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Modulation of adipogenesis-related gene expression by ethanol extracts of Detam 1 soybean and Jati belanda leaf in 3T3-L1 cells

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Abstract

In this study, we evaluated the effects of ethanol extracts of Detam 1 soybean, Jati belanda leaf, and the combination toward expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and stearoyl-CoA desaturase 1 (SCD1) genes in 3T3-L1 cells as anti-adipogenesis and anti-obesity. The differentiation of 3T3-L1 cells into adipocyte was conducted using induction medium consist of Dulbecco's Modified Eagle's Medium, 3-isobutyl-1-methylxanthine, insulin, dexamethasone, and fetal bovine serum. The expression of PPARγ, C/EBPα, and SCD1 gene was measured using real-time quantitative polymerase chain reaction (qPCR). Ethanol extract of Jati belanda at a concentration of 50 µg/mL was most effective to reduce PPARγ, C/EBPα, and SCD1 gene expression in 3T3-L1 cells. Ethanol extract of Detam 1 soybean failed to reduce PPARγ gene expression, whilst in the concentration of 50 µg/mL it was able to significantly reduce the C/EBPα and SCD1 gene expression. Both ethanol extracts of Detam 1 soybean and Jati belanda have potential as anti-adipogenesis and anti-obesity by suppressing adipogenesis-related gene expression, particularly C/EBPα and SCD1.

Introduction

Obesity is one of the largest and fastest growing health problem both in the developed and developing countries (Sergent et al., 2012; Rodgers et al., 2012). In the cellular level, obesity is characterized by a rise in the number (hyperplasia) and/or size (hypertrophy) of adipocytes, which is fat storage cells that differentiate from fibroblast-like precursor cells in adipose tissue (Bunkrongcheap et al., 2014; Lim et al., 2014). Thus, the suppression of differentiation into adipocytes, known as adipogenesis and/or promoting the intracellular lipid breakdown are potential anti-obesity mechanism (Bunkrongcheap et al., 2014). The adipogenesis process involves a cascade of transcription factors, and several factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are considered to play important role in the process (Lefterova and Lazar, 2009).

Black soybeans (*Glycine max*) have been widely used as a healthy food and medicinal herb in oriental medicine (Kim et al., 2012). Several soy isoflavonesin from soybean revealed to inhibit adipogenesis through down-regulation of adiponectin expression in 3T3-L1 adipocytes (Zhang et al., 2009; Yanagisawa et al., 2012). *Guazuma ulmifolia* Lam., known as "Jati belanda" in Indonesia has been popularly used as therapeutics for hypercholesterolemic, gastro-intestinal disorder, diabetes mellitus, and to reduce weight in some parts of the world (Feltrin et al., 2012; Magos et al., 2008). Our previous study demonstrated that ethanol extract of Detam 1 soybean seeds and ethanol extract of Jati belanda leaves have potential as anti-obesity, it

inhibited weight gain in male Wistar rat and possessed inhibitory activities toward glucose-6-phosphate dehydrogenase (G6PD), triglyceride (TG) and cholesterol (CHOL) in 3T3-L1 cell line (Hidayat et al., 2015a; Hidayat et al., 2015b).

In this research, we continue to study the anti-adipogenesis properties of ethanol extract of Detam 1 soybean (EEDS) and ethanol extract of Jati belanda leaves (EEJB) in 3T3-L1 cell line by analyzing the expression of PPARγ and C/EBPα genes which are important transcription factor in adipogenesis (Rosen et al., 2002) and SCD1 gene which plays a role in lipid storage in adipocytes (Ralston and Mutch, 2015).

Materials and Methods

Plant materials

Detam 1 black soybean variety, which is a high quality black soybean that has been approved by the Agricultural Ministry of Republic of Indonesia, was collected from the estate of Research Unit and Development of Legumes and Tuber in Malang, East Java, Indonesia. Jati belanda was collected from plantations of Bumi Herbal Dago, Bandung, West Java, Indonesia. The plant was identified by a herbarium staff from the Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia.

Preparation of the extracts

Ethanol extractions of Detam 1 soybean and Jati belanda were carried out by simple maceration method (Hidayat et al., 2015b). Detam 1 soybean (3 kg) and Jati belanda (700 g) were extracted twice by ethanol for 7 days. The solvent was evaporated and thick liquid extract was collected (71 and 56 g for Detam 1 soybean and Jati belanda, respectively). The ethanol extracts of Detam 1 soybean and Jati belanda were stored at -20°C for further use.

