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Ethanol Extract of Black Cumin Seed (*Nigella Sativa*) Reduces Expression Kidney iNOS in Mice Model Preeclampsia

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Abstract: This study aims to analyze the effect of ethanol extract of black cumin seeds (*Nigella Sativa*) on iNOS expression in the kidney of mice model preeclampsia. Design This study uses an *experimental* design with *a posttest only control group design.* Using a mice model preeclampsia. This study was divided into 6 groups: negative control, positive control (preeclampsia model), model + black cumin seed extract 500 mg/KgBB/day, 1000 mg/KgBB/day, 1500 mg/KgBB/day, and 2000 mg/KgBB/day days, after surgery on mice then examined iNOS Kidney expression by methods Immunohistochemical. Data from observations were analyzed by one way ANOVA test. The result there is an effect of ethanol extract of black cumin seeds on renal iNOS expression in mice model preeclampsia ($p < 0.05$) at doses of 500mg, 1000mg, 1500mg, and 2000mg on decreasing renal iNOS expression. The conclusion Ethanol extract of black cumin seeds (*Nigella sativa*) can reduce iNOS Kidney expression in Mice Preeclampsia.
Keyword: black cumin seeds (Nigella sativa); iNOS; kidney; mice preeclampsia

INTRODUCTION

Preeclampsia is a disorder that affects 5-10% of pregnancies, characterized by hypertension and proteinuria in the 20th week of pregnancy (Sánchez et al. 2014). Which causes high morbidity and mortality in both mother and fetus (Shah et al.2015; WHO 2011). In 2013 hypertension in pregnancy was the second leading cause of maternal mortality (MMR) in Indonesia as much as 27.1% (Ministry of Health Republic of Indonesia. 2014).

Preeclampsia is a pregnancy disorder characterized by hypertension (> 140 / 90mmHg) and proteinuria (> 300 mg / l) occurring after 20 weeks of pregnancy, which can cause morbidity and mortality (Herse et al. 2008; Matsubara et al. 2015). The Cause of preeclampsia is still unknown because preeclampsia is "the disease of theories" (Cunningham,2010). The results of several research factors that can cause preeclampsia to include an imbalance of maternal adaptation that causes systemic inflammatory reactions and endothelial dysfunction (Saucedo et al. 2014; Yi K.W. et al. 2014).

The pathogenesis of preeclampsia consists of two stages, and the first is a stage of placentation abnormality characterized by abnormal placental development during the first trimester resulting in placental insufficiency and excessive release of pl.acental particles into the maternal circulation. The second stage is a maternal syndrome characterized by hypertension, kidney damage, proteinuria, eclampsia, HELLP syndrome (hemolysis, increased liver function, thrombocytopenia), and other organ damage (Hladunewich et al. 2007).

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iNOS is one of three essential enzymes that produce NO from the amino acid L-arginine. iNOS can be expressed in various types of cells in response to lipopolysaccharides, cytokines, or other agents. iNOS produces large amounts of NO, which have a cytostatic effect on parasitic target cells and contribute to the pathophysiology of inflammatory diseases and septic shock (Förstermann, 2012). The involvement of iNOS in the environment oxidative stress will reduce the bioavailability of NO as a vasodilator (Cooke & Davidge. 2002). Independent Calcium iNOS plays a vital role in inflammation. Inflammation induces iNOS expression in blood vessel endothelium resulting in excessive NO production through increased inflammatory cytokines such as interleukin-1 (IL-1), IL-6, TNF, and interferon γ (INFγ). In preeclampsia, adhesion molecules accelerate inflammation further inflammation induces systemic and placental blood vessels causing the failure of uteroplacental perfusion (Saucedo et al. 2014).To overcome oxidative stress, it is necessary to provide antioxidants. Antioxidants are electron compounds that can capture free radicals and can reduce the negative effects of oxidants. One of the antioxidants that are well known by the public and used as a traditional treatment for coughing, asthma, abdominal pain, diarrhea, and other diseases, namely Nigella sativa/ Black cumin seeds.

