



## Effect of Soybean Extract on sFlt-1 LEVELS in Huvecs Cultures Induced by Preeclampsia Plasma

\*Wiwit Indriyani Aslina<sup>1</sup>, Luh Citrarasmi Dara Mestika<sup>1</sup>, Tatit Nurseta<sup>2</sup>,  
Eviana Norahmawati<sup>3</sup>

<sup>1</sup>Midwifery Master Study Programme, Faculty of Medicine Brawijaya University, Malang, East Java, Indonesia, <sup>2</sup> Department of Obstetrics and Gynecology, Faculty of Medicine Brawijaya University, Malang, East Java, Indonesia, <sup>3</sup> Department of Anatomical Pathology, Faculty of Medicine Brawijaya University, Malang, East Java, Indonesia. \*E-mail: [aslinawiwitindriyani@gmail.com](mailto:aslinawiwitindriyani@gmail.com).

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**Abstract:** Preeclampsia is a dangerous pregnancy issue that causes hypertension at  $\geq 20$  weeks of gestation. Oxidative stress is known to play a role in the pathophysiology of preeclampsia by raising the activity of soluble fms-like tyrosine kinase 1 (sFlt-1) and causing endothelial dysfunction. As a result, antioxidants are utilized as a therapy in preeclampsia to protect the body from the impacts of free radicals. This study aims to determine the effect of soybean extract on sFlt-1 levels in HUVECs cultures exposed to preeclamptic plasma. The benefit of this study is the consideration for clinicians to provide soybean ethanol extract supplements (*Glycine max*) to increase the potential of antihypertensive therapy. Human umbilical vein endothelial cell cultures (HUVECs) were used in the study, which was conducted in vitro. HUVECs cultures were exposed to preeclampsia plasma and subsequently treated for 24 hours with various dosages of soybean extract. The enzyme-linked immunosorbent assay (ELISA) measured the sFlt-1 levels in each observation group. The hypothesis was tested using One-Way ANOVA analysis with SPSS version 25 software. This study found a significant difference ( $p < 0,05$ ) between the mean value of the positive control group and the negative control group. A significant difference was also found ( $p < 0,05$ ) in the mean value of sFlt-1 between the positive control group and the treatment group that was given soybean extract at doses of 17,5 ppm. Soybean extract had a significant effect on decreasing sFlt-1 levels in HUVECs cultures exposed to preeclamptic plasma. Thus, soybeans isoflavones have the potential to treat preeclampsia by reducing anti-angiogenic factors.

**Keywords:** Angiogenesis; endothelial; preeclampsia; soybean.

### INTRODUCTION

Preeclampsia is a dangerous pregnancy issue characterized by hypertension at  $\geq 20$  weeks of gestation. Preeclampsia can increase morbidity and mortality in the mother, fetal, and infant because preeclampsia might be accompanied by multi-organ problems (Bouter & Duvekot, 2019; Obstetricians & Gynecologists, 2020; Perkumpulan Obstetri Ginekologi Indonesia, 2016).

Preeclampsia is responsible for more than 70,000 maternal and 500,000 fetal deaths worldwide yearly. Indonesia's maternal mortality rate (MMR) in 2020 was 4,627 cases, with hypertension in pregnancy being the second cause of maternal death, 1,110 cases. East Java's MMR in 2020 was 98.39/100,000 live births; hypertension in pregnancy was the main cause of maternal death, namely 152 individuals (26.90%)

**Corresponding Author:** Wiwit Indriyani Aslina  
Midwifery Master Study Programme, Faculty of Medicine Brawijaya University,  
Jl. Veteran, Ketawanggede, Lowokwaru, Malang, East Java, Indonesia  
Email: [aslinawiwitindriyani@gmail.com](mailto:aslinawiwitindriyani@gmail.com).

(Dinas Kesehatan Jawa Timur, 2021; Kementerian Kesehatan Republik Indonesia, 2021; Rana et al., 2019).

