Original Article

Ethanol Extract of Jati Belanda (*Guazuma ulmifolia* L.) as Therapy for Chronic Kidney Disease in *In Vitro* Model

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Abstract

Background: People with diabetes mellitus in Indonesia are predicted to increase until 2035. High glucose in body (hyperglycemia) leads to increased fibronectin synthesis. Fibronectin that gets accumulated in glomerulus (mesangial cells), at the end, will lead to diabetic glomerulosclerosis. Jati belanda (*Guazuma ulmifolia* L.) leaf is well known as an Indonesian traditional medicine to have effects as antidiabetic by the presence of its secondary metabolites such as alkaloid, tannin, saponin, flavonoid, and terpenoid, which are very important in health recovery. **Objectives:** To evaluate the activity of ethanol extract of jati belanda (EEJB) as a protection agent on induced-glucose mesangial cells of SV40 MES 13 cell line (glomerular mesangial kidney, *Mus musculus*). **Materials and Methods:** EEJB (3.125 and 6.25 µg/mL) was extracted based on maceration method using ethanol (70%) as the solvent. Proliferation and viability were performed based on (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) method. The level of transforming growth factor β1 (TGF-β1) and fibronectin in glucose-induced mesangial cells was assayed and determined using enzyme-linked immunosorbent assay kit. Reactive oxygen species (ROS) level was measured using flow cytometer. **Results:** EEJB (3.125 and 6.25 µg/mL) increased cell proliferation and viability in glucose-induced mesangial cells and significantly reduced the level of TGF-β1, fibronectin, and ROS compared to that in positive control (glucose-induced cells). **Conclusion:** Our study suggests that EEJB is able to reduce TGF-β1, fibronectin, and ROS levels in glucose-induced mesangial cells, which correlate to diabetic glomerulosclerosis condition and increase the mesangial cell proliferation and viability.

Keywords: *Fibronectin, glomerulosclerosis, Guazuma ulmifolia, reactive oxygen species, transforming growth factor β*

Introduction

In 2010, it was estimated that 285 million people worldwide had diabetes mellitus.[1] This number is predicted to rapidly increase as a result of population aging, urbanization, and associated lifestyle changes.[2] Data show that Indonesia is among the 10 countries with the largest number of people predicted to have diabetes mellitus in 2030.[1] Diabetes is characterized by hyperglycemia.[3] Despite the mechanisms remain unclear, it is believed that hyperglycemia involves in matrix expansion and generates fibrosis in mesangial interstitial space.[4,5] Hyperglycemia generates reactive oxygen species (ROS) in mesangial cells that upregulates transforming growth factor-beta (TGF-β).[6,7]

Insulin therapy and drugs such as sulphonylurea and its derivatives are used as a treatment for diabetes.[8] However, those treatments have undesirable side effects and they are unable to restore glycemic control.[8,9] For example, insulin therapy does not achieve glycemic control in patients with insulin resistance. In addition, conventional drugs are not accessible to the general population in developing countries.[10] Consequently, affordable and effective treatments in slowing the progression of kidney disease should be found.

Bioactive compounds that reduce inflammation, fibrosis, and TGF-β and possess antioxidant activity may be used in the adjuvant treatment of kidney disease.[11] Jati belanda (*Guazuma ulmifolia* L.) is a medium-sized tree widely distributed throughout the Neotropical region.[12] In traditional Mexican medicine, the bark is used as infusion for the treatment of type II diabetes.[13] *G. ulmifolia* L. is known for its capability to lower plasma glucose level by 22% in healthy rabbits.[14]

In this study, SV40 MES 13 cell line was induced with glucose as an *in vitro* model of

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kidney disease. This study aimed to investigate the ability of ethanol extract of jati belanda (EEJB) to reduce fibronectin and TGF-β and to scavenge ROS.

