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Ethanolic extract of *Moringa oleifera* leaves improve inflammation, angiogenesis, and blood pressure in rat model of preeclampsia

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ABSTRACT

The aim of this study is to investigate the effect of the ethanolic extract of *Moringa oleifera* leaves (EMOL) to improve inflammation, angiogenesis, and blood pressure by evaluating the serum level of IL-17, soluble vascular endothelial growth factor receptor 1 (sFlt-1), and blood pressure in a rat model of preeclampsia (PE). The rat model of PE was induced with Nω-nitro-L-arginine methyl ester (L-NAME) of 50 mg/kg body weight (b.w) on day 12–18 of gestation. On day 5 of gestation, three groups are given EMOL dose of 50,100, and 200 mg/kg b.w, and one group administered aspirin of 1.35 mg/200 g b.w. Blood pressure was measurement on days 0, 11, and 19 of gestation. On day 20 of gestation, the rats were sacrificed, and serum parameters were analyzed. There is a significant difference in the serum level of IL-17 in all of the groups ($p < 0.05$). EMOL doses of 50 and 100 mg/kg b.w prevent an increase in the serum level of IL-17 better than low-dose aspirin ($p < 0.05$). There was a tendency decrease in sFlt-1 serum levels with EMOL doses of 50 and 100 mg/kg b.w, but statistically there are no significant differences ($p > 0.05$). All the doses of EMOL have the same effect as low-dose aspirin to prevent blood pressure increase in the rat model of PE ($p < 0.05$).

INTRODUCTION

Preeclampsia (PE) is a core cause of maternal–fetal mortality worldwide (Weel *et al.*, 2016). The main clinical manifestation of PE is high blood pressure, which can potentially cause a serious complication for maternal and fetus (Tannetta *et al.*, 2013). The placenta is widely regarded as the core of PE growth. It started with a non-deep trophoblast attack, incomplete, and an imperfect remodeling of the spiral artery that causes immune imbalance, leading to the increased pro-inflammatory cytokine in chronic inflammation, autoantibodies to the angiotensin type 1 receptor (AT1-AA), and soluble vascular endothelial growth factor receptor 1 (sFlt-1) and the decreased nitric oxide (NO) (Cornelius, 2018).

Interleukin 17 (IL-17) is one of the pro-inflammatory cytokines which plays a role in the expansion of systemic inflammation in PE in the maternal immune response to the allogeneic fetus. There is also an imbalance between angiogenesis and antiangiogenesis factor placental hypoxia due to an incomplete placental invasion in these diseases. This imbalance in PE pathophysiology is responsible for the increase of sFlt-1 (Huppertz, 2015; Raghupathy, 2013).

The only definitive treatment is delivery; therefore, prevention is the better way to decrease the impact of this disease. The low-dose aspirin has been used during pregnancy to prevent the onset of PE in women with a high risk of PE before week 16 of gestation and continued until the delivery (Gyamfi-Bannerman and Manuck, 2018).

Natural bioactive compounds have been known to provide endothelial protection through several mechanisms, such as promote the availability of eNOS/NO, mow down oxidative stress, inhibit NF-κB pathways, and decline the expression of cell adhesion molecules (Monsalve *et al.*, 2017). Besides, mineral elements such as copper (Cu) and manganese (Mn) and Vitamins

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D, C, and E contribute to prevent the risk of PE (Azami *et al.*, 2017; Karthivashan *et al.*, 2016).

