REGULATION OF ADIPOGENESIS AND KEY ADIPOGENIC GENE EXPRESSION BY MANGOSTEEN PERICARP EXTRACT AND XANTHONES IN 3T3-L1 CELLS

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ABSTRACT

Obesity is one of the risk factors for atherosclerosis and its fat occurrence and development are associated with fat accumulation and adipocyte differentiation. Thus, the suppression of adipocyte differentiation can be a potential anti-obesity approach to this health concern. This study examined the effect of mangosteen pericarp extract (MPE) and xanthone (α-Mangostin (AM) and γ-Mangostin (GM)) on the expression of PPARγ, C/EBPα, SCD1, LPL, aP2, adipoQ and FAS in 3T3-L1 cells. Concentrations of MPE and xanthones used were based on the cytotoxic assay on 3T3-L1 cells. Three different MPE concentrations (0, 25 and 50 μg/mL) and three different AM concentrations (0, 25 and 50 μM) and GM (0, 50 and 75 μM) were used in the experiment. The expressions of PPARγ, C/EBPα, SCD1, LPL, aP2, adipoQ and FAS genes were measured using real-time quantitative PCR. The expression of the genes was down-regulated in the group of cells treated with 50 μg/mL of MPE and 50 μM of GM. However, the 25 μM and 50 μM of AM did not suppress PPARγ and SCD-1 expression. The 50 μM of AM also failed to reduce aP2 gene expression. Finally, MPE and GM showed potential anti-adipogenesis and anti-obesity effects by suppressing the expression of PPARγ, C/EBPα, SCD1, LPL, aP2, adipoQ and FAS genes in 3T3-L1 cells.

Keywords: adipogenesis, atherosclerosis, gene expression, mangosteen, obesity

INTRODUCTION

Obesity is a complex metabolic disease which can lead to various serious diseases, such as atherosclerosis (Kang *et al.* 2013). An imbalance in the energy intake and expenditure in an obese condition eventually lead to the pathological growth of adipocytes (Jou *et al.* 2010). Fat accumulation and adipogenesis are related to the occurrence and development of obesity (Song *et al.* 2013). Adipogenesis causes the differentiation of preadipocytes to adipocytes which plays an important role in fat mass growth (Sarjeant & Stephens 2012).

Some genes are responsible in adipogenesis regulation such as CCAAT/enhancer binding the beta protein (C/EBPβ), nuclear receptor peroxisome proliferation-activated receptor gamma (PPARγ), and CCAAT/enhancer binding the alpha protein (C/EBPα), and thus are playing an important roles in the complex transcriptional cascade that occurs during adipogenesis (Cristancho & Lazar 2011). Moreover, several enzymes are also involved in adipocyte differentiation such as lipogenic and glycolytic enzymes, the fatty-acid-binding protein aP2, the stearoyl-CoA desaturase (SCD), the fatty acid synthase (FAS) (Shan *et al.* 2013; Jacquemyn *et al.* 2017; Obregon 2014), the lipoprotein lipase (LPL) (Obregon 2014), and the adipoQ, nowadays known as adiponectin (Wang & Scherer 2016). Therefore, the potential strategy to prevent obesity is to control adipogenesis (Chang & Kim 2019).

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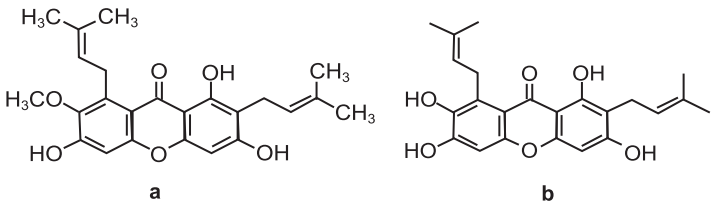


Figure 1 Chemical structure of a) α-Mangostin and b) γ-Mangostin

Once the medication is stopped, the side effects of conventional obesity drugs is usually a regained body weight. Hence, the need to find novel approaches to obesity prevention that focus on healthy foods or natural drugs without negative side effects. For decades, Mangosteen (*Garcinia mangostana* L.) has been used in traditional medicine for diarrhea, dysentery, eczema and other skin diseases (Shen *et al.* 2014). The pericarp has been known to contain abundant xanthones (α-Mangostin and γ-Mangostin) that show various bioactivities (Fig. 1) as antioxidant, antifungal, antibacterial, cytotoxic, anti-inflammatory, anti-histamine, anti-HIV, and other activities (Ibrahim *et al.* 2014; Widowati *et al.* 2016).