Adipogenesis induction

Adipogenesis induction was conducted using adipogenesis assay kit (Abcam ab133102). After cells reached 80% confluence, the medium was replaced by induction medium (DMEM + 3-isobutyl-1-methylxanthine (IBMX) + insulin + dexamethasone + fetal bovine serum (FBS)) for positive control, growth medium for negative control, and treatment medium (DMEM + IBMX + insulin + dexamethasone + FBS + EEDS or EEJB) for treatment. Cells were incubated for 3 days at 37°C, humidified atmosphere, 5% CO_2. Following that, the cells were washed with phosphate-buffered saline (PBS) 1x and the medium was replaced by insulin medium (DMEM + FBS + insulin) for positive control, culture medium for negative control, and insulin medium + EEDS or EEJB. The plate was incubated for 2 days at 37°C, humidified atmosphere and 5% CO_2. The insulin medium was replaced again and more than 80% cells were differentiated after 7 days. The adipocytes were observed using microscope (Hidayat et al., 2015a; Huang et al., 2006).

RNA extraction and cDNA synthesis

RNA extraction was performed using aurum total RNA kit (Bio-Rad) according to the manufacturer's instructions. After the extraction, the quality of RNA was checked using electrophoresis. The RNA was then used for cDNA synthesis using mix iScript cDNA synthesis kit (Bio-Rad) at 25°C for 5 min, 42°C for 30 min, with a final step of 5 min at 85°C. The product was stored at -20°C.

Quantification of PPARγ, C/EBPa, and SCD1 expression by real-time qPCR

The peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding protein alpha (C/EBPα), and stearoyl-CoA desaturase 1 (SCD1) genes expression along with the constitutively expressed β-actin gene was analyzed using real-time quantitative polymerase chain reaction (qPCR). The primers used in this study are shown in Table I. The real-time qPCR was conducted using real-time PikoReal (Thermo Scientific Inc.) with condition pre-incubation cycle at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 20 sec, and extension at 72°C for 10 sec.

Box 1: Cell culture (Mouse pre-adipocyte)

Requirements

Mouse pre-adipocytes cells, 3T3-L1 (ATCC®CL-173; CV Gamma Scientific Biolab, Indonesia); Dulbecco's Modified Eagle Medium (DMEM, Biowest) supplemented with 10% calf serum (Biowest) and 100 U/mL penicillin-streptomycin (Biowest); inverted microscope, Eppendorf micropipette; incubator with CO_2 cylinder; phosphate buffer solution

Procedure

Step 1: Collect the fresh culture medium from the water bath

Step 2: Taking out the culture cells from the incubator

Step 3: Check the confluence of the cells using the inverted microscope

Step 4: Remove the culture medium

Step 5: Add phosphate buffer solution to remove the remaining debris

Step 6: Remove the phosphate buffer solution along the remaining debris

Step 7: Add fresh medium to the cell culture

Step 8: Incubate the cell culture 37°C, humidified atmosphere, 5% CO_2

To see the **Video Clip**

Table I

Sequence of primers used in real-time quantitative PCR

Primer	Forward	Reverse
PPARγ	5′-TTATCAAGGGTCCCAGTTTC-3′	5′-TTATTCATCAGGGAGGCCAG-3′
C/EBPα	5′-GCCGAGATAAAGCCAACCAA-3′	5-CCTTGACCAAGGAGCTCTCA-3′
SCD1	5′-CTGTACGGGATCATACTGGTTC-3′	5′-GCCGTGCCTTGTAAGTTCTG-3′
β-actin	5′-TCTGGCACCACACCTTCTACAATG-3′	5′-AGCACAGCCTGGATAGCAACG-3′

Statistical analysis

Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) statistics version 17.0 software. One-way analysis of variance (ANOVA) was conducted, followed by Duncan post-hoc test and $p<0.05$ was considered to be significant. Data are presented as mean ± SD.

Results

PPARγ gene expression

The quantification of PPARγ gene expression using qPCR revealed that only several treatments than positive control, which were EEJB 50 µg/mL and combination of EEJB and EEDS (20 µg/mL, 10 µg/mL) (Figure 1). The lowest expression of PPARγ gene was found in 3T3-L1 cells treated with EEJB 50 µg/mL, while the highest expression of PPARγ gene was found in 3T3-L1 cells treated with combination of EEJB and EEDS (10 µg/mL, 5 µg/mL).