The leaves of *Moringa oleifera* has been shown to be rich in flavonoids, amino acid, multimineral vitamins, and other essential phytochemicals, which act as an antioxidant and anti-inflammatory to treat hypertension. This can use as food and medicine. A study has been done on the composition of chemical compounds of the leaves of *M. oleifera* with various solvent forms including ether, water, methanol, and ethanol. The ether extract was only found in two compounds, water and methanolic extract consisted of seven compounds, whereas by using ethanol solvents, nine compounds were found: tannins, carbohydrates, saponins, glycosides, reduced sugars, steroids, terpenoids, flavonoids, and alkaloids. The most abundant of flavonoids is along with other phytochemical compounds (Nworu *et al.*, 2013; Olson *et al.*, 2016; Ugwu *et al.*, 2013). Interestingly, flavonoids, especially quercetin, one of the compounds of extract of *M. oleifera* leaves (EMOL), are effective in all hypertension models, regardless of the origin of hypertension, the status of the renin–angiotensin system (RAS), oxidative stress, NO, and other factors. Even so, quercetin does not have a hypotensive effect on normotensive animal control (Perez-vizcaino and Duarte, 2010). The ethanolic EMOL also contains high elements such as Mn and Cu that function as catalytic cofactor and activator antioxidant enzyme superoxide dismutase in chemistry redox of free radical scavenging (Karthivashan *et al.*, 2016).

A research by (Owolabi *et al.*, 2014) suggests that EMOL mitigates effect against lead toxicity in the cortex cerebral. *M. oleifera* as a dietary supplement in food can prevent nickel-induced nephrotoxicity in Wistar rat (Adeyemi and Elebiyo, 2014). As an antioxidant and a modulative influence effect on inflammatory cytokine, EMOL has a therapeutic effect against N-acetyl-p-aminophenol in kidney tissue (Karthivashan *et al.*, 2016). EMOL was used extensively in the prevention or treatment of other disease but not in preventing PE. Therefore, this research aims to investigate the effect of EMOL on the prevention of PE by examining the serum level of IL-17, sFlt-1, and blood pressure in the rat model of PE.

MATERIALS AND METHODS

Experimental design and animals

This is an experimental *in vivo* study with a post-test only control group design. The study was conducted in the Laboratory of Bioscience Institute, Physiology Laboratory, and Biochemistry Laboratory, Universitas Brawijaya, Malang, Indonesia. All experiments in this study were carried out on 24 females Wistar rat weighing between 160 and 200 g of 12–16 weeks old (*n* = 4). The animals were obtained from Bioscience Institute Universitas Brawijaya, Malang, Indonesia. The animals were kept in the standardized animal cages, and the rats were fed with standardized rat pellets in free access to get food and water compliance with the Bioscience Institute of Universitas Brawijaya laboratory protocol (12-hour light–dark cycles, relative humidity, and stable temperature).

Preeclampsia model and treatment

All rats were acclimatized to laboratory condition for 1 week before the study. After a week of acclimatization, each female rat in the estrous cycle was housed with a male rat overnight to facilitate mating. The presence of spermatozoa plug in the vaginal lavage on the next day was counted as day 0 of gestation. On day 5 of gestation, rats were randomized into six groups (*n* = 4): no treatment (control) group, Nω-nitro-L-arginine methyl ester (L-NAME) group that was only administered with L-NAME 50 mg/kg b.w, three groups administered with EMOL doses of 50,100, and 200 mg/kg b.w, and one group which administered with aspirin dose of 1.35 mg/200 g b.w. The EMOL and aspirin were administered on day 5–18 of gestation, whereas PE-like rat model accepted L-NAME of 50 mg/kg b.w for 7 days by gavage, similar to a previous study with modification in time of administering L.NAME starting on day 12–18 of gestation (Brown *et al.*, 2013; Kaya *et al.*, 2011).

Preparation of EMOL

Moringa oleifera leaf powder was obtained from Materia Medika, Batu, East Java, Indonesia. Then, the powder was extracted in the Department of Pharmacological, Universitas Brawijaya. Extraction process was performed using the maceration method with 90% ethanol, followed by evaporation to get the EMOL that are ready to use.

Measurement of blood pressure

On days 0, 11, and 19 of gestation, blood pressure (systolic and diastolic) of the rat was measured using volume pressure recording sensor by CODA non-invasive system from Kent Scientific Corporation (Wang *et al.*, 2017).

