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

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Running title: Adipogenesis and key adipogenic gene expression

ABSTRACT

Obesity is one of the risk factors for atherosclerosis. Fat accumulation and adipocyte differentiation are associated with the occurrence and development of obesity. Thus, suppression of adipocyte differentiation provides a potential anti-obesity approach. 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 cytotoxic assay on 3T3-L1 cells. Three different MPE concentrations (0, 25, and 50 µg/ml) were used in this study. Likewise, three different concentrations of AM (0, 25, and 50 µM) and GM (0, 50, and 75 µM) were also 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, 25 µM and 50 µM of AM did not suppress PPARγ and SCD-1 expression. 50 µM of AM also failed to reduce aP2 gene expression. In conclusion, 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 cause various serious diseases, such as atherosclerosis (Kang et al., 2013). An imbalance in energy intake and expenditure in obesity 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 (Jeon et al., 2004). Adipogenesis causes the differentiation of preadipocytes to adipocytes which play an important role in fat mass growth (Choi et al., 2007; Giri et al., 2006).

Some genes have responsible in adipogenesis regulation such as CCAAT/enhancer binding protein beta (C/EBPβ), nuclear receptor peroxisome proliferation-activated receptor

gamma (PPARγ), and CCAAT/enhancer binding protein-alpha (C/EBPα), playing an important role 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) (Mackall et al., 1976; Spiegelman et al., 1983; Bernlohr et al., 1984; Ntambi et al., 1988; Obregon, 2014), the lipoprotein lipase (LPL) (Obregon, 2014; Cook et al., 1987; Flier et al., 1989), and adipoQ, nowadays known as adiponectin (Hu et al., 1996). Therefore, the potential strategy to prevent obesity is to control adipogenesis (Wang et al., 2008).

The side effects from the conventional drugs for obesity usually can lead to the regaining of body weight if the medication is stopped. Hence, it is required to find novel approaches to obesity prevention focusing on healthy foods or natural drugs without negative side effects. Mangosteen (*Garcinia mangostana* L.) has been used in traditional medicine (diarrhea, dysentery, eczema and other skin diseases) for decades (Shen et al., 2014). The pericarp has been reported to contain abundant xanthones (α-Mangostin and γ-Mangostin) that show various bioactivities (Fig 1) such as antioxidant, antifungal, antibacterial, cytotoxic, anti-inflammatory, anti-histamine, anti-HIV, and other activities (Ibrahim et al., 2014, Widowati et al., 2016).
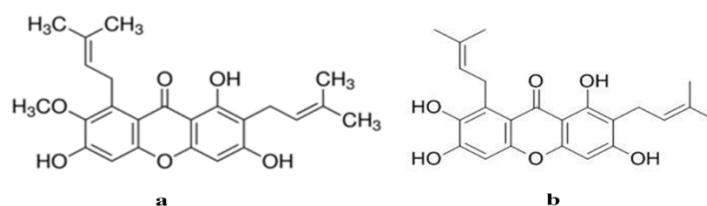
Figure 1. Chemical structure of a) α-Mangostin and b) γ-Mangostin

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 may provide better understanding regarding molecular mechanisms of MPE in controlling adipogenesis as an obesity therapy.

MATERIALS AND METHODS

Plant material preparation and extraction

G. mangostana L. was 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, Bandung Institute of Technology, West Java, Indonesia. The voucher specimen was deposited in Aretha Medika Utama (005/AMU-BBRC). The pericarps were collected, chopped, and dried using a drying device (40–45 ˚C) until a stable water level was obtained (±13%). Afterwards, the extraction was performed using maceration with ethanol 70% (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 incubated for 24 hours at 37 ˚C, 5% CO_2. Cells were collected and seeded into 6-well plates (1 x 104 cells/well) with DMEM + 10% calf serum until confluent (80-90%). Subsequently, subcultures were performed 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 positive control, growth medium for negative control, and treatment medium (DMEM + IBMX + insulin + dexamethasone + FBS + MPE or xanthones (AM and GM)). Plates were incubated for 3 d 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 negative control, and insulin medium + MPE or xanthones for treatment. The culture was incubated at 37 ˚C, 5% CO2, and fresh medium was added every 2 d to maintain the culture. After 7 d, the medium was decanted, and more than 80% cells had differentiated. The adipocytes were then observed under an inverted microscope (Hidayat et al., 2015) (Huang et al., 2006).