This study examined the effect of mangosteen pericarp extract (MPE) and xanthone compounds (α-Mangostin (AM) and γ-Mangostin (GM)) on the expression of adipogenic genes such as PPARγ, C/EBPα, SCD1, LPL, aP2, adipoQ and FAS in 3T3-L1 cells. The results were generated to provide better understanding on the molecular mechanisms of MPE in controlling adipogenesis or as an obesity therapy.

MATERIALS AND METHODS

Plant Material Preparation and Extraction

Garcinia mangostana L. fruits were collected in March 2011 from Cisalak, Subang, West Java, Indonesia. The plant was identified by Mr. Juandi in the herbarium of the School of Life Sciences and Technology, Institut Teknologi Bandung, West Java, Indonesia. The voucher specimen was deposited in Aretha Medika Utama (005/AMU-BBRC). The pericarps were collected, chopped, and dried at 40-45°C using a drying device until a stable moisture content was obtained (±13%). Afterwards, the extraction was performed using maceration with 70% ethanol (Widowati *et al.* 2014; Widowati *et al.* 2016).

3T3-L1 Cell Culture

The 3T3-L1 cell line (ATCC®CL-173) or mouse pre-adipocytes (Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biowest L0104-500), supplemented with 10% calf serum (Biowest S0400) and 100 U/mL penicillin-streptomycin (Biowest L0022), and then incubated for 24 h at 37 °C, 5% CO_2. The cells were collected and seeded into 6-well plates (1 x 10^4 cells/well) with DMEM + 10% calf serum until confluent (80-90%). Subsequently, subcultures were raised and the culture was re-incubated until it was confluent. Adipogenesis induction was conducted using an adipogenesis assay kit (Abcam ab133102). After the cells were 80% confluent, the medium was replaced with induction medium (DMEM + IBMX + insulin + dexamethasone + fetal bovine serum (FBS), Biowest S1810) for the positive control, growth medium for the negative control, and treatment medium (DMEM + IBMX + insulin + dexamethasone + FBS + MPE or xanthones (AM and GM)). Plates were incubated for 3 days at 37 °C, 5% CO_2, and humidified atmosphere. The cells were then washed with PBS 1x and the medium was decanted. Fresh medium was added: insulin medium (DMEM + FBS + insulin) for positive control, the culture medium for the negative control, and insulin medium + MPE or xanthones for the treatment. The culture was incubated at 37 °C, 5% CO_2, and fresh medium was added every 2 days to maintain the culture. After 7 days, the medium was decanted, and more than 80% cells had differentiated. The adipocytes were then observed under an inverted microscope (Hidayat *et al.* 2015; Widowati *et al.* 2017).

Viability Assay

An MTS assay was performed to investigate cell viability and to determine the non-toxic concentration of MPE or xanthones on 3T3-L1 cells. The cells were seeded into 96-well plates (5 x 10^3 cells per well) with DMEM + 10% calf serum + 100 U/mL penicillin and streptomycin, and then incubated for 24 h at 37 °C, with 5% CO_2, in a humidified atmosphere. The incubated medium was decanted and added with 90 µL serum-free medium with 10 µL MPE in various concentrations (6.25, 12.5, 25 and 50 µg/mL diluted in DMSO) to the cells. The cells in xanthone (AM or GM) treatment group, were added with 90 µL serum-free medium with 10 µL AM or GM in various concentrations (12.5, 25, 50 and 75 µM diluted in DMSO). The plates were then incubated for 24 h at 37 °C, 5% CO_2, in a humidified atmosphere. The untreated cells were presented as the control. After incubation, 20 µL MTS was added to each well. The plates were then re-incubated for 3 h at 37 °C, 5% CO_2, in a humidified atmosphere. The absorbance was then measured at 490 nm using a microplate reader (Multiskan Go, Thermo Scientific, USA) (Darsono *et al.* 2015; Laksmitawati *et al.* 2017; Novilla *et al.* 2017).

