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Antidesma thwaitesianum Pomace Extract Improves Insulin Sensitivity Via Upregulation of PPAR-γ in High Fat Diet/Streptozotocin-Induced Type 2 Diabetic Rats.

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ABSTRACT

Objectives : Diabetes mellitus (DM) is a serious disease and a global health problem. An *in vitro* antihyperglycemic activity of Thai berry (*Antidesma thwaitesianum*) called in Thai as Mamao has been reported. We aimed to investigate *in vivo* antihyperglycemic activity of Mamao pomace ethanolic extract (MPE) and its mechanism of action in high fat diet/streptozotocin (HFD/STZ)-induced type 2 diabetic rats.

Materials and Methods : Male Sprague-Dawley rats were used. The rats in the normal control group were fed normal chow, while the DM group was fed HFD (40% lard oil) throughout the experimentation period. At week 4 of HFD feeding, the animals were intraperitoneally injected with STZ 30 mg/kg. Two weeks after STZ injection, treatments were applied for further six weeks, as follows: Group I: distilled water (diabetic control); Group II-IV: MPE 250, 500 and 1000 mg/kg respectively; Group V: pioglitazone 10 mg/kg. Following this, fasting blood glucose (FBG), oral glucose tolerance test (OGTT), serum insulin, HOMA-IR, serum adiponectin, lipid profiles and expression of (PPAR-γ) mRNA in adipose tissues were determined.

Results : All doses of MPE significantly decreased the FBG and improved OGTT as compared with DM-control group ($P<0.05$). Additionally, MPE (500 and 1,000 mg/kg) significantly decreased HOMA-IR and increased serum adiponectin. Moreover, MPE significantly lowered serum total cholesterol and triglyceride, and elevated high-density lipoprotein cholesterol. Interestingly, MPE caused an increase in expression of PPAR-γ mRNA in adipose tissues.

Conclusions : These results indicate that MPE may have an antidiabetic effect, of which it improves insulin sensitivity by activating an expression of PPAR-γ in adipose tissues.

Key words : *Antidesma thwaitesianum, Thai berry, insulin resistance, type 2 diabetes mellitus, PPAR-γ*

1. Introduction

Chronic hyperglycemia in Type 2 DM patients can cause many serious complications such as retinopathy, nephropathy, neuropathy, cardiomyopathy and poor blood flow in the limbs leading to amputations (1). An important pathophysiological factor of Type 2 DM is an insulin resistance in combination with low insulin level that is too low to compensate for this resistance. Tissues that involves in glucose homeostasis and that are sensitive to insulin (liver, skeletal muscle, and adipose tissue) do not efficiently respond to insulin (2). This causes a disturbance in glucose and lipid metabolism resulting in hyperglycemia and dyslipidemia.

Diets high in saturated fats can induce insulin resistance (3). In addition to insulating the body and storing free fatty acid (FFA) after food intake, adipose tissue also secrets adipokines to regulate glucose and lipid homeostasis (4). For example, adiponectin is an adipose tissue-derived cytokine which found in peripheral blood in high concentration. In the liver, adiponectin increases fatty acid oxidation and reduces liver gluconeogenesis (5). There has been evidence indicating that adiponectin has an insulin-sensitizing effect. Adiponectin secretion from fat cells is reduced under adverse metabolic conditions such as obesity and Type 2 diabetes. In addition, insulin resistance has also been shown to downregulate the secretion of adiponectin and expression of PPAR-γ in adipose tissues (6-7).

PPAR-γ, a transcriptional factor, widely expressed in adipose tissue, regulates the expression of various critical genes involved in lipid and glucose metabolism, insulin sensitivity, immune response and adipogenesis (8-9). The activation of PPAR-γ induces adipocyte differentiation and promotes lipid accumulation in adipocytes. The down regulation of PPAR-γ is a key element in mediating the insulin-resistance effect of pro-inflammatory cytokines, such as tissue necrosis factor alpha (10). In addition, Tsuchida and coworker (11) reported that the activation of PPAR-γ enhanced adiponectin secretion and insulin sensitivity. Thus, modulation of PPAR-γ activity may be an effective tool for the treatment of disease associated with insulin resistance such as type 2 diabetes mellitus.

