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Effect of Nigella sativa Ethanol Extract on the Nitric Oxide Content and Renal Arteriole Diameter of a Pre-eclampsia Mouse Model Introduction Pre-eclampsia is a disorder diagnosed by an increase in blood pressure and proteinuria at 20 weeks or more of pregnancy [1]. The ischemic placenta produces soluble factors and cell debris in the blood-stream, causing systemic inflammation, along with maternal oxidative stress.

This is believed to be the key factor causing endothelial dysfunction and the major symptoms leading to pre-eclampsia [2]. Healthy endothelial cells produce balanced amounts of endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors to support the cardiovascular system. Nitric oxide (NO) is an EDRF that induces the relaxation of smooth muscles in blood vessels through guanylyl cyclase/cyclic guanosine monophosphate (sGC/cGMP).

This smooth muscle relaxation causes the dilatation of blood vessels that suppresses vascular resistance and optimizes tissue perfusion, including that in the arterioles [3]. NO production is decreased in pre-eclampsia, which leads to vasoconstriction, increase in vascular resistance, and elevation of blood pressure [4-8]. Moreover, the vasoconstriction in the renal arterioles decreases the renal blood flow and glomerular filtration rate.

This causes a decline in kidney performance and contributes to increases in systemic vascular resistance and blood pressure [9, 10]. Nigella sativa (black cumin) has long been used as a medication, especially in the Middle East and India. Several studies have shown that the active compounds present in N.

sativa have anti-diabetic, anti-tumor, anti-hypercholesterolemia, anti-hypertension,

anti-inflammation, and digestive protection activities [11]. In particular, thymoquinone can act as an anti-inflammatory agent by inhibiting NF-kB protein; it also exerts antioxidant activity [12-14]. Here we investigated the potential of *N. sativa* ethanol extract to increase NO levels as a marker of endothelial cell dysfunction.

We found that it can induce the dilatation of the renal arteriole in pre-eclampsia mice model.



Materials and Methods Pre-eclampsia induction was performed by injecting 0.1 mL of serum taken from severe pre-eclampsia patients on days 10 and 11 of gestation.

Thirty mice were divided into six groups: a negative control group (normal pregnant mice), a positive control group (pre-eclampsia model mice), and four treatment groups. Mice in the treatment groups for whom pre-eclampsia induction was previously performed were given *N. sativa* doses of either 500, 1000, 1500, or 2000 mg/kg BW/day. Because of being an experimental animal study, no informed consent was obtained.

*N. sativa* seed extraction was performed following published methods, with only minor modifications [15, 16]. The extract doses were based on those used by Meziti [13]. The extract was orally administered from 15 days of pregnancy until 19 days of pregnancy. Following the extract treatment, NO levels in the serum of the mice were measured using a Colorimetric Griess device (RnD Systems, Minneapolis, USA).

Next, the diameter of the renal arteriole was measured using hematoxylin-eosin staining of the renal tissue. The experimental protocol was approved by Ethical Clearance from the Research Ethics Committee (Animal Care and Use Committee) of the Brawijaya University No. 567/EC/KEPK-S3/11/2015. Statistical Analysis Statistical Package for the Social Sciences 16 for Windows (SPSS Inc.;

Chicago, IL, USA) as a statistical program application was used for data analyses. One-way analysis of variance (ANOVA) and least significance difference (LSD) were used to determine the significant differences among the data for each mice group.  $p < 0.05$  was considered to be significant.

Results NO levels were significantly higher in the normal pregnant mice (negative control group) than in the pre-eclampsia model. The renal arteriole diameter was also larger in the normal pregnant mice than in the pre-eclampsia model (Table 1).



Figure 1. The effect of **N. sativa ethanol extract on** NO levels of pre-eclampsia mice model Figure 2. The effect of N.

sativa ethanol extract on **renal arteriole diameter of** pre-eclampsia mice model  
\_One-Way ANOVA helped identify significant differences in NO levels across the six groups ( $p < 0.001$ ). The four N. sativa-treated groups had higher NO levels than the pre-eclampsia model [ $85.77 \pm 4.47 \mu\text{m}$  (500 mg/kg BW/day),  $189.04 \pm 6.01 \mu\text{m}$  (1000 mg/kg BW/day),  $226.56 \pm 2.13 \mu\text{m}$  (1500 mg/BW), and  $207.98 \pm 4.74 \mu\text{m}$  (2000 mg/kg BW/day) vs.  $70.67 \pm 4.86 \mu\text{m}$  (pre-eclampsia model)].