Measurement serum level of IL-17 and sFlt-1

The rats were sacrificed using 40 mg/kg of intraperitoneal ketamine HCL and 1 mg/kg of xylazine hydrochloride on day 20 of gestation (Kaya *et al.*, 2011). Blood was collected via the abdominal aorta in vacuum tubes without anticoagulant. Blood was centrifuged at 3,000 rpm for 20 minutes to get the serum and then stored at −80°C before analysis. The serum level of IL-17 and sFlt-1/svascular endothelial growth factor receptor 1 measured by enzyme-linked immunosorbent assay kit was obtained from Elabscience, USA (catalog no: E-EL-RO566 for IL-17 and E-EL-RO911 for sFlt-1). The procedure was carried out by following the instruction by a manufacturer.

Statistical analysis

The results of this study are expressed as mean ± standard error of the mean. A statistical analysis was conducted using the SSPS software version 24.0 Normality test data were performed by using Shapiro–Wilk test, whereas homogeneity by using the Levene's test. The differences between the control and treatment groups were analyzed using a one-way analysis of variance, followed by *post hoc* LSD (least significance different). Data were considered to be statistically significant difference at *p*-value less than 0.05 ($p < 0.05$)

Ethical consideration

This study was conducted in compliance with the principles expressed in the Declaration of Helsinki regarding ethical clearance from the Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia, No. 192/ EC/KEPK-S3/08/2018.

RESULTS

Ethanolic EMOL improves inflammation and angiogenesis in rat model of PE

The serum IL-17 level is a pro-inflammatory cytokine measured in this study. We found that serum IL-17 levels in the group received EMOL doses of 50 and 100 mg/kg b.w had almost the same effect as the control group, whereas the level of IL-17 in groups received EMOL doses of 200 mg/kg b.w and low-dose aspirin was closer to L-NAME/rat model of PE

Both the EMOL doses of 50 and 100 mg/kg b.w prevent the increase in the serum level of IL-17 better than low-dose aspirin (Fig. 1). There is a statistically significant difference in all groups that administered EMOL ($p < 0.05$). The groups received EMOL dose of 50 and 100 mg/kg b.w have significant differences compared to L-NAME group ($p < 0.05$).

Angiogenesis support in this study was measured by assessing the decrease in the serum levels of sFlt-1 or recognized as soluble fms-like tyrosine kinase (sFlt-1) that has antiangiogenic properties. The serum level of sFlt-1 is shown in Figure 2. The serum level of sFlt-1 in groups that received EMOL dose of 50 and 100 mg/kg b.w was almost the same of control group level. Hence, the serum level of sFlt-1 in EMOL dose of 200 mg/kg b.w group and low-dose aspirin group is almost the same as the group that received L-NAME to induce PE model, but statically there is no difference significant between all the groups ($p > 0.05$).

Ethanolic EMOL improves blood pressure in rat model of PE

The blood pressure was measured on day 0 and 11 of gestation to determine the initial blood pressure and the state of blood pressure before administering L-NAME to induce the rat model of PE, whereas the measurement of the blood pressure on the day 19 of gestation was to evaluate the effect of L-NAME to induced rat model PE. Besides, the measurement of the blood pressure on day 19 of gestation was also to evaluate the effect both of EMOL and low-dose aspirin against blood pressure. This study result is shown in Figure 3.

Blood pressure on day 0 and 11 of gestation within normal limits showed no increase, whereas both the systolic and diastolic blood pressures on day 19 of gestation showed an increase, especially in groups that administered L-NAME of 50 mg/kg b.w on day 12 for 7 days, which were able to induce rat model of PE. The administered L-NAME dose of 50 mg/kg b.w can induce the rat model of PE. This was revealed in the increase of blood pressure in L-NAME group compared to control group ($p < 0.05$).

In this study, we found that in all groups, administered EMOL and low-dose aspirin showed that there was no increase in both blood pressures: systolic and diastolic (Fig. 3). This means that EMOL has the effect of preventing an increase in blood pressure in the rat model of PE, and they have statistically significant differences in all groups ($p < 0.05$). Interestingly, all the doses of EMOL 50, 100, and 200 mg/kg b.w have the same effect as the low-dose aspirin to prevent the increase both of systolic and diastolic blood pressures ($p < 0.05$).

