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Review of Traditional Medicine Journal Article

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- Revise your article according to the review results
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Ethanol Extract of *Detam* 1 Soybean Seed (*Glycine Max* L. Merr) for Chronic Kidney Disease Therapy *by In Vitro* Study

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ABSTRACT

Chronic Kidney Disease (CKD) has increased incidence and prevalence in developing nations. In this *in vitro* study, we evaluated the cells proliferative effects, fibronectin (FN) , transforming growth factor β (TGF-β1), and Reactive oxygen species (ROS) - level inhibition potential of ethanol extract of detam 1 soybean seed (EEDS) on glucose-induced kidney mesangial cells (SV40 MES 13). The cells proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium (MTS) assay. FN and TGF-β1 level were measured using ELISA assay kit and ROS level using flow cytometry. Level of FN, TGF-β1 and ROS ~~level~~, on CKD cells model (5 mM, 10 mM glucose-induced mesangial cell) treated with EEDS 6.25 µg/mL ~~on 5 mM, 10 mM glucose-induced mesangial cells~~ were lower significantly compared to positive control. EEDS improve cells viability and decrease FN, TGF-β1 and ROS level in glucose-induced kidney mesangial cells as CKD cells model.
Key words: chronic kidney disease; fibronectin; ROS; soybean; TGF-β1

INTRODUCTION

Chronic Kidney Disease (CKD) is a condition when a renal injury occurs and resulting in a decreased of renal excretory function, thus causing in an accumulation of waste and metabollic products. These products accumulations in blood and organs will cause various complications. CKD presents a therapeutic challenge because of the high prevalence and high cost of dialysis. CKD can be caused by various factors such as hypertension, diabetes, infection, atherosclerosis, and others. CKD also becomes a risk factor of cardiovascular disease. Patients with CKD are restricted to protein intake as it will prevent complication and alleviate uremic symptoms in CKD (Garcı´a-Sa´nchez *et al*., 2010; Ranich and Velasquez, 2001).

The filtration unit of the kidney is the glomerulus, a capillary network supported by mesangial cells and extracellular matrix (ECM). Increased levels of the ECM protein fibronectin (FN) are also present; however, its role in diabetic nephropathy (DN) is unknown. Kidney mesangial cells cultured under high glucose conditions provide a model system for studying the effect high glucose on FN and collagen IV (COL4) (Miller *et al*.,[1] 2014), transforming growth factor-β (TGF-β) deposition (Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

ECM accumulation by enhancing mesangial cell production of COL and FN. TGF-β mediated FN expression in mesangial cells (Uchiyama-Tanaka *et al*., 2002). Reactive Oxygen Species (ROS) also underlies as a common pathogenic component and accelerates the kidney disease progression and complications (Dounousi *et al*., 2006; Widowati *et al*., 2018; Prahastuti *et al*., 2019a). Soybean seed showed an antioxidant effect and an antifibrotic effect in the remnant kidney, which resulted in the improvement of the renal function. Oxidative stress is regulated by the balance between prooxidant and antioxidant systems (Peng *et al*., 2017; Hayata *et al*., 2012; Prahastuti *et al*., 2019b).

Soybean seed (*Glycine max* L. Merr) has been known for its beneficial effects in health due to its compounds. Previous studies reported that soybean contains antinutritional factors (ANF) which include lipase inhibitors, protease inhibitors, amylase inhibitors, oxalic acid, phytic

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acid, glucosinolates, flavonoid, and saponin. The ethanol extract of soybean also known to contain natural compounds such as flavonoid, phenolic, triterpenoid, saponin, steroid, tannin, and quinon (Gemede and Ratta, 2014; Prahastuti *et al*., 2019b). In this study, we evaluated the potency of *Glycine max* L. ~~merr~~ Merr ethanol extract (EEDS) for CKD therapy by measuring the cells proliferation effect, FN, TGF-β1, and ROS level on glucose-induced kidney m~~e~~esangial cells (SV40 MES 13).

METHODOLOGY

Plants Extract Preparation

Glycine max L. (soybeans) varieties of detam 1 was collected from Unit Pengelolaan Benih Sumber (UPBS) Balai Penelitian Tanaman Aneka Kacang dan Umbi, Malang, East Java, Indonesia. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Soybeans seed were kept in drier tunnel service and the dried soybean then grinded, mashed into powder. Extraction using 70% distilled ethanol and performed based on maceration method (Widowati *et al*., 2016; Widowati *et al*., 2017; Widowati et al., 2018; Hidayat *et al*., 2016; Prahastuti *et al*., 2019b).

Glucose-induced Mesangial Cells for Viability Assay

The mesangial cells (kidney/glomerulus of *Mus musculus*) (SV40 MES 13 (ATCC® CRL-1927™) was obtained from Aretha Medika Utama, Bimolecular and Biomedical Research Center, Bandung, West Java, Indonesia. The cells 5x10^3 cells/well were platted in 96-well plate in 200 µL growth medium (DMEM) (Gibco, 11995065): F12-K Mix Nutrient (Gibco, 21127022) (with comparison 1:3), 5% of fetal bovine serum (FBS) (Gibco, 10270106), ~~15~~ 1% of Antibiotic-Antimycotic (Gibco, 1772653), 0.1 % Gentamicin (Gibco, 15750060) and incubated at 37°C, 5% CO_2 for 24 h. Medium were discarded and added 180 µL of (5 mM, 25 mM and 125 mM) glucose-induced medium and 20 µl of EEDS (6.25 µg/ml and 3.125 µg/mL). Cells were incubated at 37°C, 5% CO_2 for 5, 10 and 15 days. Viability were measured at day 5, 10 dan 15 using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium) Assay Kit (Abcam, ab197010). The absorbance at 490 nm using Multiskan GO plate reader (Thermo Scientific, 1510) for calculating the percentage of cell ~~mortality~~ viability (~~Widowati *et al*., 2016;~~ Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

Quantification of TGF-β1 Level

The quantitative determination of TGF-β1 level in the cell-free supernatant was performed using mouse TGF-β1 ELISA Kit (Elabscience, E-EL-M0051) based on manufactured protocol. Concisely, 100 µL of standard, sample and blank solution was added into each well then incubated 90 minutes at 37°C. The cell-free supernatant after treated with EEDS were served as the sample. The glucose-induced mesangial cell free supernatant without extract was used as positive control. The normal cell or untreated cell was used as negative control. Afterward, the liquid of each well was discarded and 100 µL Biotinylated Detection Ab was added then incubated for an hour at 37°C. Then the liquid was dumped and the plate was washed three times using 200 µL *wash buffer*. 100 µL HRP Conjugate was added and incubated for 30 minutes at 37°C. The liquid was dumped again and the plate was washed five times using 200 µL *wash buffer*. 90 µL Substrate Reagent was added and incubated for 15 minutes at 37°C. 50 µL Stop Solution was added and the absorbance was read at 450 nm (Widowati *et al*., 2018; Dai *et al*., 2014; Prahastuti *et al*., 2019a).