Interestingly, Thai berry or in Thai name called Mamao (*Antidesma thwaitesianum* Müll. Arg) (12) is widely cultivated in Thailand especially in n ortheastern region where its local names are *Mao, Mamao,* and *Makmao*. Several pharmacological effects of Mamao have been reported including anti-alpha amylase, anti-alpha glucosidase, anticancer, antitumor, anti-apoptotic, anti-inflammatory, antimicrobial, anti-viral and antioxidant activities (13-15). However, there is still a lack of evidence regarding the *in vivo* antidiabetic activity of Mamao. Thus, the effect of Mamao pomace extract on blood glucose and lipids, and on insulin resistance were investigated using high fat diet/STZ-induced type 2 diabetic rats. The molecular mechanism of action on PPAR-γ expression was also investigated.

2. Materials and Methods

2.1 Mamao pomace ethanolic extreact (MPE) Preparation

The pomaces of ripe Mamao Luang fruits (*Antidesma thwaitesianum* Müll. Arg.) were obtained from Mamao

juice factory in Aumphur Phuphan, Sakon Nakhon Province. The dried Mamao pomace was blended and extracted using 95% ethanol at 25°C for 2 hours. Then, the ethanol extract was filtered through nylon cloth and centrifuged at 5,000 g for 10 min. The ethanol was evaporated using a rotary evaporator. The obtained extract was freeze-dried using the lyophilization process. The yield from this procedure was 15.26%. Dried extract was stored in a sealed brown-tinted bottle at 0-4°C. The total polyphenolic content in the dried extract was determined, which was 432.18 ± 3.45 mg of gallic acid equivalent/100g dried powder.

2.2 Animals and Experimental protocols

Male Sprague-Dawley rats (200-250 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom. All animals were housed in plastic cages in an air-conditioned room (24±2 °C) with a 12 h light: 12 h dark cycle at Northeastern Laboratory Animal Center, Khon Kaen University. All experimental procedures were complied with the standards for the care and use of experimental animals and were approved by Animal Ethic Committee of Khon Kaen University (Reference No. 0514.1.12.2/81).

Animals were randomly divided into two main groups, following the type 2 diabetic-induction-in-rat model using high fat diet/low dose of streptozotocin described by Srinivasan, et al. (16) with slightly modification. The first group, the normal control group were fed a normal diet and distilled water throughout the experimental period, whereas the second group were fed a high fat diet [HFD: 40% lard oil (wt/wt) with the remaining being normal chow (Chareon Pokapan Co. Ltd., Thailand)] for the duration of the experiment. After feeding the animals with HFD for four weeks, the rats were injected with streptozotocin (STZ) 30 mg/kg. Two weeks after STZ injection, the animals with fasting blood glucose levels (FBG) over 200 mg/dl were divided into five groups, consisting of Group I: DM rats treated with distilled water; Group II-IV: DM rats treated with MPE at doses of 250, 500 and 1,000 mg/kg BW/day respectively; Group V: DM rats treated with pioglitazone, an anti-Type 2 diabetic drug as a positive control, 10 mg/kg BW/day. The animals received each treatment once daily for further 6 weeks. The FBG and oral glucose tolerance test (OGTT) were determined at before and after 6 weeks of all treatments. At the end of experiment, the blood of the fasting animals were collected in order to measure serum insulin, serum adiponectin and lipid profiles. After that, the animals were euthanized using pentobarbital sodium 100 mg/kg; the intra-abdominal fat was collected to examine the expression of PPAR-γ. Homeostasis Model Assessment-Insulin Resistance (HOMA-IR), an indicator of insulin resistance was calculated.

2.3 Determination of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

The FBG and OGTT in fasting rats were investigated before and after 6 weeks of treatment. Rats were fasted overnight (12-15 h) and blood samples were taken from lateral tail vein to determine their FBG level using glucometer (Accu-check Advantage II; Roche, Germany). Then, the animals were orally administered glucose 2 g/kg BW in order to determine glucose tolerance (OGTT). Blood glucose concentrations were determined at 30, 90 and 120 min after glucose loading.

