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Effect of Black Cumin Seeds (*Nigella Sativa*) Ethanol Extracts on Blood Pressure, Proteinuria, and ET-1 Level in Preeclampsia Mice Model

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Abstract : Preeclampsia is a pregnancy disorder characterized by systemic hypertension and endothelial dysfunction. Endothelin 1 (ET-1) plays a role in the onset of hypertension, which is the major sign of preeclampsia. Black cumin (*Nigella sativa*) serves as an anti-free radical, antihypertensive, and anti-inflammatory. The purpose of this study was to examine the effect of black cumin seeds extract against ET-1 level, blood pressure and urine protein in preeclampsia mice model. There were six groups: negative control group, positive control group (pregnant mice injected with severe preeclampsia (SPE) maternal serum without black cumin extracts), and 4-doses treatments with the addition of black cumin extracts. Steps of this study were: blood serum collection from research participants, maternal preeclampsia (SPE) serum injections on Balb/c mice, administration of black cumin extract (500mg/kg/d, 1000 mg/kg/d, 1500 mg/kg/d and 2000 mg/kg/d), ET-1 test, proteinuria measurements, blood pressure measurement, and data analysis by using One-Way ANOVA. The results showed that the average value of the ET-1 level, systolic pressure, and urine protein were significantly different in each sample, but diastolic pressure did not provide a significant difference in each sample. Treatment of black cumin (*Nigella sativa*) ethanol extract was proven to have the ability to reduce the ET-1 level, systolic pressure and lower urine protein. The 2000mg/kg/d dose of *Nigella sativa* ethanol extract was the optimum dose to reduce the ET-1 level, systolic pressure and urine protein in preeclampsia mice model.
Keywords: ET-1, black cumin (*Nigella sativa*), Balb/c mice, proteinuria, systolic pressure, diastolic pressure, preeclampsia.

Introduction

Preeclampsia is a pregnancy disorder characterized by systemic hypertension and endothelial dysfunction [1, 2]. The main symptoms of preeclampsia is hypertension (systolic blood pressure >= 140 mm Hg or

diastolic blood pressure ≥90 mm Hg), and proteinuria (300 mg or more, in 24-hour urine capacity). Hypertension and proteinuria are related to the occurrence of fetal death in the utero, stunted fetal growth and premature labor. The disease causes high morbidity and mortality on the mother and the fetal

Endothelin-1 (ET-1) plays a role in the onset of hypertension as a major sign of preeclampsia. Endothelin-1 has implications on various biochemical pathways, which is the basis of preeclampsia pathogenesis [3,4]. Conditions of endothelial dysfunction and vasodilation loss will lead to hypertension, proteinuria and systemic manifestations of preeclampsia [5, 6, 7, 8, 9]. ET-1 is produced in small quantities by endothelial cells in physiological conditions while ET-1 is produced in large quantities in the pathological condition. Increasing concentration of ET-1 induces vasoconstriction and produce hypertension and proteinuria in preeclampsia [10, 11].

Increasing oxidative stress is associated with hypertension pathogenesis in preeclampsia [12]. Meanwhile, increasing blood pressure is associated with an imbalance between antioxidant defense mechanisms and the production of free radicals. Black cumin (*Nigella sativa*) can serve as an anti-free radical, antihypertensive, and anti-inflammatory. Black cumin with active Thymoquinone has shown to have anti-oxidant effects, hypotension through Ca-channel blockers, and diuretics [13,14,15,16]. In addition, the thymoquinone (TQ) of black cumin seeds (Nigella sativa) can reduce the ET-1 level [17, 18]. Based on the above descriptions, further study on the black cumin (*Nigella sativa*) potential to reduce blood pressure, proteinuria, and ET-1 levels in patients with preeclampsia is necessary to be done.

Materials and Method

Blood serum collection from research participants

Serum was taken from the blood of patients with normal pregnancy and patients who have been diagnosed with severe preeclampsia (SPE). The gestational age ranges between 32-34 weeks. Blood was taken from the veins, under sterile conditions, by using a syringe until it reached 10 cc. Next, the identity was written and stored in plain vacutainer, left at room temperature for 12 hours to obtain normotensive pregnancy serum and severe preeclampsia pregnancy serum. Then it was centrifuged at the speed of 6000 rpm for 10 minutes. The blood serum was stored at -20°C, grouped into SPE maternal serum and normal maternal serum. Each group serum was homogenized, included in Eppendorf, and stored at -40°C until it was ready to be injected in experimental animals (mice) [19,20,21,22].