Quantification of Fibronectin Level

The quantitative determination of Fibronectin level in the cell-free supernatant was performed using mouse FN ELISA Kit (ElabScience, E-EL-M0506) based on manufactured protocol. Concisely, 100 µL of sample, standard, and blank solution was added into each well then sealed and incubated for 90 minutes at 37°C. The cell-free supernatant, after glucose induced mesangial cells and treated with EEDS was served as the sample. The glucose-induced mesangial cell free supernatant without extract was used as positive control. The untreated cell or normal cell was used as negative control. Afterward, the liquid of each well was discarded and 100 µL of Biotinylated Detection Ab was added then incubated for an hour at 37°C. Then the liquid was dumped and the plate was washed three times using 200 µL *wash buffer*. 100 µL HRP Conjugate was added and incubated for 30 minutes at 37°C. The liquid was dumped again and the plate was washed five times using 200 µL *wash buffer*. 90 µL Substrate Reagent was added and incubated for 15 minutes at 37°C. 50 µL Stop Solution was added and the absorbance was read at 450 nm (Widowati *et al*., 2018; Dai *et al*., 2014; Pankov *et al*., 2004; Prahastuti *et al.*, 2019a).

Quantification of Reactive Oxygen Species

The ROS generation of SV40 MES 13 cells induced with glucose were measured using the DCFDA – Cellular Reactive Oxygen Species Detection Assay

Kit (Abcam, ab113851,) and flow cytometry. SV40 MES13 cells were suspended into buffer DCFDA in FACS tube at final concentration of 250,000 cells per 500 µL. DCFDA was added to cells suspension at final concentration 20 µM on each tube. The cells was then incubated at 37°C, 5% CO_2 (dark room) for 45 minutes. Glucose 5 mM and 10mM (Amresco, 0188) and EEDS (6.25 µg/mL and 3.125 µg/mL) were added to each tube. The TBHP-induced mesangial cell free supernatant without extract was used as positive control. The normal cell or untreated cell was used as negative control. Cells were incubated at 37°C, 5% CO_2 (dark room) for 4 hours. ROS was measured by flow cytometer (Gilmore *et al*., 2017; Prahastuti *et al*., 2019a).

Statistical Analysis

SPSS 16 (SPSS Inc., Chicago, IL, USA) was used for analyzing data to perform one-way ANOVA to verify the results of different treatments and Duncan post hoc and T-Test was used to validate significant differences for all treatments ($p<0.05$). The results are displayed as means±standard deviation.

Effect EEDS toward Kidney Mesangial Cells Viability

Table I. Effect of EEDS towards viability of glucose-induced kidney mesangial cells ~~viability in glucose-induced mesangial cells~~ at 5 days of incubation

Sample ~~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%^{cd}
Positive Control		96.71±0.91%^{a}	84.78±1.71%^{a}	74.92±1.30%^{a}
EEDS 3.125 µg/mL	176.85±28.81%^{c}	193.66±29.67%^{c}	142.59±21.84%^{b}	99.03±2.03%^{c}
EEDS 6.25 µg/mL	147.34±16.30%^{b}	163.42±24.59%^{b}	158.42±9.80%^{c}	95.16±3.85%^{b}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM (a,b,c), 5 mM (a,b,c), 25 mM (a,b,c), 125 mM (a,b,c,cd,) glucose concentration show significant differences among treatments of EEDS concentrations ($P<0.05$) analyzed using ANOVA and Duncan post hoc test.

Table II. Effect of EEDS towards viability of glucose-induced kidney mesangial cells ~~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^{a}	100.00±5.84^{bc}	100.00±5.84^{c}
Positive Control		96.64±0.43^{a}	86.84±1.50^{a}	73.26±2.25^{a}
EEDS 3.125 µg/mL	147.24±15.42^{b}	111.10±4.43^{ab}	99.63±0.49^{b}	88.70±0.66^{b}
EEDS 6.25 µg/mL	110.11±1.87^{ab}	133.98±21.57^{b}	99.07±1.62^{b}	99.08±1.32^{c}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM (a,ab,), 5 mM (a,ab,b), 25 mM (a,b,bc), 125 mM (a,b,c,) glucose concentration show significant differences among treatments of EEDS concentrations ($P<0.05$) analyzed using ANOVA and Duncan post hoc test.

Table III. Effect of EEDS towards kidney mesangial cells 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^{b}	100.00±5.84^{d}	100.00±5.84^{d}
Positive Control		76.89±2.13^{a}	70.94±1.80^{a}	54.12±4.18^{a}
EDS 3.125 µg/mL	110.44±11.69^{b}	102.72±16.21^{b}	91.44±7.35^{c}	67.77±12.59^{c}
EEDS 6.25 µg/mL	117.40±8.35^{bc}	100.62±9.04^{b}	82.59±3.86^{b}	63.61±6.77^{b}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM (a,b,bc,), 5 mM (a,b), 25 mM (a,b,c,d), 125 mM (a,b,c,d) glucose concentration show significant differences among treatments of EEDS concentrations ($P<0.05$) analyzed using ANOVA and Duncan post hoc test.

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RESULT AND DISCUSSION

Excessive amount of glucose in renal leads to an activation of many metabolic pathways which further leads to an increase of ROS (Yu *et al.,* 2011). These activated pathways induce release of ECM components such as FN and TGF-β1. These increase of and TGF-β1 are closely linked to glomerulosclerosis which further lead to CKD (Loeffler and Wolf, 2013; Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

The viability of glucose-induced SV 40 MES 13 and treated with EEDS were assessed for 5, 10, and 15 days of incubation. The results were shown in Table I, II, and III. Based on the result, the viability of EEDS 3.125 µg/mL and 6.25 µg/mL are significantly higher than positive control at some levels of glucose-induced and days of incubation. However, the viability of samples at day 15 of incubation showed lower viability compared to other incubation time. The viability of each sample is decreased when the incubation time is longer. This result was in line with previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts improved cell viability on glucose-induced mouse kidney cells (Widowati et al., 2018; Prahastuti *et al*., 2019a)