Viability assay

An MTS assay was performed to investigate cell viability and 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, 5% CO_2, in a humidified atmosphere. The incubated

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medium was decanted, and 90 µl serum-free medium with 10 µl MPE in various concentrations (6.25, 12.5, 25, and 50 µg/ml diluted in DMSO) was added to the cells. For the cells in xanthone (AM or GM) treatment group, 90 µl serum-free medium with 10 µl AM or GM in various concentrations (12.5, 25, 50, and 75 µM diluted in DMSO) were added. The plates were then incubated for 24 h at 37 ˚C, 5% CO_2, in a humidified atmosphere. Untreated cells were presented as a 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., 2016; 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. 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). The product was stored at -20 ˚C. RNA concentrations and purities are shown in Table 1.

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

[*]The data are presented as a mean $\pm$ 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. The primers

used in this study can be seen in 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).

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

Primer	Forward	Reverse
PPAR-	5'-TTT TCA AGG GTC CCA GTT TC-3'	5'-TTA TTC ATC AGG GAG GCC AG-
C/EBPα	5'-GCC GAG ATA AAG CCA AAC AA-3'	5'-CCT TGA CCA AGG AGC TCT CA-
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 GGG TTC CAT GTT T-3'
LPL	5'-CTG CTG GCG TAG CAG GAA GT-5'	5'-GCT GGA AAG TGC CTC CAT TG-
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-
β-actin	5'-TCT GGC ACC ACA CCT TCT ACA -3'	5'-AGC ACA GCC TGG ATA GCA -3'

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 software. To compare negative and positive control, the data was analyzed using unpaired-T test. The differences among treatment, the data were analyzed using one-way analysis of variance (ANOVA) with SPSS 20.0 statistical package. Only probability values of P<0.05 were considered statistically significant and later subjected to Tukey HSD post hoc test. Data are presented as mean ± SD.

RESULTS AND DISCUSSION

Nowadays, adverse effects after therapy have been threatening patients suffering from obesity. Therefore, medicines obtained from natural sources have the potential to substitute the commercial drugs because are much safer and more effective. Mangosteen pericarp extract (MPE) contains various beneficial secondary metabolites such as prenylated and oxygenated xanthones which are promising anti-obesity drugs (Pedraza-Chaverri et al., 2008; Adnyana et al., 2016). Xanthone founded in mangosteen fruit has derivatives such as α-Mangostin (AM) and γ-Mangostin (GM) that showed several pharmacological activities such as antioxidant, antitumor, anti-inflammatory, antibacterial, anti-allergy, antifungal, and antiviral activities

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173 (Pedraza-Chaverri et al., 2008; Widowati et al., 2014). A previous study reported that MPE and

174 xanthones (AM and GM) exhibited anti-inflammatory properties by inhibiting the COX-2

175 gene, IL-6, IL-1β, and NO activities in LPS-induced RAW264.7 (Widowati et al., 2016).

176   MTS assay showed that MPE, AM, and GM concentrations used in this study were non-

177 toxici to cells due to the percentage of viable cells more than 85%. The results of the viability

178 assay are related to the adverse effects of bioactive substances on living organisms prior to

179 their use as drugs or chemicals in clinical settings (Depress et al., 1989; Lalitha et al., 2012).

180 In this study, two concentrations of MPE (25 and 50 µg/ml), AM (50 and 25 µM), and GM (75

181 and 50 µM) were used for further treatments on 3T3-L1 cells. Cytotoxic or viability assays

182 showed that the viability of cells was concentration dependent. The percentage of cell viability

183 was determined by comparing treatment (MPE, AM, GM) to control groups. All sample

184 concentrations were safe for the 3T3-L1 cell, except 75 µM of AM, which resulted in less than

185 85% of cells being viable. Based on these results, all concentrations of MPE, AM, and GM,

186 except 75 µM of AM, can be used for further treatment in 3T3-L1 cells (Table 3).