Materials and Methods

Plants extract preparation

G. ulmifolia, also known as jati belanda, was collected from the plantation of Bumi Herbal Dago, Bandung, West Java, Indonesia and identified by a staff of herbarium of the Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia (0020218-A002). The jati belanda leaf was collected, chopped, and kept in drier tunnel service. Extraction was performed based on the maceration method, using ethanol (70%) as the solvent to collect EEJB.[15-17]

Glucose-induced mesangial cells for proliferation and viability

A total of 5×10^3 cells/well of SV40 MES 13 cells were plated in 96-well plate in 200 µL growth medium and incubated at 37°C, 5% CO_2 for 24 h. The medium was discarded and added with 180 µL of glucose-induced medium (0, 5, 25, and 125 mM) and 20 µL of EEJB (6.25 and 3.25 µg/mL). Cells were incubated at 37°C, 5% CO_2 for 5, 10, and 15 days. The viability was measured at day 5, 10, and 15 using MTS Proliferation Assay Kit (ab197010; Abcam, Cambridge, Massachusetts). The absorbance at 490 nm using Multiskan GO plate reader (Thermo Scientific, Massachusetts, USA) was used for calculating the percentage of cell mortality).[18,19]

Quantification of transforming growth factor β 1 level

TGF-β1 level in the cell-free supernatant was measured using Mouse TGF-β1 ELISA Kit (Elabscience, E-EL-M0051, Wuhan, China) based on the manufacturer's protocol with modified method. The mixture solution using 100 µL of sample, standard, and blank solution was added into each well and then incubated at 37°C for 90 min. The cell-free supernatant after treating with EEJB served as the sample. The glucose-induced mesangial cell–free supernatant without extract was used as positive control, whereas negative control was the normal cell (untreated cell). Subsequently, the liquid of each well was discarded and 100 µL biotinylated detection antibody (Ab) was added and then incubated for an hour at 37°C. Later, the liquid was discarded and the plate was washed three times using 200 µL wash buffer. Horseradish peroxidase (HRP) conjugate (100 µL) was added and incubated at 37°C for 30 min. After the liquid was discarded again, the plate was washed five times using 200 µL wash buffer. Substrate reagent (90 µL) was added and incubated for 15 min at 37°C. Briefly, stop solution (50 µL) was added and the absorbance was read at 450 nm.[19,20]

Quantification of fibronectin level

The fibronectin level in the cell-free supernatant was measured using Mouse fibronectin (FN) Elisa Kit (Elabscience, E-EL-M0506, Wuhan, China) based on the manufacturer's protocol. A total of 100 µL of standard, blank, and sample solution was added into each well, then sealed, and incubated at 37°C for 90 min. The glucose-induced mesangial cell–free supernatant after treating with EEJB served as the sample. The positive control was glucose-induced mesangial cell–free supernatant without extract, whereas negative control was the normal cells or the untreated cells. Subsequently, the liquid of each well was discarded and 100 µL of biotinylated detection Ab was added and then incubated for an hour at 37°C. Then, the liquid was discarded and the plate was washed three times using 200 µL wash buffer. A total of 100 µL HRP conjugate was added and incubated for 30 min at 37°C. The liquid was discarded again and the plate was washed five times using 200 µL wash buffer. A total of 90 µL substrate reagent was added and incubated for 15 min at 37°C. Later, 50 µL stop solution was added and the absorbance was read at 450 nm.[19-21]

Quantification of reactive oxygen species level

The ROS level was measured using the 2',7'–dichlorofluorescin diacetate (DCFDA)–Cellular Reactive Oxygen Species Detection Assay Kit (Abcam) and flow cytometry. SV40 MES 13 cells were suspended into buffer DCFDA in fluorescence-activated cell sorting tube at final concentration of 250,000 cells per 500 µL. DCFDA was added to cell suspension at final concentration of 20 µM in each tube. The cells were then incubated at 37°C, 5% CO_2 (dark room) for 45 min. Glucose (5 and 10 mM) (Amresco, Cat. No. 0188, Australia) and EEJB (6.25 µg/mL) were added to each tube as sample. The positive control was tert-butyl hydroperoxide (TBHP)-induced Mesangial cells–free supernatant without extract, whereas the negative control was the normal cell or the untreated cell. Cells were incubated at 37°C, 5% CO_2 (dark room) for 4 h. ROS was then measured by flow cytometer.[20,22]

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 16 (SPSS, Chicago, Illinois) to perform one-way analysis of variance (ANOVA) to verify the results of different treatments, and Duncan *post hoc* and *t*-test were used to validate significant differences for all treatments ($P < 0.05$). The results are displayed as mean values ± standard deviation.