Preeclampsia pathogenesis in the first stage is based on several causal factors, including genetic, immunological, and pre-existing risk factors. These factors cause the remodeling of the spiral arteries to be disrupted, resulting in placental ischemia, which triggers an increase in oxidative stress through increased free radicals (ROS). In the second stage, increased oxidative stress causes an imbalance of angiogenic factors, namely increased sFlt-1. Increased production of sFlt-1 will inhibit the production of placental growth factor (PlGF), which will trigger an increase in vasoconstrictors, namely ET-1 and TxA<sub>2</sub>, a decrease in vasodilators, namely PGI<sub>2</sub> and NO, resulting in endothelial dysfunction in the maternal vascular system and leading to clinical manifestations of preeclampsia (Bokslag et al., 2016; Ives et al., 2020; Kadife et al., 2022; Keman, 2014; Phipps et al., 2020; Rana et al., 2019).

Treatments for preeclampsia, including nifedipine and magnesium sulfate, have side effects such as tachycardia, palpitations, headache, leg edema, nausea or vomiting, muscular weakness, and an increased risk of respiratory depression. Meanwhile, the administration of methyldopa is expensive but ineffective in decreasing blood pressure. Treatment of preeclampsia is often discontinued due to high costs and negative effects. Additional therapy as supplementation, from natural ingredients containing isoflavones, is needed to increase the potency of antihypertensive therapy (Ives et al., 2020; Perkumpulan Obstetri Ginekologi Indonesia, 2016; Safitri & Dewi, 2021).

Soybean is a common healthy food in Indonesia that includes many isoflavones. The isoflavone component in soy with the strongest estrogen-like action is genistein. In preeclampsia, the antioxidant characteristics of the phytoestrogen genistein can reduce the degree of oxidative stress by suppressing ROS generation and enhancing antioxidants, thereby balancing anti- and pro-angiogenic forces. Furthermore, isoflavones, which operate similarly to estrogens, have antihypertensive properties and improve endothelial function by promoting the generation of endothelial nitric oxide synthase (eNOS), increasing nitric oxide (NO) (Ekambaram et al., 2016; Labiba et al., 2020; Odigboegwu et al., 2018; Pabich & Materska, 2019; Wening et al., 2020).

There has been research on the potential of soybean isoflavone against preeclampsia, whereas soybean isoflavone has an antioxidant action that can reduce free radicals. However, little research indicates a direct link between soybeans isoflavones and preeclampsia biomarkers, particularly sFlt-1. A literature review conducted by Cahyani et al. (2020); Sahroh et al. (2020); Tohalifah et al. (2020) found that genistein as part of soybeans isoflavones significantly increased antioxidant levels, then reducing clinical symptoms of preeclampsia through a balance of biomarkers that contribute to the cause of preeclampsia, suggesting that isoflavones have potential as adjuvant therapy in preeclampsia. Further research is needed to expand the literature that can assess the effect of soybean isoflavones on sFlt-1 levels associated with preeclampsia in endothelial cell culture models, so this study aims to determine the effect of soybean ethanol extract on sFlt-1 levels in HUVECs cultured exposed to preeclampsia plasma.

## **MATERIALS AND METHODS**

### **Research Design**

The research design used by the author in this study, namely true experimental with an approach post-test only control group design. The samples used in this study

were the umbilical cord of newborns from vaginal delivery of healthy pregnant women, blood plasma samples of healthy pregnant women and pregnant women with preeclampsia. This study has passed an ethical review from The Health Research Ethics Commission of General Hospital Dr. Saiful Anwar Malang with an ethical approval number of 400/058/K.3/102.7/2023. Before sampling, all research participants were explained and requested to sign an informed consent form willingly.

### **Soybean Ethanol Extract**

Soybeans with Anjasmoro variety were obtained from Balai Penelitian Tanaman Aneka Kacang dan Umbi (BALITKABI) Malang Regency, East Java. At the Pharmacy Laboratory of the University of Brawijaya in Malang, two kilograms of soybean simplisia were extracted with 96% ethanol. The soybean ethanol extract was tested for phytochemicals at the UPT Laboratorium Herbal Materia Medica Batu before being utilized for therapy.