NO **levels tended to increase with increasing** N. sativa dose (Figure 1). One-Way ANOVA helped identify significant differences in renal arteriole diameter across the six groups ( $p < 0.001$ ). Moreover, using LSD test, it was found that the **renal arteriole diameter of** the pre-eclampsia model ( $8.59 \pm 1.21 \mu\text{m}$ ) was significantly smaller than those of mice treated with **N. sativa ethanol extract** (1000 mg/kg BW/day, 1500 mg/kg BW/day, and 2000 mg/kg BW/day ( $15.15 \pm 2.21 \mu\text{m}$ ,  $16.35 \pm 2.52 \mu\text{m}$ , and  $15.76 \pm 3.03 \mu\text{m}$  respectively). These data show that N.

sativa ethanol extract causes the renal arteriole to dilate, up to a dose of 1500 mg/BW/day (Figure 2).



Discussion Pre-eclampsia induction in mice can be monitored by following the progress of key symptoms, such as **increase in blood pressure**, proteinuria, increase in placental secretions (sEng), kidney abnormality, and renal arteriole vasoconstriction [17].

Angiotensin II type I receptor agonistic autoantibodies (AT1-AAs) induce **reactive oxygen species (ROS)** production through nicotinamide adenine dinucleotide phosphate oxidase. An AT1-AA-containing serum sample was injected into pregnant mice [18, 19]. Moreover, this serum also contains higher TNF- $\alpha$  concentrations that can further induce NF- $\kappa$ B production [20, 21].

Along with ROS, NF- $\kappa$ B as **the transcription factor** activates HIF1- $\alpha$ , which then leads to an increase in the expression of soluble FMS-like tyrosine kinase 1 in the placenta [2, 23, 24]. **Soluble FMS-like tyrosine kinase 1** is an angiogenic factor that enters the maternal blood stream and binds **vascular endothelial growth factor (VEGF)**.

Under these conditions, VEGF levels decrease, causing endothelial cell damage due to the inability of VEGFR to bind VEGF [25]. VEGF levels are also related to the activation of endothelial nitric oxide synthase (eNOS), which induces NO synthesis [26]. Thus, the reduced VEGF levels **lead to a decrease in** NO synthesis [27].

Furthermore, NO easily reacts with superoxide, forming peroxynitrite, and reduces NO bioavailability [28, 29]. NO is an EDRF that induces the dilatation of smooth muscles in blood vessels. This activates the soluble guanylyl cyclase (sGC) enzyme, which catalyzes the formation of cyclic guanosine 3',5'-monophosphate (cGMP) from guanosine 5'-triphosphate [30].

Moreover, NO/sGC/ cGMP acts as an intracellular signal and leads **to the activation of** several effector molecules, ultimately resulting in vasodilatation and vascular resistance [31]. Thus, **this can account for lower NO levels and larger renal arteriole diameter detected in pre-eclampsia mice than in the control group in the present study** [31-35].

We suspect that thymoquinone, which scavenges superoxide, is the principal active compound responsible for the changes in NO levels **and renal arteriole diameter** observed in the present study [36, 37]. Thymoquinone is also known to act as an anti-inflammation agent by inhibiting NF- $\kappa$ B in a dosage-dependent manner [13, 38-40].

Thymoquinone, as the inhibitor of NF- $\kappa$ B, causes a decrease in HIF1- $\alpha$  activation in cases of pre-eclampsia. Furthermore, thymoquinone **plays an important role in** sFLT-1 synthesis, which triggers VEGF to bind its receptor on endothelial cells. This increases

the activation of the eNOS cascade and NO synthesis in endothelial cells. Previous studies have shown that N.

sativa extract can prevent tissue damage caused by ROS. The scavenger effect of thymoquinone and its derivatives has been investigated on several ROS; and they have been shown to have a strong antioxidant activity. Thymol scavenger effect is related to singlet oxygen, while both thymoquinone and dithymoquinone have the similar activity to superoxide dismutase [14].

Therefore, N. sativa treatment prevents NO from interacting with superoxide so that the product peroxynitrite is prevented from causing harm to the endothelial cells.

Otherwise, N. sativa might inhibit several different targets, including calcium channels, inositol triphosphate, and intracellular Ca<sup>2+</sup> release [41]. N. sativa induces smooth muscle relaxation by blocking voltage-operated Ca<sup>2+</sup> channels, which leads to the dilatation of blood vessels [42].

The main limitation of this study is that the results focused only on the effect of N. sativa ethanol extract on NO levels and renal arteriole diameter of a pre-eclampsia mouse model dependent on the dose that we used. Because this was an in vivo study, we could not find the exact underlying mechanism of how N.

sativa ethanol extract increases NO levels and enlarges the renal arteriole diameter. Besides we did not test the chemical substances or chemical compounds contained in our extracts; thus, we could not estimate the chemical compound that played a significant role in the mechanism. In summary, N.

sativa ethanol extract treatment increases NO levels and enlarges the renal arteriole diameter of a pre-eclampsia mouse model in a dose-dependent manner. Furthermore, NO levels and renal arteriole diameters of pre-eclampsia mouse model (without N. sativa ethanol extract treatment) were less than those of other mouse groups.

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