Result

Viability assay

The viability of glucose-induced SV40 MES 13 cells, during 5, 10, and 15 days of incubation time, treated with EEJB (3.125 and 6.25 µg/mL) is shown in Tables 1–3.

At 5 days of incubation [Table 1], the viability of EEJB was higher than that of control and positive control, which was induced with 0, 5, 25, and 125 mM glucose. In 0 mM glucose induction, 6.25 µg/mL EEJB had the highest viability (160.38 ± 7.92) compared to other but not significantly higher than 3.125 µg/mL EEJB (145.35 ± 14.61). In 5 mM glucose induction, the highest viability was found in EEJB (3.125 µg/mL) (191.39 ± 15.61%), whereas EEJB (6.25 µg/mL) had a viability of 181.52 ± 28.36%. In 25 mM glucose induction, EEJB (6.25 µg/mL) had the highest

Table 1: Effect of ethanol extract of jati belanda (EEJB) toward cell viability (%) in glucose-induced mesangial cells at 5 days of incubation

Concentration	Glucose concentration			
	0 mM	5 mM	25 mM	125 mM
Control	100.00 ± 5.84[a]	100.00 ± 5.84[a]	100.00 ± 5.84[a]	100.00 ± 5.84[b]
Positive control		96.71 ± 0.91[a]	84.78 ± 1.71[a]	74.92 ± 1.30[a]
EEJB (3.125 µg/mL)	145.35 ± 14.61[b]	191.39 ± 15.61[b]	135.91 ± 21.65[b]	94.16 ± 2.11[b]
EEJB (6.25 µg/mL)	160.38 ± 7.92[b]	181.52 ± 28.36[b]	167.87 ± 32.26[b]	96.25 ± 2.86[b]

Data are presented as average ± standard deviation. Different superscript letters in the same column of 0 mM (a and b), 5 mM (a and b), 25 mM (a and b), and 125 mM (a and b) glucose concentrations show significant differences among treatments per induction (P<0.05) analyzed using analysis of variance (ANOVA) and Duncan *post hoc* test

Table 2: Effect of ethanol extract of jati belanda (EEJB) toward cell viability (%) in glucose-induced mesangial cells at 10 days of incubation

Concentration	Glucose concentration			
	0 mM	5 mM	25 mM	125 mM
Control	100.00 ± 5.84[a]	100.00 ± 5.84[ab]	100.00 ± 5.84[b]	100.00 ± 5.84[b]
Positive control		94.64 ± 0.43[a]	86.84 ± 1.50[a]	73.26 ± 2.25[a]
EEJB (3.125 µg/mL)	120.17 ± 8.75[a]	111.57 ± 6.60[bc]	97.61 ± 0.75[ab]	92.27 ± 0.45[b]
EEJB (6.25 µg/mL)	143.93 ± 6.34[b]	119.21 ± 4.55[c]	95.08 ± 3.52[ab]	90.75 ± 1.67[b]

Data are presented as average ± standard deviation. Different superscript letters in the same column of 0 mM (a and b), 5 mM (a, ab, bc, and c), 25 mM (a, ab, b), 125 mM (a and b) glucose concentrations show significant differences among treatments per induction (P<0.05) analyzed using analysis of variance (ANOVA) and Duncan *post hoc* test

Table 3: Effect of ethanol extract of jati belanda (EEJB) toward cell viability (%) in glucose-induced mesangial cells at 15 days of incubation

