ANTI-DIABETIC EFFECT OF Anredera cordifolia (Ten.) Steenis (BINAHONG) ON DIABETES GENE MARKERS EXPRESSION

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Abstract

The evidence of molecular mechanism of Anredera cordifolia (Ten.) Steenis (binahong) as an anti-diabetic has not been elucidated. In this study, we investigated the potential of 70% ethanolic extract of binahong as anti-diabetic by evaluating its effect on two diabetic gene markers expression, including Glucose Transporter 4 (GLUT4) and Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) in 3T3-L1 adipocytes. The investigation has been carried out by semi quantitative real time PCR. Our finding showed the GLUT4 mRNA levels were dose dependent excess in adipocyte which was treated by 6.25-12.5 μg/ml of 70% ethanolic extract. However, the GLUT4 expression level went down to 1.2-fold at 50 μg/ml dose. In addition, the pattern of PPAR-γ mRNA levels in 3T3-L1 were almost the same. Every extract produced an increment on PPAR-γ mRNA levels except at 3.125, 6.25, and 50 μg/ml doses. In undifferentiated adipocytes, GLUT4 and PPAR-γ mRNA were barely expressed. We assumed that the up regulation of GLUT4 and PPAR-γ mRNA levels on adipocytes due to the effect of binahong active compounds. However, the exact compounds have not been discovered yet. We hypothesize that these compounds act as a ligand for PPAR-γ transcriptional factor supporting the expresion of the GLUT4 in glucose homeostasis at molecular level.

Keywords: Anredera cordifolia, anti-diabetic, expression, GLUT4, PPAR-γ

Introduction

Anredera cordifolia (Ten.) Steenis, known as Madeira vine, is a South American native plant that has been globally distributed to other countries, including Indonesia. A. cordifolia, called as binahong in Indonesia, is commonly used as vegetables and ornamental vine in some tropical countries. However, people in China and Brazil utilize its leaves to cure wound from animal bites. In Indonesia, people use this herb for the treatment of several diseases such as diabetes, stroke, and obesity (Azahari et al., 2015; Chao et al., 2010).

Binahong has the ability to cure a variety of diseases, since it has several active compounds with a specific mechanism for targeting cells as a therapeutic agent. Phytochemical screening, resulted from stem, leaves, and tuber of A. cordifolia showed that this plant has rich of terpenoids, steroid, glycoside and alkaloid contents. Whilst, its flower contains terpenoids, steroid, and glycoside (Azahari et al., 2015; Chao et al., 2010; Djamil et al., 2017).

Many studies have successfully found the effect of binahong as anti-diabetic through in-vivo and in vitro assays (Fidrianny et al., 2013; Garmana et al., 2016; Hajiaghaalipour et al., 2015). From in vivo studies, it was found that methanolic extract of binahong can reduce blood glucose levels in diabetic mice model (Fidrianny et al., 2013; Leliqia et al., 2017). Moreover, in vitro test was conducted towards α-glucosidase, α-amylase and dipeptidyl peptidase IV (DPP IV) enzymes. α-glucosidase and α-amylase inhibition, which can reduce hyperglycemic condition after meal by delaying glucose absorption process. It is because both enzymes had role in carbohydrate hydrolysis process. DPP IV had role in the incretion degradation process, especially GLP-1 (Glucagon Like Peptide-1) that stimulated insulin production (Azahari et al., 2015; Makalalag & Wullur, 2013).
However, the evidence of molecular mechanism of *binahong* extract as anti-diabetic has never been demonstrated. In this study, we aim to investigate the potential of 70% of *binahong* ethanolic extract as anti-diabetic by evaluating its effect on the expression of *Glucose Transporter 4* (GLUT4) and *Peroxisome Proliferator-Activated Receptor Gamma* (PPAR-γ) genes expression in 3T3-L1 adipocyte cell line. GLUT4 is glucose homeostasis regulator in insulin target tissues, such as skeletal muscle and fat tissue. GLUT4 is a 12-transmembrane protein that permits peripheral blood glucose to move into the cell across the plasma membrane (Mulia et al., 2017; Muzaffar et al., 2013).

PPAR-γ is predominantly expressed in adipocyte tissues and plays a central role in adipocyte tissue functions. PPAR-γ regulates the expression of genes associated with insulin signaling and glucose and lipid metabolism immature adipocytes. Reduced expression of PPAR-γ has been shown to be effective in inhibiting the adipogenesis of 3T3-L1 cells. GLUT4 and PPAR-γ are two gene markers for glucose homeostasis in cells and expressed in insulin-sensitive tissues. Increased expression of GLUT4 and PPAR-γ has been found to lower blood glucose and enhance glucose transport and utilization (Muzaffar et al., 2013).

