [3].

Excessive lipid accumulation in the nonadipose tissues, especially in the liver, can lead to the development of fatty liver and insulin resistance. Fatty liver is a reversible condition that is marked by increased accumulation of triglyceride (TG) in the hepatocytes. The liver and adipose tissues have been found to remain dysfunctional in obesity condition [4–6]. Adipose tissue plays an important role in the regulation of energy homeostasis and insulin resistance development. Long-term high-fat diet (HFD) feeding can cause adipocyte hypertrophy and its dysfunction, which is characterized by an excessive release of free fatty acids into the circulation [7]. Adipose tissue produces adipokine, including adiponectin, which is responsible

for controlling glucose and lipid metabolism. Studies on rodents and human subjects have shown that an increased plasma adiponectin level can improve the insulin resistance [8–12]. Moreover, the glucose uptake and insulin sensitivity in peripheral tissues are involved in the stimulation of glucose transporter 4 (GLUT4) signaling cascade [13–17].

Pandanus amaryllifolius (*P. amaryllifolius*) is a tropical plant found in India, South China, and Southeast Asia including Thailand. It exhibits several bioactivities such as antiviral [18], antioxidant [19], and antihyperglycemic activities [20]. However, the effects of *P. amaryllifolius* leaf extract on the obesity-induced insulin resistant state have not been clearly demonstrated. Therefore, given the increasing incidence of obesity, the aim of this study was to investigate the effect of *P. amaryllifolius* leaf extract on impaired insulin sensitivity in HFD-fed mice.

2. Materials and methods

2.1. Plant extraction

The leaves of *P. amaryllifolius* were collected from Buriram, Thailand between July and September 2013. A voucher specimen (SKP 138 16 01 01) was deposited at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The dried leaves (100 g) were extracted three times with distilled water (1 L) at 100 °C for 30 min. This extract was concentrated and subsequently freeze-dried. By using this procedure, the yield obtained was 10.11% of the starting dry weight of the leaves. The *P. amaryllifolius* extract obtained during this procedure was kept at −20 °C until further use.

2.2. Animals and induction of obesity

All experimental procedures involving animals were conducted in accordance with the standards of Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD, USA) and approved by the Animal Ethics Committee, Faculty of Medicine, Thammasat University, Thailand (Rec. No. AE 010/2014). Male ICR mice weighing 20–25 g were purchased from the National Laboratory Animal Center of Mahidol University, Nakhon Pathom, Thailand. They were maintained in an air-conditioned room [(25 ± 2) °C] with a 12 h light: 12 h dark cycle and fed with a low-fat diet and water *ad libitum* for a week. After random selection, the mice were fed with low-fat diet containing 10% fat (D12450H, Research Diets, New Brunswick, NJ, USA) with a total energy of 3.85 kcal/g or HFD containing 45% fat (D12451, Research Diets, New Brunswick, NJ, USA) with a total energy of 4.73 kcal/g for 12 weeks.

2.3. Treatment design

After the completion of the first six weeks on diets, the animals were divided into four groups with eight mice in each group. Group 1 consisted of normal control mice treated with 5% gum arabic; Group 2 comprised obese control mice treated with 5% gum arabic; and Groups 3 and 4 consisted of obese mice treated with 125 and 250 mg/kg of *P. amaryllifolius* extracts, respectively. The doses selection was based on our preliminary study, and the high blood glucose was reduced in the obese mouse model after one-week treatment with *P. amaryllifolius* extracts. The *P. amaryllifolius*

extracts were dissolved in 5% gum arabic. The body weight and food intake of the mice were measured every week. After five weeks of treatment, the mice were fasted for 6 h and orally administered with glucose (2 g/kg) for oral glucose tolerance test (OGTT). The blood glucose levels were determined from the tail vein blood, before and after the glucose loading at regular intervals of 20, 60, and 120 min.

2.4. Blood and tissue collection

After six weeks of treatment, the mice were fasted for 6 h and sacrificed with isoflurane anesthesia. Whole blood samples were collected from the heart for determining the blood glucose level, and the remaining blood samples were centrifuged at 3000 r/min for 10 min at 4 °C. Subsequently, the serum was collected for measuring the lipid profiles, insulin, leptin, and adiponectin levels. The liver, gastrocnemius muscle, and epididymal fat tissues were then removed for the determination of biochemical parameters, protein expression, and histological examination.