2.4 Determinations of serum insulin and adiponectin levels, lipid profiles, and HOMA-IR

At the end of all treatments, blood was collected to determine fasting basal insulin and adiponectin levels using the rat insulin ELISA kit (Millipore, USA) and Rat/Mouse adiponectin ELISA kits (Millipore, USA). Lipid profiles [Total cholesterol (TC), triglyceride (TG), low density lipoprotein-cholesterol (LDL-c) and high density lipoprotein –cholesterol (HDL-c)] were measured using the enzymatic and colorimetric method (Roche diagnostics, Bangkok, Thailand).

Insulin resistance was evaluated according to Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) method described by Matthews *et al.* (17). The HOMA-IR index was calculated as follows: (fasting insulin (μIU/ml) x fasting glucose (mmol/l)/ 22.5. Insulin 1 μIU/ml is equal to 6.945 pmol/l (18).

2.5 Analysis of mRNA Expression by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

At the end of the experiment, visceral fat tissue was immediately removed and stored at -80 ^{o}C for subsequent RNA extraction for determination of PPAR-γ expression. Total RNA was extracted from frozen visceral fat tissues by using TRIzol® reagents according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 1 μl of total RNA (cDNA iScript kit, BioRad) by priming 5 min at 25 ^{o}C, reverse transcription 30 min at 42 ^{o}C, RT inactivation 5 min at 85 ^{o}C and holding at 4 ^{o}C in a C1000 Thermal cycler (BioRad, USA). The RT-qPCR analysis was performed as described previously (19) using a cDNA template (4μl), forward and reverse primers (1.5 μl), 2x QPCR Green Master Mix (Fluorescein) (7.5 μl, including Taq polymerase, reaction buffer, $MgCl_2$, SYBR green dye and dNTP mix), and sterile water (2 μl) in a final reaction volume of 15 μl. The PCR reactions for *β-actin* and PPAR-γ were performed using 7500 Fast Real-Time PCR System instrument (ABI; Applied Biosystems) with the following condition: denaturation at 95 ^{o}C for 3 min and amplification by cycling 40 times at 95 ^{o}C for 15 sec and 60 ^{o}C for 30 sec. The specific primers used for PCR are described in Table 1. Levels of specific types of mRNA were expressed relative to *β-actin*. Relative fold change for target mRNA was calculated using the standard curve method. Amplification of specific transcripts was confirmed by melting curved profiles generated at the end of each run.

2.6 Statistical analysis

All results are expressed as mean ± standard error of the mean (S.E.M). The effect of MPE on all parameters were analyzed by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test to show specific group difference using SPSS. The level of significance was uniformly set at $P<0.05$.

3. Results

3.1 Effect of MPE on body weight of HFD/STZ-induced diabetic rats

The average initial body weight of all groups were similar (Fig.1), however at the end of 6 weeks of treatment, the average body weight of HFD/STZ-induced diabetic rats was less than that of the normal rats. MPE had no effect on the body weight of DM rats.

Table 1 Nucleotide sequences of primers used for PCR *(Rattus norvegicus)*.

Genes	Forward Primer	Reverse Primer	PCR Product
β-actin (NM_031144.3)	5'- GGAGATTACTGCCCTGGCTCCTA-3'	5'- GACTCATCGTACTCCTGCTTGCTG-3'	150 bp
PPAR-γ (NM_031144.3) (NM_001145366.1) (NM_001145367.1)	5'- ATTCTGGCCCACCAACTTCGG-3'	5'- TGGAAGCCTGATGCTTTATCCCCA-3'	339 bp

