Alpha Mangostin and Xanthone from Mangosteen (*Garcinia mangostana* L.) Role on Insulin Tolerance and PPAR-γ in Preclinical Model Diabetes Mellitus

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Abstract: *Objective*: This research elaborated role of alpha mangostin and xanthone on insulin resistance and peroxisome proliferator–activated receptor (PPAR)-γ by measuring blood glucose level and PPAR- γ expression on adipocyte cell culture.

Methods: Insulin tolerance test were conducted using male wistar rat divided into 9 groups, which were normal, control (D-Glucose induced only), glibenclamide, various doses of α-mangostin and xanthone (5, 10, 20 mg/kgbw). All group induced by D-glucose 3 g/kg orally 30 minutes later. Blood glucose levels changes were observed at 90th and 150th minute. While other study observed PPAR-γ expression on adipocyte cell culture that treated with α-mangostin/xanthone/pioglitazone in various concentration.

Results: KITT in all treatment groups were significantly different ($p<0.05$) when compared to the positive control group, except xanthone 5 mg/kgbw. This suggests that α-mangostin 5, 10 and 20 mg/kgbw, xanthone 10 and 20 mg/kgbw, as well as metformin, have the effect of lowering insulin resistance in white rats given a 10-day fatty emulsion. Almost similar with thiazolinedione, alpha mangostin and xanthone increase PPAR-γ expression in adipocyte when the concentration bigger. But xanthone effect not as good as α-mangostin or thiazolinedione effect.

Conclusion: Alpha mangostin and xanthone are two substances that showed potential effect to improve insulin tolerance by increasing PPAR-γ in adipocyte.

Keywords: α-mangostin, xanthone, thiazolinedione, insulin tolerance, PPAR-γ.

INTRODUCTION

The prevalence of diabetes mellitus (DM) risen dramatically in worldwide [1]. DM will affect 438 million people by 2030, with 70% of cases occur in low-middle income countries. Without proper treatments, DM may cause cardiovascular disease which was the major cause of morbidity and mortality [2-4]. During the development of type-2 diabetes, insulin's ability to stimulate the cellular uptake of glucose from the blood was impaired [5-6].

Prolonged elevation of blood glucose concentration as in poorly controlled diabetes, may cause blindness, renal failure, cardiac and peripheral vascular disease, and neuropathy. Therefore, blood glucose concentrations need to be maintained within narrow limits. Rising in blood glucose levels after meal rapidly stimulates insulin secretion, which results within minute in increased glucose transport, metabolism, and storage by muscle and adipocytes [7].

Diabetes mellitus is a pathological process affecting the whole body system. Skeletal muscle, fat, and liver are considered as the insulin-sensitive tissues [8-12]. Alterations of the functional status of these tissues may result in insulin resistance of the body. The main manifestation is the dysfunction of glucose absorption and utilization, metabolism disturbance of glucose in liver cells and lipid metabolism disturbance in adipose cells [11-12]. Several papers have reported increases in adipose cell volume and number accompanied with redistribution of these cells over the body in insulin-resistant animals and humans. Nevertheless, it is more important that adipose cell volume increases when insulin resistance occurs [10].

Because the lipid bilayers which built cell membranes were impermeable to carbohydrates, carbohydrate-transport systems are required. In recent years, two distinct molecular families of cellular transporters of glucose have been cloned, The sodium-linked glucose transporters are largely restricted to the intestine and kidney, where they actively transport glucose against a glucose-concentration gradient by using sodium co-transport as an energy sourcewhere they actively transport glucose against a glucose-

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concentration gradient by using sodium co-transport as an energy source [13].

The other group of transporters convey glucose by facilitated diffusion down glucose-concentration gradients. This group consists of five homologous transmembrane proteins, GLUT-1, 2, 3, 4, and 5, that are encoded by distinct genes. The GLUT proteins have distinct substrate specificities, kinetic properties, and tissue distributions that dictate their functional roles. Muscle is the principal site of insulin-stimulated glucose disposal *in vivo*; less glucose is transported into adipose tissue [14]. Previous studies have indicated that α-mangostin and xanthone increase GLUT-4 expression on cardiac cell muscle and adipocyte.