Quantification of Adipogenesis Gene Expression by Real-Time qPCR

RNA extraction was performed using an Aurum Total RNA Kit (Bio-Rad 732-6820) according to the manufacturer's instructions. The RNA yield was estimated spectrophotometrically at 260/280 nm (Table 1). Subsequently, the RNA was used for cDNA synthesis using a Mix iScript cDNA Synthesis Kit (Bio-Rad 170-8841) with three incubation steps: at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min (Hidayat *et al.* 2016; Afifah *et al.* 2019; Widowati *et al.* 2019). The product was stored at -20 °C. Then RNA concentrations and purities were computed.

Table 1 RNA purity of adipogenesis-induced 3T3L1, non-induced 3T3L1, MPE-treated 3T3L1, AM-treated 3T3L1, GM-treated 3T3L1

Sample	RNA purity (260/280 nm)
Negative Control	2.7079 ± 0.3202
Positive Control	2.5700 ± 0.4403
MPE 50 µg/mL	2.4128 ± 0.4230
MPE 25 µg/mL	2.3358 ± 0.2921
AM 50 µM	2.7036 ± 0.3432
AM 25 µM	2.3858 ± 0.3469
GM 75 µM	2.7203 ± 0.3703
GM 50 µM	2.5279 ± 0.2364

Notes: The data are presented as a mean ± standard deviation. The experiment was conducted in triplicate.

The expression of PPARγ, C/EBPα, SCD1, LPL, aP2, adipoQ and FAS genes along with the constitutively expressed β-actin gene was analyzed using real-time qPCR (Table 2). PCR amplification was carried out using a PikoRealTM Real-Time PCR System (Thermo Scientific Inc.) with preincubation cycle at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 20 s and elongation at 72 °C for 10 s (Hidayat *et al.* 2016; Afifah *et al.* 2019; Widowati *et al.* 2019).

Statistical Analysis

The statistical analysis was performed using SPSS version 16.0 software. To compare the negative and positive control, the data were analyzed using unpaired-T test and the differences among treatment were analyzed using one-way analysis of variance (ANOVA) with SPSS 16.0 statistical package and later subjected to Tukey HSD post hoc test.

Table 2 Sequence of primers used in real-time quantitative PCR

Primer	Forward	Reverse
PPAR-G	5'-TTT TCA AGG GTC CCA GTT TC-3'	5'-TTA TTC ATC AGG GAG GCC AG-3'
C/EBPα	5'-GCC GAG ATA AAG CCA AAC AA-3'	5'-CCT TGA CCA AGG AGC TCT CA-3'
SCD1	5'-CTG TAC GGG ATC ATA CTG GTT C-3'	5'-GCC GTG CCT TGT AAG TTC TG-3'
FAS	5'-GCG ATG AAG AGC ATG GTT TAG-3'	5'-GGC TCA AGG GTT CCA TGT T-3'
LPL	5'-CTG CTG GCG TAG CAG GAA GT-5'	5'-GCT GGA AAG TGC CTC CAT TG-3'
aP2	5'-CTG AAA TGG GGA TTT GGT CA-3'	5'-TCG ACT TTC CAT CCC ACT TC-3'
AdipoQ	5'-CCT GGT GAG AAG GGT GAG AA-3'	5'-CAA TCC CAC ACT GAA TGC TG-3'
β-actin	5'-TCT GGC ACC ACA CCT TCT ACA ATG-3'	5'-AGC ACA GCC TGG ATA GCA ACG-3'

