Ficus elastica Leaf Extract Effect Toward IL-10 and TNF-α as a Preeclampsia Model on Hypoxia-induced EA.hy926

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Abstract. Preeclampsia is a pregnancy complication characterized by proteinuria, hypertension, hypoxia as well an increased systemic inflammatory response. It affects 5 to 10% of all births and is a leading factor in fetal and maternal morbidity and death. *Ficus elastica* L. leaves are known to have natural compounds with anti-inflammatory and antioxidant properties. This research assessed the potential of *F. elastica* leaf extract (FEE) as anti-inflammatory agent in a hypoxic-induced human endothelial cell line (EA.hy926) as preeclampsia model. *F. elastica* leaves were extracted using 70% ethanol. Cytotoxic assay of the FEE in different concentrations (3.13 to 100 µg/ml) against EA.hy926 was done using MTS assay. The FEE's anti-inflammatory activity was measured with ELISA for the TNF-α and IL-10 parameters. The FEE did not cause cytotoxicity to the EA.hy926 cells, with viability above 80% at the highest concentration. The FEE could decrease the pro-inflammatory TNF-α level and increase the IL-10 level as anti-inflammatory markers in the preeclampsia model at significance level $p < 0.05$. This study suggests that FEE is a safe alternative herbal medicine for preeclampsia treatment.

Keywords: *EA.hy926; Ficus elastica; hypoxia; IL-10; preeclampsia; TNF-α.*

1 Introduction

Preeclampsia (PE) is a pregnancy-related condition with clinical symptoms of proteinuria and hypertension that occur after a gestational age of 20 weeks [1,2]. In Europe and America, 2% to 5% of pregnancies are complicated by PE [1]. In Asia, the World Health Organization (WHO) estimates that 10% of maternal mortality is directly caused by PE [3]. PE may affect pregnancy outcome, cause higher risk of premature birth as well as fetal and perinatal morbidity and mortality [4].

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Tumor necrosis factor-α (TNF-α), known as a main proinflammatory cytokine, is embroiled in the pathogenesis of chronic inflammatory diseases [5]. TNF-α concentrations are elevated in PE condition [6]. Hypoxia has been proposed as a major cause of preeclampsia. Hypoxia is a condition when the cells do not receive an adequate amount of oxygen. Under hypoxic conditions, several genes, such as hypoxia-inducible factor (HIF)-1, a hypoxia-dependent transcription factor, are activated. HIF-1 is provoked to induce glycolytic activity by growth factors and oxidative stress [7]. Hypoxia also activates the NFκB pathway, which escalates production of TNF-α, triggering an inflammatory response [8].

WHO recommends the use of an antihypertensive agent for treating high blood pressure in pregnant women with PE [9]. However, common anti-hypertensive drugs, such as angiotensin-converting enzyme (ACE) inhibitors, cannot be safely used by pregnant woman because this type of drug may cause fetal development abnormalities [10].

Herbal medicines have potential as alternative and safe therapies for PE. Several plants have been extensively studied in different countries and found to have antihypertensive properties. A previous study found that *Ficus elastica* leaf contains various compounds, such as flavonoid quercetin and myricitrin [11]. Flavonoid compounds are able to decreased the TNF-α level by *in vitro*, *in vivo*, and clinical studies [12]. Various flavonoids such as quercetin, hesperidin and catechin can upregulate IL-10, an anti-inflammatory cytokine [13]. This suggest that *F. elastica* leaves may have potential in treating PE. Therefore, this study aimed to assess the potential of *F. elastica* leaf ethanolic extract (FEE) as PE medication through TNF-α and IL-10 level parameters in hypoxic-induced EA.hy926 cells as PE cell model.

2 Methods

2.1 Preparation of Extracts

Rubber plant *(Ficus elastica)* leaves were collected from Raya Village, Karo, Sumatera Utara, Indonesia. They were extracted based on the method described in Ginting *et al.* (2020) using 70% ethanol solvents and maceration until FEE was obtained. The FEE was then stored at -20 °C. Various concentrations of FEE were prepared using DMSO 10% solution for further assay [14].