At 5 days of incubation, the viability of 5 mM glucose-induced EEDS 3.125 µg/mL was the highest among other samples (193.66±29.67%) while EEDS at concentration 6.25 µg/mL was lower compared to EEDS 3.125 µg/mL (163.42±24.59%) (Table I). Both treatments of EEDS (3.125; 6.25 µg/mL) were significantly different compared to positive control. At 10 days incubation, EEDS 6.25 µg/mL with 5 mM glucose-induced had the highest viability (133.98±21.57%) compared to other treatment then the viability gradually decrease. EEDS 3.125 µg/mL also shows the same pattern (Table II). However higher level of glucose induction, decreased mesangial cell viability. This pattern is also shown at 10 days incubation, the highest viability was EEDS 6.25 µg/mL at 5 mM glucose induction (133.98±21.57%). The highest cells viability (100.62±9.04%) was 15 days incubation (Table III), ~~which~~ on 5 mM glucose-induced cells and treated with EEDS 3.125 µg/mL, ~~then~~ the cells viability decreased at higher level of glucose induction. Moreover, the cells viability of EEDS treatment was significantly different compared to positive control. This result was in line with previous study which shows that EEDS also has high viability (about >90%) on 3T3-L1 cells (Hidayat *et al*., 2015). The cells viability decreased as the level of increased-glucose induction. The glucose induction can decreased the cell viability, induced apoptosis, and elevated level of intracellular reactive species in a concentration and time-dependent manner (Hou *et al*., 2015). The high level of cells viability indicates that the EEDS can protect the cell from damage of glucose induction in renal cell (Prahastuti *et al*., 2019a).

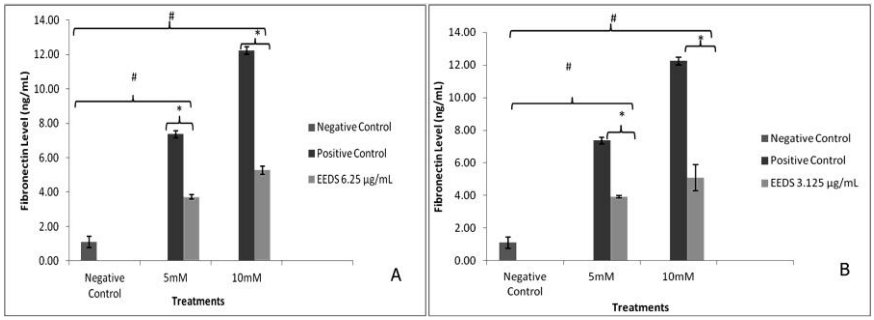

Figure 1. Effect EEDS (3.125 µg/mL, 6.25 µg/mL) toward FN level on 5 mM and 10 mM ~~of~~ glucose-induced kidney mesangial cells

*The histogram are presented as mean ± standard deviation of negative control, positive control and EEDS 6.25 µg/mL (A) and EEDS 3.125 µg/mL (B). Asterisk symbols (*) show significant difference between positive control (5 mM, 10 mM glucose induction) and EEDS 6.25µg/mL treatment, between positive control (5 mM, 10 mM glucose induction) and 3.125 µg/mL treatment on cells ($P < 0.05$) (independent *T*-test). Pound symbol (#) show significant difference between negative control and positive control (5 mM glucose induction), negative control and positive control (10 mM glucose induction) ($P < 0.05$) (independent *T*-test).

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Current study, SV40 MS13 kidney mesangial cells were cultured in a high concentration of glucose which is related to diabetic glomerulosclerosis condition. To study the effect of EEDS on CKD cells model toward, cells viability, FN TGF-β1, ROS level which were used as a parameter due to their role in glomerulosclerosis disease. ~~Compared to EEDS 3.125,~~ The EEDS 6.25 µg/mL was able to reduce the FN level especially at 5 mM glucose induction as low as 3.72±0.13 ng/mL. However the difference between EEDS 3.125 and 6.25 µg/mL treatments was not significant. At this point, it also indicated that higher glucose induction increased FN level. However, the EEDS treatment decreased the FN level significantly compared to positive control both after induced 5 mM and 10 mM of glucose (Figure 1). This results was in line with previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts decreased FN level on glucose-induced glomerulus cells (Widowati *et a*l., 2018; Prahastuti *et al*., 2019a).

Previous study exhibited that significant increase of FN level in higher level of glucose induction (Nahman *et al*., 1992). Glucose can increase the FN level which is key protein matrix accumulation in excess in kidney disease (Wang *et al*., 2008). The accumulated level of FN on glucose-induced cell can be mediat~~i~~ed by oxygen-free radical, therefore the antioxidant can lower the FN accumulation. The result exhibited that EEDS was able to suppress the FN expression in hyperglyc~~i~~emic cell model (Figure 1).

The EEDS 6.25 µg/mL and 3.125 µg/mL decreased TGFβ1. EEDS 6.25 µg/mL at 5 mM ~~of glucose~~ glucose-induced cells showed lowest TGFβ1 level (62.73 ± 10.89 pg/mL), it was the best treatment compared to 10 mM of glucose induction.

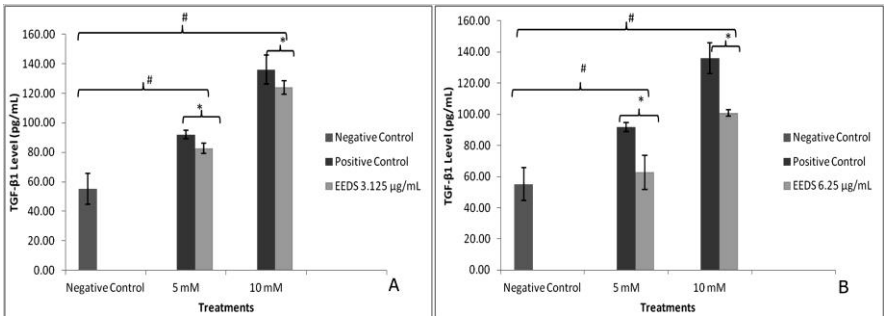

Figure 2. Effect EEDS (3.125 µg/mL, 6.25 µg/mL) toward TGF-β1 level on 5 mM and 10 mM of glucose-induced SV 40 MES 13

* The histogram are presented as mean ± standard deviation of negative control, positive control and EEDS 6.25 µg/mL (A) and EEDS 3.125 µg/mL (B). Asterisk symbols (*) show significant difference between positive control (5 mM, 10 mM glucose induction) and EEDS 6.25µg/mL treatment , between positive control (5 mM, 10 mM glucose induction) and 3.125 µg/mL treatment on cells ($P < 0.05$) (independent *T*-test). Pound symbol (#) show significant difference between negative control and positive control (5 mM glucose induction), negative control and positive control (10 mM glucose induction) ($P < 0.05$) (independent *T*-test).