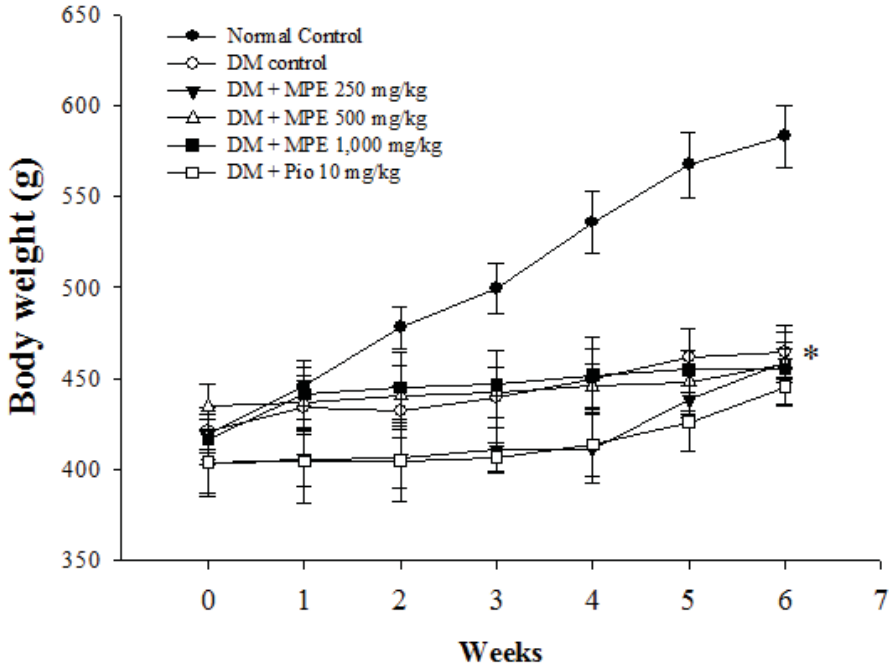

Figure 1. Effect of MPE on the body weight of HFD/ STZ-induced diabetic rats. MPE had no effect on the body weight of DM rats. Results are expressed as mean ± S.E.M.,
*: P<0.05; significant decrease as compared to normal diet feeding rats.

3.2 Effect of MPE on FBG and OGTT in HFD/STZ-induced diabetic rats

The FBG levels before treatment of all DM groups were significantly higher than that of normal rats and there was no significant difference among the DM groups (Table 2). For the DM control group receiving DW, FBG levels were still high after 6 weeks of DW administration (Table 2). This indicated that the FBG of DM group had been at a high level throughout the period of the experiment. Interestingly, MPE at doses of 250, 500 and 1,000 mg/kg BW/day significantly decreased FBG of DM rats (Table 2, $P<0.05$). The percent decrease in blood glucose of MPE was dose dependent.

Pioglitazone treatment, a positive control, also significantly (P<0.05) reduced the blood glucose of DM rats.

In the assessment of OGTT, it showed that the area under the curve (AUC) of total blood glucose (from 0 min to 120 min) of DM control rats was significantly higher than that of normal rats (Fig. 2). This implied that the HFD/STZ-induced diabetic rats had an impaired OGTT. The increased AUC of DM rats was significantly decreased in all MPE-treated groups (Fig. 2), which indicated that MPE could improve the impaired OGTT of DM rats.

Table 2. Effect of MPE on fasting blood glucose level of HFD/STZ-induced diabetic rats.

Treatment groups	Fasting Blood Glucose		
	Before treatment (mg/dL)	**After 6 weeks of treatment**	
		(mg/dL)	(% Changes)
Normal Diet control	104.6 ± 2.11	100.8 ± 3.92	-3.6
DM +Distilled water	314.6 ± 24.07*	380.3 ± 3.88*	27.2
DM +MPE 250 mg/kg	311.3± 17.03*	235.1 ± 23.87#	-24.8
DM +MPE 500 mg/kg	300.1 ± 16.88*	216.6 ± 9.68#	-30.2
DM +MPE 1,000 mg/kg	301.6 ± 7.84*	134.6 ± 11.66#	-54.2
DM +Pioglitazone 10 mg/kg	309.9 ± 16.77*	168 ± 15.07#	-44.4

Results are expressed as mean ± S.E.M., *: P<0.05; significant increase in FBG as compared to normal control rats, #: P<0.05; significant decrease as compared to DM control rats.