187

188 Table 3. 3T3-L1 cell viability in various concentrations of MPE, AM, and GM measured in
189 triplicates

| 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]	-

190 *The data are presented as a mean ± standard deviation. Different superscript small letters (a,
191 ab,b) in the same row (among various concentrations of MPE, AM, GM toward cells viability)
192 are significant at p < 0.05 based on Tukey HSD post hoc test. The experiment was conducted
193 in triplicate.

194

195   In certain conditions, 3T3-L1 cells may differentiate into adipocytes. (Fig. 2) shows the

196 normal cells and induced adipocyte differentiation. After induction using insulin for five days,

197 lipid droplets were formed. The accumulation of fat and adipogenesis are the sign of obesity

198 development (Jeon et al., 2004). Adipogenesis is a complex process that involves specific genes

199 and enzymes. Those genes are involved in the regulation of adipocyte differentiation, thus the

200 potential strategy to prevent obesity is to inhibit the adipogenesis genes and enzymes (Obregon,

201 2014; Gwon et al., 2013). In this study, it was found that 50 µM of GM were the most effective

concentrations to suppress 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 (Rosen et al., 2000). MPE, AM, GM were sufficient to suppress C/EBPα, 50 μg/ml of MPE, while 75 μM and 50 μM of GM were 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 differentiated-3T3-L1 cells.
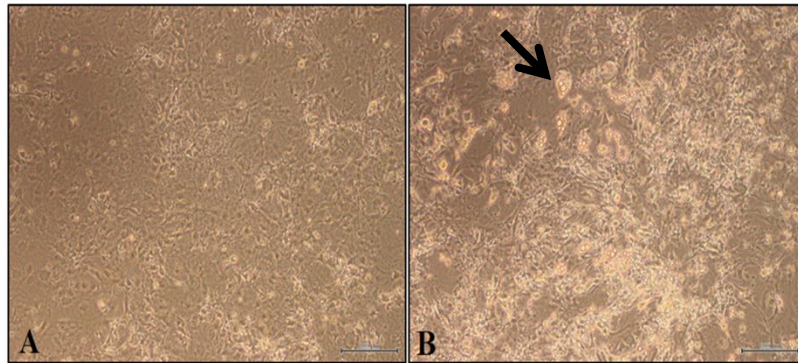

Figure 2. Morphology of 3T3-L1 cell culture. (A) Non-induced cell; (B) Adipogenesis-induced cells.
*Black arrow shows accumulated intracellular lipid droplet which show higher lipid droplet.

PPARγ gene is activated by fat accumulation (Tontonoz et al., 1994; Hidayat et al. 2016), which has an important role in inducing adipocyte differentiation (Tontonoz et al., 2008; Lefterova & Lazar, 2009). PPARγ and C/EBPα are master regulators of adipogenesis (Christy et al., 1991; Hidayat et al. 2016), and the activation of those genes is important for the progression of the terminal stage of adipogenesis (Christy et al., 1991; Tamori et al., 2002; Hidayat et al. 2016). Fifty μM of GM significantly suppressed PPARγ gene expression, whilst the other concentrations of GM and extracts showed no significant difference in suppressing PPARγ gene expression compared to the positive and/or negative control (Fig. 3a). PPARγ expression is maintained by C/EBPα which regulates insulin sensitivity in adipocytes as well (Rosen et al., 2002; Hidayat et al. 2016). C/EBPα is commonly expressed in the adipose tissue, liver, lung, adrenal, and placenta (Birkenmeier et al., 1989; Yeh et al., 1995; Moseti et al., 2016). C/EBPα mRNA in 3T3-L1 cells treated with MPE, AM, or GM was significantly suppressed compared to the positive control. MPE, AM, GM were active in reducing C/EBPα gene expression (Fig. 3b). Transient expression of C/EBPβ and C/EBPδ occurs during the early stages of differentiation, followed by expression of PPARγ and C/EBPα that induce the expression of specific genes in terminal adipocyte differentiation (Kudo et al., 2004). Insulin