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Table IV. Effect EEDS towad ROS level on glucose-induced mesangial cells

Treatment	ROS level (%)	ROS level (MFI)
Positive control (Cell + TBHP)	82.51 ± 2.83^{d}	19.68±0.67^{e}
Negative control (cell + DCFDA)	6.54 ± 0.31^{b}	10.90±0.49^{bc}
Glucose 5 mM	8.26 ± 1.48^{b}	11.40±0.31^{c}
Glucose 10 mM	50.99 ± 3.42^{c}	15.24±0.56^{d}
Glucose 5 mM + EEDS 6.25 µg/mL	4.58 ± 1.02^{ab}	10.06 ± 0.20^{bc}
Glucose 10 mM + EEDS 6.25 µg/mL	2.26 ± 0.418^{a}	10.03 ± 0.32^{ab}

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

TGF-β plays a crucial role in mesangial matrix expansion. According to previous study, the injured glomeruli express more TGF-β1 mRNA, synthesize more TGF-β1 protein, and secrete many fold greater amounts of FN and proteoglycans than normal glomeruli (Okuda *et al.*, 1990). Thus the damage in glomeruli can lead to a renal disease. The excessive amount of TGF- β1 due to glucose induction can be suppressed by the the treatment with EEDS.

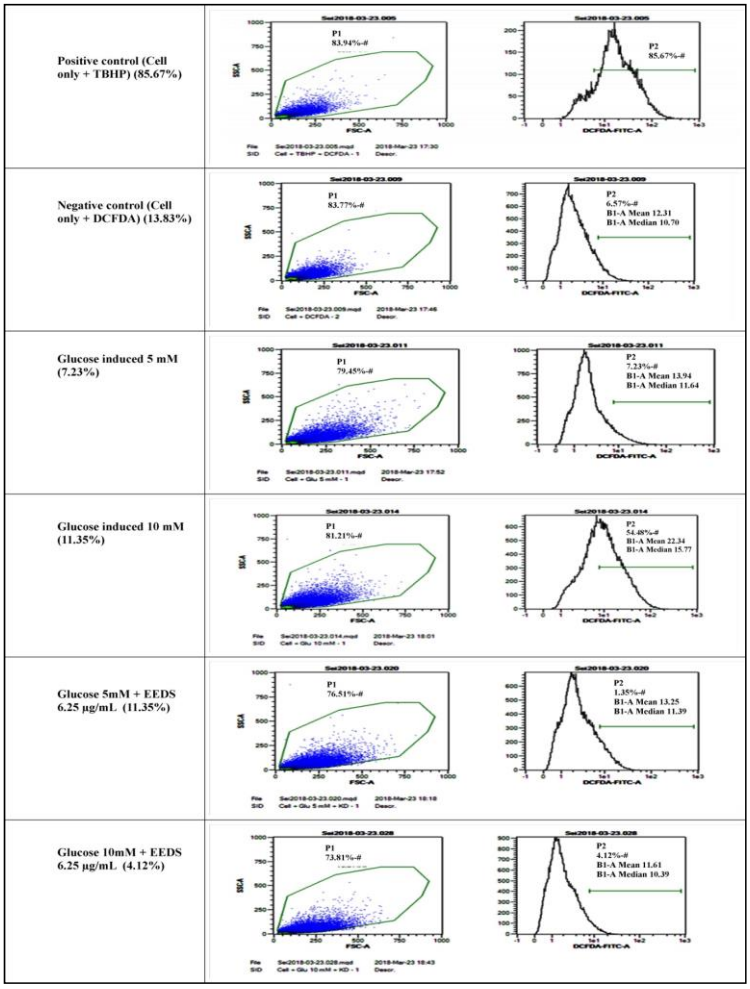

Figure 3. Dot blot of EEDS 6.25 µg/mL on glucose-induced mesangial cell toward ROS level. This figure is the dot blot representative of ROS level in glucose-induced mesangial cells treated with EEDS 6.25 µg/mL

*EEDS = ethanol extract of detam 1 soybean
TBHP = tert-butyl hyroperoxide
DCFDA = 2',7' –dichlorofluorescin diacetate

The EEDS treatment resulted in significant decrease of TGF-β1. This result was supported by previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts decreased TGF-β1 level on glucose-induced glomerulus cells (Widowati *et al*., 2018; Prahastuti *et al*., 2019a). The reduction of and TGF-β1 level are associated with antioxidant activity of EEDS. Ethanol extract of soybean is reported to contain phenolic and flavonoid contents which have good potential as antioxidant (Juan and Chou, 2010; Prahastuti et al., 2019a).

Antioxidant compounds inhibit activation the protein kinase c, which induce diabetic

nephropathy and also TGF-β1 synthesis in mesangial cell 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 FN synthesis (Ha *et al*., 1998).

The ROS (%) level of 10 mM glucose-induced mesangial cells was higher than 5 mM glucose induction, EEDS treatment (6.25 µg/mL and 3.125 µg/mL) decreased ROS level both 5 mM and 10 mM glucose induction were comparable with negative control. These results indicate that the EEDS treatment can significantly lower the ROS level on CKD cells model (Table 4, Figure 1).

Soybeans showed much higher free-radical scavenging activity, inhibition of lipid peroxidation and ability to chelate metals. Soy isoflavones as antioxidants have been reported to reduce the risk of coronary heart disease. (Ramdath *et al*., 2017). To inhibit lipid peroxidation, to prevent low density-lipoprotein oxidation and to reduce oxidative DNA damage. Soybean also reported to have phenolic compounds which is also a great antioxidant source (Han and Baik, 2008; Prahastuti *et al*., 2019a). Antioxidants are able to improve cell growth and regenerate a damaged extracellular matrix under high glucose concentration (hyperglycemic) through glycation of proteins (Chen *et al*., 2012; Kurtz and Oh, 2012; Widowati *et a*l., 2018; Prahastuti *et al*., 2019a).

Oxidative stress is associated with other disease related to a reduced antioxidant defense which led to a decreased renal function, therefore, it can be said that the antioxidants might exert a key role to protect mesangial cells and might be applied therapeutically as nephroprotective agent as well as prevent renal scarring (Lee and Song, 2009; Tylicki *et al*., 2003). The presence of antioxidant according to Jha *et al.* (2016) can ROS into nonreactive oxygen molecules which is harmless to cell (Jha *et al*., 2016). Also, it obstruct glucose absorption through inhibition of carbohydrate-hydrolyzing enzymes such as α-glucosidase and α-amylase and down regulates the TGF-β expression and FN level by decreasing NADPH oxidase expression (Widowati *et al*., 2018; Manaharan *et al*., 2012; Han *et al*., 2017; Prahastuti *et al*., 2019a). Table IV shows that EEDS is capable to lower ROS level mesangial cells treated with 5 and 10 mM glucose compared to positive control. EEDS is able to reduce ROS level I due to antioxidant activity, therefore it might be considered to be a nephroprotective strategy with further studies.