2.2 Cytotoxicity Assay

The human endothelial cells (EA.hy926, ATCC®CRL-2922™) used in this study were obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. The cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Biowest, L0104), with additional Fetal Bovine Serum (FBS) 10% (v/v) (Biowest, S181G), 1% (v/v) antibiotic/antimycotic 100× (Biowest, L0010), and 1% (v/v) Amphotericin B 100× (Biowest, L0009) under controlled condition (37 °C, 5% CO_2, humidified environment).

For cytotoxicity assay the cells were cultured in a 96-well plate with 10 x 10^4 cell density. Cells were treated with various levels of FEE (100.00; 50.00; 25.00; 12.50; 6.25; 3.13 µg/ml) and left inside an incubator (37 °C, 5% CO_2, humidified) for 24 h. Furthermore, 3-4,5-dimethylthiazol-2-(yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Abcam, 197010) was added and the cells were re-incubated for 3 h. The color intensity, as indicator of cell viability was quantified as absorbance at 490 nm wavelength (Multiskan GO, Thermo Fisher) [15,16,17].

2.3 Hypoxic-induced Human Endothelial Cells

The PE *in vitro* model used hypoxic-induced EA.hy926 cells. The cells were grown in a six-well plate (5×10^5 cell density/well) and incubated in hypoxic condition (2% O_2, 5% CO_2 and 37 °C) for 24 h. Safe concentrations based on cytotoxicity assay (25; 100 µg/ml) were used for the next treatment and the cells were re-incubated for 24 h. The conditioned medium was separated and centrifuged for 10 min at 1600 rpm (MPW-260R). The supernatant was taken and stored at -80 °C for ELISA assay [15,17,18,19].

2.4 Bradford Assay

The total protein content in the endothelial cells was quantified with Bradford assay. The total protein standard curve was made using Bovine Standard Albumin (BSA, Sigma Aldrich, A9576) in serial dilution. 20 µl of protein standard, the samples, and 200 µl Quick Start Dye Reagent 1× (Bio Rad, 5000205) were introduced in a 96-well plate for 5 min at room temperature. The absorbance was measured with a microplate reader at 595 nm wavelength [20].

2.5 TNF-α Level Assay

The level of TNF-α was measured using an enzyme-linked immunosorbent assay (ELISA) kit (BioLegend, ELISA kit 421701). A microplate was coated with an antibody solution and incubated overnight at 4 °C. 300 µl of wash buffer was used to wash out unbound antibodies four times and then incubated for an hour in an orbital shaker. 100 µl of each sample and standard were inserted in the well and then 50 µl matrix C and assay buffer were added. After that, 100 µl antibody detection solution was applied and incubated on an orbital shaker at room temperature for 1 h. Then the plate was washed again and 100 µl of diluted Avidin-HRP solution was applied and incubated for 30 min. Then it was washed again 5 times, replaced with 100 µl of substratum solution, and incubated in a dark room for 10 min. The application of around 100 µl of stop solution stopped the reaction. The level of TNF-α was measured at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher) [17,21,22,23].

2.6 IL-10 Level Assay

IL-10 level was determined in each treatment using ELISA assay (BioLegend ELISA kit, 430604). In the first step, the microplate was covered with capture antibody solution and

incubated overnight at 4 °C. Then, the microplate was washed using around 300 µl of wash buffer for four times and placed on an orbital shaker for 1 h. Standard and samples were inserted into the wells, later matrix C and assay buffer as much as 50 µl were added successively. Thereafter, 100 µl of antibody detection solution was applied and re-incubated with the same condition. The plate was washed again, and 100 µl of diluted Avidin-HRP solution was inserted and left at room temperature for 30 min. It was later washed again for 5 times, then filled with around 100 µl of substrate solution. Hereafter, it was placed for 10 mins in a darkroom. The reaction was stopped briefly when 100 µl of stop solution added. After that, the absorbance of the samples was read using a microplate reader at 450 nm [17,21,22,23].