Concentration	Glucose concentration			
	0 mM	5 mM	25 mM	125 mM
Control	100.00 ± 5.84[a]	100.00 ± 5.84[ab]	100.00 ± 5.84[c]	100.00 ± 5.84[b]
Positive control		76.89 ± 2.13[a]	70.94 ± 1.80[a]	54.12 ± 4.18[a]
EEJB (3.125 µg/mL)	123.34 ± 12.81[a]	112.12 ± 4.65[b]	85.78 ± 7.33[b]	66.02 ± 8.16[a]
EEJB (6.25 µg/mL)	119.05 ± 14.26[a]	108.39 ± 15.10[b]	83.08 ± 4.64[b]	64.21 ± 4.19[a]

Data are presented as average ± standard deviation. Different superscript letters in the same column of 0 mM (a), 5 mM (a, ab, b), 25 mM (a, b, and c), and 125 mM (a and b) glucose concentrations show significant differences among treatments per induction (P < 0.05) analyzed using analysis of variance (ANOVA) and Duncan *post hoc* test

viability among all treatments (167.87 ± 32.26%), whereas EEJB (3.125 µg/mL) had a viability of 135.91 ± 21.65%. Meanwhile, in 125 mM glucose induction, EEJB (3.125 µg/mL) had a viability of 94.16 ± 2.11% and EEJB (6.25 µg/mL) had a viability of 96.25 ± 2.86%.

At 10th day [Table 2], EEJB also showed high viability compared to control and positive control. In 0 mM glucose-induced treatment, the highest viability was EEJB (6.25 µg/mL) with a viability of (143.93 ± 6.34%), whereas EEJB (3.125 µg/mL) had (120.17 ± 8.75%); EEJB (6.25 µg/mL) also had the highest viability (119.21 ± 4.55%) compared to that of other treatment. In 25 mM glucose induction, the highest viability was found in EEJB (3.125 µg/mL) (97.61 ± 0.75%) and in 125 mM glucose induction, the highest was in EEJB (3.125 µg/mL) (92.27 ± 0.45%).

At day 15 of incubation, the highest viability was found in EEJB-treated cells. In 0 mM glucose induction, the highest viability was in EEJB (3.125 µg/mL) (123.34 ± 12.81%) and so was in EEJB (3.125 µg/mL) with 5 mM glucose induction (112.12 ± 4.65%). In 25 mM glucose induction, the highest viability was in EEJB (3.125 µg/mL), whereas in 125 mM glucose induction, the highest viability was in EEJB (3.125 µg/mL) (66.02 ± 8.16%).

Transforming growth factor β 1 level

The TGF-β1 level in glucose-induced mesangial cells as positive control and glucose-induced mesangial cells treated with EEJB (3.125 and 6.25 µM) is shown in Figure 1.

Figure 1 showed the concentration of TGF-β1 by ELISA method after treatment of glucose-induced SV40 MES 13 cells with EEJB at a concentration of 3.125 and 6.25 µg/mL. The lowest TGF-β1 (66.64 pg/ml) were obtained when the cells were induced by 10 mM glucose and treated by 6.25 µg/ml EEJB among the others treatment.