resistance can be caused by the lack of C/EBPα expression in terminal adipocyte differentiation (Moseti et al., 2016; El-Jack et al., 1999; Linhart et al., 2001). PPARγ and C/EBPα genes can activate some other specific genes in adipogenesis, such as aP2, FAS, and LPL (Song et al., 2013). Fig. 3c shows that only MPE and GM reduced SCD1 gene expression. The expression of proteins involved in adipogenesis is regulated by specific genes. Adipogenesis can be inhibited by suppressing LPL gene expression (Merkel et al., 2002; Linehan et al., 2012). Fig. 3d demonstrates that LPL gene expression was inhibited by MPE, AM, or GM in this study.
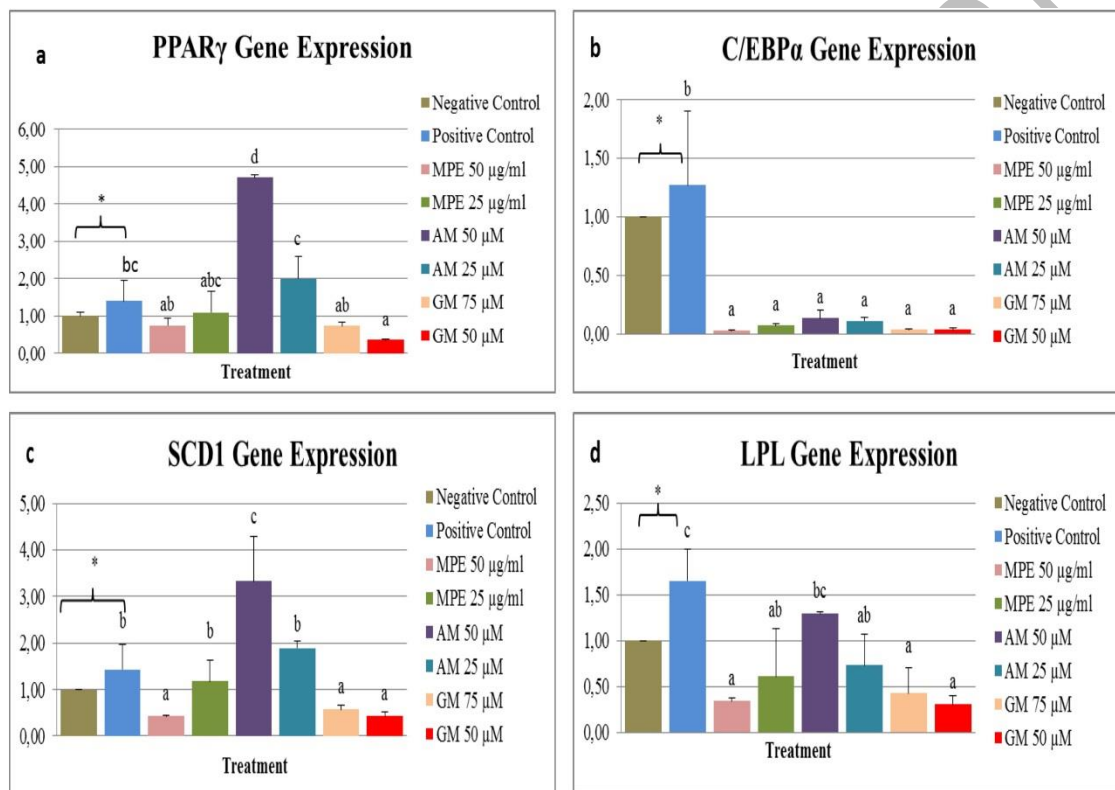
Figure 3. Histogram of gene expressions in 3T3-L1 cell. a) Relative expression of PPARγ in 3T3-L1; b) Relative expression of C/EBPα in 3T3-L1; c) Relative expression of SCD1 in 3T3-L1; d) Relative expression of LPL in 3T3-L1.