2.5. Determination of serum insulin, leptin, and adiponectin levels

After six weeks of treatment, the fasting serum insulin, leptin, and adiponectin concentrations were measured using the ELISA kit (EMD Millipore, Billerica, MA, USA).

2.6. Determination of serum and liver lipid profiles

After six weeks of the treatment with *P. amaryllifolius* extracts, the serum total cholesterol (TC), TG and non-esterified fatty acid (NEFA), and liver TG levels were measured using the enzymatic colorimetric kit (Wako, Osaka, Japan). The protocols for liver TG and NEFA extractions were used as described previously [21].

2.7. Determination of hepatic glycogen synthesis

The glycogen content was determined as described previously [21]. Briefly, the liver was homogenized in 30% KOH solution. The homogenized liver was then dissolved in a boiling water-bath (100 °C) for 30 min and precipitated with 95% ethanol. The pellet was suspended in distilled water, mixed with 0.2% anthrone reagent, and absorbance was measured at 620 nm.

2.8. Western blot

Plasma membrane proteins of skeletal muscle and epididymal adipose tissues were homogenized and extracted in TPER® mixed with Halt® protease inhibitor cocktail (Thermo Scientific, IL, USA). The plasma membrane GLUT4 protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked and incubated with anti-GLUT4 and anti-actin primary antibodies (dilution 1:200, Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:2000, Santa Cruz Biotechnology, Dallas, TX, USA), and immunoreactive bands were developed in Clarity™ Western ECL substrate (Bio-Rad, CA, USA). The band intensities were analyzed

by densitometry using the Odyssey® Fc imaging system (LI-COR Bioscience, Lincoln, NE, USA).

2.9. Histological examination

Liver and epididymal fat tissues were fixed with 10% formalin and embedded in paraffin. The paraffin sections (3 μm) were cut, mounted on glass slides and stained with hematoxylin and eosin. The area of adipocyte was calculated using an ImageJ software program (National Institute of Health, Bethesda, MD, USA).

2.10. Statistical analyses

All data were expressed as mean ± SEM, and statistical analysis was performed using One-way ANOVA, followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered to be statistically significant. The statistical analyses were performed using computer-based software SigmaStat (Systat Software, CA, USA).

3. Results

3.1. Glucose and lipid metabolic parameters

After six weeks of treatment, the body weight of obese control mice was found to be significantly higher than that of normal control mice (Figure 1A) ($P < 0.05$). The body weight of obese mice treated with *P. amaryllifolius* extract at 125 and 250 mg/kg, respectively also followed an increasing trend. No significant difference in food intake was observed among the groups (Figure 1B). The energy intake in obese mice treated with 250 mg/kg *P. amaryllifolius* was significantly increased as compared to normal control mice (Figure 1C). However, the epididymal fat weight in all obese groups was significantly

increased as compared to normal control group (Figure 1D) ($P < 0.05$). No reduction in the fat weight was observed in obese mice treated with *P. amaryllifolius* extract. Interestingly, their adipocyte size was significantly smaller as compared to obese control mice (Figure 1E,F) ($P < 0.05$).

After six weeks of treatment, the fasting blood glucose level of obese control mice was found to be significantly higher than that in normal control mice (Figure 2A) ($P < 0.05$). Comparatively, a significant reduction in fasting blood glucose was observed in obese mice treated with *P. amaryllifolius* extracts at 125 and 250 mg/kg, respectively ($P < 0.05$). Obese control mice showed a high serum insulin level as compared to normal control mice (Figure 2B), whereas a significant reduction in insulin level was noted in *P. amaryllifolius*-treated obese mice ($P < 0.05$). As shown in Figure 2C, the *P. amaryllifolius*-treated obese mice showed increased adiponectin levels as compared to obese control mice. The serum leptin level of obese control mice was also found to be significantly higher than that of normal control mice (Figure 2D) ($P < 0.05$) while obese mice treated with *P. amaryllifolius* at 125 and 250 mg/kg showed a significant reduction in the leptin level as compared to obese control mice ($P < 0.05$). In comparison to normal control mice, the OGTT for obese control mice showed significantly higher levels of blood glucose in the fasting state and during 120 min after the glucose loading (Figure 2E) ($P < 0.05$). In the case of *P. amaryllifolius*-treated obese mice, a significant reduction in the high blood glucose was observed at 60 and 120 min after glucose loading as compared to obese control mice ($P < 0.05$). In addition, obese control mice showed a decreased liver glycogen content as compared to normal control mice; however, the treatment with *P. amaryllifolius* at 250 mg/kg restored this reduction (Figure 2F).