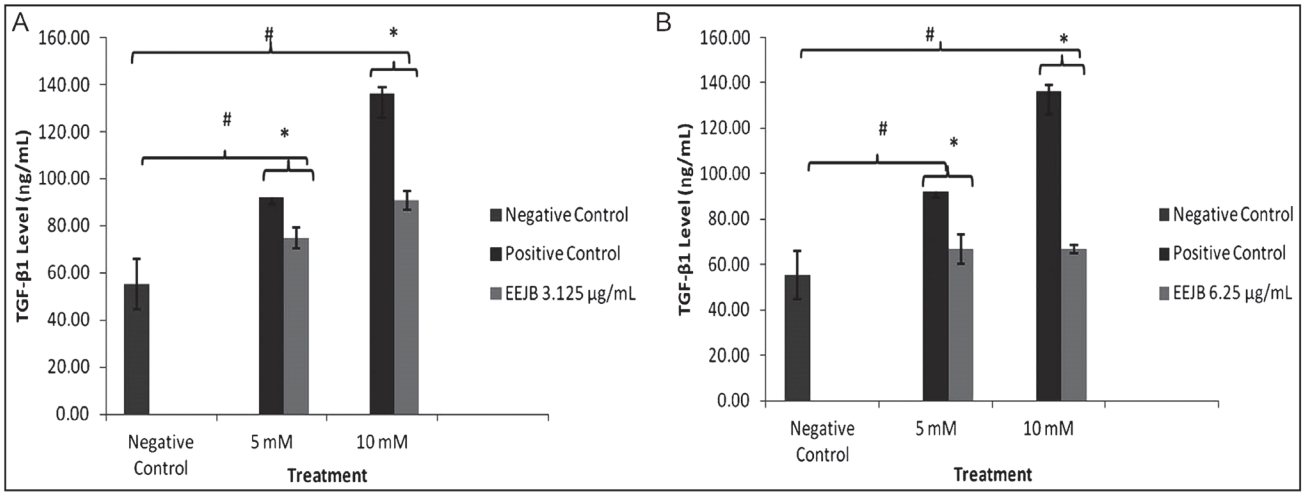

Figure 1: Transforming growth factor β 1 (TGF-β1) level of glucose-induced SV40 MES 13 cells treated with EEJB (A) 3.125 µg/mL and (B) 6.25 µg/mL compared to that of control

The histogram is presented as mean value ± standard deviation of negative control, positive control, and EEJB, (A) 3.125 µg/mL and (B) 6.25 µg/mL

***Significant difference among positive control and EEJB-treated cell (3.125 and 6.25 µg/mL) (*P* < 0.05) (independent *t*-test), #significant difference among negative control and EEJB-treated cell (3.125 and 6.25 µg/mL) (*P* < 0.05) (independent *t*-test)**

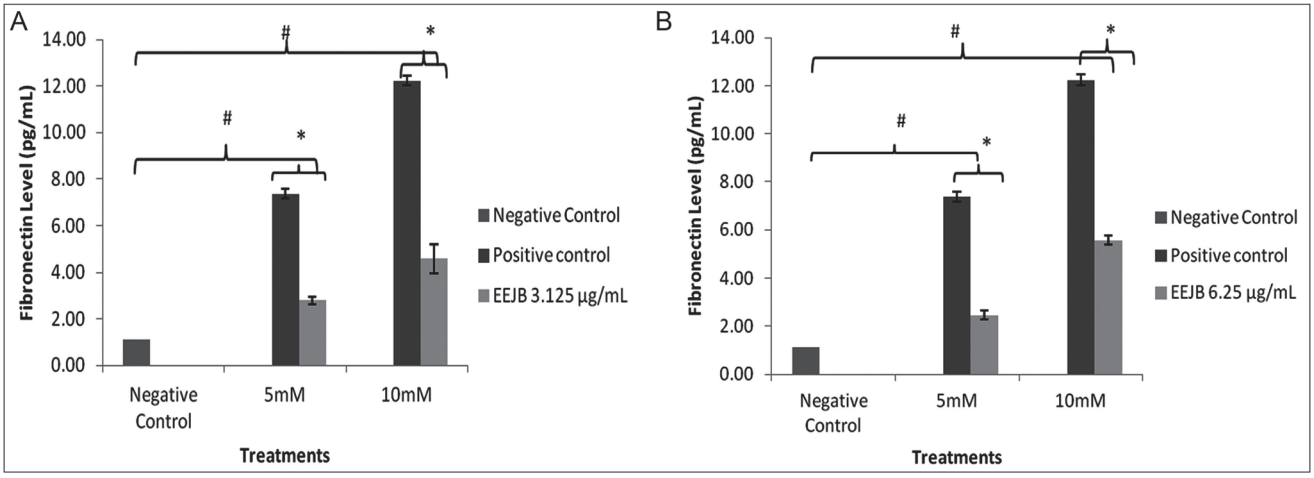

Figure 2: Fibronectin level of glucose-induced SV40 MES 13 cells treated with EEJB (A) 3.125 µg/mL and (B) 6.25 µg/mL compared to that of control

