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Inhibitory Potential of Ethanol Extract of *Detam* 1 Soybean *(Glycine max)* Seed and Jati Belanda (*Guazuma ulmifolia)* Leaves on Adipogenesis and Obesity Models in 3T3-L1 Cell Line

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Authors' contributions

This work was carried out in collaboration between all authors. Author MH identified the species of plant, designed the study performed, the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SS and SP designed and conducted the the study. Authors PPE and WW managed the literature searches, conducted the the study and managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Background: Obesity is considered an emergency health problem through out the world and is characterized by an increase in the number and size of adipocytes in adipose tissue. Some phytochemical bioactive have been shown to inhibit adipocyte differentiation and induce adipocyte apoptosis.
The Objective: In this *ex vivo* study, we evaluated the cytotoxic effects, antiobesity and antidiapogenesis potential of ethanol extract of *Detam* 1 soybean seed (EEDS), *Jati Belanda*

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leaves (EEJB) and their combinations on 3T3-L1 cells.
Experimental Approach: The cytotoxic effect of EEDS and EEJB were assayed using MTS assay. Triglyceride (TG) level and inhibitory activity were assayed using a TG assay kit. Glucose 6-phosphate dehydrogenase (G6PD) activity and inhibitory activity were determined by using G6PDH assay kit. The cholesterol level was measured according to the Chol Kit Randox protocol.
Results: The lowest cytotoxic activity and safe substances on 3T3-L1 cell were EEDS and EEJB in 50 and 10 µg/ml of concentration. EEJB in the concentration of 50 µg/ml was the most active to inhibit G6PD, TG, and cholesterol activity with inhibition activities 47.30%, 37.93% and 73.91% respectively compared to the control (differentiated 3T3-L1 adipocyte).
Conclusion: Ethanol extract of *Detam* 1 soybean seed and *Jati Belanda* leaves posses the inhibitory potential on G6PD, triglyceride and cholesterol activities in 3T3-L1 cell line and the most active compound showed by ethanol extract of *Jati Belanda* leaves.

Keywords: Obesity; adipogenesis; Detam 1 soybean; Jati belanda; 3T3-L1; G6PD; triglyceride; cholesterol.

1. INTRODUCTION

Recently, obesity becomes one of the most serious problem worldwide [1]. It is the most common global metabolic disorders defined as a condition of excessive body adipose tissue determined by standardized measuring [2]. Obesity is a chronic dissease characterized by excess of body fat caused by imbalance between energy intake and expenditure [1]. It is caused by excessive energy intake for a long period and commonly followed by hormonal imbalances, therefore it has a strong association with chronic diseases such as diabetes, cardiovascular diseases, hypertension, osteoarthritis, some cancer and inflammation-based pathologies [3]. At cellular level, obesity is characterized by an increase in the number and size of adipocytes in adipose tissue [4,3]. Formation of new adipocytes from precusor cells- is defined as adipose tissue growth, which further leading to an increase in adipocyte size [5]. Adipocytes have a particularly large capacity to synthesize and store triglycerides in overfeeding condition, as well as to hydrolise and release triglycerides as FFAs and glycerol during fasting [6]. Developing antiobesity drugs that are efficacious and have minimal side effects is highly needed [2]. There are several mode of action from natural compounds to treat obesity such as metabolic stimulants, appetite suppresants, starch blockers, glucose/insulin metabolism, lipid metabolism, and adipocyte-specific effects [5]. Some phytochemical bioactive have been shown to inhibit adipocyte differentiation and induce adipocyte apoptosis [7,8].

Black soybean [*Glycine max* (L) Merr., *Fabaceae*] is one of the most important crops for human and animal consumption, and the most important organic components of soybean seed are the proteins (40%) and oil (20%) [9]. Black soybean contains high-quality proteins and isoflavones, the seed coats contain anthocyanin [10]. Black soybean contain phytochemical bioactive that has antilipemic, lipase inhibitory, lipoxygenase inhibitory and antithrombotic activities [11]. *Detam* 1 black soybean which were used in this study contains proteins higher than average soybean, 41.82% and oil 35.61% [12].