2.7 Statistical Analysis

The results were analyzed using SPSS version 20.0. One-way analysis of variance (ANOVA) at a 95% confidence interval was carried out to show significant differences between groups. A Tukey HSD post hoc test was performed to show statistically significant differences between the groups.

3 Results

3.1 Effect of FEE Toward Human Endothelial Cell Viability

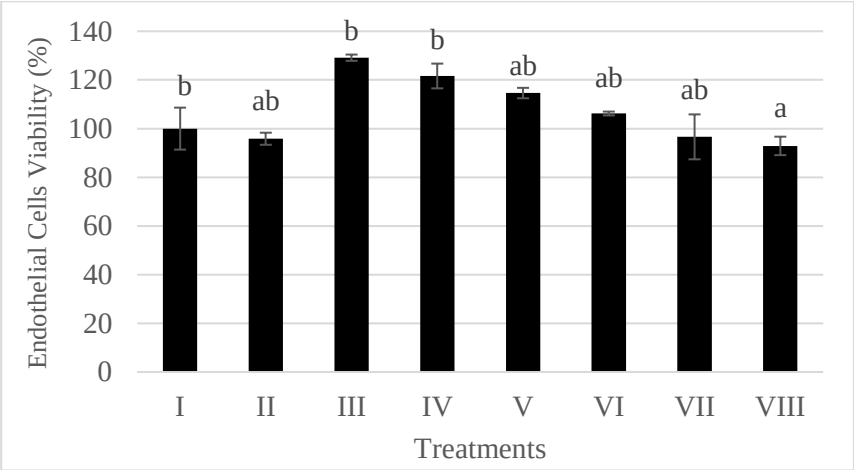

Figure 1 Effect of various concentrations of FEE toward endothelial cell viability. The histograms are shown as mean ± STD. ANOVA followed by a Tukey HSD post hoc test was used to analyze the data. Different letters (a,ab,b) indicate significant differences between various concentrations of FEE. I: Untreated endothelial cells. II: Endothelial cells treated with 1% DMSO. III: Endothelial cells treated with 3.13 µg/ml FEE. IV: Endothelial cells treated with 6.25 µg/ml FEE. V: Endothelial cells treated with 12.50 µg/ml FEE. VI: Endothelial cells treated with 25.00 µg/ml FEE. VII: Endothelial cells treated with 50.00 µg/ml FEE. VIII: Endothelial cells treated with 100.00 µg/ml FEE.

Figure 1 shows that human endothelial (EA.hy926) cells treated with different FEE concentrations exhibited cell viability more than 80% relative to control (untreated EA.hy926). Statistical analysis showed that 100 µg/ml FEE had a significant effect on lowering the viable cell number compared to control. However, it did not differ from DMSO control. Therefore, it can be concluded that FEE itself does not affect cell viability. It indicates that FEE does not have a cytotoxic effect on endothelial cells. Safe concentrations based on the cytotoxic assay were chosen for the next assay. A 25 µg/ml concentration was chosen, because it gave the same viability number as DMSO control, thereby minimizing the risk of bias. As comparison 100 µg/ml was chosen as an extreme value.

3.2 Protein Total Assay

The Bradford assay is a fast and accurate method for measuring protein concentrations and is important for various areas of biochemistry and biology [20]. The total protein content results are shown in Table 1.

Table 1 Total Protein Content

Treatments	Average (mg/ml)
Normal cells	0.157 ± 0.001
Normal cells +1% DMSO	0.155 ± 0.009
Hypoxia-induced endothelial cells	0.155 ± 0.102
Hypoxia-induced endothelial cells + FEE 25 µg/ml	0.146 ± 0.012
Hypoxia-induced endothelial cells + FEE 100 µg/ml	0.156 ± 0.006

The total protein was used for calculating TNF-α, IL-10 assay.