### **Blood Plasma Isolation of Pregnant Women**

Normotensive plasma was taken from healthy pregnant women at 37 weeks of gestation, while preeclampsia plasma was taken from pregnant women diagnosed with severe preeclampsia at 36 weeks of gestation. Samples were taken with informed consent, while blood samples were collected using a 3,2% citrate vacutainer with an aseptic technique, then centrifuged at 1000g for 10 minutes. Aliquot plasma samples into the Eppendorf tube and store them at -40oC refrigerator before use.

### **Isolation of Umbilical Cord Samples**

Newborn cord samples were collected during the vaginal delivery of healthy pregnant women as soon as the placenta was born, with previous agreement. The umbilical cord was cut 15-20 cm long and immediately placed in a cord solution. Endothelial cell culture (HUVECs) was performed up to 8 hours after obtaining the sample.

### **Endothelial Cells Isolation and Culture**

HUVECS culture was created using a method adapted by the Biomedical Laboratory of the Medicine Faculty, Brawijaya University. To remove the remaining blood, insert the cannula into the end of the umbilical vein and rinse the vein with phosphate-buffered saline using a syringe via the cannula. After cleaning the umbilicus, clamp the end of the umbilicus that is not attached to the cannula and insert 8 mL collagenase type II into the umbilical vein through the cannula, then clamp the cannula to keep the fluid inside. Incubate for 5-8 minutes at 37°C, occasionally massaging the umbilicus. Collect the liquid that contains the collagenase and endothelial cells into a tube. Centrifuge at 700 g for 8-10 minutes, remove the supernatant and resuspend the pellet in 10 ml of serum-free medium, which contains 20% fetal bovine serum. The pellets were planted in a 25 cm<sup>2</sup> flask with 0.2% gelatin. Endothelial cells culture were incubated at 37°C until a full monolayer formed, and the media was replenished every 3-4 days. This study used subculture passage 1; subculture was performed when the cells were 80-90% confluent.

### **Treatment**

The treatment given to subculture passage 1 was (1) plasma of normal pregnant women as much as 2% (0.2 mL) in 10 mL of culture medium as a negative control group, (2) plasma of preeclampsia as much as 2% (0.2 mL) in 10 mL of culture medium as the positive control group and (3) administration of soybean ethanol extract doses of 1.75 µl, 3.5 µl, 7 µl, 14 µl and 28 µl, as well as 2% (0.2 mL) preeclampsia plasma in 10 mL of culture medium. According to the observation group, the HUVECs culture was incubated for 24 hours (overnight) after receiving the therapy. In addition,

the supernatant was collected into an Eppendorf tube and kept at -40°C until the levels of sFlt-1 were determined.

**Analysis of sFlt-1 Levels**

Analysis of sFlt-1 levels was carried out after the treatment was given according to the observation group. The levels of sFlt-1 were analyzed using the Enzim-Linked Immunosorbent Assay (ELISA) technique with a sample from HUVECs culture supernatant, and the results were expressed in ng/mL. The method is based on standard protocols in the Human sFlt-1 kit brand BT Lab (Bioassay Technology Laboratory, China, Catalogue Series number E0277Hu).

**Statistic analysis**

Hypothesis testing was carried out using One-Way ANOVA analysis, and the results showed a significant difference, so the analysis was continued with Tukey's HSD Post hoc test. Statistical analysis was assisted with the help of SPSS software version 25.

**RESULTS AND DISCUSSION**

**Phytochemical Screening of Soybean Ethanol Extract**

Before administering the medication, phytochemical screening was performed at the UPT Herbal Materia Medica Batu Laboratory to determine the concentration of bioactive components in an ethanol extract of soybean seeds (*Glycine max*). Table 1 shows the findings of the phytochemical screening related to the amount of bioactive chemicals in the ethanol extract of soybean seeds.

Table 1. Results of Qualitative Phytochemical Screening of Soybean Ethanol Extract

No	Compound Identification	Parameter	Results
1	Flavonoid	Orange, brick red and dark red	Positive
2	Alkaloid	White sediment, orange sediment and brown sediment	Positive
3	Tanin/Fenol	Dark brown or dark blue	Positive
4	Saponin	Permanent foam	Positive

According to the results of the phytochemical screening, the soybean ethanol extract utilized in this study contains a variety of chemicals, including flavonoids (isoflavones, flavonols, and flavones), alkaloids, tannins/phenols, and saponins.