Jati Belanda-is belonging to the family Sterculiaceae and commonly called Bastard cedar. *Jati Belanda* leaves is used to treat ailments like diarrhoea, disentery, cold, cough, diuretic, astringent, and venereal disease [13]. The leaves also known to possess antimicrobial activities, antiulcer activity related to the presence of anthocyanidin and promising antioxidant capacity related to the presence of phenolic compounds and flavonoids [14-16]. This plant extract also extensively used in Mexico for the empirical treatment of type 2 diabetes melitus [17]. According to the previous study, ethanol extract of *Detam* 1 soybean seed (EEDS) contained many secondary metabolites, like: phenolic, flavonoid NaOH, triterpenoids, steroids, saponins, quinones and tannins, but did not contain alkaloids, while ethanol extract of *Jati Belanda* Bumi Herbal Dago variety (EEJB) which were used in this study contained: phenolic, flavonoid H2SO4 triterpenoids, quinones and tannins, but did not contain steroid alkaloids, saponins [12,18]. Therefore, the aim of this study is to evaluate the anti-adipogenesis and anti-obesity activities of EEJB and EEDS in 3T3-L1 cells. 3T3-L1 preadipocytes cells is one of the most useful and established cell lines for adipogenesis proccess [2].

2. MATERIALS AND METHODS

2.1 Plant Material Preparation and Extraction

Detam 1 soybean seed variety, which is a high quality of black soybean that has been approved by the Agricultural Ministry of Republic of Indonesia; grown on the estate of Research Unit and Development of Legumes and Tuber in Malang, East Java Indonesia and *Jati Belanda* plant grown in plantations of Bumi Herbal Dago, North Bandung, Indonesia. The plant was identified by herbarium staff, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Extraction was performed based on the simple maceration method [12,18].

2.2 3T3-L1 Cell Culture and Adipocyte Differentiation Induction

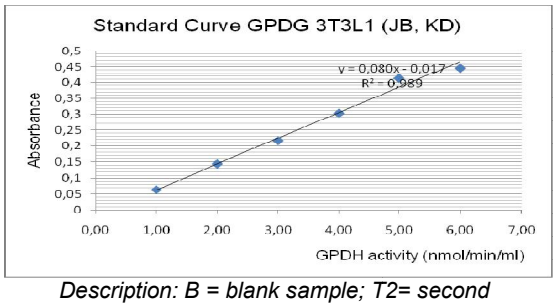
The mouse preadipocytes cells, 3T3-L1 (ATCC®CL-173) was obtained from CV Gamma Scientific Biolab, Malang-East Java, Indonesia. The 3T3-L1, were grown and maintained in Dulbecco's Modified Eagle Medum (DMEM, Biowest) supplemented with 10% calf serum (Biowest) and 100 U/mLpenicillin-streptomycin (Biowest) then incubated for 24 hours at 37 ^{o}C, humidified atmospheres and 5% CO_2. After the cells was confluence, medium was discharged and cells were seeded in 96 well plate (3 x 10^4 cells/ well) with DMEM suplemented with 10% calf serum medium and then incubated for 48 hours. After cells reached 80% confluence, cells were induced to differentiate using Millipore ECM 950 kit. Medium was replaced by initiation medium (DMEM containing FBS 10% and 1:10000 dexamethasone), and incubated for 48 hours. Insulin medium was replaced with progression medium (DMEM containing FBS 10% and 1 : 1000 insulin) and placed in incubator for 48 hours. The medium then replaced again with maintenance medium (DMEM supplemented with 10% calf serum) and incubated for 2-4 days in 37ºC incubator [8]. Cell then observed under the inverted light Olympus microscope after Oil-red O staining (Millipore ECM 950) [19,20].