Glucose transporter (GLUT)-4 is a high-affinity glucose transporter predominantly expressed in insulin-sensitive tissues such as muscle and adipocytes. Increasing GLUT-4 expression and plasma membrane translocation of GLUT-4 have been found in low blood glucose. This transporter enhance glucose transport and utilization [15]. In normal muscle cells and adipocytes, GLUT-4 will be recycled between the plasma membrane and intracellular storage pools. GLUT-4 is different from others because 90% of them will be broken on the intracellular network when there is no stimulation of insulin, physical activity or other stimuli. The presence of insulin or other stimuli will trigger the translocation of GLUT-4 from the plasma membrane to intracellular network. At cardiac muscle, GLUT-4 translocation will lead to the transverse tubules [7].

Insulin stimulates translocation of GLUT-4 by initiating insulin binding to receptors on the plasma membrane. This bond will activate tyrosine phosphorylation of the receptor intracellular kinase. Stimulation of glucose transport in muscle cells and adipocytes need the phosphoinositide-3 kinase. Phosphoinositide-3 kinase will activate the protein kinase B (serine-threonine kinase). Stimulation of glucose transport in diabetic subjects impaired by the change of insulin levels, while activation of protein kinase remained normal [7].

One alternative way that can be used to overcome the disease of diabetes is to utilize native medicinal plants. One of Asia native plant is Mangosteen (*Garcinia mangostana* L.). Phytochemical studies show that mangosteen contains oxygenated and prenylated xanthones. Xanthone is believed to have anti-cancer effects, anti-inflammatory, anti-viral and cardiovascular protection with the antioxidant effects. Xanthone most researched are alpha, beta and gamma mangostin, garcinone E, 8-deoksigartanin and gartanin. Xanthone could be found on the skin of fruit, fruit, bark and leaves of mangosteen [16-19].

MATERIAL AND METHODS

Insulin Tolerance Test

Preparation of Fat Emulsion

A constant volume of 100 mL fat emulsion containing 20 g lamb's fat, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 5 g sucrose and 5 g saccharose, 20 mL Tween 80, 30 mL propylene glycol was prepared by adding distilled water and stored at 4^0C [20].

Animal Treatment

Fourty five Wistar rats were randomly divided into normal group, control group which only got high fat emulsion, treatment group that got high fat diet and α-mangostin/xanthone 5, 10, 20 mg/kgbw respectively, or metformin. Rats in normal group received common water. Rats in control and treatment group got high fat emulsion group received fat emulsion for 10 d.

Insulin resistance sensitivity assay by short insulin tolerance test using capillary blood glucose [21]. Rats were weighed and placed into mouse cage after fasting overnight. Blood sugar in rats was detected six times after insulin (0.05 U/kgbw) intraperitoneally, every 30 minutes for 150 minutes. Blood glucose were checked using glucose oxidase method with a glucometer.

Absis indicates time and ordinate expresses nature logarithm of blood sugar. Regression coefficient (**r**) or slope was determined by linear regression and **K**ITT was calculated by multiplying **r** by 100. **K** value indicates insulin sensibility with smaller **K** values for lower sensibilities.

PPAR- γ Expression on Adipocytes

Preadipocytes were isolated from rat retroperitoneal tissue [22]. Fibrous tissue and blood vessels were removed first. Tissue was washed and chopped. Tissue suspension incubated with 0.2% collagenase type 1 (Sigma) for 45 minutes, at 37 ^{0}C on shaking condition. Incubation was stopped by the addition of culture medium, Dulbecco’s Modified Eagle Media (DMEM/F12 (1:1) supplemented with 15 mmol/l HEPES, 14 mmol/NaHCO3, 33 μmol/l of biotin, 17 μmol l D-panthotenate and 10% Fetal Bovine Serum

(FBS). The filtration using a nylon mesh (250 μm). Cell suspension then rotated 1500 rpm for 7 minutes. Fat layers (mature adipocyte and fat droplets) in the supernatant was discarded. Pellet containing fibroblast-like preadipocyte then resuspended in cell culture media, rotated 1500 rpm for 7 minutes and then resuspended again using the culture medium.

Cell Culture

Preadipocytes were grown in Dulbecco's Modified Eagle Media (DMEM) containing 10% Fetal Bovine Serum (FBS), 1% penicillin (10.000 U/mL), and 1% streptomycin (10.000 μg/mL supplemented in 37^0C incubator in a humidified atmosphere of 5% CO_2). Cells were subcultured every 3 to 4 days at approximately 80% confluence. Mature adipocytes were seeded in 96-well plates and grown until confluence. Alpha mangostin/xanthone/pioglitazone was dissolved in dimethyl sulfoxide (DMSO) and treated for 48 h. Cells were then washed two times with PBS [23].