RESULTS AND DISCUSSION

Adverse effects after therapy have been currently threatening to patients who are suffering from obesity. Therefore, medicines obtained from natural sources are needed to substitute for commercial drugs as these are much safer and more effective. Mangosteen pericarp extract (MPE) contains various beneficial secondary metabolites such as prenylated and oxygenated xanthones which have the potential as anti-obesity drugs (Adnyana *et al.* 2016). Xanthone found in mangosteen fruit has derivatives such as α-Mangostin (AM) and γ-Mangostin (GM) that showed several pharmacological activities as antioxidant, antitumor, anti-inflammatory, antibacterial, anti-allergy, antifungal, and antiviral (Widowati *et al.* 2014). MPE and xanthones (AM and GM) had exhibited anti-inflammatory properties by inhibiting the COX-2 gene, IL-6, IL-1β and NO activities in LPS-induced RAW264.7 (Widowati *et al.* 2016).

MTS assay showed that MPE, AM, and GM concentrations used in this study were non-toxic to cells due to the presence of viable cells that was more than 85%. The results of the viability assay are related to the adverse effects of bioactive substances on living organisms prior to their use as drugs or chemicals in clinical settings (Lalitha *et al.* 2012). In this study, two concentrations of MPE (25 and 50 μg/mL), AM (50 and 25 μM) and GM (75 and 50 μM) were used for further treatments on 3T3-L1 cells. Cytotoxic or viability assays showed that the viability of cells was concentration dependent. The percentage of cell viability was determined by comparing treatment (MPE, AM, GM) to control groups. All sample concentrations were safe for the 3T3-L1 cell, except 75 μM of AM, which resulted in less than 85% of cells remaining viable. Apparently, all concentrations of MPE, AM and GM, except 75 μM of AM, can be used for further treatment in 3T3-L1 cells (Table 3).

In certain conditions, 3T3-L1 cells may differentiate into adipocytes (Fig. 2). After induction using insulin for five days, normal cells differentiate into adipocyte and form lipid droplets. The accumulation of fat and adipogenesis are the sign of obesity development (Song *et al.* 2013; Widowati *et al.* 2018). Adipogenesis is a complex process that involves specific genes and enzymes in the regulation of adipocyte differentiation. Thus, the potential strategy to prevent obesity is to inhibit the adipogenesis genes and enzymes (Obregon 2014; Gwon *et al.* 2013). In this study, a concentration of 50 μM of GM was most effective in suppressing the expression of adipogenesis-related genes, such as PPARγ. This gene is one of the main regulators of adipogenesis, which is induced during adipocyte differentiation (Cao 2013). MPE, AM and GM were sufficient to suppress C/EBPα. A concentration of 50 μg/mL of MPE, and 75 μM and 50 μM of GM were also capable of reducing the expression of C/EBPα, SCDI, LPL, adipoQ and FAS genes. These results indicated the potential of MPE and GM as anti-obesity agents in the differentiated-3T3-L1 cells.

Table 3 3T3-L1 cell viability in various concentrations of MPE, AM and GM

Samples	Viability (%)				
	75 (μg/mL or μM)	50 (μg/mL or μM)	25 (μg/mL or μM)	12.5 (μg/mL or μM)	6.25 (μg/mL or μM)
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
MPE	-	87.77 ± 6.69^{a}	95.86 ± 3.25^{a}	103.19 ± 7.76^{a}	130.73 ± 7.25^{b}
AM	79.48 ± 6.29^{a}	87.33 ± 9.81^{a}	109.71 ± 2.11^{b}	113.98 ± 3.93^{b}	-
GM	97.91 ± 10.39^{a}	111.79 ± 3.90^{ab}	112.20 ± 4.16^{ab}	122.24 ± 10.06^{b}	-

Notes: The data are presented as a mean ± standard deviation. Means with different superscripts across the same row are significantly different at $p < 0.05$ based on Tukey HSD post hoc test.