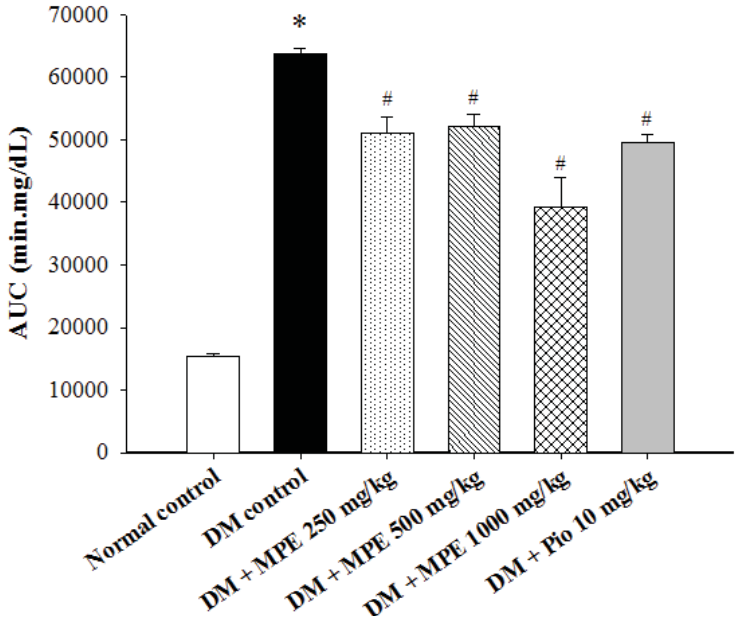

Figure 2. Effect of MPE on oral glucose tolerance (OGTT) of DM rats. The total blood glucose (from 0 to 120 min) was demonstrated as the area under the curve (AUC). Results are expressed as mean ± S.E.M., *: P<0.05; significant increase as compared to normal control rats, #: P<0.05; significant decrease as compared to DM control rats.

3.3 Effect of MPE on insulin level and HOMA-IR in HFD/STZ-induced diabetic rats

In the diabetic control group, the basal insulin level was significantly (P<0.05) lower and the HOMA-IR score was significantly higher than that of normal control rats (Figure 3A and 3B). The results indicated insulin resistance in HFD/STZ-induced DM rats.

Interestingly, treatment with MPE (500 and 1,000 mg/kg) improved the insulin resistance of DM rats as shown by their HOMA-IR scores, which were significantly (P<0.05) lower than that of DM control rats (Fig. 3A and 3B). Pioglitazone also showed the ability to reverse the impaired insulin responsiveness of DM rats (Fig. 3A and 3B).

3.4 Effect of MPE on the secretion of adiponectin in HFD/STZ-induced diabetic rats.

The serum adiponectin concentration in DM control group was significantly decreased (P<0.05) as compared to the normal control group. MPE (500 and 1,000 mg/kg) and pioglitazone significantly enhanced the secretion of adiponectin in DM rats (Fig. 4). The adiponectin secretion enhancing activity of MPE was dose-dependent.

3.5 Effect of MPE on serum lipid profile in HFD/STZ-induced diabetic rat.

The DM control group had dyslipidemia; significantly high levels of TC, LDL and TG, and low levels of HDL, as compared to normal control group (Fig. 5). After 6 weeks of MPE (250, 500 or 1,000 mg/kg) or pioglitazone treatment, the serum TC, LDL and TG levels of DM rats were significantly (P<0.05) decreased, and the serum HDL was significantly increased (Fig. 5).