Induction of Adipocytes Differentiation

Before and after incubated in adipogenic media (DMEM/F12) 100 U/ml penicillin and 100 U/ml streptomycin, 66 nM insulin, 100 nM dexamethasone, 0.5 mM Methyl Iso buthyl Xantine (IBMX) and 10 μg ml transferrin were added for adipocytes differentiation. Suspension cells grew in culture plates, incubated at 37 ^{0}C, 5 % CO_2 and 95 % humidity for 24 hours. Cells were washed once every 3 days.

Adipocyte Differentiation

Cells were seeded into 22-well plates at a density of $2×10^4$ cells/well. Two days after confluence (defined as day-0), cells were stimulated to differentiate with differentiation medium containing DMEM with 10% FBS and MDI [0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone, and 1 μg/mL insulin] for 2 days. In the course of screening adipocyte differentiation inhibitory activity, preadipocytes were treated with differentiation medium in the presence of various concentrations of test compound (0.78 μM, 1.56 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 and 50 μM of α-mangostin/xanthone/ pioglitazone) at day-0. At day-2, differentiating medium was replaced with 10% FBS/DMEM medium containing 1 μg/mL insulin and incubated for another two days (day-4). Thereafter, the cells were maintained in 10% FBS/DMEM medium for an additional 4 days (day-8) with medium changes every 2 days. Before and after incubated in adipogenic media, adipocytes were calculated. Calculating the number of cells in Neubauer Improved counting chamber was performed using a light microscope. The data were calculated by the formula k = n x p x 2500, with k = cell density (cells/ml), n = the total number of cells in the four counting chamber, and p is the dilution rate used [24].

Immunocytochemistry Staining

Adipocyte fixed in 10% formalin (v/v) in PBS (pH 7.4) for 20 minutes. Cells then washed with PBS (pH 7.4) three times, and treated by 0.02% (w/v) sodium azide. Cells then washed again with PBS (pH 7.4) three times for 5 minutes, then treated by H_2O_2 in PBS for 10 minutes. Reaction then stopped with 0.25% Triton-X blocking serum added in 5% FBS for 1 h, then washed with PBS. Adipocytes were treated with anti-PPAR- γ antibody diluted in serum 1:500 for 24 h, then incubated at 4^0C for 24 h. After incubated they were washed with PBS 3 times for 5 minutes each. Cells were incubated in anti-rabbit secondary antibody 1:500 for 1 h at room temperature, then washed with PBS 3 times for 5 minutes each. Cells treated by SA-HRP for 40 minutes, then washed with PBS 3 times for 5 minutes each. Cells then treated with Diamino benzidine (DAB) in the DAB buffer. Cells treated with courstexin with Mayer hematoxilin for 10 minutes, then washed with tap water, followed by distilled water for 10 minutes. Oil Red O staining was used to confirm that cells that were differentiated were adipocytes.

Oil Red O Staining

Eight days after the differentiation induction, cells were washed three times with PBS and fixed with 10% formalin for 1 h at room temperature. After fixation, cells were washed once with PBS and stained with freshly diluted Oil Red O solution (3 parts of 0.6% Oil Red O in isopropanol and 2 parts of water) for 1 h. Cells were then washed twice with distilled water and visualised under a microscope [25]. Images were collected on an Olympus microscope.

RESULT AND DISCUSSION

Insulin tolerance test was performed on insulin resistant animal models. Resistant animals were obtained with a fatty emulsion diet for 10 days. This diet will induce oxidative stress, which increases blood glucose levels, which are immediately followed by an increase in blood insulin levels, leading to insulin resistance. Insulin-resistant animals are then given intraperitoneal insulin (0.05 U/kgbw), then glucose levels of experimental animals are measured every 15 minutes.