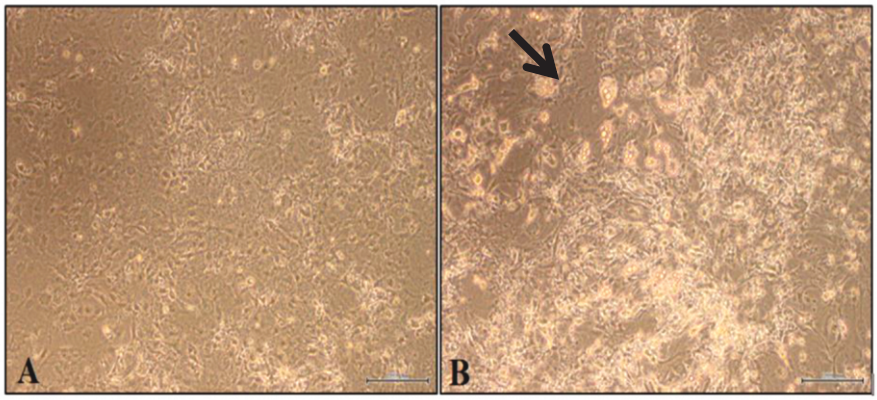


Figure 2 Morphology of 3T3-L1 cell culture: (A) Non-induced cell; (B) Adipogenesis-induced cells (black arrow pointing towards higher accumulation of intracellular lipid droplet)

PPARγ gene is activated by fat accumulation (Li *et al.* 2014), which is important in inducing adipocyte differentiation (Moseti *et al.* 2016). PPARγ and C/EBPα are master regulators of adipogenesis (Park *et al.* 2012) and the activation of those genes is important in the progression until the terminal stage of adipogenesis (Yang *et al.* 2020). Fifty μM of GM significantly suppressed PPARγ gene expression, whilst the other concentrations of GM and extracts did not significantly suppress the PPARγ gene expression as compared with the positive and/or negative control (Fig. 3a). PPARγ expression is maintained by C/EBPα which also regulates insulin sensitivity in adipocytes (Wang 2010). C/EBPα is commonly expressed in the adipose tissue, liver, lung, adrenal, and placenta (Moseti *et al.* 2016). When compared with the positive control, MPE, AM, or GM significantly reduced the C/EBPα mRNA in 3T3-L1 cells (Fig. 3b). Transient expressions of C/EBPβ and C/EBPδ occur during the early stages of differentiation, followed by PPARγ and C/EBPα expressions that induced specific gene expressions during terminal adipocyte differentiation (Chen *et al.* 2011) whose absence can cause insulin resistance (Moseti *et al.* 2016). PPARγ and C/EBPα genes can activate some other specific genes in adipogenesis, such as aP2, FAS, and LPL (Song *et al.* 2013). However, only MPE and GM reduced the SCD1 gene expression (Fig. 3c). The expression of proteins involved in adipogenesis is regulated by specific genes, like LPL gene expression that inhibits adipogenesis (Linehan *et al.* 2012). In this study, LPL gene expression was inhibited by MPE, AM, or GM in this study (Fig. 3d).

C/EBPα regulates normal adipocyte differentiation by expressing LPL, SCD, and FAS. C/EBPα induced the activation of some adipogenesis genes including SCD and aP2 (Moseti *et al.* 2016). SCD is associated with several disorders including diabetes and obesity and its suppression can result in loss of body fat (Dobrzyn *et al.* 2010). aP2 is a member of the cytoplasmic fatty acid binding protein family which is highly expressed during the adipogenesis process (Gwon *et al.* 2013). The presence of PPARγ and C/EBPα activate the aP2 gene expression in early adipocytes differentiation (Obregon 2014). MPE and GM were found to suppress aP2 gene expression. Fifty μg/mL of MPE showed the highest and most significant suppression activity. The suppression of AdipoQ gene expression in all 3T3-L1 cells treated with MPE, AM or GM was lower than for the positive control (Fig. 4b).