Black seed/Nigella Sativa is one type of herbal plants. Cumin Seed extracts black, there are main components, namely thymoquinone (TQ) has therapeutic effects as antioxidants, anti-inflammatory, antineoplastic, analgesic, antimicrobial, and anti-hypertensive. Nigella sativa (NS) has strong antioxidant properties. Previous studies have documented that in treatment with TQ, organs are protected against oxidative damage caused by various free radical agents such as carbon tetrachloride and include alkylating agents, cisplatin, and doxorubicin. The free radical scavenging effect of TQ, thymoquinone, and thymol has been tested against several ROS. The compounds tested from Nigella sativa provide a powerful antioxidant effect, namely thymol acts as a single oxygen sequencer, while TQ and thymoquinone show the activity of superoxide dismutase (SOD) (Leong et al. 2014).

Research on the administration of Nigella sativa to Renal iNOS expression in rat preeclampsia models has never done. Therefore, this study aims to prove the effect of Nigella sativa ethanol extract on decreased iNOS Renal expression in mouse preeclampsia models.

MATERIALS AND METHOD

Design The study used in this study was experimental (experimental), with a post-test only control group design. The sample used was a model of preeclampsia in pregnant mice under a research ethics permit approved by the UB institutional review board No. 567 / EC / KEPK / 11/2015. This study divided into six groups: with the details of the control group in group 1 of normal pregnant mice, group 2 of preeclampsia mice, and the treatment group, which given a group of black cumin ethanol extract. 3: dose of 500 mg/kg body weight/ day, in group 4: dose 1000 mg/kg body weight/ day, in group 5: dose 1500 mg/kg body weight/ day and in group 6: dose 2000 mg/kg body weight/day.

Procedure for making mice preeclampsia model: Serum of severe preeclampsia of the mother is injected intraperitoneally on pregnant mice at gestational 10^{th} & 11^{th} day. To ensure the making of preeclampsia mice by measuring blood pressure using CODA and urine protein using a quantichrom protein assay kit at gestational days 15 (Teixeira, 2014; Wicaksono et al. 2015).

Procedure for making cumin extract black: Black cumin seeds are cleaned. The process of making black cumin extract through 3 stages: the drying process, the extraction process (the results of this extract will be used in the study of this extract in the form of thick liquid (dark oil), and dark brown close to 100 cc black, active substance concentration this extract is 100%) and, the evaporation process.

Measurement of iNOS

Mice kidney organs are taken, put into formalin 10% for at least 72 hours, a process of cutting the blockage tissue and preparations cut with a microtome 4-5 microns thick, and affixed to a glass object and then examined by the immunohistochemical method (IHC). In the process of painting, deparaffinization is done by heating at a temperature of 60^{O}C for 60 minutes. Then it is soaked in sequence with xylol, absolute ethanol, 90%, 80%, 70% for 5 minutes each, then washed with sterile distilled water three times 5 minutes. Perform retrieval antigens with citrate buffer and heating, immunohistochemical staining with primary antibody NOS2 (N-20) (SANTA CRUS BIOTECHNOLOGY with catalog no. Sc-7271) and CPC of Merck Biocare Medical kits and then incubated overnight at 4°C for 18 hours. Incubation with secondary antibodies for 60 minutes, incubation of Streptavidin Horseradish Peroxidase (SA-HRP) for 40 minutes, application of Chromagen Diaminobenzidine (DAB), counterstain with Mayer's hematoxylin, and mounting with etellan, and observed under a microscope at 400x magnification in 10 field of view photographed by using a camera (Canon DSLR) and transferred to a computer for measurement of iNOS expression in the kidney. Observation data were analyzed by one way ANOVA test with SPSS

RESULTS AND DISCUSSION

Picture of iNOS Placental Expression in Mice Model of Preeclampsia iNOS Expression on Immunohistochemistry Examination