3.3 Effect of FEE on TNF-α Level

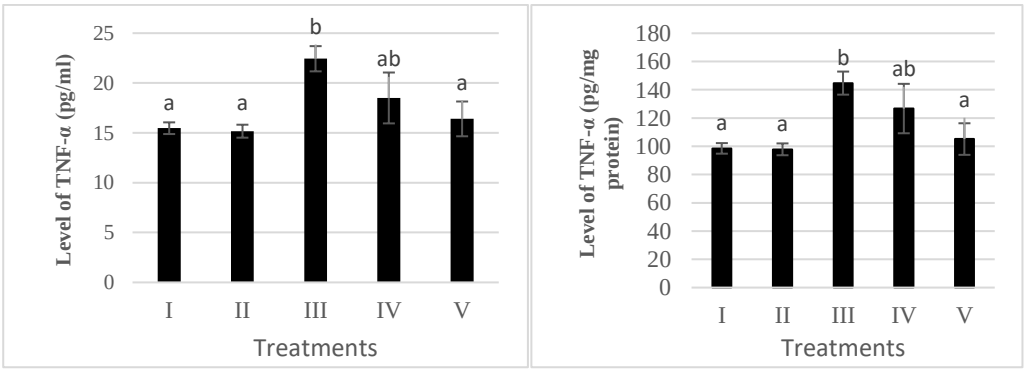

Figure 2 Effect of FEE toward TNF-α levels on PE cell model. The histograms are shown as mean ± STD. ANOVA followed by a Tukey post hoc test was continued. Different letters (a, ab, b) indicate significant differences between treatments. I: Control provided for uninduced endothelial cells. II: Cells treated with 1% DMSO. III: Hypoxia (2% O_2) represents hypoxic cells as PE model. IV: Hypoxic cell model treated with FEE 25 µg/ml. V: Hypoxic cell model treated with FEE 100 µg/ml.

The effect of the FEE on the TNF-α level in hypoxic-induced endothelial cells was shown in Figure 2. The treatment with 25 μg/ml of FEE was able to decrease the TNF-α level. FEE concentration higher than 100 μg/ml was more active in lowering the TNF-α level, comparable to control.

3.4 Effect of FEE on IL-10 Level

IL-10 is known as an anti-inflammatory cytokine that can reduce inflammation in PE condition. The IL-10 level is lowered in hypoxic condition. FEE can upregulate IL-10 secretion in a dose-dependent manner, as shown in Figure 3.

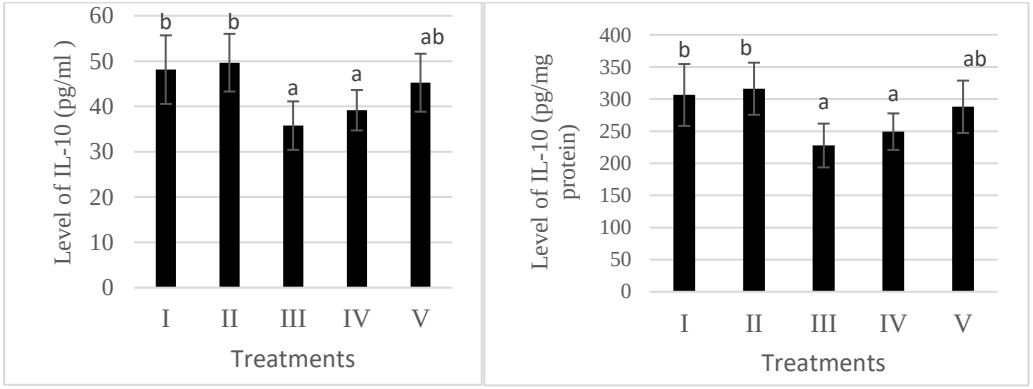

Figure 3 Effect of FEE on IL-10 levels in PE cell model. The histograms are shown as mean ± STD. ANOVA followed by a Tukey post hoc test was continued. Different letters (a, ab, b) indicate significant differences between treatments. I: Control provided to uninduced endothelial cells. II: Cells treated with 1% DMSO. III: Hypoxia (2% O_2) represents hypoxic cells as PE model. IV: Hypoxic cell model treated with FEE 25 μg/ml. V: Hypoxic cell model treated with FEE 100 μg/ml.