FAS plays an important role in the regulation of de novo lipogenesis by converting acetyl-CoA and malonyl-CoA into palmitate, which is subsequently esterified into triacylglycerols and stored in adipose tissue (Strable & Ntambi 2010). When treated with MPE, AM, or GM, the FAS gene expression in all 3T3-L1 cells was lower than the positive control. The lowest expression of FAS gene was found in 3T3-L1 cells treated with 50 μM of GM (Fig. 4c). These findings confirmed the results of Quan *et al.* (2012), that AM could induce apoptosis of 3T3-L1 preadipocytes via inhibition of FAS. This process results in decreasing intracellular lipid accumulation during adipocyte differentiation and stimulates lipolysis in mature adipocytes.

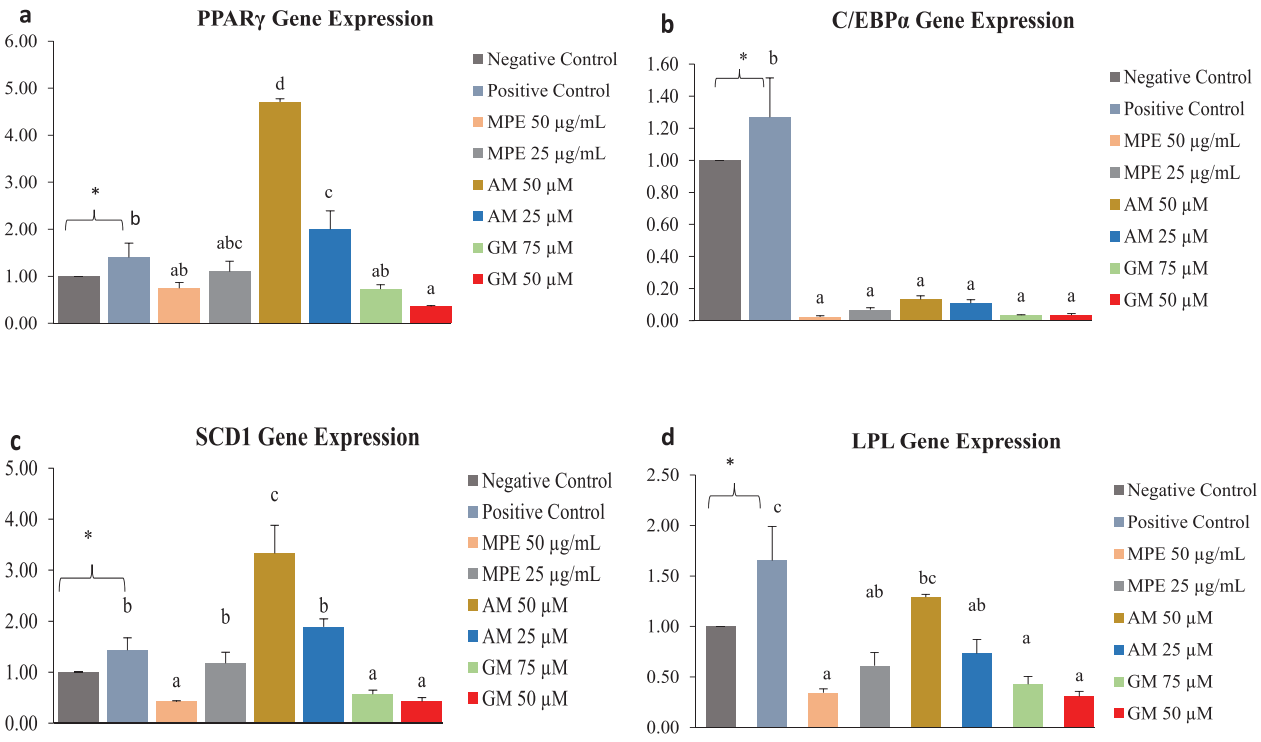
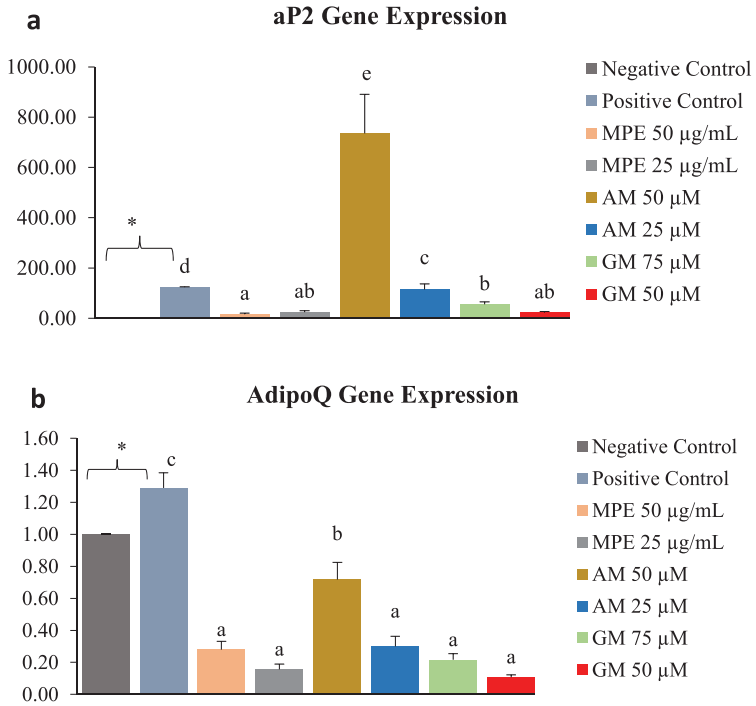