C/EBPα gene expression

All 3T3-L1 cells treated with the EEJB and/or EEDS were found to express C/EBPα mRNA lower than positive control, but the all combination of extracts treatments did not able to reduce the expression significantly (Figure 2). EEJB 50 µg/mL was the most active to reduce C/EBPα gene expression, whilst the combination of EEJB and EEDS (10 µg/mL, 5 µg/mL) was the least active.

SCD1 gene expression

In this research, EEJB and EEDS at the concentration of 50 µg/mL showed lower SCD1 gene expression by approximately twice than positive control (Figure 3). EEJB at the concentration of 50 µg/mL was able to reduce SCD1 gene expression the most, with relative expression value of 4.9. Meanwhile, the combination of EEJB and EEDS (10 µg/mL and 5 µg/mL) resulted in highest SCD1 gene expression, with relative expression value of 15.8.

Discussion

In this research, only EEJB and the combination of EEJB

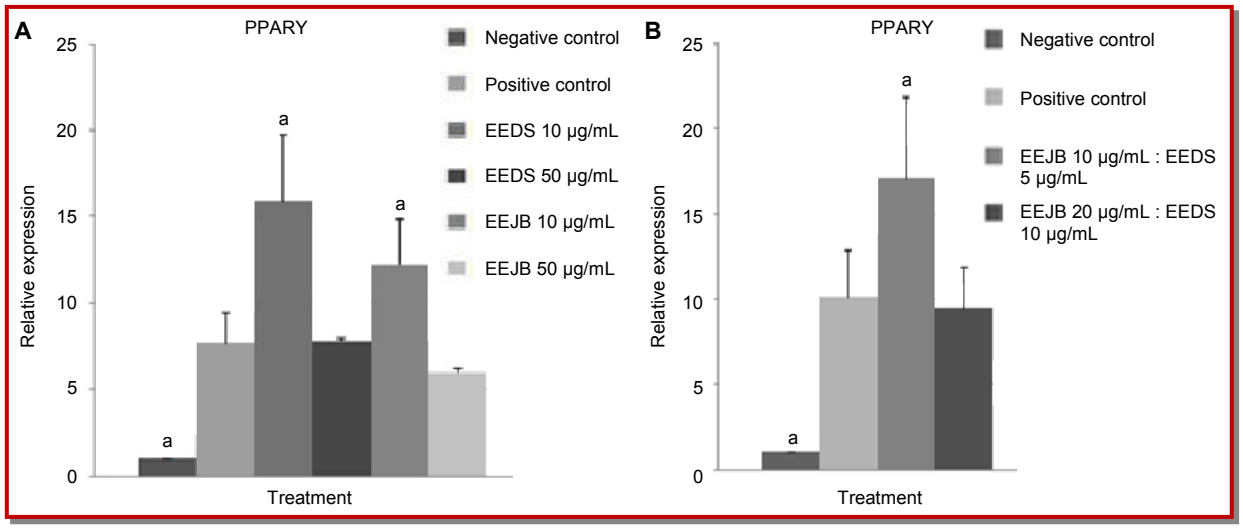

Figure 1: Relative expression of adipogenesis-related gene, PPARγ in 3T3-L1 cells. (a) Effects of single ethanol extract of Detam 1 soybean (EEDS) or ethanol extract of Jati belanda leaves (EEJB) treatments in concentration of 10 µg/mL and 50 µg/mL; (b) Effects of combination of ethanol extract of Jati belanda leaves and ethanol extract of Detam 1 soybean treatments in concentration of 10 µg/mL, 5 µg/mL and 20 µg/mL, 10 µg/mL (*$p<0.05$, significant compared to positive control, Duncan post-hoc test)