PE maternal serum injection in experimental animals

Maternal serum injections were administered twice on the 10^{th} and 11^{th} days of gestation. The injections were performed by injecting each pregnant women serum into 1 ml syringe until it reached 0.1 CC. Mice's tail until the base of the tail was held, the rear body was held by hand palm and index finger and thumb gently placed on the left and right side of the neck, then the other hand was used to handling specified location for intraperitoneal injection [20, 22, 23, 24].

Proteinuria measurement

Mice were placed in a special cage on the 15^{th} and 20^{th} gestation days, urine was collected within 24 hours, stored at -800 ^{0}C and urine protein level was checked with spectrophotometry [20, 25, 26, 27].

Blood pressure measurement

Blood pressure measurement was performed on the 15^{th} and 20^{th} gestation days by using Digimed Blood Pressure analyzer, mice adaptation was done for 5 minutes, measurements were taken for 3 times at 1 minute interval, average value was calculated and a measurement was done by using Digimed Blood Pressure analyzer with a Rasia scale in Balb/c mice on the 15^{th} and 20^{th} gestation days [22, 23].

Preparation of black cumin extracts

The preparation of black cumin (*Nigella sativa*) extracts was divided into several steps: drying process, extraction process, and evaporation process. The drying process was done by drying and cleaning black cumin

seeds and placed it in an oven at a temperature of 40-60°C or by drying it under the sun. The extraction process was done by refining dried black cumin seeds in a blender, black cumin seeds that have been refined was weighed at 100 grams, refined black cumin seed was inserted in an ± 1L Erlenmeyer glass, soaked with 900 ml ethanol, shaken for 30 minutes until homogeneous, left for one night until it precipitated, the top layer of a mixture of ethanol (solvent) with active substances that have been mixed with filtration was taken by using filter paper, and soaked for 3 times. The evaporation process was done by entering the top layer of ethanol with active substances that have been taken from the 1L evaporation flask, evaporation flask was placed in the evaporator, filled water bath with water to the brim, all series of tools were assembled including the rotator evaporator, water bath heater was connected with electricity, ethanol solution was left until it was separated with the evaporation flask active substances, waited until ethanol stopped dripping from the ±900 ml flask shelter (± 1.5 to 2 hours for 1 flask), the extraction were inserted into the a plastic bottle, stored in a freezer and 100 cc of black cumin seed extract in the form of a viscous (oil) dark-brown-black fluid was 100% ready to be used as concentration of active substances extract [28, 29, 30].

Administration of black cumin extract

Female Balb/c mice with 16 weeks pregnancy and with preeclampsia were used in this study. Preeclampsia was identified through signs of hypertension and positive urine protein. Females and males Balb/c mice were mated on the evening at 19.00 pm and checked on the next morning at 06.00 am to determine the occurrence of fertilization by looking at the female Balb/c mice's vaginal white plague. Before administering the black cumin extract, the weighing of mice was done with ohaus balance. Black cumin extract was given orally on the 15^{th}-20^{th} days of gestation with different doses (500 mg/kg/d, 1000 mg/kg/d, 1500 mg/kg/d and 2000 mg/kg/d) [31] by using a sonde. Sonde was inserted into the mouth of Balb/c mice through the ceiling and slowly moved up to the pharynx and esophagus, and black cumin extract contained in the syringe was pushed into the esophagus until it reached the gastric.