Figure 3 Histogram of gene expressions in 3T3-L1 cell: a) Relative expression of PPARγ in 3T3-L1; b) Relative expression of C/EBPα in 3T3-L1; c) Relative expression of SCD1 in 3T3-L1; d) Relative expression of LPL in 3T3-L1

Notes: This histogram represents the mean ± SD values.

* indicates significant differences between the negative and positive controls based on un-paired T-test ($p < 0.05$). Different letters (a-d) indicate significant differences among the means (concentrations of MPE, AM, GM and positive control) on a) Relative expression of PPARγ in 3T3-L1; b) Relative expression of C/EBPα in 3T3-L1; c) Relative expression of SCD1 in 3T3-L1; d) Relative expression of LPL in 3T3-L1) based on Tukey HSD post-hoc test ($p < 0.05$).



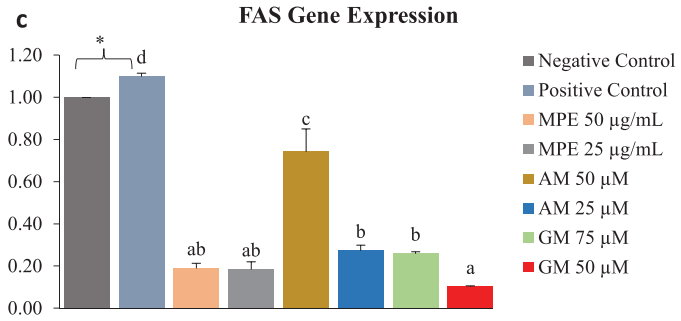


Figure 4 Histogram of gene expressions in 3T3-L1: a) Relative expression of aP2 in 3T3-L1; b) Relative expression of AdipoQ in 3T3-L1; c) Relative expression of FAS in 3T3-L1
Notes: This histogram represents mean ± SD value.
* indicates significant difference at ($p < 0.05$) between negative and positive controls according to unpaired T-test. Different letters (a-e) indicate significant differences among the means (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test toward gene expressions of a) Relative expression of aP2 in 3T3-L1; b) Relative expression of AdipoQ in 3T3-L1; c) Relative expression of FAS in 3T3-L1.

CONCLUSION

MPE and xanthones (AM and GM) showed potentials as anti-obesity agents, through down-regulation of genes involved in adipogenesis. Fifty µg/mL of MPE and 50 µM of GM were the most suitable concentrations for suppressing the gene expression involved in adipogenesis. AM also reduced the expression of those genes, except for PPARγ, SCD1, LPL and aP2. However, further pre-clinical and clinical investigations are recommended prior to the application of MPE, AM and GM for obesity therapy.

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