In order to clarify if the insulin-resistant animal model was established in our study, we detected the dynamic characteristics of blood sugar after insulin injection by short insulin tolerance test using capillary blood glucose. The result demonstrated that the **K**ITT value decreased markedly after high fat emulsion for 10 d compared to normal group, indicating that the rats are insensitive to exogenous insulin, i.e. insulin resistance. Insulin resistance refers to the insensitivity of tissues (such as sceletal muscle, liver, kidney, and adipose tissue) to insulin action, i.e. the weaker glucose utilization of body after insulin action that results in hyperglycemia. Absis shows time and ordinate showing blood glucose levels. Lower insulin tolerance test (KITT) co-efficient indicates low insulin sensitivity

Table 1: Insulin Tolerance Test

Group	*Mean* K_{ITT} ± SD
Normal	94.82 ± 3.70[a]
Control	56.62 ± 11.25
Alpha Mangostin 5 mg/kgbw	90.59 ± 2.40[a]
Alpha Mangostin 10 mg/kgbw	93.39 ± 2.96[a]
Alpha Mangostin 20 mg/kgbw	93.69 ± 2.92[a]
Xanthone 5 mg/kgbw	72.40 ± 9.50[b]
Xanthone 10 mg/kgbw	90.23 ± 3.33[a]
Xanthone 20 mg/kgbw	91.76 ± 2.59[a]
Metformin	94.55 ± 2.77[a]

Note: [a]Mann-Whitney test, $p < 0.05$ compared to control group; [b]compared to normal group.

The results of the insulin tolerance test showed that the lowest KITT was seen in the positive control group, which was given only 10 days of fatty emulsion. The Kruskal Wallis results ($p < 0.05$), than it was analized using Mann-Whitney test and showed that there's significant difference between positive control group when compared to the normal group who were not given a high-fat diet. This suggests that the induction of insulin resistance states using a fatty emulsion for 10 days was success.

KITT in all treatment groups were significantly different when compared to the positive control group, except xanthone 5 mg/kgbw. This suggests that alpha mangostin 5, 10 and 20 mg/bw, xanthone 10 and 20 mg/kgbw, as well as metformin, have the effect of lowering insulin resistance in animal model that given a 10-day fatty emulsion.

Peroxisome proliferator–activated receptors (PPARs) belong to a subfamily of the nuclear receptors superfamily and are ligand-activated transcription factors. There are 3 subtypes of PPAR, PPAR-α, δ, and γ. PPAR-α reduces the triglyceride level, but increase plasma HDL-cholesterol. PPAR-δ is potential therapeutic target for metabolic syndrome, insulin resistance, and obesity. PPAR-γ agonist are being developed to increase insulin sensitivity and to simultaneously prevent diabetic cardiovascular complication [26].

PPAR-γ also having a key role in adipogenesis. The PPAR-γ has been identified as the receptor for the thiazolidinediones, a new class of oral antidiabetic agents which improves glycaemic control by lowering peripheral insulin resistance. In patients with Type II (non-insulin-dependent) diabetes mellitus, thiazolidinediones reduce peripheral insulin resistance, but also seem to improve the pattern of insulin secretion [27].

From Figure **1** we could see that PPAR-γ expression in adipocyte will increase when the concentration bigger. Thiazolidinediones are potent antidiabetic that lower the hyperglycemia, and hypertriglyceridemia observed in human and animal models of NIDDM [28]. In contrast to sulfonylureas, which improve insulin secretion, thiazolidinediones act by enhancing the peripheral sensitivity to insulin. Some insights into their mode of action has been provided by the finding that thiazolidinedione compounds are high-affinity ligands for PPAR-γ, a subtype of the nuclear receptor superfamily of ligand-activated transcription factors.

Almost similar with thiazolinedione, in Figure **2** we could see that PPAR-γ expression in adipocyte will increase when the consentration bigger. The antihyperglycemic activity of various thiazolidinediones is closely linked to their PPAR-γ agonist activity [29]. The PPAR-γ gene codes for two PPAR-γ isoforms, PPAR-γ1 and PPAR-γ2, with PPAR-γ2 being the predominant form expressed in adipose tissue [30]. This suggests that adipose tissue might be an important target for the effect of thiazolidinediones and α-mangostin on insulin sensitivity, as suggested previously [31-32].

In Figure **3** we can see that xanthone also increase PPAR-γ expression in adipocyte. But its effect not as good as α-mangostin or thiazolinedione effect. We may suggest that adipose tissue might be an important target for the effect of xanthone, like thiazolinedione and α-mangostin.