**Effect of Soybean Ethanol Extract on sFlt-1 Levels in HUVECs Cultures Exposed to Preeclampsia Plasma**

Based on the study's findings using One Way Anova, the derived p-value is 0,025, less than  $\alpha = 0,05$  ( $p < 0,05$ ). It was possible to conclude that H0 was rejected, meaning that supplying soybean extract had a significant effect on sFlt-1 levels; in other words, giving soybean extract was proved to reduce sFlt-1 levels in preeclampsia HUVECs models.

Figure 1 shows a significant rise in sFlt-1 levels in the positive control group compared to the negative control group based on the post hoc Tukey HSD test. This is demonstrated by the positive control group having a higher sFlt-1 level ( $21.65 \pm 2.73$ ) than the negative control group ( $15.86 \pm 2.08$ ), as seen in Table 2.

Figure 1 shows the results of a post hoc Tukey HSD test that revealed a significant decrease in sFlt-1 levels in the treatment group that received a dose of 17.5 ppm of soybean ethanol extract. This is demonstrated by the fact that the mean value of sFlt-1 in the treatment group was 17.5 ppm ( $16.43 \pm 2.03$ ) lower than in the positive control group ( $21.65 \pm 2.73$ ), as shown in Table 2. Based on Figure 1 and Table 2, there

was no significant difference in sFlt-1 levels between the positive control group and the treatment group, which was administered soybean ethanol extract at doses of 35 ppm, 70 ppm, 140 ppm, and 280 ppm. According to the findings of this study, treatment of soy ethanol extract at a concentration of 17.5 ppm effectively reduced sFlt-1 levels in HUVECs culture exposed to plasma from preeclampsia patients and even approached the negative control group.

Table 2. Effect of Soybean Ethanol Extract on sFlt-1 Levels

Observational Groups	Mean±SD (ng/mL)	p-value
HUVECs Normotensive	15.86±2.08	
HUVECs Preeclamptic	21.65±2.73	
HUVECs Preeclamptic + 17,5 ppm dose	16.43±2.03	0,025*
HUVECs Preeclamptic + 35 ppm dose	19.02±1.46	
HUVECs Preeclamptic + 70 ppm dose	19.21±2.47	
HUVECs Preeclamptic + 140 ppm dose	17.76±1.11	
HUVECs Preeclamptic + 280 ppm dose	18.42±2.69	

\*Significance (p< 0.05) with One Way ANOVA test.