**Materials and Methods**

**Plant extraction**

Anredera cordifolia (*binahong*) leaves were obtained from Center for Pharmaceutical and Medical Technology medicinal plant garden (Lampung, Indonesia). Determination of plant was carried out by Research Center for Biology, Indonesian Institute of Science (No. B-104/ IV/ DI.01/1/2021). *Binahong* leaves were dried and ground into fine powders. One kg of grounded medicinal herb was extracted with 10 folds of 70% ethanol by shaking and stirring at room temperature for 6 hours. This ethanolic leave extract was evaporated using a rotary evaporator at 40°C until concentrated. The 70% of *binahong* ethanolic extract was then stored at -20°C for further analysis.

**3T3-L1 cell culture and cytotoxicity assay**

The 3T3-L1 cells were maintained in DMEM high glucose supplemented with 10% FCS, 100U/ml penicillin, 100 µg/ml streptomycin, and 0.5% amphotericin (DMEM Hg + FCS Complete) at 37°C in humidified incubator with 5% CO2 atmosphere. The medium was replaced every 2-3 days. To investigate the cytotoxicity of 70% *binahong* ethanolic extracts in 3T3-L1 cells, the viability of cells was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The 3T3-L1 preadipocytes were treated with 6 variant concentrations (1000; 500; 250; 125; 62.5; 31.25 µg/ml) of each extract for 24 h. After medium was removed, the MTT media (DMEM including 0.2 µg/ml of MTT) were added into each well. The plate was then stored in a CO2 incubator for 4 h at 37 °C. The reaction medium was completely removed, and the insoluble formazan was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a micro-plate reader. All experiments were performed in triplicates, and percentage of cell viability at each concentration of extract was calculated as follows:

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\% \text{ Cell viability} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100
\]

**3T3-L1 differentiation and sample treatment**

Adipocyte differentiation was performed as described on Zebisch et al. 2012 (Zebisch et al., 2012). Briefly, mouse 3T3-L1 fibroblasts (about 0.2 million cells/2-ml medium/well) were grown in 6-well plates under the same conditions for 48 h until cell confluent, and medium was replaced with differentiation medium containing DMEM Hg + FCS complete with 0.25 µM Dexamethasone, 0.5 mM IBMX, 1 µg/ml insulin, and 2 µM Rosiglitazone. Following incubation for 48-60h, the medium was replaced with DMEM Hg supplemented by 10% FBS, 100U/ml penicillin, 100 µg/ml streptomycin, and 0.5% amphotericin, and 1 µg/ml insulin (DMEM Hg + FBS complete).

After 48-60 h additional incubation, medium was replaced again with DMEM Hg + FBS complete and cells were grown for an additional 4-6 days. Accumulated lipids dropin a cell is a sign of successful differentiation from preadipocytes to adipocytes. For additional sample, the cells were treated by 70% of *binahong* ethanolic extracts for 24 h. All experiments were repeated three times in triplicates.
RNA Isolation and real time PCR
The total RNA was extracted from the earlier treated 3T3-L1 adipocytes with the plant extracts using Genezol® reagent kit. The quantity and quality of RNA in this study was measured by using the NanoDrop UV- VIS spectrophotometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. The amount of each RNA sample used in this procedure was initially standardized. Quantitative real-time PCR reaction was performed by using SensiFAST™ SYBR® No-ROX One-Step Kit was used according to manufacturer’s instructions. This quantitative real-time PCR was performed using Eco Ilumina 4.1.

Gene expression and statistical analysis
The relative expression level of the PPAR-γ and GLUT4 mRNA was normalized by the amount of β-actin, as a housekeeping gene. All data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using the GraphPad Prism 5.0 for Windows. The significance of differences between the groups was assessed with one-way analysis of variance (ANOVA) followed by Post Hoc with Tukey's Multiple Comparison Test. A P-value < 0.05 was used for significance.

Results
Yields of extraction
The total extract of 70 % binahong ethanolic extract was 142.37 g with yield percentage of 14.2%.

Cytotoxicities of 70% binahong ethanolic extract on 3T3-L1 cells
Before we evaluated the anti-diabetic effect of binahong extracts, we examined the cytotoxicities of 70% of binahong ethanolic extracts in 3T3-L1 cells using MTT assay. The 3T3-L1 preadipocytes were treated with 7.5, 15.625, 31.25, 125, and 250 μg/ml extracts for 24 h. The increased concentration of extract caused a decrease in percentage of cell viability (Figure 1). This study found that the IC₅₀ of 70% ethanolic extracts of binahong were 54.22 μg/ml. In addition, above the IC₅₀ value, the binahong extract treatment significantly reduced the viability of 3T3-L1 cells. Thus, the concentrations below IC₅₀ value were chosen for further experiments.