DISCUSSION

IL-17 is a fascinating inflammatory biomarker that is in the center of discussion lately because it is a sensitive biomarker that takes a big part in the pathologic of PE. Serum IL-17 level rises in patients with PE compared with healthy pregnant and healthy women who are not pregnant (El Shahaway *et al.*, 2019; Poordast *et al.*, 2017).

The study shows that administering EMOL doses of 50 and 100 mg/kg b.w can prevent the increase in the serum IL-17 level, and its level on both doses is almost the same as the control group. The mechanism to explain how EMOL prevents the increase in the serum IL-17 level is through the pathway of NF-κB. The NF-κB is the main pathway that regulates the inflammatory response through the production of cytokines and other inflammatory mediators, T-cell proliferation and differentiation, and dendritic cell maturation (Ginwala *et al.*, 2019). Besides, hypoxia condition strengthens the NF-κB pathway by increasing toll-like receptor
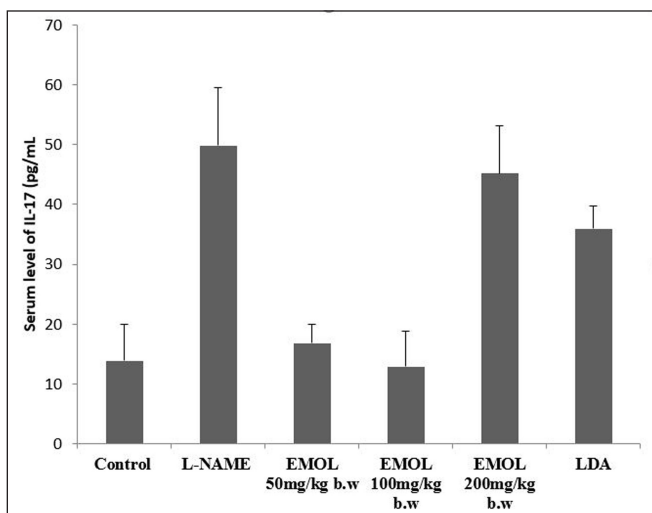
Figure 1. Effect of EMOL on serum level of IL-17 in rat model of PE. Value expressed as mean ± SEM. Serum level of IL-17 significant different between all treatment ($p < 0.05$). EMOL 50 mg.kg b.w and 100 mg.kg b.w have significant different with L-NAME group ($p < 0.05$).
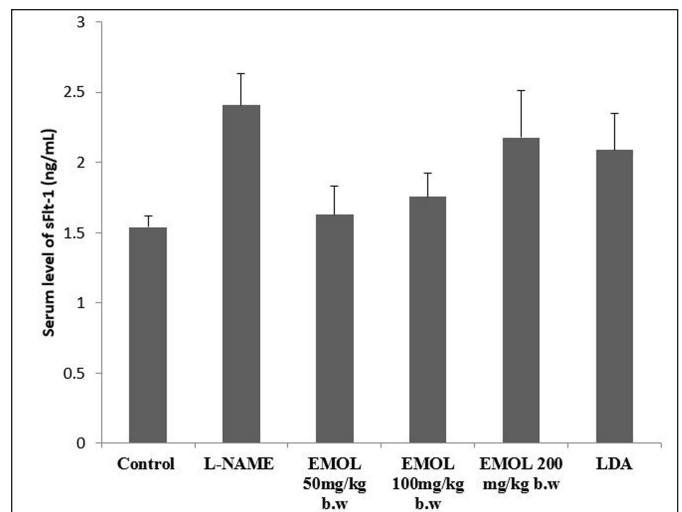
Figure 2. Effect EMOL on sFlt-1 level in rat model of PE. There in trend decrease of serum level sFlt-1 in EMOL 50 mg/kg and 100 mg/kg groups, but no Significant statistically ($p < 0.05$).
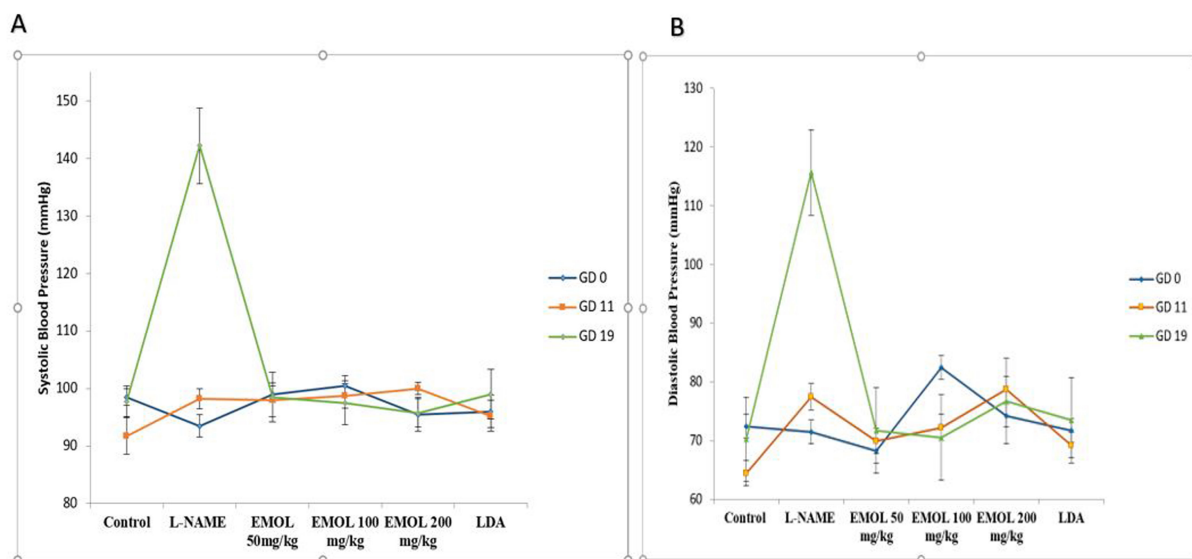
Figure 3. Blood pressure of the different groups on day 0,11 and 19 of gestation. A. Systolic blood pressure. B. Diastolic blood pressure, EMOL and LDA have the same effect to prevent increase of systolic blood pressure and diastolic blood pressure in rat model of PE $p < 0.05$.