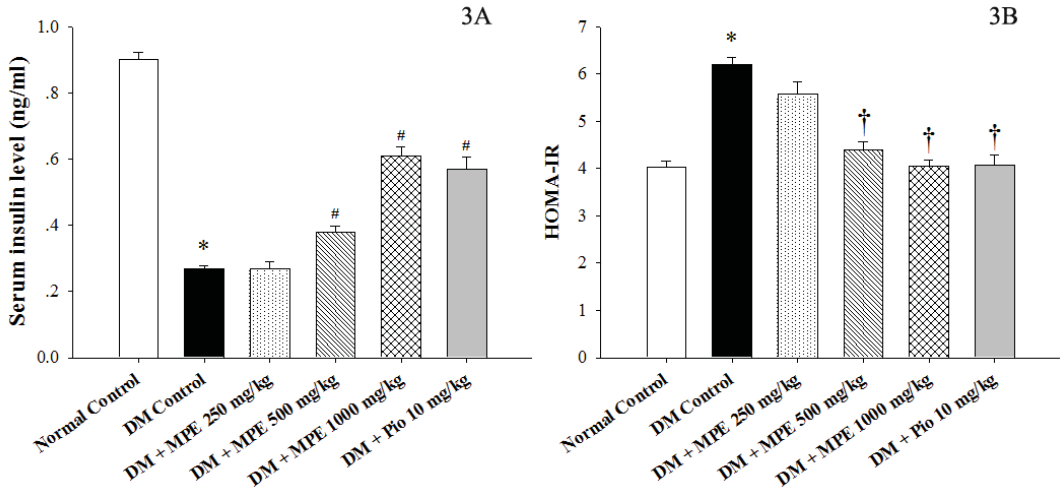

Figure 3. Effect of MPE on insulin level (**A**) and HOMA-IR (**B**) in HFD/STZ-induced diabetic rats. Results are expressed as mean ± S.E.M., *: P<0.05; significant decrease as compared to normal control rats, #: P<0.05; significant increase as compared to DM rats, **: P<0.05; significant increase as compared to normal control rats, †: P<0.05; significant decrease as compared to DM control rats

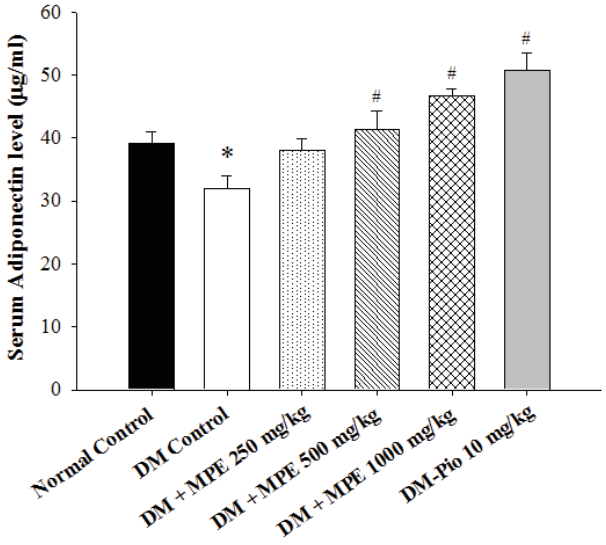

Figure 4. Effect of MPE on serum adiponectin level in HFD/STZ-induced diabetic rats. Results are expressed as mean ± S.E.M., *: $P<0.05$; significant decrease as compared to normal control group, #: $P<0.05$; significant increase as compared to DM control group.