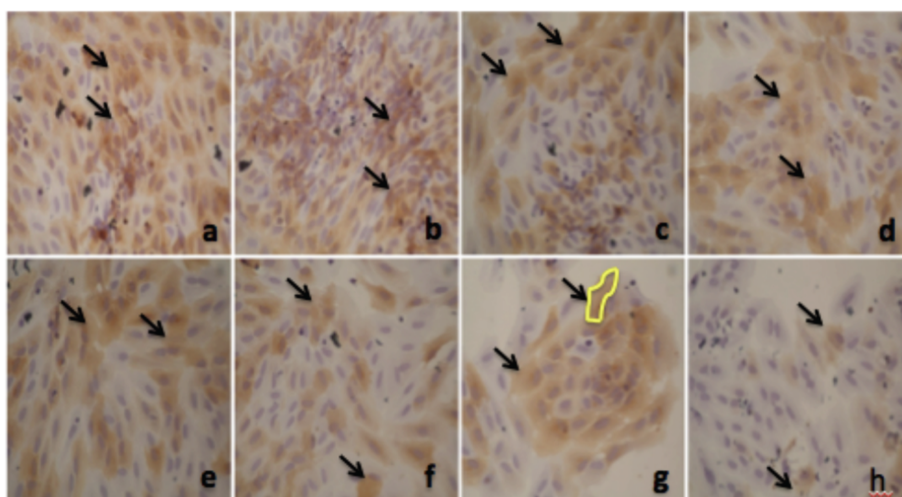

Figure 1: Thiazolinedione effect on PPAR-γ expression in adipocyte.

a. 50 μM; **b**. 25 μM; **c**. 12.5 μM; **d**. 6.25 μM; **e**. 3.125 μM; **f**. 1.56 μM; **g**. 0.78 μM, **h**. 0 μM.

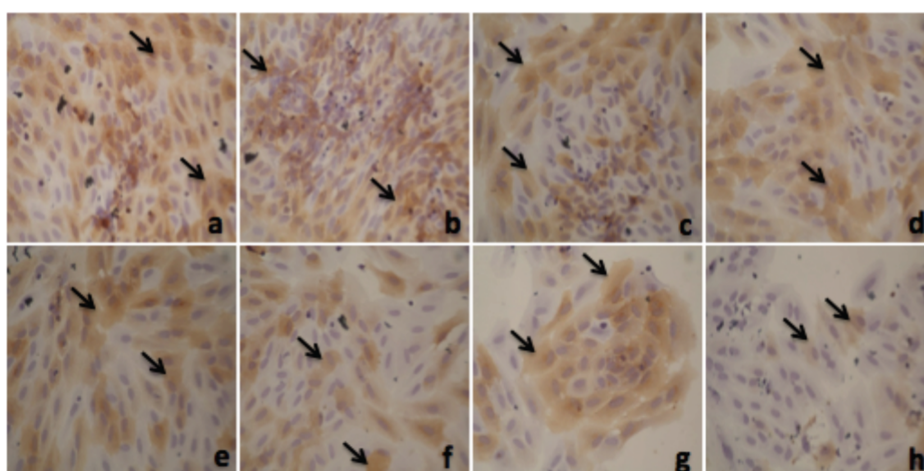

Figure 2: Alpha mangostin effect on PPAR-γ expression in adipocyte.

a. 50 μM; **b**. 25 μM; **c**. 12.5 μM; **d**. 6.25 μM; **e**. 3.125 μM; **f**. 1.56 μM; **g**. 0.78 μM, **h**. 0 μM.

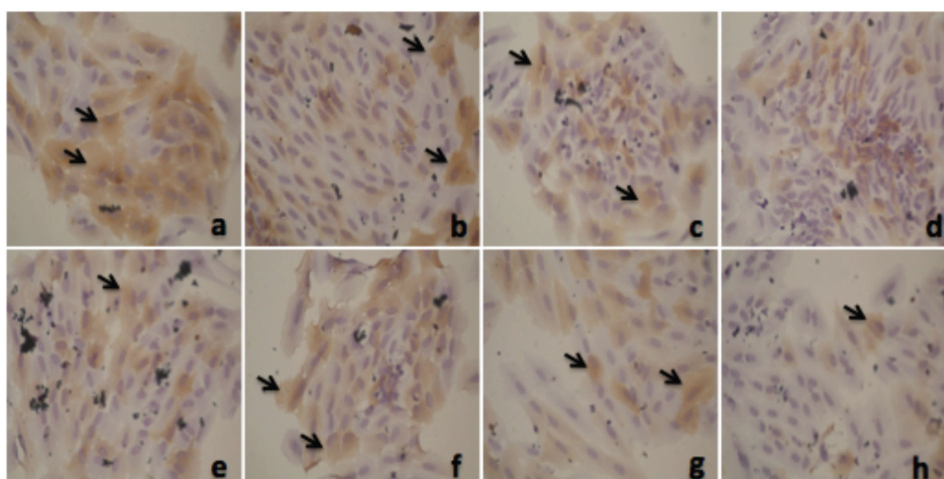

Figure 3: Xanthone effect on PPAR-γ expression in adipocyte.

a. 50 μM; **b**. 25 μM; **c**. 12.5 μM; **d**. 6.25 μM; **e**. 3.125 μM; **f**. 1.56 μM; **g**. 0.78 μM, **h**. 0 μM.