The histogram is presented as mean value ± standard deviation of negative control, positive control, and EEJB (A) 3.125 µg/mL and (B) 6.25 µg/ml

***Significant difference among positive control and EEJB-treated cell (3.125 and 6.25 µg/mL) (*P* < 0.05) (independent *t*-test), #significant difference among negative control and EEJB-treated cell (3.125 and 6.25 µg/mL) (*P* < 0.05) (independent *t*-test)**

Fibronectin level

The reduction of fibronectin level in glucose-induced mesangial cells as positive control and glucose-induced mesangial cells treated with EEJB (3.125 and 6.25 µg/mL) is shown in Figure 2.

Figure 2 shows the concentration of fibronectin level by ELISA method after treating glucose-induced SV40 MES 13 cells with EEJB. The fibronectin level of EEJB-treated SV40 MES cells was lower compared to that of the controls (5 and 10 mM glucose-induced cells). The lowest among the treatment was EEJB-treated cells at a concentration of 6.25 µg/mL with 5 mM glucose induction (2.46 ng/mL).

Reactive oxygen species level

The level of fluorescence intensity is an indicator of ROS production. The ROS level (MFI) of 5 mM glucose-induced mesangial cells treated with EEJB 6.25 µg/mL was higher than 10 mM glucose-induced mesangial cells treated with EEJB 6.25 µg/mL (10.40±0.73 compared to 9.55±0.07) (Table 4); however, the difference was not significant. Also, if it was compared to controls, significant differences were observed in ROS level and they were lower than that in negative control. These results indicate that the treatment with EEJB can lower the ROS level of glucose-induced cells. The dot blot