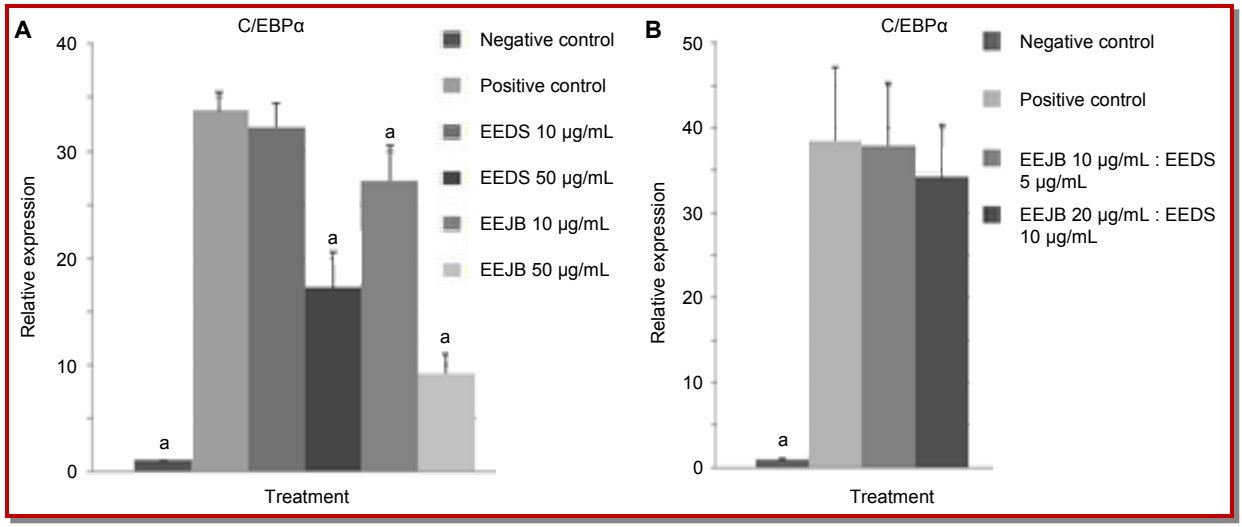

Figure 2: Relative expression of adipogenesis-related gene, C/EBPα in 3T3-L1 cells. (a) Effects of single ethanol extract of Detam 1 soybean (EEDS) or ethanol extract of Jati belanda leaves (EEJB) treatments in concentration of 10 µg/mL and 50 µg/mL; (b) Effects of combination of ethanol extract of Jati belanda leaves and ethanol extract of Detam 1 soybean treatments in concentration of 10 µg/mL, 5 µg/mL and 20 µg/mL, 10 µg/mL (*p<0.05, significant compared to positive control, Duncan post-hoc test)

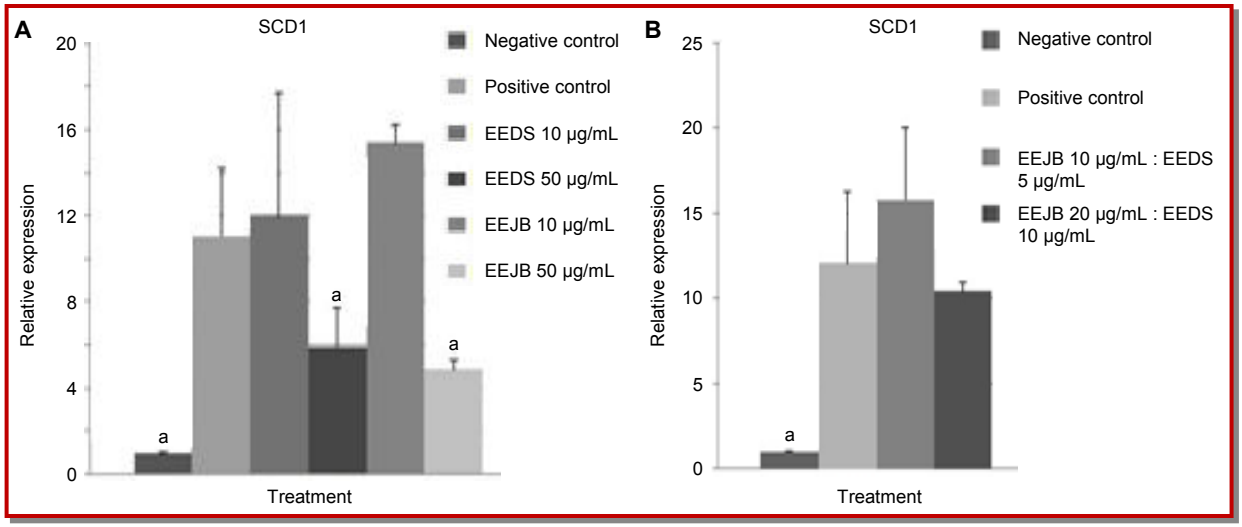

Figure 3: Relative expression of adipogenesis-related gene, SCD1 in 3T3-L1 cells. (a) Effects of single ethanol extract of Detam 1 soybean (EEDS) or ethanol extract of Jati belanda leaves (EEJB) treatments in concentration of 10 µg/mL and 50 µg/mL; (b) Effects of combination of ethanol extract of Jati belanda leaves and ethanol extract of Detam 1 soybean treatments in concentration of 10 µg/mL, 5 µg/mL and 20 µg/mL, 10 µg/mL (*p<0.05, significant compared to positive control, Duncan post-hoc test)

and EEDS treatments were able to reduce PPARγ gene expression, but the reduction by combination of extracts was lowed compared to the reduction by EEJB alone. EEDS resulted in relative expression level similar to positive control, while other treatments seemed to induce the expression rather than suppress it. These results indicated that EEJB and EEDS can both reduce and increase PPARγ gene expression depend on the concentration used, high concentration of extract resulted in low expression of PPARγ mRNA while low concentration of extract given to the 3T3-L1 cells resulted in high expression of PPARγ mRNA. PPARγ belongs to the nuclear receptor super family of ligand-activated transcription factors, which is needed in sufficient quantity so adipocyte differentiation can take place (Lefterova and Lazar, 2009).