2.3 Viability Assay

MTS (3 - (4, 5 - dimethylthiazol - 2 - yl) - 5 - (3 - carboxymethoxyphenyl) - 2 - (4 - sulfophenyl) - 2H - tetrazolium) assay (Promega, Madison, WI, USA) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays [20,21]. The 3T3-L1 cells were seeded in 96 well plates (5 x 10^3 cells/ well) in 100 µL medium (DMEM containing 10% calf serum and 100 U/mL penicilin-streptomycin) for 24 hours at 37ºC humidified atmospheres and 5% CO_2. The medium then washed and supplemented with 99 µL new medium and 1 µL of EEDS and EEJB in various concentrations (10, 50, and 100 µg/ml) and incubated for 48 hours at 37ºC and 5% CO_2. After 48 hours medium was replaced by 20 µl MTS and incubated for 3 hours at 37ºC. The absorbance was measured at 490 nm wavelength [20]. The viability assay was performed to determine the safe concentrations for available concentrations on the next assay.

2.4 G6PD (Glucose-6-Phosphate Dehydrogenase) Assay

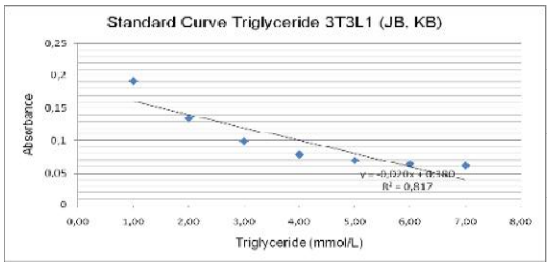
The 3T3-L1 cells were seeded in 96 well plates (5 x 10^3 cells/ well) in 100µL medium (DMEM containing 10% calf serum and 100 U/mL penicilin-streptomycin) for 24 hours at 37ºC humidified atmosphere and 5% CO_2. G6PD was assayed using G6PD kit (Abnova Cat KA 0880). According to the cytotoxicity of the EEDS and EEJB on 3T3-L1, we concluded that EEDS and EEJB were safe and nontoxic. 20 µL samples of medium from cell culture after EEDS and EEJB treatment (10 and 50 µg/mL of concentration) and G6PD positive kit for positive control were added into the well and then 30 µl assay buffer and 50 µl developer work were added. The assay buffer without samples was used for blank. The absorbance was measured at 450 nm. Then the samples were incubated at 37ºC for 30 min in dark room. After 30 min, the samples were measured again using at 450 nm of wavelength. G6PD concentration was determined by equation 1 (Fig. 1).



Description: B = blank sample; T2= second absorbance measurment result; T1= first absorbance measurement; result V= total volume

2.5 Triglyceride (TG) Assay

The triglyceride level was measured according to Randox protocol using Randox TR 210 assay kit. 500 µL mix reaction contained 450 µL reagen with 5 µL sample (cell lysate after treatment in concentration of 10 and 50 µg/mL) was incubated in 37ºC for 5 minutes. ddH2O was used for blank well and standard reagent was used for standard well. Standard reaction was prepared in seven different concentrations using serial dilution (2.180; 1.090; 0.545; 0.273; -0.136; 0.068 and 0.034 mmol/L). The absorbance was measured in 500 nm of wavelength. Triglyceride concentration was calculated using the equation 2 (Fig. 2).

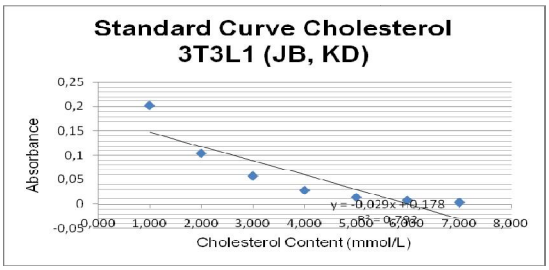


2.6 Cholesterol Assay

The cholesterol level assay was measured according to the Chol Kit Randox CH 200 protocol. 500 µL mix reagent was added into 24 well plate. 5 µL sample (cell lysate after EEDS and EEJB treatment in concentration of 10 and 50 µg/mL) was added into the sample well. 5 µL of ddH_2O was used as blank sample. 5 µL standard solution was added into the standard well. Standard reaction was prepared in seven different concentration using serial dilution (5.170; 2.585; 1.293; 0.646; 0.323; 0.162; and 0.081 mmol). The reaction then incubated at 37ºC for 5 minutes. The absorbance was measured in 500 nm of wavelength. The cholesterol concentration was calculated using the equation 3 (Fig. 3).