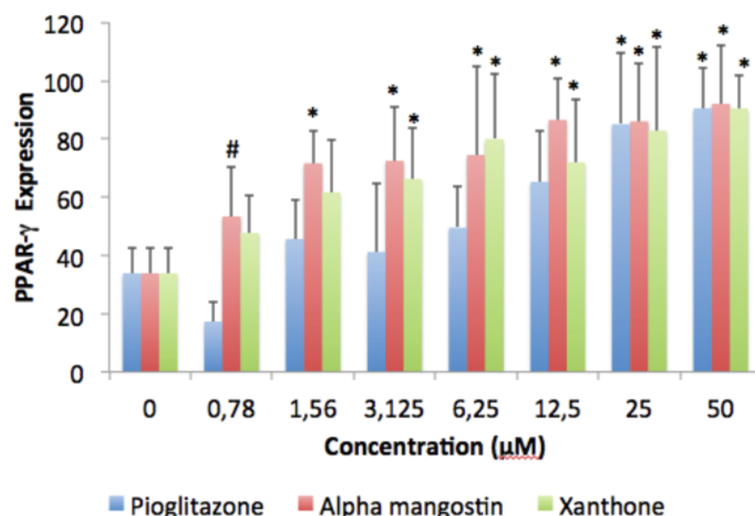

Figure 4: Alpha mangostin and xanthone effect on PPAR-γ expression in adipocyte cell culture (counting by immunoratio-JPEG2000 virtual slide microscope).

*) $p < 0.05$ compared to control; #) $p < 0.05$ compared to pioglitazone.

From this figure we can see that alpha mangostin effect not differ significantly to pioglitazone, except on consentration 0.78 μM, on which alpha mangostin effect bigger than pioglitazone. Xanthone effect on each consentration also not differ significantly if compared to pioglitazone. But it's effect still below mangostin's. On the other hand, PPAR-γ is a key factor for adipocyte differentiation, as shown using cell lines [33] and thiazolidinediones are efficient promoters of adipocyte differentiation *in vitro* [2,14-16, 34-37]. Thus, it could be questioned whether a thiazolidinedione therapy aimed at improving insulin sensitivity would promote the recruitment of new adipocytes *in vivo*, an effect that could be deleterious, since most of the NIDDM patients were obese. Glucose utilization is obviously linked to an increased capacity of taking up and metabolizing glucose through the activation of genes involved in glucose transport (GLUT-4) and metabolism into lipids (FAS and PEPCK).

Garcinia mangostana Linn or mangosteen found in South East Asia. Its pericarps have been used as traditional medicine. Phytochemical studies shown that mangosteen contained secondary metabolite as oxygenated and prenylated xanthones (α, β, γ mangostin). Previous study has shown that α-mangostin and xanthone isolated from mangosteen (*G. Mangostana* L.) are two substance that showed protective effect to glucose tolerance and also potential to improve insulin resintency by increasing GLUT-4 on muscle and adipocyte [38].

Adipose is not only known for its capacity to store the excess of dietary energy in the form of triglyceride [39], but has also been acknowledged to play an important role in the control of energy metabolism [40-41]. A number of transcription factors have been documented to be involved in the adipogenesis, glucose uptake, and glycolysis pathway [42]. These transcription factors include PPAR-γ, glucose transporter-4 (GLUT-4), and adipokines such as leptin.

PPAK-γ is predominantly expressed in adipose tissues and plays a central role in adipose tissue functions [43]. PPAK-γ regulates the expression of genes associated with insulin signalling and glucose and lipid metabolism in mature adipocytes [11]. Reduced expression of PPAK-γ has been shown to be effective in inhibiting the adipogenesis of 3T3-L1 cells [44-45].

CONCLUSION

In conclusion, the present study has shown that α-mangostin and xanthone isolated from mangosteen (*Garcinia mangostana* L) are two substance that showed potential effect on preventing tolerance, and increasing PPAR-γ expression on adipocyte.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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