CONCLUSION

The ethanol extract of *Detam* 1 black soybean seed has ability to protect kidney cells from hyperglycemic damage and possess the potential to lower FN TGF-β1, and ROS level in CKD cells model

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Ethanol Extract of *Detam* 1 Soybean Seed (*Glycine Max* L. Merr) for Chronic Kidney Disease Therapy *by In Vitro* Study

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ABSTRACT

Chronic Kidney Disease (CKD) has increased incidence and prevalence in developing nations. In this *in vitro* study, we evaluated the cells proliferative effects, fibronectin (FN) , transforming growth factor β (TGF-β1), and Reactive oxygen species (ROS) - level inhibition potential of ethanol extract of detam 1 soybean seed (EEDS) on glucose-induced kidney mesangial cells (SV40 MES 13). The cells proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium (MTS) assay. FN and TGF-β1 level were measured using ELISA assay kit and ROS level using flow cytometry. Level of FN, TGF-β1 and ROS ~~level~~, on CKD cells model (5 mM, 10 mM glucose-induced mesangial cell) treated with EEDS 6.25 µg/mL ~~on 5 mM, 10 mM glucose-induced mesangial cells~~ were lower significantly compared to positive control, EEDS improve cells viability and decrease FN, TGF-β1 and ROS level in glucose-induced kidney mesangial cells as CKD cells model.
Key words: chronic kidney disease; fibronectin; ROS; soybean; TGF-β1

INTRODUCTION

Chronic Kidney Disease (CKD) is a condition when a renal injury occurs and resulting in a decreased of renal excretory function, thus causing in an accumulation of waste and metabollic products. These products accumulations in blood and organs will cause various complications. CKD presents a therapeutic challenge because of the high prevalence and high cost of dialysis. CKD can be caused by various factors such as hypertension, diabetes, infection, atherosclerosis, and others. CKD also becomes a risk factor of cardiovascular disease. Patients with CKD are restricted to protein intake as it will prevent complication and alleviate uremic symptoms in CKD (Garcı´a-Sa´nchez *et al*., 2010; Ranich and Velasquez, 2001).

The filtration unit of the kidney is the glomerulus, a capillary network supported by mesangial cells and extracellular matrix (ECM). Increased levels of the ECM protein fibronectin (FN) are also present; however, its role in diabetic nephropathy (DN) is unknown. Kidney mesangial cells cultured under high glucose conditions provide a model system for studying the effect high glucose on FN and collagen IV (COL4) (Miller *et al*.,[1] 2014), transforming growth factor-β (TGF-β) deposition (Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

ECM accumulation by enhancing mesangial cell production of COL and FN. TGF-β mediated FN expression in mesangial cells (Uchiyama-Tanaka *et al*., 2002). Reactive Oxygen Species (ROS) also underlies as a common pathogenic component and accelerates the kidney disease progression and complications (Dounousi *et al*., 2006; Widowati *et al*., 2018; Prahastuti *et al*., 2019a). Soybean seed showed an antioxidant effect and an antifibrotic effect in the remnant kidney, which resulted in the improvement of the renal function. Oxidative stress is regulated by the balance between prooxidant and antioxidant systems (Peng *et al*., 2017; Hayata *et al*., 2012; Prahastuti *et al*., 2019b).

Soybean seed (*Glycine max* L. Merr) has been known for its beneficial effects in health due to its compounds. Previous studies reported that soybean contains antinutritional factors (ANF) which include lipase inhibitors, protease inhibitors, amylase inhibitors, oxalic acid, phytic

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acid, glucosinolates, flavonoid, and saponin. The ethanol extract of soybean also known to contain natural compounds such as flavonoid, phenolic, triterpenoid, saponin, steroid, tannin, and quinon (Gemede and Ratta, 2014; Prahastuti *et al*., 2019b). In this study, we evaluated the potency of *Glycine max* L. ~~merr~~ Merr ethanol extract (EEDS) for CKD therapy by measuring the cells proliferation effect, FN, TGF-β1, and ROS level on glucose-induced kidney m~~e~~esangial cells (SV40 MES 13).

METHODOLOGY

Plants Extract Preparation

Glycine max L. (soybeans) varieties of detam 1 was collected from Unit Pengelolaan Benih Sumber (UPBS) Balai Penelitian Tanaman Aneka Kacang dan Umbi, Malang, East Java, Indonesia. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. Soybeans seed were kept in drier tunnel service and the dried soybean then grinded, mashed into powder. Extraction using 70% distilled ethanol and performed based on maceration method (Widowati *et al*., 2016; Widowati *et al*., 2017; Widowati et al., 2018; Hidayat *et al*., 2016; Prahastuti *et al*., 2019b).

Glucose-induced Mesangial Cells for Viability Assay

The mesangial cells (kidney/glomerulus of *Mus musculus*) (SV40 MES 13 (ATCC® CRL-1927™) was obtained from Aretha Medika Utama, Bimolecular and Biomedical Research Center, Bandung, West Java, Indonesia. The cells 5x10^3 cells/well were platted in 96-well plate in 200 µL growth medium (DMEM) (Gibco, 11995065): F12-K Mix Nutrient (Gibco, 21127022) (with comparison 1:3), 5% of fetal bovine serum (FBS) (Gibco, 10270106), ~~15~~ 1% of Antibiotic-Antimycotic (Gibco, 1772653), 0.1 % Gentamicin (Gibco, 15750060) and incubated at 37°C, 5% CO_2 for 24 h. Medium were discarded and added 180 µL of (5 mM, 25 mM and 125 mM) glucose-induced medium and 20 µl of EEDS (6.25 µg/ml and 3.125 µg/mL). Cells were incubated at 37°C, 5% CO_2 for 5, 10 and 15 days. Viability were measured at day 5, 10 dan 15 using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium) Assay Kit (Abcam, ab197010). The absorbance at 490 nm using Multiskan GO plate reader (Thermo Scientific, 1510) for calculating the percentage of cell ~~mortality~~ viability (~~Widowati *et al*., 2016;~~ Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

Quantification of TGF-β1 Level

The quantitative determination of TGF-β1 level in the cell-free supernatant was performed using mouse TGF-β1 ELISA Kit (Elabscience, E-EL-M0051) based on manufactured protocol. Concisely, 100 µL of standard, sample and blank solution was added into each well then incubated 90 minutes at 37°C. The cell-free supernatant after treated with EEDS were served as the sample. The glucose-induced mesangial cell free supernatant without extract was used as positive control. The normal cell or untreated cell was used as negative control. Afterward, the liquid of each well was discarded and 100 µL Biotinylated Detection Ab was added then incubated for an hour at 37°C. Then the liquid was dumped and the plate was washed three times using 200 µL *wash buffer*. 100 µL HRP Conjugate was added and incubated for 30 minutes at 37°C. The liquid was dumped again and the plate was washed five times using 200 µL *wash buffer*. 90 µL Substrate Reagent was added and incubated for 15 minutes at 37°C. 50 µL Stop Solution was added and the absorbance was read at 450 nm (Widowati *et al*., 2018; Dai *et al*., 2014; Prahastuti *et al*., 2019a).