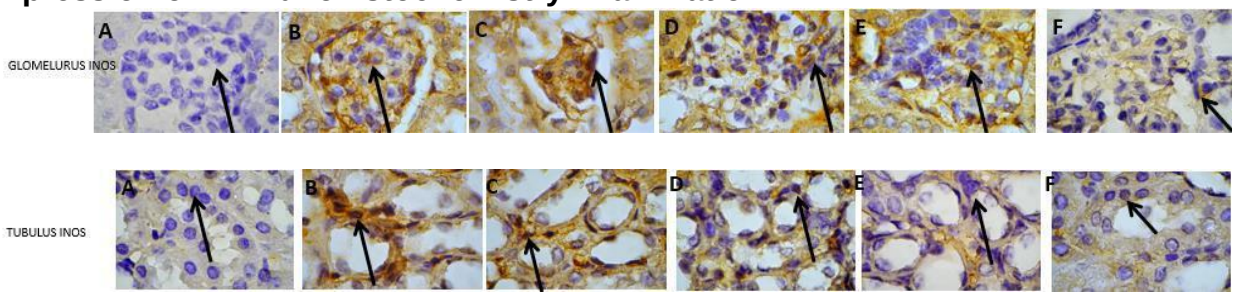

Figure 1. Results of Comparison of Kidney

Figure 1 shows in the arrow that the expression of iNOS increased in the positive control group (B) brown color in kidney tissue when compared with negative control (A), whereas in the treatment group showed that in treatment 1 (C) the dose 500mg / KgBB / day and treatment 2 (D) dose 1000 mg / KgBB / day the expression began to decrease compared to the positive control (B), while in the third treatment (E) dose 1500 mg / KgBB / day and the fourth treatment (F) dose of 2000 mg / Kg Bodyweight/day of expression is close to negative control, indicating that expression decreases with Ns (400x magnification).

Parametric prerequisite test results

Table 1. Test results for normality of data Kidney iNOS expression

observation group	*p-value* of iNOS expression	Distribution
Negative control	0,492	normal
Positive control (PEB)	0,737	normal
PEB + ethanol extract NS 500mg	0,967	normal
PEB + ethanol extract NS 1000mg	0,899	normal
PEB + NS ethanol extract 1500mg	0,777	normal
PEB + ethanol extract NS 2000mg	0,314	normal

Table 1 shows the Shapiro-Wilk test results obtained that iNOS expression data for each observation group has shown *p-values* that are all greater than the significance level α = 0.05 proven normally distributed

Table 2. Results Comparison of iNOS Kidney expression

Observation group	n	Average ± stan.dev	*p-value*
Positive control (PEB)	5	18.80 ± 3.35^{a}	
PEB + NS ethanol extract 500mg	5	14.00 ± 1.58^{b}	0,000
PEB + NS ethanol extract 1000mg	5	8.80 ± 2.39^{c}	
PEB + NS ethanol extract 1500mg	5	4.80 ± 1.48^{d}	
PEB + NS ethanol extract 2000 mg	5	3.20 ± 0.837^{d}	

Table 2 shows the results of the One Way Anova test on iNOS expression data in the kidney obtained that there were significant differences in the mean expression of iNOS in the kidneys of the six groups of the observed sample group, this indicated by the value (*p-value*= 0.000 < α .

There were significant differences in the mean iNOS exposure in the kidneys between negative control groups (2.60 ± 1.52) and positive controls (18.80 ± 3.35) and similarly there were significant differences between positive controls with the treatment group giving ethanol extract Nigella sativa dose 500mg, 1000mg, 1500mg and 2000mg. This means that there is an effect of ethanol extract of Nigella sativa on mice of the preeclampsia model to decrease iNOS expression in the kidney.

Table 3.Significance Levels Of Differences In Nigella Sativa Doses Between Treatment Groups Based On Post Hoc Tests

GROUP	PEB + NS500MG	PEB+ NS1000MG	PEB+ NS1500MG	PEB+ NS2000MG
PEB + NS500MG		p=0,005	p=0,000	p=0,000
PEB+NS1000MG	p=0,005		p=0,047	p=0,003
PEB+NS1500MG	p=0,000	p=0,047		p=0,808
PEB+NS2000MG	p=0,000	p=0,003	p=0,808	

Table 3 shows the significant differences in the administration of Nigella sativa at doses of 500mg with 1000mg, 1500mg and 2000mg, at doses of 1000mg with

500mg, 1500mg and 2000mg which are said to be significantly different when p = <0.005 while those at doses of 1500mg with 2000mg were not significantly different.