Effect of 70% ethanolic extract of binahong to the GLUT4 and PPAR-γ gene expression
To study the effect of ethanolic binahong extract, the 3T3-L1 adipocytes were treated by several doses ranged from lower dose to nearly IC₅₀ value (3.125, 6.25, 12.5, 25, 50 μg/ml). The amount of GLUT4 and PPAR-γ mRNAs were analyzed by semi quantitative real time PCR. In order to control the successful of adipogenesis process, investigation of GLUT4 and PPAR-γ mRNA levels were also conducted including on adipocyte only and undifferentiated cells. The relative expression level of the PPAR-γ and GLUT4 mRNA was also normalized by the amount of β-actin, as a housekeeping gene. The aim was to avoid the fluctuation results during treatments under normal and pathophysiological conditions. It has been known that β actin was expressed in all cells of organism which were required for the maintenance of basic cellular function (14).

Our finding showed an undifferentiated cell had less of GLUT4 mRNA levels in all groups (1.0 Fold). For control system, we found that this GLUT4 mRNA levels were narrower than in adipocytes group (1.3 fold). Even though, the level of GLUT4 mRNA levels in adipocyte was smaller than adipocytes treated by 1 μg/ml insulin as positive control (2.0 fold). However, the GLUT4 mRNA levels on cells treated by all variation doses of 70% ethanolic extracts of binahong were higher than positive control group, with dose dependent from 3.125 to 25 μg/ml of 70% binahong ethanolic extracts. GLUT4 mRNA levels in 31.25, 6.25, 12.5, 25

Figure 1. Effect of various concentrations of binahong ethanolic extract on the cell viability in 0.2 μg/ml of MTT assay.
μg/ml concentrations treated cells were 2.0, 2.4, 3.1, 6.4-fold, respectively.

In contrast, the GLUT4 expression went down to 1.2-fold at 50 μg/ml dose (Figure 1). It is presumed that the dose was close to the IC<sub>50</sub> value (Figure 2). At this point, the possibility of its small gene expression was caused by the reduction of 50% of 3T3-L1 adipocyte cells. The mRNA expression levels at 25 μg/ml extract was statistically significant to undifferentiated, adipocytes and 50 μg/ml dose groups (p<0.5) (Figure 2).

Furthermore, the mRNA expression level of PPAR-γ in 3T3-L1 has a similar pattern as GLUT4. Every binahong extract resulted an increment on PPAR-γ mRNA levels except at 3.125, 6.25, and 50 μg/ml doses. The highest PPAR-γ mRNA levels were at adipocytes treated by 25 μg/ml of binahong extract (2.92 fold), followed by 12.5 (1.89 fold) and 50 μg/ml (0.89 fold) doses. Statistical analysis found that 25 μg/ml dose had significantly different to undifferentiated, 3.125, 6.25, and 50 μg/ml doses groups (p< 0.05). At the same time, in undifferentiated adipocyte, GLUT4 and PPAR-γ mRNA were barely expressed than in adipocytes only and adipocyte cells treated by binahong extract (0.05 fold) (Figure 3).

Discussion

Studies found that Anredera cordifolia (Ten.) Steenis (binahong) contains many active compounds, such as phenolic acids, flavonoids, stilbenes, and lignans which has beneficial effects to treat various diseases such as, cancer, cardiovascular diseases, inflammatory diseases, obesity, and diabetes (Nugroho et al., 2012). In our study, we successfully investigated the anti-diabetic effect of 70% ethanolic extract of binahong on diabetic genes marker, including GLUT4 and PPAR-γ utilizing 3T3-L1 adipocyte cells. The 3T3-L1 preadipocytes undergo differentiation process known as adipogenesis, convert preadipocytes to mature adipocytes to perform highly specialized function. The observed effect of the extracts on diabetic gene markers in the adipocytes is clinically important for glucose homeostasis and energy storage. Thus, it might lead to more effective strategies for the treatment of diabetes and metabolic diseases (Ratna et al., 2008).

In this study, the expression of diabetic gene markers was compared between adipocyte treated by various concentration of 70% binahong ethanolic extract and insulin as positive control. In addition, the successful of adipogenesis process was also validated by investigating the GLUT4 and PPAR-γ mRNA levels on adipocyte only and undifferentiated...
The relative expression level of the PPAR-γ and GLUT4 mRNA was also normalized by the amount of β-actin, as a housekeeping gene. The aim was to avoid the fluctuation results during treatment under normal and pathophysiological conditions. It has been known that β actin is expressed in all cells of organism required for the maintenance of basic cellular function (Seabi et al., 2016).