*This histogram as the mean ± SD value. The experiment was conducted in triplicate.

Figure 3a, the asterisk symbol (*) indicate significant difference between negative and positive control according un-paired T-test (P<0.05). Different letters (a,ab,abc,bc,c,d) indicate significant differences among the means of groups (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test (P< 0.05) toward gene expression of PPARγ in 3T3-L1 cells.

Figure 3b, the asterisk symbol (*) indicate significant difference between negative and positive control according to un-paired T-test (P<0.05), Different letters (a,b,c) indicate significant differences among the means of groups (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test (P< 0.05) toward gene expression of C/EBPα in 3T3-L1 cells.

Figure 3c, the asterisk symbol (*) indicate significant difference between negative and positive control according un-paired T-test (P<0.05). Different letters (a,b) indicate significant

255 differences among the means of groups (concentrations of MPE, AM, GM and positive
256 control) based on Tukey HSD post-hoc test (P< 0.05) toward gene expression of SCD1 in
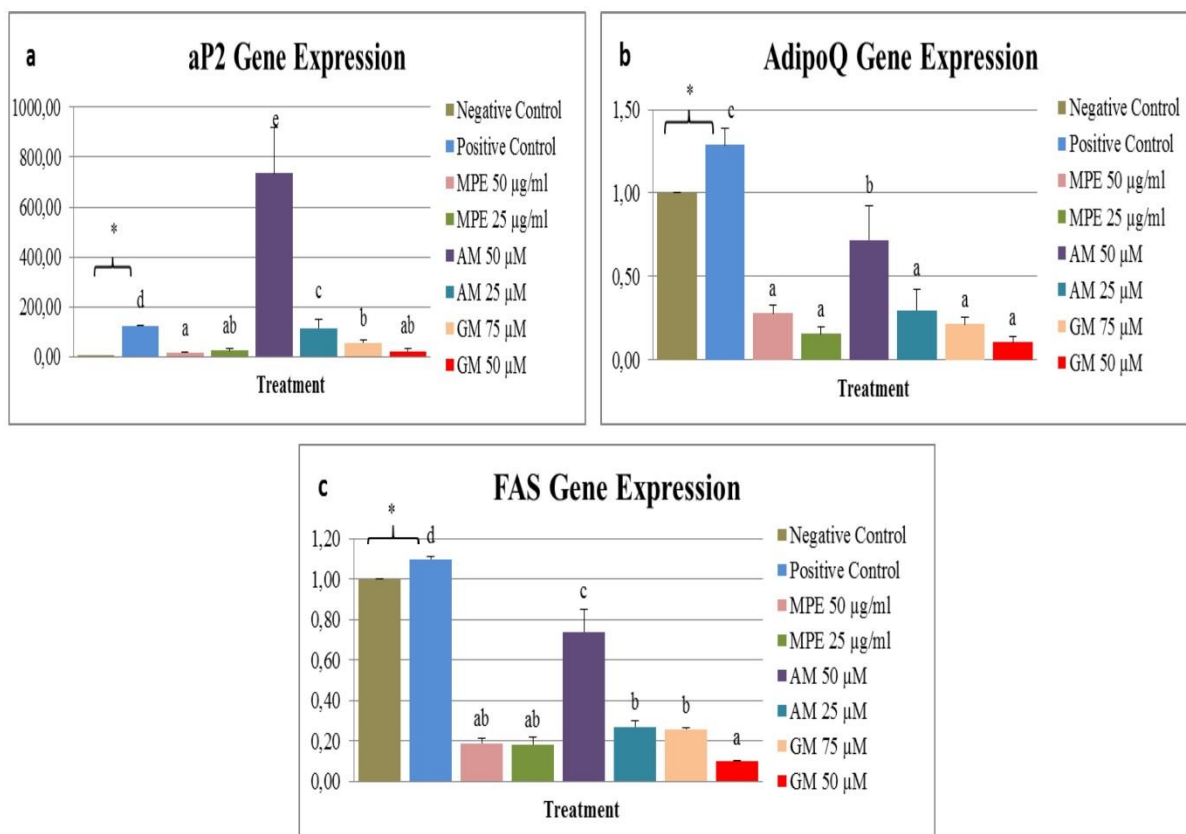257 3T3-L1 cells.
258 Figure 3d, the asterisk symbol (*) indicate significant difference between negative and positive
259 control according un-paired T-test (P<0.05). Different letters (a,ab,b,c) indicate significant
260 differences among the means of groups (concentrations of MPE, AM, GM and positive
261 control) based on Tukey HSD post-hoc test (P< 0.05) toward gene expression of LPL in
262 3T3-L1 cells.
263