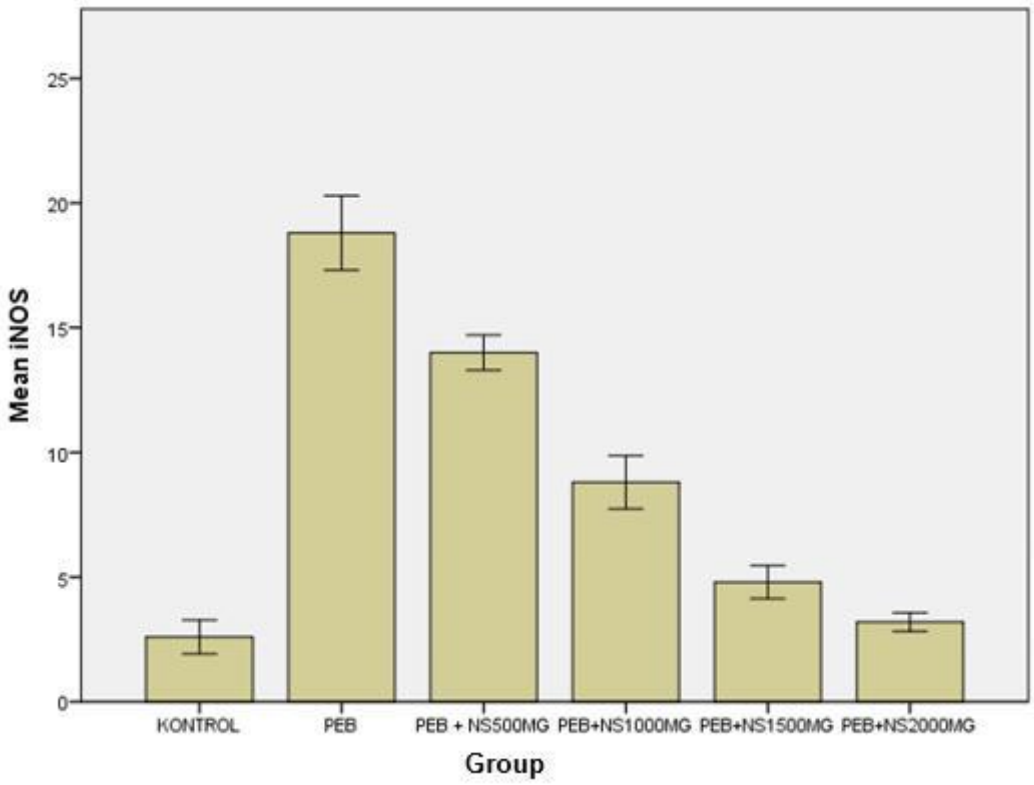

Figure 2. Histogram of iNOS expression of kidneys in mice preeclampsia model

Histogram of iNOS expression in kidneys in the negative control (healthy mice), positive control (mice model of preeclampsia), and four groups of preeclampsia mice and administration of ethanol extract control Nigella sativa at a dose of 500mg, 1000mg of, 1500mg, and 2000mg.

There was a decrease in the mean expression of iNOS in the kidneys in various doses compared to the mean in the positive control group. The mean value of iNOS expression in the kidneys was lowest in the treatment group giving ethanol extract Nigella sativa at a dose of 2000 mg and the closest value in the negative control group. It can also be said that the higher the dose is given, the lower iNOS expression in the kidney.

The effect of maternal serum preeclampsia on iNOS expression in the kidneys

About Figure 1 shows that the expression of iNOS increased in the positive control group brown color in kidney tissue when compared with the negative control. This means that in mice the preeclampsia model is characterized by high iNOS expression when compared to the negative control group.

iNOS is one of three essential enzymes that produce NO from the amino acid L-arginine. iNOS plays a vital role in physiological and pathophysiological conditions, for example, regulation of blood pressure, inflammation, infection, and the onset of disease development(Matsubara et al.2015). iNOS has a vital role in hemodynamic changes in pregnancy kidney, the expression of the renal protein in pregnant women induced by iNOS (Alexander et al.2002).