2.7 Statistical Analysis

All data were derived from three independent experiments. Statistical analysis was conducted using SPSS software (version 17.0). Value were presented as mean±standard deviation. Significant differences between the groups were determined using the Analysis of Variat (ANOVA) and Duncan Post Hoc Test. Statistical significance was set at $p \leq 0.05$.



3. RESULTS

3.1 Adipocyte Differentiation Induction, Viability Assay

Cell culture of 3T3-L1 cell is a prefibroblast cell which in some conditions can propagated become adipocyte cell. Five (5) days insulin induction transformed cell to accumulate intracellular lipid become lipid droplet. Fig. 4 showed the normal cells and adipocyte differentiation induction cells with red lipid droplet.

Cytotoxic or viability activity was assayed using the MTS assay. The percent of cells viability was determined by comparing the cells viability value of among samples (EEJB, EEDS and also the combination of both extract) to the control. This viability assays (Table 1) showed that viability of samples were concentration dependent manner. EEJB, EEDS in 50 and 10 µg/mL concentration were more safe compared to the other fractions, so it can be used for the next assays in 3T3-L1 cells. The combination of both extract was potentially toxic to the 3T3-L1 cells showed by less than 90% viable cells in 50, 10 and 5 µg/mL concentration but in concentration of 2.5 µg/ml, viability of combination of both extract was safe, mean 90.85%, showed in Table 1.

3.2 G6PD Inhibition Activity

EEJB and EEDS were used to determine G6PD inhibition activity in 3T3-L1 cells. The highest G6PD inhibitory activity was showed by EEJB in the concentration of 50 µg/ml (Table 2). Glucose-6-phosphate dehydrogenase (G6PD) is the cytosolic enzyme that plays a role in penthose phospate pathway. The high activity of G6PD in adipocyte cells caused the lipid metabolism disregulation and insulin resistance [22].

3.3 Triglyceride (TG) Inhibition Activity

Table 3 showed that EEDS and EEJB could reduce TG–level in 3T3L1 cells. EEJB had the

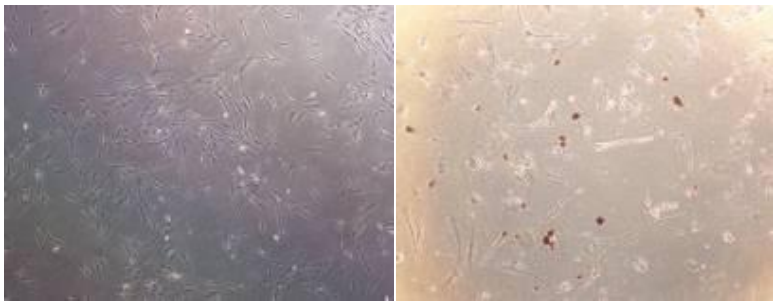


Fig. 4. 3T3-L1 cell culture. The figure was obtained from inverted olympus microscop 10 x 100 magnification

(a). cell without adipogenesis induction; (b). adipogenesis induction cells with red lipid droplet

Table 1. Mean and *Duncan post hoc* test 3T3L1 cells' viability of single and combination extract were measured in triplicate

Samples	Absorbance				
	100 µg/ml	50 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml
EEJB	81.77±1.36 a	90.79±1.21 b			
EEDS	85.39±1.12 a	93.79±0.44 b			
Combination	76.66±1.23 a	81.64±2.32 b	84.85±1.62 c	88.18±1.30 d	90.85±0.79 e

Data were presented as mean ± standard deviation. Different letters in the same row (concentrations) are significant at p ≤ 0.05 (Duncan post hoc test)

highest activity to inhibit TG for both concentrations. Triglyseride is one kind of molecule that affect insulin sensitivity. The higher the TG level exhibited the lower the insulin sensitivity [23].