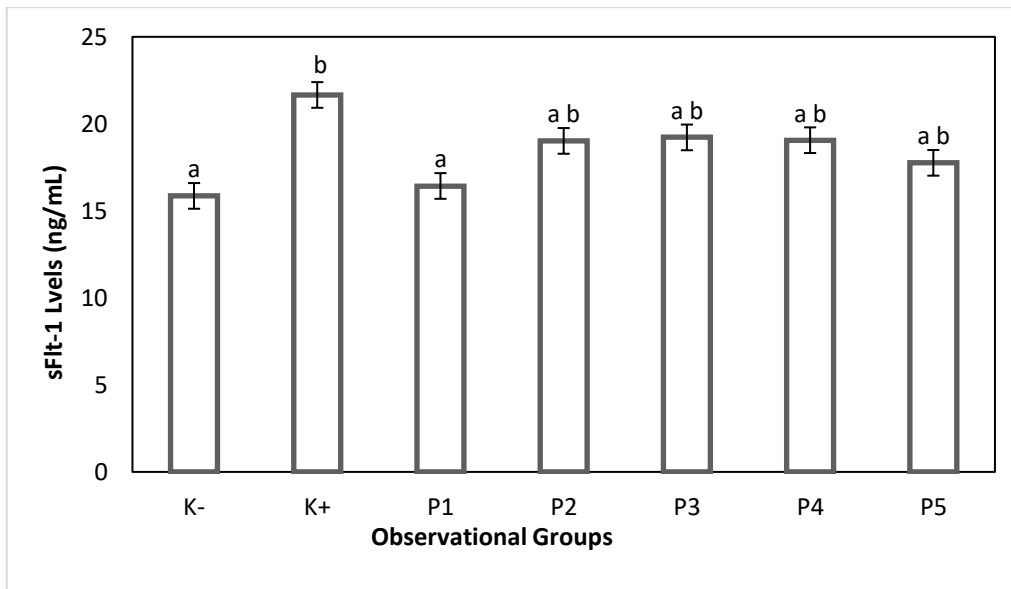


Figure 1. Groups with different letters on histogram bars are statistically significant differences with Tukey's HSD test (p<0,05). K-: negative control group (HUVECs culture + normotensive plasma 2%); K+: positive control group (HUVECs culture + preeclampsia plasma 2%); P1: first treatment (HUVECs Preeclamptic + Soybean Ethanol Extract with a concentration of 17,5 ppm); P2: second treatment (HUVECs Preeclamptic + Soybean Ethanol Extract with a concentration of 35 ppm); P3: third treatment (HUVECs Preeclamptic + Soybean Ethanol Extract with a concentration of 70 ppm); P4: fourth treatment (HUVECs Preeclamptic + Soybean Ethanol Extract with a concentration of 140 ppm); P5: fifth treatment (HUVECs Preeclamptic + Soybean Ethanol Extract with a concentration of 280 ppm).

The cause of preeclampsia was assumed to be an imbalance of angiogenic factors between sFlt-1 and PlGF produced by oxidative stress. Oxidative stress occurs when free radicals surpass antioxidant defense capacity. Placental ischemia causes oxidative stress, characterized by increased free radicals (ROS). High levels of free

radicals reduce the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione, and glutathione peroxidase (GPx) because these enzymatic antioxidants directly neutralize free radicals and are metabolized in the body. The imbalance between free radicals and system scavenging results in oxidative stress, which can cause cell damage because cells in the body have some defenses against free radical damage (Keman, 2014; Rana et al., 2019; San Juan-Reyes et al., 2020).

Based on the results of statistical analysis using One-Way Anova and Tukey HSD, it was found that preeclampsia plasma had a significant effect ( $p < 0.05$ ) on increased sFlt-1 levels in HUVECs culture. Other research conducted by Yeni et al. (2020) stated that sFlt-1 levels in HUVECs cultures exposed to plasma of preeclamptic patients experienced a significant increase compared to HUVECs exposed to plasma of normal pregnant women ( $p = 0.001 < \alpha$ ). Ambarwati et al. (2017) discovered that HUVECs cultures exposed to plasma from preeclamptic patients produced anti-angiogenic factors such as sFlt-1 and sEng considerably more than HUVECs cultures exposed to plasma from normal pregnant women ( $p < 0,05$ ). Other in vitro research claimed that when compared to HUVECs exposed to plasma from normal pregnant women, sFlt-1 levels in HUVECs cultures exposed to preeclampsia plasma increased significantly, and the cell structure looked to be harmed (Ambarwati et al., 2017; Chielie et al., 2020; Christanto et al., 2021; Gunardi et al., 2016; Kadife et al., 2022; Sánchez-Aranguren et al., 2014; Yeni et al., 2020).