264   C/EBPα regulates normal adipocyte differentiation by expressing LPL, SCD, and FAS.

265 C/EBPα has been reported to induce the activation of some adipogenesis genes including SCD

266 and aP2 (Moseti et al., 2016). SCD is associated with several disorders including diabetes and

267 obesity and its suppression can result in loss of body fat (Ntambi et al., 2002). aP2 is a member

268 of the cytoplasmic fatty acid binding protein family which is highly expressed during the

269 adipogenesis process (Gwon et al., 2013). The presence of PPARγ and C/EBPα activates the

270 aP2 gene expression in early adipocytes differentiation (Lin & Lane, 1992; Mandrup & Lane,

271 1997; Obregon, 2014). MPE and GM were found to suppress aP2 gene expression. Fifty µg/ml

272 of MPE was again shown to have the highest suppression activity. The suppression of AdipoQ

273 gene expression in all 3T3-L1 cells treated with MPE, AM, or GM was lower than for the

274 positive control (Fig. 4b).

275   FAS has an important role in the regulation of de novo lipogenesis by converting acetyl-

276 CoA and malonyl-CoA into palmitate, which is subsequently esterified into triacylglycerols

277 and stored in adipose tissue (Griffin & Sul, 2004; Ranganathan et al., 2006). FAS gene

278 expression in all 3T3-L1 cells treated with MPE, AM, or GM was lower than the positive

279 control. The lowest expression of FAS gene was found in 3T3-L1 cells treated with 50 µM of

280 GM (Fig. 4c). These findings are in accordance with Quan et al. (2012), where AM could

281 induce apoptosis of 3T3-L1 preadipocytes via inhibition of FAS. This process results in the

282 decreasing of intracellular lipid accumulation during adipocyte differentiation and stimulates

283 lipolysis in mature adipocytes.

284

285

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.

*This histogram as the mean ± SD value. The experiment was conducted in triplicate.

Figure 4a, the asterisk symbol (*) indicate significant difference between negative and positive control according un-paired T-test ($P<0.05$). Different letters (a,ab,b,c,d,e) indicate significant differences among the means of groups (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test ($P< 0.05$) toward gene expression of aP2 in 3T3-L1 cells.

Figure 4b, the asterisk symbol (*) indicate significant difference between negative and positive control according un-paired T-test ($P<0.05$), Different letters (a,b,c) indicate significant differences among the means of groups (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test ($P< 0.05$) toward gene expression of AdipoQ in 3T3-L1 cells.

Figure 4c, the asterisk symbol (*) indicate significant difference between negative and positive control according un-paired T-test ($P<0.05$). Different letters (a,ab,b,c,d) indicate significant differences among the means of groups (concentrations of MPE, AM, GM and positive control) based on Tukey HSD post-hoc test ($P< 0.05$) toward gene expression of FAS in 3T3-L1 cells.

CONCLUSION

MPE and xanthones (AM and GM) showed the potential as anti-obesity agents, through down-regulation of genes involved in adipogenesis. Fifty µg/ml of MPE and 50 µM of GM

310 was found to be the most suitable concentrations for suppressing the expression of genes
311 involved in adipogenesis. AM was also shown to reduce the expression of those genes, except
312 for PPARγ, SCD1, LPL and aP2. Further preclinical and clinical investigations should be
313 performed prior to the application of MPE, AM, and GM for obesity therapy.

314

315 **ACKNOWLEDGMENTS**

321

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