(TLR) expression and signaling, which increases the production of antimicrobial factors and stimulates phagocytosis, leukocyte recruitment, and adaptive immunity. Hypoxia-inducible factor 1α (HIF-1α) and HIF-1β translocate to the nucleus, and they were binded as HIF to hypoxia response element, then inducing transcription genes including NF-κB and TLR in hypoxia condition (Schwartz *et al.*, 2011).

Excessive systemic inflammatory responses, oxidative stress, and placental hypoxia in the progress of PE cause the activation of NF-κB signaling and CD4 + differentiation, which, in turn, will increase IL-17 secretion by TH17, AT1-AA, and HIF-1α. An increased IL-17 can stimulate the production of other cytokines such as IL-6, IL-8, TNF-α, granulocytes, and macrophage colony factors in PE (Cornelius, 2018; Fu *et al.*, 2014). This condition worsens the development of this disease. Therefore, prevention is a better choice to reduce the fatal impact of PE which is maternal and fetal mortality.

The EMOL can inhibit the increase in the serum level of IL-17 in the rat model of PE because the active compounds of EMOL have anti-inflammatory activity (Molvarec *et al.*, 2015; Pal *et al.*, 2012). Anti-inflammatory bioactivity EMOL, which are flavonoid compounds, also work through an effective inhibition of IL-17A to their receptors (IL-17RA), which, in turn, causes the inhibition signaling of inflammation induced by TH17 cells that produce IL-17 (Arulselvan *et al.*, 2016). The interaction between IL-17 and its receptors and NF-KB signaling is inhibited, and scavenging free radicals by EMOL flavonoids together with other pro-inflammatory cytokines and oxidative stress that worsens PE can be prevented.