No reduction in serum TC was observed even after six weeks of treatment with *P. amaryllifolius* (Figure 3A). However, a significant reduction ($P < 0.05$) in serum TG and NEFA
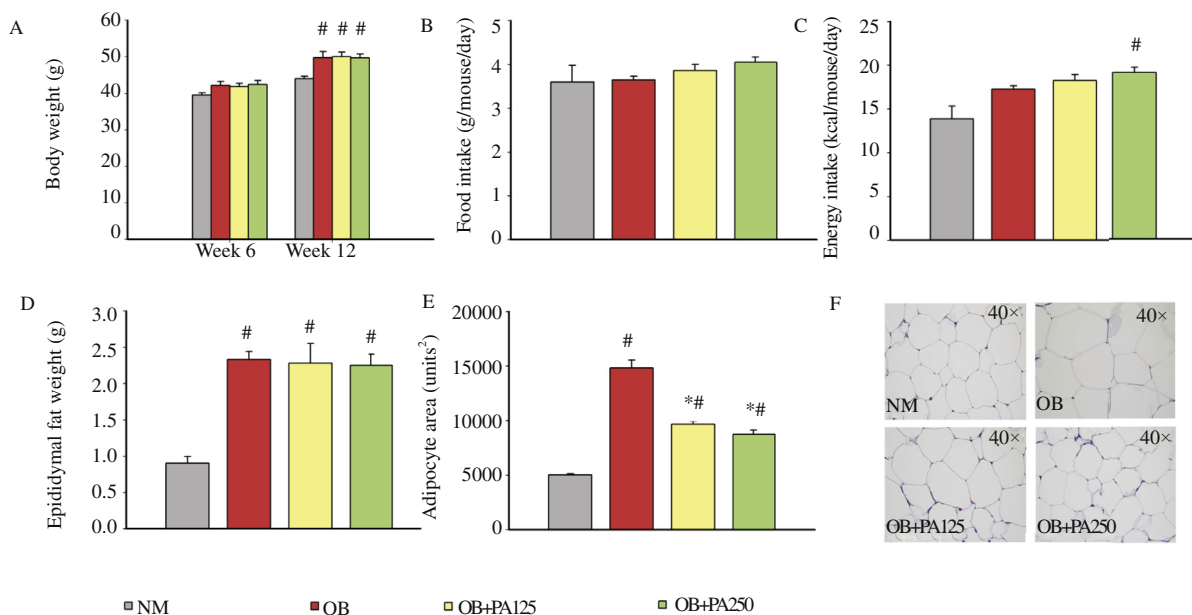
Figure 1. Effect of *P. amaryllifolius* on body weight (A), food intake (B), energy intake (C), epididymal fat weight (D), adipocyte size (E), and histology of epididymal fat tissues (F) in HFD-induced obese mice.
Data are presented as mean ± SEM ($n = 8$). #: $P < 0.05$ when compared to normal control mice. *: $P < 0.05$ when compared to obese control mice. NM: Normal control mice; OB: Obese control mice; OB + PA125: Obese mice treated with *P. amaryllifolius* at 125 mg/kg; OB + PA250: Obese mice treated with *P. amaryllifolius* at 250 mg/kg.
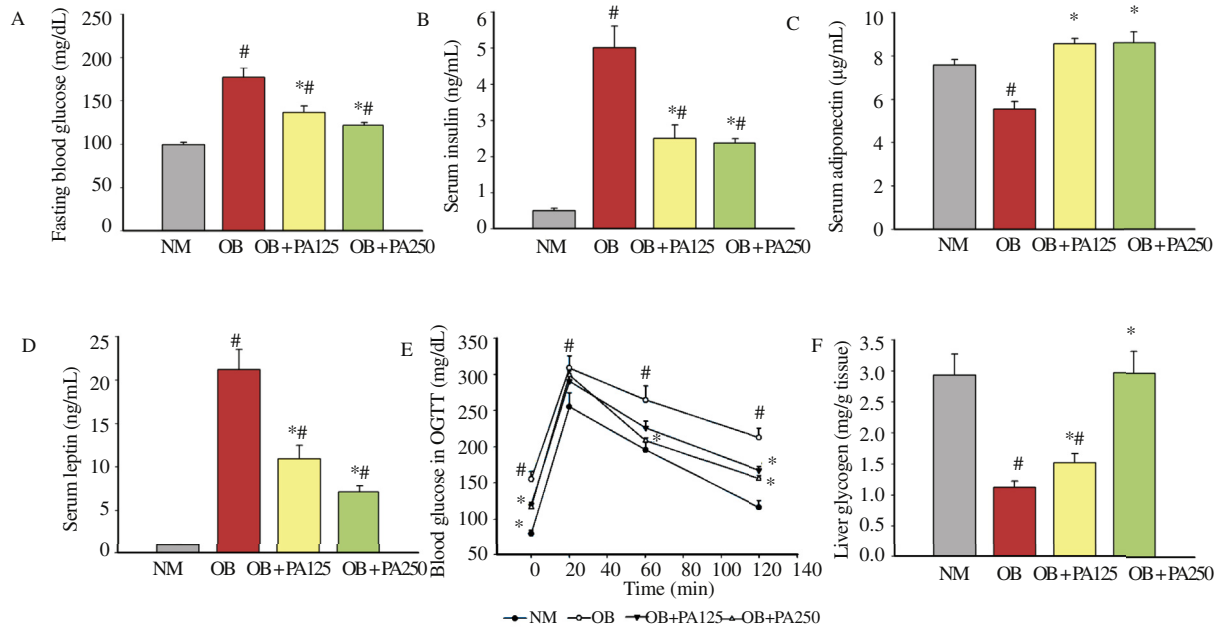