PPARγ is abundant in adipose tissue, and serves as essential regulator of adipocyte differentiation and maintenance of mature adipocyte. It also controls several adipocyte genes involved in all pathways of lipid metabolism (Kershaw et al., 2007). Suppression of PPARγ expression can block adipogenesis and lipogenesis (Huang et al., 2006), therefore the reduction of PPARγ gene expression by EEJB in high concentration (50 µg/mL) in this study could be one mechanism in inhibiting adipogenesis in 3T3-L1 cells.

C/EBPα is one of adipogenic transcription factors that

plays a role in adipogenesis (Rosen, 2005). Based on the result, all 3T3-L1 cells treated with EEJB, EEDS, and combination of both resulted in C/EBPα expression lower than cells without treatment (positive control), but the treatment of EEJB or EEDS alone showed more reduction than the combination of EEJB and EEDS. Single treatment of both EEDS and EEJB in high concentration was able to significantly down-regulate the C/EBPα expression compared to the low concentration of extract (10 µg/mL), indicating that the properties of both EEDS and EEJB in down-regulating C/EBPα expression were in concentration-dependent manner. C/EBPα is major contributor of adipsin and leptin expression in fat cells (Rosen et al., 2002), and plays a role in maintaining PPARγ levels and insulin sensitivity in differentiated adipocytes (Rosen, 2005). C/EBPα and PPARγ induce expression of each other and can act synergistically to promote adipogenesis, by activate promoters of some adipocyte-specific genes (Harmon et al., 2002). Therefore, suppression of C/EBPα gene expression by EEJB and EEDS in this study also could inhibit adipogenesis in 3T3-L1 cells (Gwon et al., 2013).

Stearoyl-CoA desaturase (SCD) is enzyme which catalyzes the introduction of the *cis* double bond in the Δ9 position of fatty acyl-CoA substrates in the biosynthesis of monounsaturated fatty acids, increase of SCD activity in the body has been found to correlate with several disorders including diabetes and obesity (Ntambi et al., 2002). SCD1 activity can be enhanced in the presence of insulin, thus 3T3-L1 cells which induced by insulin without given any treatment was used as positive control and have high SCD1 gene expression. In this study, both EEJB and EEDS in high concentration showed to be able to down-regulate SCD1 gene expression in 3T3-L1 cells significantly, and the single extract treatments suggested have better result in suppressing the expression rather than combination of extracts treatment. The low concentration of EEJB and EEDS used as treatments resulted in higher SCD1 gene expression than positive control, indicating that EEJB and EEDS were able to both down-regulate and up-regulate the expression depend on the concentration used. In mice, it was revealed that SCD1 gene deficiency promotes fat oxidation by induces a signal that activates the PPARα pathway and reduce lipid synthesis and storage by down-regulates SREBP-1 expression (Ntambi et al., 2002). SCD1 inhibition in fully differentiated 3T3-L1 adipocytes reported to down-regulated the expression of several genes involved in triacylglycerol (TAG) biosynthesis (Ralston et al., 2014), which is important factor in increasing cell volume of adipocytes (Kim et al., 2012). Previous study has demonstrated that EEJB and EEDS were able to decrease cholesterol and triglyceride levels in 3T3-L1 cells (Hidayat et al., 2015a), these might strongly related to ability of EEJB and EEDS in suppressing SCD1 mRNA level as shown in this study, since SCD1 inhibition can down-regulate biological pathway and genes associated with triglyceride synthesis (Ralston et al., 2014).

Conclusion

EEDS and EEJB in high concentration had anti-adipogenesis and anti-obesity properties by down-regulated C/EBPα and SCD1 gene expression in 3T3-L1 cells. EEJB in the concentration of 50 µg/mL was the most active in suppressing expression of C/EBPα and SCD1 gene. Moreover, it was also able to reduce the PPARγ gene expression.

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Conflict of Interest

All contributing authors declare no conflicts of interest.

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