Flavonoids can avoid cell damage by scavenging free radicals directly and thereby avoid their deleterious effects (Mahmoud *et al.*, 2019). An increase in the serum level of IL-17 in EMOL dose of 200 mg/kg b.w in groups in this study might have been because the cause of this dose is outside the effective dose for rat condition, and thus, it is a disadvantage.

The result of the serum levels of sFlt-1 in this study is shown in Figure 2. There is a significant difference in the level of sFlt-1 in L-NAME group compared to control group ($p < 0.05$). The serum level of sFlt-1 in groups that administered EMOL dose of 50 and 100 mg/kg b.w is almost the same as control group, whereas in EMOL dose of 200 mg/kg b.w and low dose aspirin (LDA) approaches to the level of the L-NAME group, but there was no statistically significant difference. Although there is no statistically significant difference, there is a trend decrease of the serum level of sFlt-1 after administered EMOL. There are two possibilities for this result. First, sFlt-1 levels in the maternal circulation are reduced after Moringa leaf extract was given, but sFlt-1 was still produced and released by the placenta into the maternal circulation. Palmer *et al.* (2017) stated that the primary source of sFlt-1 is secretion of the placenta, which is secreted into the maternal circulation. Second, the prevention mechanism of increased blood pressure and excessive inflammation in a rat model of PE by EMOL does not occur via angiogenesis pathway and that is a need to be explored furthermore.

Ethanolic EMOL provides antihypertension in this study. EMOL prevents an increase in the systolic and diastolic blood pressure. Interestingly. EMOL and low-dose aspirin provide the same effect to prevent an increase in blood pressure. L-NAME has been observed to induce NO deficient, and a state of NO deficiency has been known to impair the relaxation vascular in human and animal models of PE. In this study, L-NAME was used to induce the rat model of PE (Korokin *et al.*, 2011; Liu *et al.*, 2017).

Hypertension is due in large part to increased oxidative stress, endothelin-1 overproduction, reduced production and/or bioavailability of NO, and/or RAS overstimulation. The antihypertensive effects of flavonoids are reduced oxidative stress, inhibition activity of angiotensin-converting enzyme, enhanced endothelial function, active effect on the vascular smooth muscle, and/or cell signaling modulation (Larson *et al.*, 2010). Although we do not measure the mechanism of action of the bioactive

compounds of EMOL in blood vessels such as the availability of eNO/NO, oxidative stress, and cell adhesion molecules in this study, we can argue that EMOL prevents an increase in blood pressure through the ability of the bioactive component EMOL, which maintains a balance of vasodilation and vasoconstriction factors of blood vessel (Amaral *et al.*, 2017; Li *et al.*, 2012).

Flavonoids also increase baroreflex sensitivity by reducing oxidative stress and modifying blood vessel elasticity and resistance, body fluid volume, autonomic nervous system, and angiotensin–renin system through anti-inflammatory and antioxidant activity (Monteiro *et al.*, 2012).

EMOL containing essential elements such as Cu and Mn are also considered to be catalytic factors of the Cu/Zn-SOD and Mn-SOD antioxidant enzymes that can increase the activity of free radical scavenging and thus boost metabolism, whereas Cu is required for the metabolism of Fe and energy and can also act as a reduction agent in lysil oxidation enzymes cytochrome oxidase, superoxide dismutase, and dopamine hydroxylase (Karthivashan *et al.*, 2016).

In this study, EMOL and low-dose aspirin are given early in pregnancy, on day 5 of gestation. Therefore, both EMOL and low-dose aspirin can provide an effect to reduce the risk factor in a rat model of PE by preventing an increase in the serum level of IL-17 and blood pressure. The low-dose aspirin was also known to more effectively reduce the risk of PE if used in early gestational age (before 16 weeks of pregnancy) until delivery (Roberge *et al.*, 2016; Xu *et al.*, 2015).

CONCLUSION

The ethanolic EMOL can improve inflammation and blood pressure by preventing increased serum level of IL-17 and blood pressure in the rat model of PE. EMOL also supports angiogenesis in a trend decrease of serum level of sFlt-1 but not statistically different.

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CONFLICT OF INTEREST

Authors declared that there is no conflicts of interest.

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