Quantification of Fibronectin Level

The quantitative determination of Fibronectin level in the cell-free supernatant was performed using mouse FN ELISA Kit (ElabScience, E-EL-M0506) based on manufactured protocol. Concisely, 100 µL of sample, standard, and blank solution was added into each well then sealed and incubated for 90 minutes at 37°C. The cell-free supernatant, after glucose induced mesangial cells and treated with EEDS was served as the sample. The glucose-induced mesangial cell free supernatant without extract was used as positive control. The untreated cell or normal cell was used as negative control. Afterward, the liquid of each well was discarded and 100 µL of Biotinylated Detection Ab was added then incubated for an hour at 37°C. Then the liquid was dumped and the plate was washed three times using 200 µL *wash buffer*. 100 µL HRP Conjugate was added and incubated for 30 minutes at 37°C. The liquid was dumped again and the plate was washed five times using 200 µL *wash buffer*. 90 µL Substrate Reagent was added and incubated for 15 minutes at 37°C. 50 µL Stop Solution was added and the absorbance was read at 450 nm (Widowati *et al*., 2018; Dai *et al*., 2014; Pankov *et al*., 2004; Prahastuti *et al.*, 2019a).

Quantification of Reactive Oxygen Species

The ROS generation of SV40 MES 13 cells induced with glucose were measured using the DCFDA – Cellular Reactive Oxygen Species Detection Assay

Kit (Abcam, ab113851,) and flow cytometry. SV40 MES13 cells were suspended into buffer DCFDA in FACS tube at final concentration of 250,000 cells per 500 µL. DCFDA was added to cells suspension at final concentration 20 µM on each tube. The cells was then incubated at 37°C, 5% CO_2 (dark room) for 45 minutes. Glucose 5 mM and 10mM (Amresco, 0188) and EEDS (6.25 µg/mL and 3.125 µg/mL) were added to each tube. The TBHP-induced mesangial cell free supernatant without extract was used as positive control. The normal cell or untreated cell was used as negative control. Cells were incubated at 37°C, 5% CO_2 (dark room) for 4 hours. ROS was measured by flow cytometer (Gilmore *et al*., 2017; Prahastuti *et al*., 2019a).

Statistical Analysis

SPSS 16 (SPSS Inc., Chicago, IL, USA) was used for analyzing data to perform one-way ANOVA to verify the results of different treatments and Duncan post hoc and T-Test was used to validate significant differences for all treatments ($p<0.05$). The results are displayed as means±standard deviation.

Effect EEDS toward Kidney Mesangial Cells Viability

Table I. Effect of EEDS towards viability of glucose-induced kidney mesangial cells ~~viability in glucose-induced mesangial cells~~ at 5 days of incubation

Sample ~~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%^{cd}
Positive Control		96.71±0.91%^{a}	84.78±1.71%^{a}	74.92±1.30%^{a}
EEDS 3.125 µg/mL	176.85±28.81%^{c}	193.66±29.67%^{c}	142.59±21.84%^{b}	99.03±2.03%^{c}
EEDS 6.25 µg/mL	147.34±16.30%^{b}	163.42±24.59%^{b}	158.42±9.80%^{c}	95.16±3.85%^{b}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM (a,b,c), 5 mM (a,b,c), 25 mM (a,b,c), 125 mM (a,b,c,cd,) glucose concentration show significant differences among treatments of EEDS concentrations ($P<0.05$) analyzed using ANOVA and Duncan post hoc test.

Table II. Effect of EEDS towards viability of glucose-induced kidney mesangial cells ~~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^{a}	100.00±5.84^{bc}	100.00±5.84^{c}
Positive Control		96.64±0.43^{a}	86.84±1.50^{a}	73.26±2.25^{a}
EEDS 3.125 µg/mL	147.24±15.42^{b}	111.10±4.43^{ab}	99.63±0.49^{b}	88.70±0.66^{b}
EEDS 6.25 µg/mL	110.11±1.87^{ab}	133.98±21.57^{b}	99.07±1.62^{b}	99.08±1.32^{c}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM (a,ab,), 5 mM (a,ab,b), 25 mM (a,b,bc), 125 mM (a,b,c,) glucose concentration show significant differences among treatments of EEDS concentrations ($P<0.05$) analyzed using ANOVA and Duncan post hoc test.

Table III. Effect of EEDS towards kidney mesangial cells 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^{b}	100.00±5.84^{d}	100.00±5.84^{d}
Positive Control		76.89±2.13^{a}	70.94±1.80^{a}	54.12±4.18^{a}
EDS 3.125 µg/mL	110.44±11.69^{b}	102.72±16.21^{b}	91.44±7.35^{c}	67.77±12.59^{c}
EEDS 6.25 µg/mL	117.40±8.35^{bc}	100.62±9.04^{b}	82.59±3.86^{b}	63.61±6.77^{b}

*Data is served in average±standard deviation. Different superscript letters in the same column of 0 mM

RESULT AND DISCUSSION

Excessive amount of glucose in renal leads to an activation of many metabolic pathways which further leads to an increase of ROS (Yu *et al.*, 2011). These activated pathways induce release of ECM components such as FN and TGF-β1. These

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increase of and TGF-β1 are closely linked to glomerulosclerosis which further lead to CKD (Loeffler and Wolf, 2013; Widowati *et al*., 2018; Prahastuti *et al*., 2019a).

The viability of glucose-induced SV 40 MES 13 and treated with EEDS were assessed for 5, 10, and 15 days of incubation. The results were shown in Table I, II, and III. Based on the result, the viability of EEDS 3.125 µg/mL and 6.25 µg/mL are significantly higher than positive control at some levels of glucose-induced and days of incubation. However, the viability of samples at day 15 of incubation showed lower viability compared to other incubation time. The viability of each sample is decreased when the incubation time is longer. This result was in line with previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts improved cell viability on glucose-induced mouse kidney cells (Widowati et al., 2018; Prahastuti *et al*., 2019a)

At 5 days of incubation, the viability of 5 mM glucose-induced EEDS 3.125 µg/mL was the highest among other samples (193.66±29.67%) while EEDS at concentration 6.25 µg/mL was lower compared to EEDS 3.125 µg/mL (163.42±24.59%) (Table I). Both treatments of EEDS (3.125; 6.25 µg/mL) were significantly different compared to positive control. At 10 days incubation, EEDS 6.25 µg/mL with 5 mM glucose-induced had the highest viability (133.98±21.57%) compared to other treatment then the viability gradually decrease. EEDS 3.125 µg/mL also shows the same pattern (Table II). However higher level of glucose induction, decreased mesangial cell viability. This pattern is also shown at 10 days incubation, the highest viability was EEDS 6.25 µg/mL at 5 mM glucose induction (133.98±21.57%). The highest cells viability (100.62±9.04%) was 15 days incubation (Table III), ~~which~~ on 5 mM glucose-induced cells and treated with EEDS 3.125 µg/mL, ~~then~~ the cells viability decreased at higher level of glucose induction. Moreover, the cells viability of EEDS treatment was significantly different compared to positive control. This result was in line with previous study which shows that EEDS also has high viability (about >90%) on 3T3-L1 cells (Hidayat *et al*., 2015). The cells viability decreased as the level of increased-glucose induction. The glucose induction can decreased the cell viability, induced apoptosis, and elevated level of intracellular reactive species in a concentration and time-dependent manner (Hou *et al*., 2015). The high level of cells viability indicates that the EEDS can protect the cell from damage of glucose induction in renal cell (Prahastuti *et al*., 2019a).