The results of this study are by Sánchez-aranguren et al. (2014) In an in vitro study iNOS mRNA expression increased in cells treated with serum from preeclampsia women(Sánchez et al. 2014)

Other studies conducted by Zhen et al. (2007) showed that increased regulation of eNOS and iNOS in the kidneys and aorta was the impact of increased ROS activity in in vitro studies of human coronary artery endothelial cells (HCAECs), this effect was partly mediated by a decrease in NO so as to exert the influence of negative feedback on NOS expression through NFKB activation (Zhen et al.2007)

The results of Amaral et al. (2013) model Reducing Uteroplacental Perfusion Pressure (RUPP, showing that vascular expression of iNOS is an up-regulation of a mouse model of preeclampsia (RUPP), there is an increase in iNOS expression as well as oxidative and nitrosative stress associated with RUPP. iNOS promotes oxidative and nitrosative stress and hypertension in the RUPP animal model (Amaral et al. 2013).

Effect of ethanol extract of cumin seeds on renal iNOS expression.

About tables 2, There was a significant effect of ethanol extract of cumin seeds on decreasing renal iNOS expression with a p-value of 0,000 ($p < 0.05$). the treatment group given ethanol extract of cumin seeds, there was a decrease in the mean expression of iNOS along with the increasing dose of ethanol extract of Nigella sativa at doses of 500mg, 1000mg, 1500mg and 2000mg, the most optimal dosage to reduce renal iNOS expression is dose 1500mg / KgBB / day and 2000mg / KgBB / day. Black Cumin (Nigella sativa) or Black Seed has the main active ingredient TQ that can prevent organ damage due to free radicals and free radicals and able to prevent reactive oxygen species (ROS) (Mansour et al. 2002).

In the study of Chehl et al (2009) Nigella sativa containing Thymoquinone, TQ is a significant component of oil extract Nigella sativa, inhibits proliferation in the pancreatic ducts of adenocarcinoma cells, can inhibit the transcription of NF-kB through reducing its promoter activity, first inhibiting the signaling pathway of NF-kB and inhibits transcription, TQ as an inhibitor of the inflammatory pathway which is a merging as an anti-inflammatory and antiapoptotic (Chehl et al. 2009).

The results of this study are by the research of Sethi et al. (2008) reporting that Nigella Sativa containing Thymoquinone, TQ has been shown to suppress the proliferation of various tumor cells, including colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, and pancreatic carcinoma. With TQ treatment after 4 hours of administration almost maximally inhibits activation of NF-kB p65 even after 6 hours does not activate NF-kB. TQ works by activating NF-κB correlated with inhibition of activation of IκBα kinase, phosphorylation of IκBα, degradation of IκBα, p65 phosphorylation, p65 nuclear translocation, and gene expression.

Research conducted by Gado & Yassen 2012 on iNOS activity in pulmonary toxicity due to oxidative damage produced by the generation of free radicals then treated with Nigella sativa shows that thymoquinone significantly weakens the development of pulmonary toxicity through inhibition of iNOS activity thereby reducing the formation of peroxynitrite (Gado et al. 2012). Similarly, research conducted by Fathy & Nikaido 2013 on the involvement of iNOS in various tumors, one of which is hepatocellular carcinoma, which then treated with Nigella sativa shows that Ns have antitumor benefits and can suppress the activity of iNOS mediated by inflammation (Fathy et al. 2013).

The results of this study indicate that there is a decrease in the mean expression of iNOS along with the increasing dose of Nigella sativa ethanol extract in preeclampsia mice.

CONCLUSION

The administration of extract ethanol Nigella sativa various doses had a significant effect on lowering the expression of renal iNOS in preeclampsia model mice, especially at doses of 2000 mg/kg/ day. Ethanol extract of black cumin seeds (Nigella Sativa) can reduce the expression of renal iNOS in preeclampsia model mice, especially at doses of 1500 and 2000 mg/kg/ day.

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