Table 2. Mean and *Duncan post hoc* G6PD activity (nmol/min/ml) and G6PD inhibition activity (%) of EEJB and EEDS in 3T3-L1 cells, were measured in triplicate

Samples	G6PD (nmol/min/ml)
EEJB 50 µg/ml	0.14±0.01 a
EEJB 10 µg/ml	0.21±0.01 b
EEDS 50 µg/ml	0.22±0.00 bc
EEDS 10 µg/ml	0.24±0.01 c
Control	0.27±0.02 d

Control: induced 3T3-L1Cell without treatment. Data are presented as mean ± standard deviation. Different letters in the same column (Among samples) are significant at p ≤ 0.05 (Duncan post hoc test)

3.4 Cholesterol Inhibition Activity assay

Both of EEJB and EEDS showed cholesterol inhibition activity over 50% (Table 4). EEJB had the highest activity. Obesity has an association with decreasing high-density lipoprotein cholesterol (HDL-C) concentration [24].

4. DISCUSSION

Obesity is considered an emergency health problem throughout the world. Primarily, obesity is a disorder of lipid metabolism and the enzyme involved in this process could be selectively targeted to develop antiobesity drugs. [25] Different parts of medicinal plants like stem, flower, seed, root, fruit, etc. are used to obtain pharmacologically active metabolite. [26] In the previous study, combination of EEDS and EEJB showed good effects in inhibition of lipase pancreas activity, and the best combination is EEDS : EEJB = 1 : 2.[18] In this *ex vivo* study, we evaluated the antiobesity and antiadipogenesis potential of EEJB and EEDS on 3T3-L1 cells. 3T3-L1 preadipocytes, which can be induced to differentiate into adipocytes according to the coordinated program are one of the most useful and established cell lines for researching the adipogenesis process [2]. Our results demonstrated that EEJB and EEDS exhibited potent to inhibit G6PD, trygliceride (TG) and cholesterol activity to prevent obesity. EEJB and EEDS exhibited positive result as antiadipogenesis and antiobesity and non-toxic to the 3T3-L1 cells. Viability test is important aspect to test the bioactive toxicity. Viability test is important aspect of pharmacology that deals with the adverse effect of bioactive substance on

Table 3. Mean and *Duncan post hoc* triglyceride activity (nmol/min/ml) and triglyceride inhibition activity (%) of single and combination extract in 3T3-L1 cells, were measured in triplicate for sample

Samples	Triglyceride	
	Triglyceride (mmol/L)	Triglyceride (mg/dL)
EEJB 50 µg/ml	0.79±0.01 a	70.34±0.86 a
EEJB 10 µg/ml	0.92±0.01 b	81.81±0.10 b
EEDS 50 µg/ml	1.04±0.01 c	92.51±0.56 c
EEDS 10 µg/ml	1.17±0.02 d	103.28±1.73 d
Control	1.28±0.02 e	113.51±1.48 e

Control: induced 3T3-L1Cell without treatment.
Data are presented as mean ± standard deviation.
Different letters in the same column
(among samples) are significant at $p \leq 0.05$ (Duncan post hoc test)

Table 4. Mean and *Duncan post hoc* cholesterol activity (nmol/min/ml) and cholesterol inhibition activity (%) of single and combination extract in 3T3L1 cells, were measured in triplicate

Samples	Cholesterol	
	Cholesterol (mmol/L)	Cholesterol (mg/dL)
EEJB 50 µg/ml	0.75±0.06 a	28.93±2.49 a
EEJB 10 µg/ml	1.20±0.13c	46.38±5.07c
EEDS 50 µg/ml	1.00±0.13b	38.73±4.99b
EEDS 10 µg/ml	1.27±0.13 c	49.22±4.96 c
Control	2.87±0.01 d	110.89±0.35 d