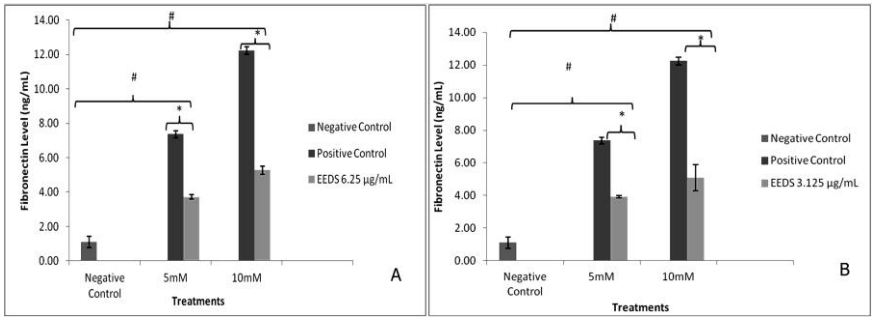

Figure 1. Effect EEDS (3.125 µg/mL, 6.25 µg/mL) toward FN level on 5 mM and 10 mM ~~of~~ glucose-induced kidney mesangial cells

*The histogram are presented as mean ± standard deviation of negative control, positive control and EEDS 6.25 µg/mL (A) and EEDS 3.125 µg/mL (B). Asterisk symbols (*) show significant difference between positive control (5 mM, 10 mM glucose induction) and EEDS 6.25µg/mL treatment, between positive control (5 mM, 10 mM glucose induction) and 3.125 µg/mL treatment on cells ($P < 0.05$) (independent *T*-test). Pound symbol (#) show significant difference between negative control and positive control (5 mM glucose

Current study, SV40 MS13 kidney mesangial cells were cultured in a high concentration of glucose which is related to diabetic glomerulosclerosis condition. To study the effect of EEDS on CKD cells

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model toward, cells viability, FN TGF-β1, ROS level which were used as a parameter due to their role in glomerulosclerosis disease. ~~Compared to EEDS 3.125,~~ The EEDS 6.25 µg/mL was able to reduce the FN level especially at 5 mM glucose induction as low as 3.72±0.13 ng/mL. However the difference between EEDS 3.125 and 6.25 µg/mL treatments was not significant. At this point, it also indicated that higher glucose induction increased FN level. However, the EEDS treatment decreased the FN level significantly compared to positive control both after induced 5 mM and 10 mM of glucose (Figure 1). This results was in line with previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts decreased FN level on glucose-induced glomerulus cells (Widowati *et a*l., 2018; Prahastuti *et al*., 2019a).

Previous study exhibited that significant increase of FN level in higher level of glucose induction (Nahman *et al*., 1992). Glucose can increase the FN level which is key protein matrix accumulation in excess in kidney disease (Wang *et al*., 2008). The accumulated level of FN on glucose-induced cell can be mediat~~i~~ed by oxygen-free radical, therefore the antioxidant can lower the FN accumulation. The result exhibited that EEDS was able to suppress the FN expression in hyperglyc~~i~~emic cell model (Figure 1).

The EEDS 6.25 µg/mL and 3.125 µg/mL decreased TGFβ1. EEDS 6.25 µg/mL at 5 mM ~~of glucose~~ glucose-induced cells showed lowest TGFβ1 level (62.73 ± 10.89 pg/mL), it was the best treatment compared to 10 mM of glucose induction.

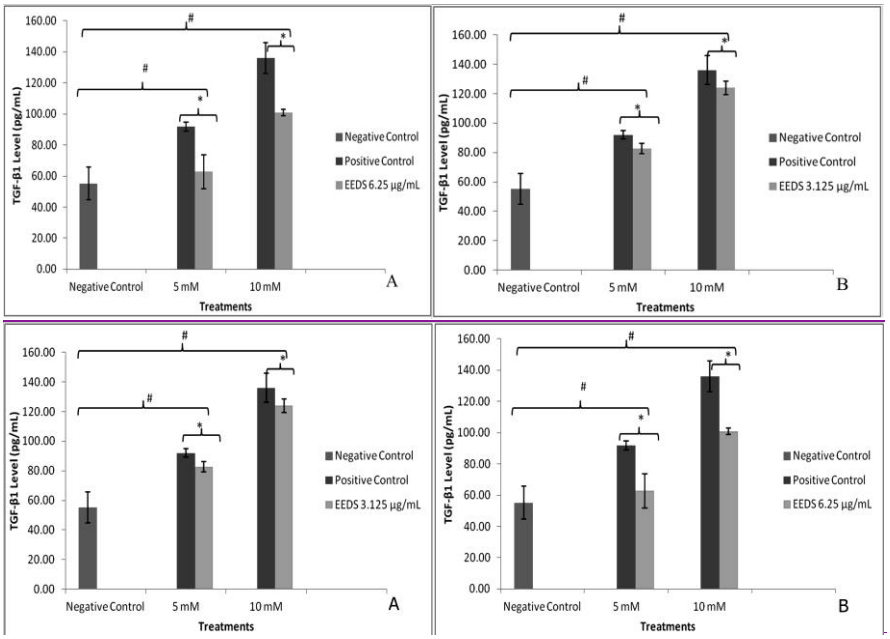

Figure 2. Effect EEDS (3.125 µg/mL, 6.25 µg/mL) toward TGF-β1 level on 5 mM and 10 mM of glucose-induced SV 40 MES 13

* The histogram are presented as mean ± standard deviation of negative control, positive control and EEDS 6.25 µg/mL (A) and EEDS 3.125 µg/mL (B). Asterisk symbols (*) show significant difference between positive control (5 mM, 10 mM glucose induction) and EEDS 6.25µg/mL treatment , between positive control (5 mM, 10 mM glucose induction) and 3.125 µg/mL treatment on cells *(P* < 0.05) (independent *T*-test). Pound symbol (#) show significant difference between negative control and positive control (5 mM glucose induction), negative control and positive control (10 mM glucose induction) (*P* < 0.05) (independent *T*-test).