Previous studies stated that increased sFlt-1 in preeclampsia was driven by oxidative stress, characterized by increased ROS. Several studies have found that blood plasma from pregnant women with preeclampsia contains several circulating factors with cytotoxic properties, such as syncytiotrophoblast microvilli (STBM), which can inhibit proliferation, vasodilation in endothelial cells, and induce the production of pro-inflammatory cytokines. In addition, preeclampsia plasma also contains ROS, pro-inflammatory cytokines (TNF $\alpha$ , IL-6, IL-4 and IL-8), anti-angiogenesis factors (sFlt-1 and sEng), HIF-1 $\alpha$ , AT1-AA and vasoconstrictor factors (ET-1 and TxA2). As a result, when HUVECs cells are exposed to preeclampsia plasma containing harmful chemicals such as oxidative stress and inflammation, they create more superoxide (O $_2^-$ ). Mechanism of oxidative stress in endothelial cells after exposure to preeclampsia plasma, namely increased ROS including superoxide (O $_2^-$ ), hydrogen peroxide (H $_2$ O $_2$ ), hydroxyl radicals (OH $^-$ ) and peroxynitrite (ONOO $^-$ ) exceeding antioxidants which act as protective agents to prevent oxidative damage caused by high levels of ROS. Superoxide (O $_2^-$ ) rapidly transforms nitric oxide (NO) to peroxynitrite (ONOO $^-$ ), a powerful cytotoxic anion that eliminates NO's angiogenesis capacity. Meanwhile, NO is a vasodilator that reduces hypertension during pregnancy by maintaining a balance of angiogenic factors, decreasing sFlt-1 levels and raising PIGF levels. NO inactivation produces endothelial dysfunction via oxidative stress as the primary mediator of preeclampsia by increasing sFlt-1 production (Huang et al., 2013; Jones et al., 2013; Keman, 2014; Rahardjo et al., 2014; Rana et al., 2019; Sánchez-Aranguren et al., 2014).

A good defense mechanism that includes both endogenous (enzymatic) and exogenous (non-enzymatic) antioxidants was required to decrease free radicals. The enzymatic antioxidants in question are antioxidants produced in the body, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Non-enzymatic antioxidants can be received from outside sources, such as diet, vegetables, and fruits containing vitamin C, vitamin E, Zn, polyphenols, and flavonoids (isoflavones). Isoflavones are polyphenolic chemicals with a structure similar to

estrogen. Hence they have antioxidant properties similar to estrogen. Isoflavones are found in many legumes, particularly soybeans (Fawwaz et al., 2017; San Juan Reyes et al., 2020).

This study found a significant difference in sFlt-1 level between the negative control group ( $15.86 \pm 2.08$ ) and the positive control group ( $21.65 \pm 2.73$ ). The results also found a significant difference in the sFlt-1 level between the positive control group ( $21.65 \pm 2.73$ ) to the treatment group by giving soybean ethanol extract at a dose of 17.5 ppm ( $16.43 \pm 2.03$ ). This is consistent with the findings of Varinska et al. (2015), who discovered that sFlt-1 expression was reduced in HUVECs cells after treatment with genistein soybean extract (Varinska et al., 2015).

Other studies explain that isoflavones might lessen the damaging effects of free radicals by decreasing lipid peroxidation via the activation of endogenous (enzymatic) antioxidant peroxidases such as SOD and CAT. Peroxidase is useful to prevent the accumulation of H<sub>2</sub>O<sub>2</sub>, whose existence becomes dangerous in the company of O<sub>2</sub> because it can create OH radicals, the most reactive and hazardous free radicals, which can damage cell membranes by inducing the dissolution of unsaturated fatty acids. Isoflavone content is a fat-soluble chain-breaking antioxidant that operates on cell membranes to break the lipid peroxidation chain (Tarnajaya et al., 2018).

In addition, the activity of soybean isoflavones as antioxidants in preeclampsia, among others, by attenuating free radical reactivity, lowering serum levels of Low-Density Lipoprotein (LDL), as a ROS scavenger, increasing NO production, which is a potent vasodilator to induce smooth muscle relaxation in blood vessels, activity and increase expression of antioxidant enzymes such as SOD, catalase (CAT), and glutathione peroxidase (GPx), so that antioxidants have stronger potential in fighting free radical reactions under conditions of oxidative stress and do not form prolonged lipid peroxidation and can protect cells from damage caused by free radical oxidation. Soybean has the most estrogen-like isoflavone chemicals, particularly daidzein and genistein. In preeclampsia, genistein's antioxidant capabilities can reduce oxidative stress by decreasing ROS generation and enhancing antioxidants. The reduction in sFlt-1 levels after treatment of soy ethanol extract to HUVECs cells exposed to preeclamptic plasma demonstrates the potential of soy isoflavones as antioxidants by restoring the balance of angiogenesis (Ekambaram et al., 2016; Fawwaz et al., 2017; Labiba et al., 2020; Pabich & Materska, 2019; Yudiono, 2020).