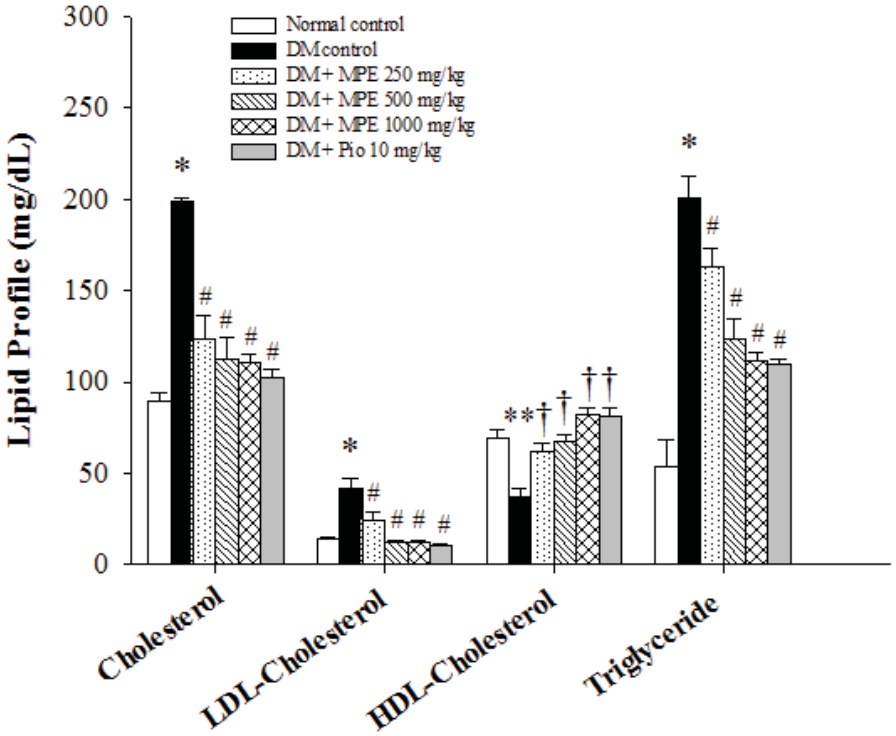

Figure 5. Effect of MPE on serum lipid profile in HFD/STZ-induced diabetic rats. Results are expressed as mean ± S.E.M., *: $P<0.05$; significant increase as compared to normal control rats, **: $P<0.05$; significant decrease as compared to normal control rats, #: $P<0.05$; significant decrease as compared to DM control rats, †: $P<0.05$; significant increase as compared to DM control rats.

3.6 Effect of MPE on PPAR-γ expression in adipocytes of HFD/STZ-induced diabetic rats

To gain insights into the molecular basis of the effect of MPE on the lipid and glucose metabolism, we examined the alteration of expression level of the transcriptional factor PPAR-γ. The expression of PPAR-γ ($P<0.05$) was significantly decreased in the DM control rats as compared to normal control rats. Interestingly, the expression of PPAR-γ was significantly increased in DM rats receiving MPE at the doses of 250, 500 and 1,000 mg/kg BW. This effect was dose dependent. Also in DM rats receiving pioglitazone, the expression of PPAR-γ was significantly increased (Fig. 6).

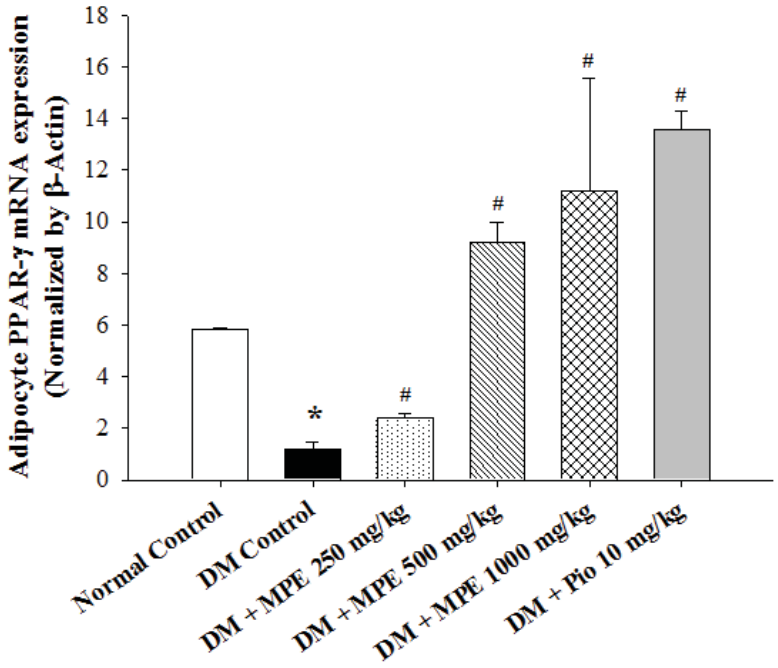

Figure 6. The effect of MPE on PPAR-γ expression in the fat cells of HFD/STZ-induced diabetic rats. The represented amount of each PPAR-γ mRNA was normalized by the amount of *β*-actin. Results are expressed as mean ± S.E.M., *: $P<0.05$; significant decrease as compared to normal rats, #: $P<0.05$; significant increase as compared to DM control rats.

4. Discussion

The present study demonstrated that HFD with low dose STZ could cause changes in glucose and lipid metabolism which are comparable to type 2 diabetes, *i.e.*, hyperglycemia, impaired glucose tolerance test and increased HOMA-IR score together with dyslipidemia. All of these values indicated an insulin resistant condition. Previous study has also reported that HFD/STZ can induce insulin resistance and dyslipidemia in animals (20). Furthermore, HFD also cause a decreasing in serum adiponectin and down-regulating PPAR-γ in adipose tissue. This is the first

report that MPE administration could improve insulin resistance and increase the expression of PPAR-γ in adipose tissue in HFD/STZ-induced diabetic rats.