Figure 2. Effect of *P. amaryllifolius* on fasting blood glucose (A), serum insulin (B), serum adiponectin (C), serum leptin (D), blood glucose in OGTT (E), and liver glycogen (F) in HFD-induced obese mice.
Data are presented as mean ± SEM ($n = 8$). #: $P < 0.05$ when compared to normal control mice. *: $P < 0.05$ when compared to obese control mice. NM: Normal control mice; OB: Obese control mice; OB + PA125: Obese mice treated with *P. amaryllifolius* at 125 mg/kg; OB + PA250: Obese mice treated with *P. amaryllifolius* at 250 mg/kg.
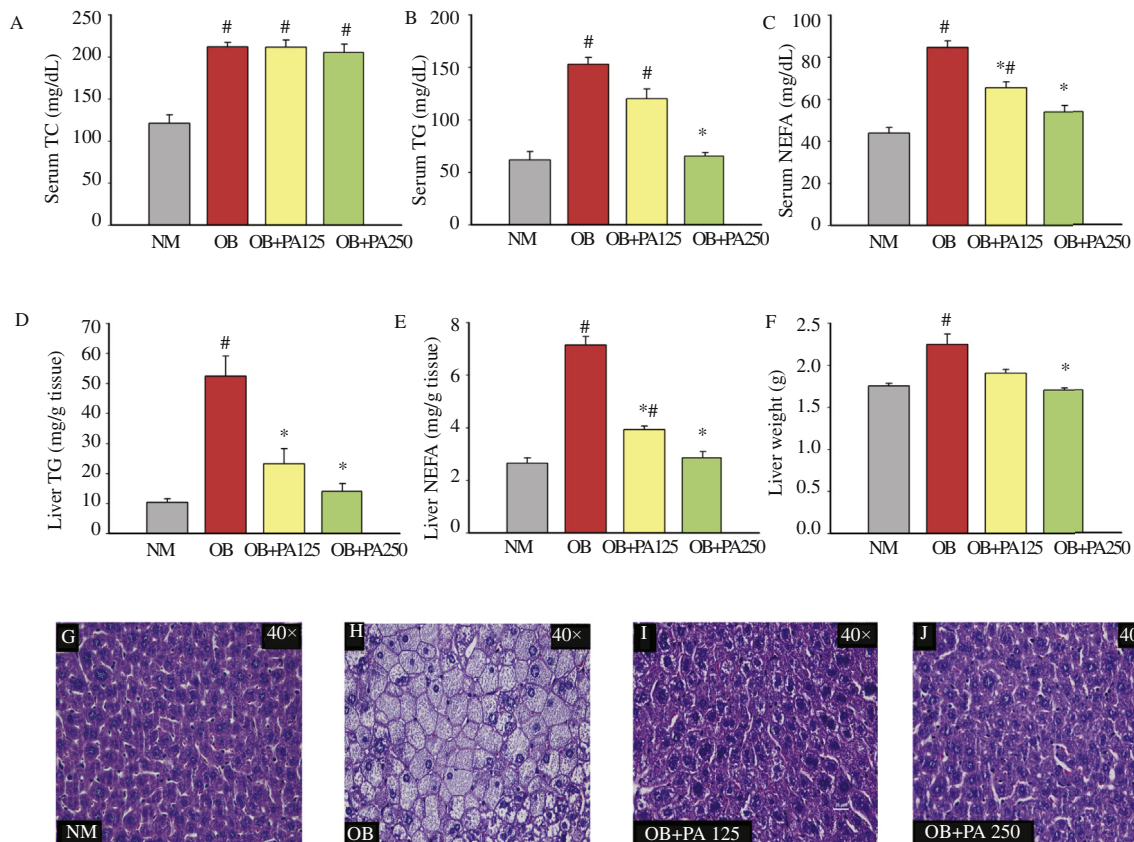
Figure 3. Effect of *P. amaryllifolius* on serum TC (A), serum TG (B), serum NEFA (C), liver TG (D), liver NEFA (E), liver weight (F), and histology of liver (G–J) in HFD-induced obese mice.
Data are presented as mean ± SEM ($n = 8$). #: $P < 0.05$ when compared to normal control mice. *: $P < 0.05$ when compared to obese control mice. NM: Normal control mice; OB: Obese control mice; OB + PA125: Obese mice treated with *P. amaryllifolius* at 125 mg/kg; OB + PA250: Obese mice treated with *P. amaryllifolius* at 250 mg/kg.