According to earlier research, the action of isoflavones on ROS occurs via two mechanisms: trapping free radicals or neutralizing and boosting endogenous antioxidants such as SOD. Isoflavones have been shown in vitro to increase endogenous antioxidants by boosting the transcription factor Nrf2, which plays a key role in protecting cells from oxidative stress. Nrf2 activates key genes that encode detoxification enzymes and antioxidant proteins, maintaining the balance of pro and anti-angiogenic factors, particularly through the target protein heme oxygenase-1 (HO-1). The function of HO-1 is to metabolize heme to biliverdin, iron, and carbon monoxide (CO). Carbon monoxide (CO) promotes increased synthesis of VEGF, which in turn causes vasodilation and weakens hypertension. HO-1 was also found to inhibit sFlt-1 production in vitro studies. In addition, isoflavones can reduce the activation of p38 MAPK will trigger an increase in anti-angiogenic activity (Jena et al., 2020; Tarnajaya et al., 2018).

According to the Tukey HSD test results in Figure 1, there was a significant drop in sFlt-1 levels in the treatment group that received soybean ethanol extract at a dose of 17.5 ppm. This is demonstrated by the fact that the mean value of sFlt-1 in the treatment group was 17.5 ppm ( $16.43 \pm 2.03$ ) lower than in the positive control

group (21.65±2.73). However, there was no significant difference in sFlt-1 levels between the treatment group and the positive control group when soybean ethanol extract was administered at doses of 35 ppm, 70 ppm, 140 ppm, and 280 ppm.

According to research by Asshidiqy et al. (2020) stated, the strength level of antioxidant activity comprises extremely active (<50 ppm), active (50-100), moderate (101-250 ppm), weak (250-500 ppm), and inactive (>500 ppm). The lower of IC50 value, the greater the antioxidant activity (Asshidiqy et al., 2020). If it is connected with the strength of antioxidant activity, a dose of 17.5 ppm can considerably reduce sFlt-1 levels since it has very active antioxidant activity. Based on the present study's result, it can be concluded that giving a dose of 17.5 ppm soybean ethanol extract reduced sFlt-1 levels in HUVECs cultures exposed to preeclampsia plasma similar to the negative control group.

Based on the hormesis theory, which states that there is a relationship between a therapy's dose and a biphasic reaction. According to the notion of hormesis, administering stress agents such as medications and natural chemicals in low quantities might create a favorable response or provide significant protection. Meanwhile, excessive dosages can have no cytoprotective effect and may even be harmful (Calabrese et al., 2010). According to the hormesis theory, raising the dose to 35 ppm, 70 ppm, 140 ppm, and 280 ppm induces a decrease in the cytoprotective impact of antioxidants on endothelial cells, resulting in a rise in the average value of sFlt-1 levels at higher doses.

This study did not carry out phytochemical screening with quantitative tests, so the total concentration of bioactive compounds in each component contained in soybean ethanol extract was not known. No toxicity test was carried out on the dose of soya bean extract before the study was carried out on HUVECs culture, so the maximum toxic dose on endothelial cells was unknown. Further research is needed to verify that soybean ethanol extract can suppress the rise in sFlt-1 by activating the Nrf2 transcription factor and reducing p38 MAPK. Furthermore, research can be expanded by evaluating biomarkers or circulating components identified in the blood plasma of preeclamptic patients, such as pro-inflammatory cytokines (IL-4, IL-6 dan IL-8), STBM, ROS, AT1-AA and ET-1 among others, which can activate endothelial cells.

## CONCLUSION

Soybean ethanol extract (*Glycine max*) reduced sFlt-1 levels in HUVECs cells exposed to preeclampsia plasma. This study demonstrates the potential of soybean extract to be used as a supplement to increase the potential of antihypertensive therapy by restoring the balance of anti-angiogenic (sFlt-1).

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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