In our study, the degree of insulin resistance was found to be very high in HFD/STZ-induced diabetic rats, as indicated by a high HOMA-IR score. The HOMA-IR score is a model of interactions between glucose and insulin dynamics that is used to predict fasting steady-state glucose and insulin concentrations of a wide range of possible combinations of insulin resistance and β-cell function (17). This score assumes a feedback loop between the liver and β-cell (21-22); i.e., blood glucose levels are regulated by insulin-dependent hepatic glucose production, whereas insulin levels depend on the pancreatic β-cell response to glucose concentrations.

HFD has been proposed to induce insulin resistance in muscles and liver. In the case of over intake of lipids, the buffering capacity for lipid storage in adipocytes is decreased and has reached a near-maximum level. Consequently, non-adipose tissues, such as skeletal muscle, pancreas and liver, are exposed to an excessive influx of triacylglycerol (TAG) and fatty acids, which may play an important role in the development of insulin resistance and/or impaired insulin secretion (23). It has also been proposed that increased intracellular content of fatty acid metabolites, such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA) and ceramides, cause an inactivation of insulin-signaling pathways (24). Interestingly, treatment of HFD/STZ-induced diabetic rats with MPE (250, 500 and 1000 mg/kg) caused the decreases in blood glucose and HOMA-IR scores, and the improvement in OGTT. This indicated that MPE may improve insulin resistance in animals with type 2 diabetes mellitus. This finding corresponds to the report that crude water and methanol extracts of *Antidesma madagascariense* reduced the increase in blood glucose in response to glycogen loading in mice (25). Dyslipidemia (high in TC, TG and LDL levels, and low in HDL levels) was also observed in HFD/STZ-induced diabetic rats. Insulin resistance can cause dyslipidemia. In the case of insulin resistance, enhanced lipolysis and increased fatty acid flux from adipose tissue, hypersecretion and hypocatabolism of chylomicron and VLDL remnants, and de novo hepatic lipogenesis, all of these may finally result in dyslipidemia (26). Interestingly, we found that MPE treatment caused an improvement in the lipid profiles of diabetic rats, decreases in TC, TG and LDL levels with an increase in HDL levels. These effects of MPE may be related to its ability to improve insulin resistance in peripheral tissue, *i.e.*, liver, skeletal muscle and adipose tissue.

Adiponectin is well known as an anti-diabetic and insulin sensitizing adipokine (6, 27). It causes increases in glucose uptake and fatty acid oxidation, and decreases in hepatic gluconeogenesis. The plasma adiponectin concentration and mRNA expression level are decreased in the obese and insulin resistant state (28). In addition, decreases in adiponectin secretion and expression of PPAR-γ in adipose tissues have been shown in insulin-resistant subjects as well (6-7). PPAR-γ, a nuclear receptor that is highly abundant in adipose tissue, is the master regulator of adipocyte differentiation and controls many adipocyte genes including adipokine synthesis. Many studies have suggested

that the PPAR-γ activation stimulates the secretion of adiponectin (29-31). The insulin-sensitizing effects of thiazolidinediones (antidiabetic drugs such as pioglitazone) are thought to be mediated through PPAR-γ activation and stimulating the secretion of adiponectin (32). In the present study we also demonstrated that pioglitazone (10 mg/kg) improved insulin resistance, and increased the level of adiponectin and the expression of fat cells PPAR-γ mRNA in HFD/STZ-induced diabetic rats. Interestingly, our results demonstrated that MPE treatment upregulated the expression of PPARγ mRNA in fat cells and increased serum adiponectin level of HFD/STZ-induced diabetic rats. Thus, these effects may result in improving in insulin sensitivity, which could eventually lead to the lowering of blood glucose and improvement of dyslipidemia.

In conclusion, this study demonstrated that MPE treatment improves glucose and lipid metabolism and insulin resistance in HFD/STZ-induced diabetic rats. This most likely occurs through the upregulation of the PPAR-γ gene and stimulation of adiponectin secretion. Thus, mamao pomace extract may be useful as an alternative medicine for type 2 diabetes associated with hypercholesteremic patients.

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