Table IV. Effect EEDS toward ROS level on glucose-induced mesangial cells

TGF-β plays a crucial role in mesangial matrix expansion. According to previous study, the injured glomeruli express more TGF-β1 mRNA, synthesize more TGF-β1 protein, and secrete many fold greater amounts of FN and proteoglycans than normal glomeruli (Okuda *et al*., 1990). Thus the damage in glomeruli can lead to a renal disease. The excessive amount of TGF- β1 due to glucose induction can be suppressed by the the treatment with EEDS.

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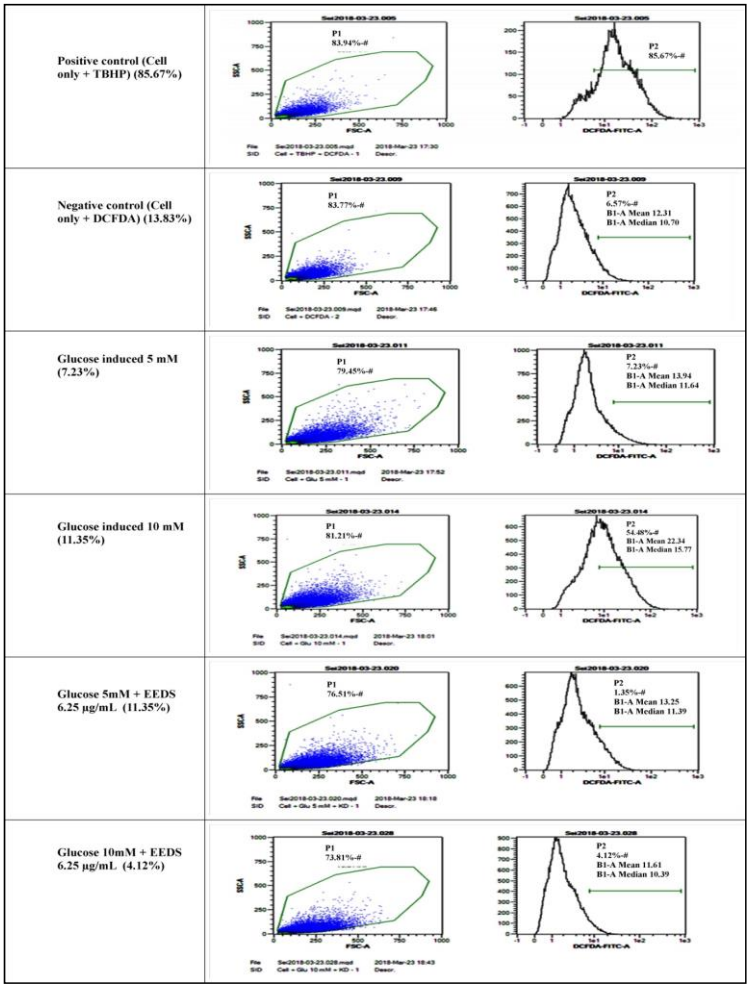

Figure 3. Dot blot of EEDS 6.25 µg/mL on glucose-induced mesangial cell toward ROS level. This figure is the dot blot representative of ROS level in glucose-induced mesangial cells treated with EEDS 6.25 µg/mL

*EEDS = ethanol extract of detam 1 soybean
TBHP = tert-butyl hyroperoxide
DCFDA = 2',7' –dichlorofluorescin diacetate

The EEDS treatment resulted in significant decrease of TGF-β1. This result was supported by previous research that ~~Jati~~ jati ~~Belanda~~ belanda leaf and mangosteen peel extracts decreased TGF-β1 level on glucose-induced glomerulus cells (Widowati *et al*., 2018; Prahastuti *et al*., 2019a). The reduction of and TGF-β1 level are associated with antioxidant activity of EEDS. Ethanol extract of soybean is reported to contain phenolic and flavonoid contents which have good potential as antioxidant (Juan and Chou, 2010; Prahastuti et al., 2019a).

Antioxidant compounds inhibit activation the protein kinase c, which induce diabetic

nephropathy and also TGF-β1 synthesis in mesangial cell 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 FN synthesis (Ha *et al*., 1998).

The ROS (%) level of 10 mM glucose-induced mesangial cells was higher than 5 mM glucose induction, EEDS treatment (6.25 µg/mL and 3.125 µg/mL) decreased ROS level both 5 mM and 10 mM glucose induction were comparable with negative control. These results indicate that the EEDS treatment can significantly lower the ROS level on CKD cells model (Table 4, Figure 1).

Soybeans showed much higher free-radical scavenging activity, inhibition of lipid peroxidation and ability to chelate metals. Soy isoflavones as antioxidants have been reported to reduce the risk of coronary heart disease. (Ramdath *et al*., 2017). To inhibit lipid peroxidation, to prevent low density-lipoprotein oxidation and to reduce oxidative DNA damage. Soybean also reported to have phenolic compounds which is also a great antioxidant source (Han and Baik, 2008; Prahastuti *et al*., 2019a). Antioxidants are able to improve cell growth and regenerate a damaged extracellular matrix under high glucose concentration (hyperglycemic) through glycation of proteins (Chen *et al*., 2012; Kurtz and Oh, 2012; Widowati *et a*l., 2018; Prahastuti *et al*., 2019a).

Oxidative stress is associated with other disease related to a reduced antioxidant defense which led to a decreased renal function, therefore, it can be said that the antioxidants might exert a key role to protect mesangial cells and might be applied therapeutically as nephroprotective agent as well as prevent renal scarring (Lee and Song, 2009; Tylicki *et al*., 2003). The presence of antioxidant according to Jha *et al.* (2016) can ROS into nonreactive oxygen molecules which is harmless to cell (Jha *et al*., 2016). Also, it obstruct glucose absorption through inhibition of carbohydrate-hydrolyzing enzymes such as α-glucosidase and α-amylase and down regulates the TGF-β expression and FN level by decreasing NADPH oxidase expression (Widowati *et al*., 2018; Manaharan *et al*., 2012; Han *et al*., 2017; Prahastuti *et al*., 2019a). Table IV shows that EEDS is capable to lower ROS level mesangial cells treated with 5 and 10 mM glucose compared to positive control. EEDS is able to reduce ROS level I due to antioxidant activity, therefore it might be considered to be a nephroprotective strategy with further studies.

CONCLUSION

The ethanol extract of *Detam* 1 black soybean seed has ability to protect kidney cells from hyperglycemic damage and possess the potential to lower FN TGF-β1, and ROS level in CKD cells model

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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