Control: induced 3T3-L1Cell without treatment.
Data are presented as mean ± standard deviation. Different letters in the same column
(Among samples) are significant at $p \leq 0.05$ (Duncan post hoc test)

living organism prior to the use as drug or chemical in clinical use [27-29]. EEDS and EEJB at concentration 50 ug/ml and 10 ug/ml were approved to be safe, showed by the results of cells viability is more than 90%. However the viability of combination of both extract at the same concentration did not show the same results. Combination of EEDS and EEJB showed the safe viability to 3T3-L1 cells at concentration 2.5 ug/ml. One of possibility reason is there were potentiation effects of combination of EEDS and EEJB in lipase pancreas inhibition. The active substance in each extract were sinergistically produced better results than single extract, EEDS EEJB, but this condition may lead to potentiation of the toxic effects also. Therefore it is needed an investigation of their effects in subchronic toxicity test on experimental animal.

G6PD can accelerate adipogenesis through generating ligand peroxisome proliferator-activated receptor γ (PPARγ) which is transcription factor in adipogenesis [22,30,31]. In the present study, EEJB and EEDS showed an ability to inhibit G6PD in a concentration dependent manner. These results suggest that EEJB and EEDS have anti-adipogenesis effect. There have been some report that soybean decreased body weight, serum lipid, and adipose tissue [32,33].

High plasma TG is associated with obesity [23]. In present study EEDS and EEJB could lower TG level. Triglyceride metabolism is activated by expression of Acetyl-CoA carboxylase (ACC), FAS, and adipocyte-specific fatty acid binding protein (aP2) genes. Our result demonstrated that EEDS and EEJB inhibit the TG synthesis. The decrease TG content may result from decreasing lipid synthesis [34].

Protein in soybean had been reported as lipase inhibitor [18,34,35]. Soybean (*Glycine max*) contains protein, fat, esential amino acids, and phytochemicals including isoflavones, tocopherols, saponins and anthocyanins [36] that have been found to inhibit low-density lipoprotein (LDL) oxidation *in vitro* [37,38]. Several recent

studies using soybean powder have reported its blood lipid lowering effects in diabetic patients with hyperlipidemia or hypercholesterolaemic otherwise healthy subjects [39]. Soybean protein isolate mainly composed of β-conglycinin and glycinin and well known to decrease plasma cholesterol level [40]. *Detam 1* soybean protein extract using Deak method has been approved that contains high β-conglycinin level [41]. Previous study showed that EEDS contained secondary metabolites: flavonoid and tannin (18). Genistein as one of soybean flavonoid also could inhibit the adipocyte differentiation [42]. In ethyl acetate fraction of EEDS, contained the highest level of genistein [12]. *Jati Belanda* leaves have already known to contained alkaloids, flavonoid, tannin, and steroid/triterpenoid [43] and in the EEJB also contained flavonoid and tannin, but did not contain steroid alkaloids and saponnin [18]. Tannin were hypothesized to reduce fat absorption in gastrointestinal tract by inhibit lipase pancreas enzyme [44]. Tannic acid as a major component of tannin stimulates glucose transport and inhibits adipocyte differentiation in 3T3-L1 cells [45]. EEJB with these two mechanisms of action of tannin, showed the best result in this present study. Based on our *ex vivo* study, we recomended EEJB and EEDS have beneficial effects as antiadipogenesis and antiobesity potential agent. Another previous study that conducted combination EEDS and EEJB in therapeutic dose to male Wistar rats showed good results in reducing body weight and are safe to be consumed according to the observation of histopathology jejunum mucosa [46]. However, further mode of action test, preclinical and clinical studies should be pursued before pharmaceutical applications.

5. CONCLUSION

Ethanol extract of *Detam 1* soybean seed and *Jati Belanda* leaves posses inhibitory potential on G6PD, triglyceride and cholesterol activities in 3T3-L1 cell line line and the most active compound showed by ethanol extract of *Jati Belanda* leaves.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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