The finding showed that the GLUT4 mRNA levels were dose dependent excess in adipocyte when treated by 6.25-12.5 μg/ml of 70% binahong ethanol extract. Though, at 50 μg/ml dose, the GLUT4 expression went down to 1.2-fold. It was probably because 50 μg/ml dose was close to the IC50 of binahong extract (54.22 μg/ml). At this point, the possibility of small gene expression because 50% of 3T3-L1 adipocyte cells were already eliminated by the binahong extract. In addition, the pattern of PPAR-γ mRNA levels in 3T3-L1 were almost same. Almost all binahong extracts gave increment on PPAR-γ mRNA levels. In undifferentiated adipocytes, GLUT4 and PPAR-γ mRNA were barely expressed than in adipocytes only and adipocyte cells treated by binahong extract. It showed that induction media successfully made the differentiation from pre-adipocyte 3T3-L1 to 3T3-L1 adipocytes (Sukandar et al., 2011).

Normally, insulin cascades maintain glucose homeostasis by tightly modulating glucose transporter (GLUT), trafficking in a spatially and temporally dependent fashion (Seabi et al., 2016). GLUT4 is the main insulin-responsive glucose transporter and is located primarily in skeletal muscle cells, cardiac muscle cells and adipocytes. In the basal state, about 95% of GLUT4 are present in small intracellular vesicles; the rest reside in the plasma membrane. After a meal, insulin is secreted from pancreatic β cells and circulated to insulin-responsive tissues, such as adipocyte, skeletal muscle, and liver tissues. Then, it interacts with the insulin receptor and triggers activation of the insulin receptor substrate/PI3K/Akt signaling pathway. This ultimately promotes GLUT4 translocation to the plasma membrane to allow glucose uptake into the cells. Defects in both insulin signaling and GLUT4 trafficking pathways impair insulin stimulated glucose uptake, contributing to insulin resistance and development of type 2 diabetes. (Mulia et al., 2017; Muzaffar et al., 2013).

The up regulation of GLUT4 mRNA levels on adipocytes treated by binahong extracts were probably because of the effect of active compounds in the leaves. The phytochemicals screening studies showed that binahong leaves contain flavonoids, saponine, sterol/triterpenes, and aetheric oil (Djamil et al., 2017; Sukandar et al., 2016). From the previous studies, binahong leaves which comprise oleanolic acid from triterpenes as an anti-oxidant, is able to treat many diseases, including diabetes mellitus. While, other studies suggested that the flavonoid and saponine of the ethyl acetate extracts of binahong play as anti-diabetic in alloxan induced mice (Leliqia, Sukandar, Fidrianny, 2017; Nugroho et al., 2012; Sukandar et al., 2011). The flavonoid glycoside in binahong leaves is known as vitexin (8-β-D-Glucopyranosyl-apigenin) (Vinayagam & Xu, 2015). However, it has not been revealed the mechanism of anti diabetic property in cellular level (Vishnu et al., 2010). Therefore, it is not yet known which one of the active compounds of binahong that regulate the expression of GLUT4, as a protein factor involved in the signaling pathway of insulin-mediated glucose transport in 3T3-L1 adipocytes model (Mulia et al., 2017).

Moreover, this study also showed the similar effect of binahong extracts on the promotion of PPAR-γ gene expression, which might be due to increased transcriptional level as well as GLUT4 expression. In agreement with the GLUT4 examination in this study, it was shown that the cells treated with binahong extracts significantly increased the expression of PPAR-γ mRNA. PPAR-γ plays an important role in adipocytes differentiation, glucose metabolism as well as regulating fatty acid storage. The increase in activity of PPAR-γ mRNA simultaneously increases adipocyte differentiation or adipogenesis (Ratna et al., 2008). Translocation of glucose transporter GLUT4 to the adipocyte cell plasma membrane underlies glucose uptake induce fat accumulations in cell which need to be regulated by PPAR-γ (Zang et al., 2016). Studies found that in 3T3-L1 adipocyte differentiation, GLUT4 gene expression is activated by PPAR-γ resulting in increased GLUT4 protein levels during adipocyte differentiation (Zebisch et al., 2012). We hypothesize that active compound in binahong leaves act as ligand for PPAR-γ transcriptional factor which activate the GLUT4 transcription.
and play a role in glucose homeostasis at molecular level (Zhang et al., 2014).

**Conclusion**

*A. cordifolia* (Ten.) Steenis has potential as medicinal plant. This research suggests the potential of this plant to cure diabetes, shown by *in vitro* study whereas diabetic gene markers, including GLUT4 and PPAR-γ were successfully expressed in model cell line, 3T3-L1 adipocyte cells. However, it needs further investigation to prove the active compounds of *A. cordifolia* (Ten.) Steenis as anti-diabetic based medicinal plant. In addition, exploration of molecular mechanisms underlying the enhancement of glucose uptake produced by PPAR agonists in 3T3-L1 adipocytes is also required.

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**References**


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