4 Discussion

PE is a chronic inflammatory and antiangiogenic condition [24]. In this analysis, an EA.hy926 cell line was used and cultured throughout PE under hypoxic conditions (2% O_2) to simulate a hypoxic placenta and treated with FEE. Zhou *et al*. [25] have shown that PE may be caused by a persistent condition of low oxygen tension. Hence, inflammation and hypoxia have an interdependent relationship with one another [26].

The hypoxic cell model showed that the TNF-α level increased and IL-10 levels decreased. Both are principally involved in PE pathogenesis [6, 27]. IL-10 plays an important role during pregnancy because it can hinder inflammatory cytokine secretion by TH-1 and accordingly, in the fetal-maternal interface, provide an important balance for controlling inflammation [28]. During PE, placental inflammation and chronic peripheral vascular

conditions lead to further complications in the pregnancy [29]. *F. elastica* is beneficial for treating PE due to antioxidant and anti-inflammatory activities and inhibition of beta-adrenergic receptor 2 (ADRB2) [11]. In another study, the methanolic extract of *F. elastica* also had anti-inflammatory activities through inhibition of 15-lipoxygenase (15-LOX) with an IC_{50} value of 3.47 ± 0.07 µg/mL compared to quercetin ($IC_{50} = 24.60 \pm 0.07$ µg/mL). The secondary metabolites from high-phenolic-content natural products have been shown to decrease the inflammatory response by inhibiting molecular targets of inflammation, such as 15-LOX, TNF-α, nitrogen species (NO), interleukin 1-β (IL-1β), cyclooxygenase (COX), and the ROS/RNS pathways [30,31].

In accordance with Hung *et al.* [6], TNF-α in EA.hy926 cultured under hypoxic conditions significantly increased. Under hypoxic conditions, the mitochondrial complex III rapidly generates ROS. Increased cellular ROS production can induce increased NF-κB expression, which leads to an increase in inflammatory factors such as TNF-α [32,33]. Bioactive compounds in *F. elastica* extract may involve biological effects by inhibiting components of major signaling pathways such as NF-κB and mitogen-activated protein kinases (MAPKs), which are important in the generation of pro-inflammatory mediators [34].

Our results show that FEE can reduce pro-inflammatory TNF-α and increase proinflammatory IL-10 in PE cells model. Previous studies by Ginting *et al.* [11,35] found that FEE contains flavonoid compounds, including quercitrin and myricitrin. These flavonoids belong to the polyphenol group and have an important anti-inflammatory effect by influencing cytokine secretion. Quercitrin in the *F. elastica* extract was able to inhibit pro-inflammatory cytokines like TNF-α and increased the IL-10 cytokine level [11]. Based on Ginting *et al.* [36], in hypoxia-induced endothelial cells as a preeclampsia model, quercitrin had antioxidant and anti-inflammatory effects that could reduce the percentage of apoptotic cells, inhibit FGF2 and MDA levels, and enhance the percentage of viable cells.

This study showed that FEE can influence the balance between anti-inflammatory and pro-anti-inflammatory production. FEE suppresses TNF-α secretion and increases the level of IL-10, which indicates its potential as a preeclampsia therapeutic drug.

5 Conclusion

Our research provides information on the potentiation of a local plants that has rarely been explored before. *F. elastica* extract can suppress TNF-α and increases IL-10 in hypoxic-induced human endothelial cells without causing a cytotoxic effect. Thus, it can be concluded that FEE is a viable and safe alternative as a candidate therapy for treating preeclampsia. To the best of our knowledge this is the first study on the usage of the extract of a local Indonesian plant, *F. elastica*, as preeclampsia medicine.

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