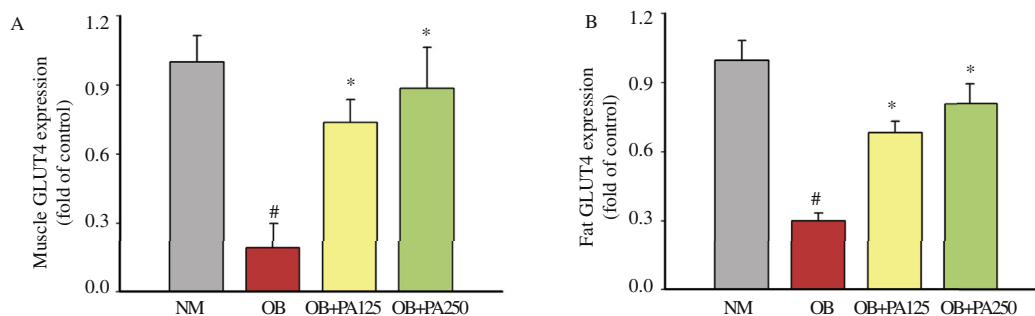
was observed in the case of *P. amaryllifolius*-treated obese mice (125 and 250 mg/kg) as compared to obese control mice (Figure 3B,C). In addition, the treatment with *P. amaryllifolius* significantly decreased the accumulation of TG and NEFA in the liver tissue (Figure 3D,E) ($P < 0.05$). The liver weight of obese control mice increased as compared to normal control mice, and the increased liver weight was significantly reduced after the treatment with *P. amaryllifolius* (Figure 3F) ($P < 0.05$). In the case of liver histology, more lipid droplets were found in obese control mice compared to those in the *P. amaryllifolius*-treated obese mice (Figure 3G–J), which was consistent with the results of liver TG and NEFA.