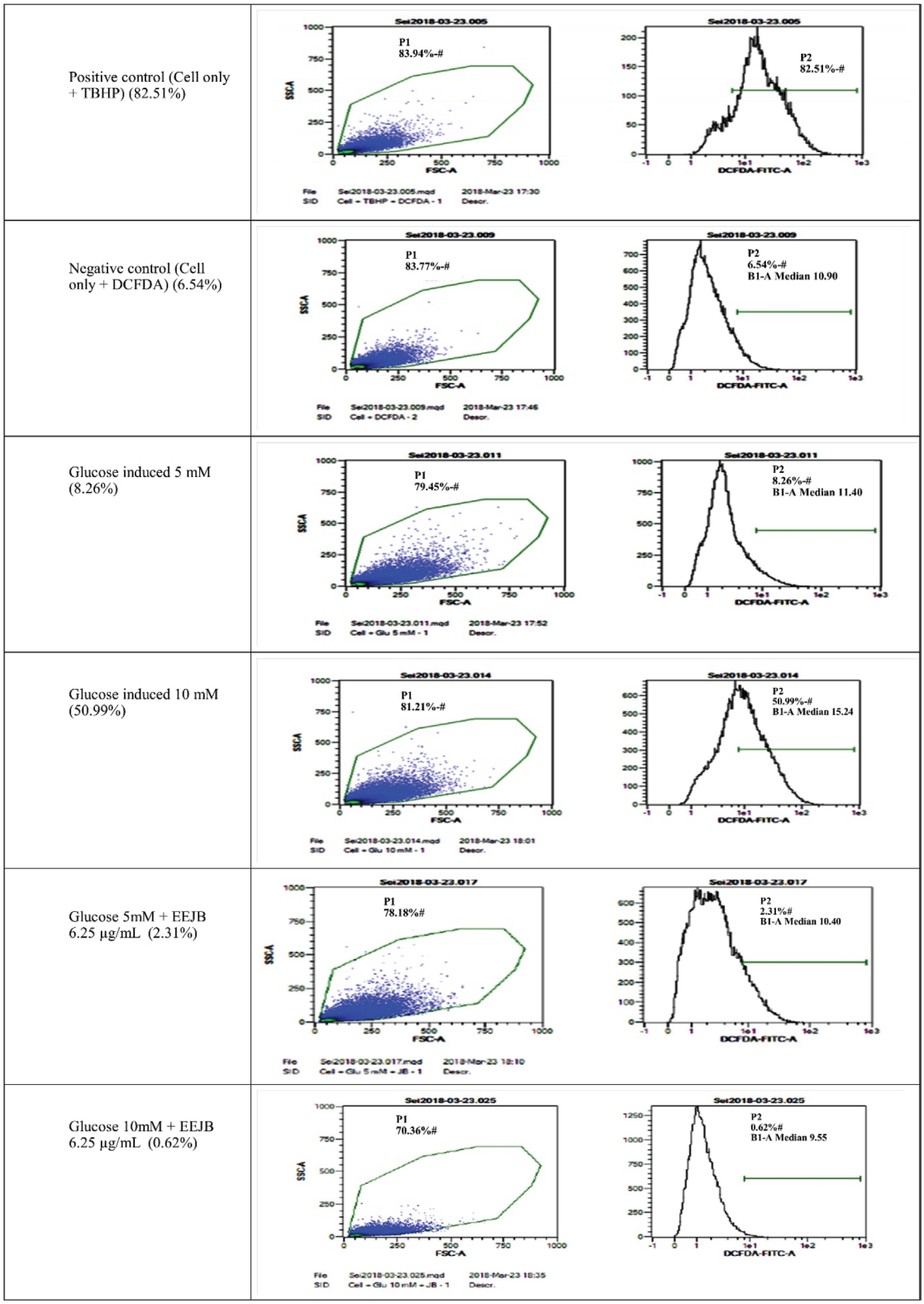

Figure 3: Dot blot of various concentrations of EEJB (6.25 µg/mL) treatment on glucose-induced SV40 cell toward ROS level. This figure is the dot blot representative of ROS level in SV40 cells, which was induced by glucose and treated using EEJB (6.25 µg/mL)

TBHP = tert-butyl hydroperoxide, DCFDA = 2',7'–dichlorofluorescin diacetate

representative of ROS level on glucose-induced SV40 cell which induced by glucose and treated used EEJB (6.25 µg/ml) was performed in Figure 3.

Discussion

Jati belanda, especially the leaves, is known for its benefits in health such as for antidyslipidemic and antioxidative stress, also in some

Table 4: Effect EEJB toward ROS level in glucose-induced mesangial cells

Treatment	ROS level (%)	ROS level (MFI)
Positive control (cell + TBHP)	82.51 ± 2.83^{e}	19.68 ± 0.67^{d}
Negative control (cell + DCFDA)	6.54 ± 0.31^{bc}	10.90 ± 0.49^{ab}
Glucose (5 mM)	8.26 ± 1.48^{c}	11.40 ± 0.31^{b}
Glucose (10 mM)	50.99 ± 3.42^{d}	15.24 ± 0.56^{c}
Glucose (5 mM) + EEJB (6.25 µg/mL)	2.31 ± 0.28^{ab}	10.40 ± 0.73^{ab}
Glucose (10 mM) + EEJB (6.25 µg/mL)	0.62 ± 0.09^{a}	9.55 ± 0.07^{a}

TBHP = tert-butyl hydroperoxide, DCFDA = 2',7'–dichlorofluorescin diacetate, MFI = mean fluorescence intensity
Data are presented as mean ± standard deviation. Different superscripts in the same column (a, ab, b, bc, c, d, e) indicate significant differences among treatment in ROS level (%) and different superscript (a,ab,b,c,d) indicate significant differences among treatment in ROS level (MFI) based on Duncan's *post hoc* test ($P < 0.05$)

countries, it is used to treat kidney, gastrointestinal disorder, and other types of diseases.[23] In this study, we measured the viability of glucose-induced cells treated with EEJB. EEJB shows higher viability compared to positive control after many days of incubation [Tables 1–3]. The highest viability of the cell was obtained in 5 days of incubation, especially in cell treated with 3.125 µg/mL of EEJB and induced by 5 mM glucose (191.39 ± 15.61%). In line with previous study, EEJB has high viability that is about >90%.[21] Cells under hyperglycemic condition can decrease the cell viability, induce apoptosis, and elevate the level of intracellular ROS in a concentration- and time-dependent manner.[24] The high level of viability indicates that the EEJB both in the concentration of 3.125 and 6.25 µg/mL can protect the cells from damage caused by glucose induction in renal cell.