3.2. Protein expressions of plasma membrane GLUT4

The expressions of plasma membrane GLUT4 proteins in the muscle and fat cells were significantly reduced in obese control mice (0.19- and 0.30-fold, respectively compared with the basal level) (Figure 4A,B). However, treatment with 125 and 250 mg/kg *P. amaryllifolius* significantly increased the muscle GLUT4 protein by 0.78- and 0.88-fold of control, respectively when compared with obese control group (Figure 4A). GLUT4 protein expression in adipose tissue was also up-regulated by 0.68- and 0.81-fold after treatment with 125 and 250 mg/kg *P. amaryllifolius*, respectively (Figure 4B).

level tends to be elevated and the adiponectin level is slightly reduced in dietary obese rodents [23]. However, in this study, it was observed that the serum leptin was significantly reduced in obese mice treated with *P. amaryllifolius*.

Moreover, the treated obese mice showed a significant increase in the serum adiponectin level. These results suggest that this treatment may improve insulin resistance in HFD-induced obese mice.

GLUT4 is considered to be the most important glucose transporter in the peripheral tissues, such as the fat and muscle cells. Improved GLUT4 function can control the blood glucose level. However, the deficiency of insulin or insulin-resistant condition plays a crucial role in the suppression of GLUT4 translocation, leading to decreased uptake of glucose into the fat and muscle cells, which contributes significantly to the elevated glucose levels [24]. Obesity is strongly related to insulin resistance that can lead to reduced GLUT4 expression in the insulin-sensitive tissues [14,16]. Our results showed that the treatment with *P. amaryllifolius* restored the decreased GLUT4 protein expression leading to enhanced glucose uptake into the muscle and fat cells. This action could be useful in controlling the hyperglycemic condition.

The cholesterol absorption from the small intestine shows increased cholesterol level in HFD feeding [25]. The HFD model can increase the risk of hypertriglyceridemia possibly due to the

Figure 4. Effect of *P. amaryllifolius* on muscle (A) and fat (B) plasma membrane GLUT4 protein expressions in HFD-induced obese mice.
Data are presented as mean ± SEM ($n = 8$). [#]: $P < 0.05$ when compared to normal control mice. [*]: $P < 0.05$ when compared to obese control mice. NM: Normal control mice; OB: Obese control mice; OB + PA125: Obese mice treated with *P. amaryllifolius* at 125 mg/kg; OB + PA250: Obese mice treated with *P. amaryllifolius* at 250 mg/kg.

4. Discussion

In the present study, HFD-induced insulin resistant state with decreased glucose tolerance and insulin sensitivity and increased insulin level in mice. This model also showed dyslipidemia and increased hepatic fat accumulation. Furthermore, this model was found to have reduced hepatic glycogen contents. Interestingly, the treatment with *P. amaryllifolius* extract can significantly improve the conditions of hyperinsulinemia, glucose intolerance, hyperlipidemia, and fatty liver in obese mice.

Insulin resistance is a characteristic feature of type 2 diabetes mellitus development. This condition is related to hyperglycemia and decreased glycogen synthesis in type 2 diabetes mellitus [22]. The present study showed a significant reduction in hyperglycemia and improved insulin sensitivity in the *P. amaryllifolius*-treated obese mice. Moreover, the treatment with *P. amaryllifolius* also improved glucose metabolism by restoring glycogen synthesis. Adipokines, such as leptin and adiponectin, are recognized as systemic factors affecting insulin sensitivity. The leptin

increased absorption and formation of TG and decreased TG uptake into the fat tissues [26]. Other studies showed that the increased fat accumulation in the liver tissue and the elevated circulating FFA are the indexes in insulin resistance cases [27,28]. Although treatment with *P. amaryllifolius* could not decrease the elevated serum TC, all doses of the treatment effectively reduced the levels of serum TG and NEFA. Obese mice treated with *P. amaryllifolius* also showed reduced liver TG and NEFA levels with decreased fat accumulation in the liver. The reduction of hyperlipidemia during treatment with *P. amaryllifolius* could be the result of stimulated lipid storage in fat tissue and suppressed TG and NEFA concentrations in the serum and liver.

In conclusion, the administration of *P. amaryllifolius* improved the insulin resistance in HFD-induced obese mice by reducing the blood glucose level, stimulating insulin sensitivity, decreasing serum and hepatic TG and NEFA, stimulating protein expression of GLUT4, and reducing adipocyte size in epididymal fat tissues. Therefore, these results support the useful

action of treatment with *P. amaryllifolius* leaf extracts in the improvement of insulin action in the obesity condition.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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