TGF-β1 and fibronectin are used as a parameter correlated with glomerulosclerosis because of their existence in glomerulosclerosis disease. TGF plays an important role in mesangial matrix expansion. The injured glomeruli resulted in TGF-β1 messenger ribonucleic acid (mRNA) expression and TGF-β1 synthesis, and also secreted a great amount of fibronectin. This injury in glomeruli can lead to renal disease.[25] This study shows an increase of TGF-β1 levels in SV40 cell after induced with glucose. However, after treatment with EEJB (3.125 and 6.25 µg/mL), the TGF-β1 level decreased. On the basis of this study, 6.25 µg/mL EEJB can reduce the level of TGF-β1 and it was found to be lower than other treatments [Figure 1]. Thus, this research shows that the excessive amount of TGF- β1 because of glucose induction can be suppressed by the treatment with EEJB, which resulted in significant decrease.

In line with an earlier study conducted by Nahman *et al*.[17] (1992), a significant increase in fibronectin level in higher level of glucose induction was reported. Previous research showed the ability of glucose to increase the fibronectin level, which is the key matrix protein accumulated in excess in kidney disease.[26] However, the significant decrease is presented after the cell was treated with EEJB both in the concentration of 3.125 µg/mL and 6.25 µg/mL [Figure 2]. This data shows that EEJB is able to suppress the fibronectin expression in hyperglycemic cell model, which prevents kidney disease occurrence.

These reductions in fibronectin and TGF-β1 levels are associated with antioxidant activity of EEJB. Antioxidant compounds in jati belanda can inhibit the activation of protein kinase c, which induce diabetic nephropathy and also TGF-β1 synthesis in Mesangial cells in response to high glucose induction. Inhibition of protein kinase c effectively blocks high glucose-, phorbol ester-, and H_2O_2-induced TGF-β1 and fibronectin synthesis.[27]

Jati belanda is reported to have antioxidant activity due its compounds. The antioxidant components are reported to be higher than α-tocopherol.[28] The major compounds of EEJB, which have activity as antioxidant are chlorogenic acid, flavonoid, and quercetin. It is also found that some phenolic compound such as quercetin glucosides (rutin and quercitrin) also contribute to the antioxidant effect.[29] Under hyperglycemic conditions, we suggest that antioxidants are able to regenerate a damaged extracellular matrix and improve cell growth as a result of oxidative stress through nonenzymatic glycation of proteins.[30,31] Because oxidative stress is associated with glomerulosclerosis and other diseases related to a reduced antioxidant defense, therefore, it can be postulated that the antioxidants, which can reduce the oxidative stress and prevent the progression of the disease, may exert a key role to protect mesangial cells in glomerulosclerosis.[32] According to Jha *et al*.[33] (2016), antioxidants are able to convert ROS into nonreactive oxygen molecules, which are harmless to cells. They also have an effect on retarding glucose absorption through the inhibition of carbohydrate-hydrolyzing enzymes such as α-glucosidase and α-amylase[34] and downregulate the TGF-β expression and fibronectin level by decreasing nicotinamide adenine dinucleotide phosphate oxidase expression.[20] The result shows that ROS level of EEJB-treated cells at 5 and 10 mM glucose induction decreased compared to that of positive control. The ROS level of EEJB-treated cells was even lower than that of negative control. This indicates that EEJB is able to reduce ROS level as it contains antioxidant compounds.

Conclusion

EEJB is able to reduce TGF-β1, fibronectin, and ROS levels in glucose-induced mesangial cells, which correlates to diabetic glomerulosclerosis condition and leads to chronic kidney disease. Moreover, it can increase the mesangial cell viability.

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Conflicts of interest

There are no conflicts of interest.

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