Examination of Endothelin-1

All reagents, standard and samples were prepared, excess microplate strips were disposed from the frame disc, the foil pouch containing desiccant packs was returned and resealed, 150 µL of RD1-105 Assay diluents was added, 75 µL of standard was added, closed with adhesive strips, incubated for 1 hour at room temperature in a set of micro disc horizontal orbital shaker (0.12' orbit), set at a speed of 500 ± 50 rpm, the disc provided for recording standard and sample was set, suctioned on each testing well, and washed for four times. The washing process was done by filling each Buffer (400 µL) test well by using spray bottle, manifold dispenser, or automatic washers. After the washing procedure, washing Buffer was removed by using aspiration or mixing, the back of disc was wiped with a paper towel until it cleaned, 200 µL of endothelin-1 was added for each test, covered with new adhesive strip, incubated in a shaker for 3 hours at room temperature, washing procedure was repeated again, 200 µL of substrate solution was added to each test wells, incubated for 30 minutes at room temperature in a benchtop, protected from light, and 50 µL of solution was added. Well color has to change from blue to yellow. If the well was green or the color change did not appear universally, then the disc has to be pressed to ensure a thorough mixing. Furthermore, the optical density was determined in each well test within 30 minutes by using 450 nm micro disc readers. If the wave correction was available, set it to 540 nm or 570 nm. If the correction wave was not available, then at 540 nm or 570 nm reading reduced into 450 nm reading. Reading was done directly at 450 nm without correction might be higher.

Data Analysis

Data were statistically analyzed by using SPSS 19.0 software program for Windows. Prior to data analysis, normality test was done by using Shapiro-Wilk test to assess the probability of empirical error in p-value. If the p-value>0.05, then data have a normal distribution, and if p-value<0.05 then the data doesn't have a normal distribution, and thus parametric tests cannot be used [32]. Furthermore, One Way ANOVA was used to compare the mean of measured variables between the positive control groups, that was the group of pregnant mice injected with serum of SPE pregnant women without black cumin extracts (*Nigella sativa*) and the group of pregnant mice injected with SPE maternal serum with black cumin extracts (*Nigella sativa*). Black cumin extracts were administered in 4 different doses to obtain data from each variable.

Results

Effect of Nigella sativa on the systolic pressure

The result showed that there were significant differences in the average value of systolic pressure on the six observation groups. It was indicated by the p-value=0.000<α.

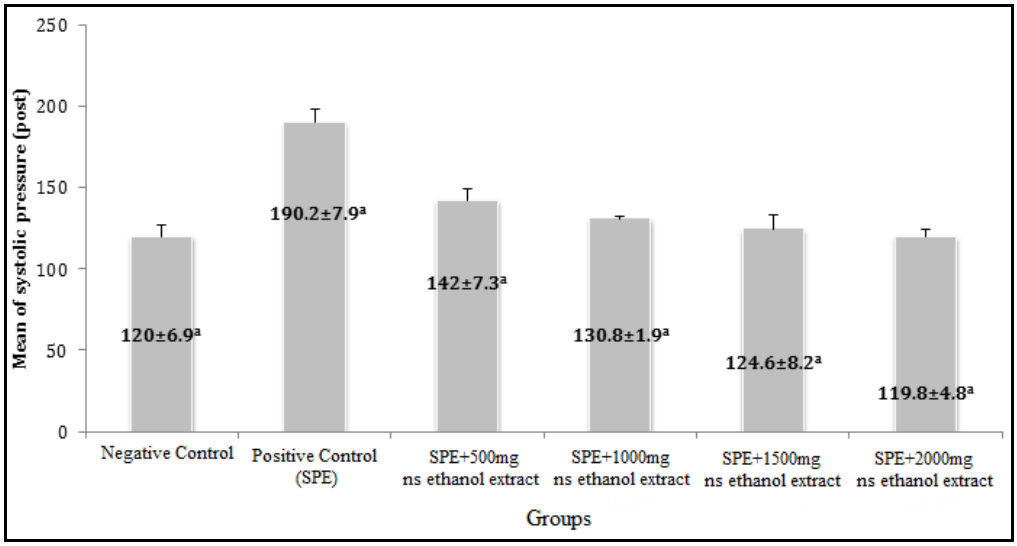

Figure 1. Average value of systolic pressure on the six observation groups

Figure 1 showed that the average value of systolic pressure decreased as the doses of *Nigella sativa* ethanol extract increased. The group with the lowest average value of systolic pressure was the 2000mg (119.8±4.8) *Nigella sativa* ethanol extract. It can be concluded that 2000mg *Nigella sativa* ethanol extract was the most optimum dose in lowering systolic pressure in mice with preeclampsia. In other words, the optimum dose of 2000mg *Nigella sativa* ethanol extract can significantly lower the systolic pressure in mice with preeclampsia.

Effect of Nigella sativa on the diastolic pressure

The result showed that there were no significant differences in the average value of diastolic pressure on the six observation groups. It was indicated by the p-value=0.248<α.

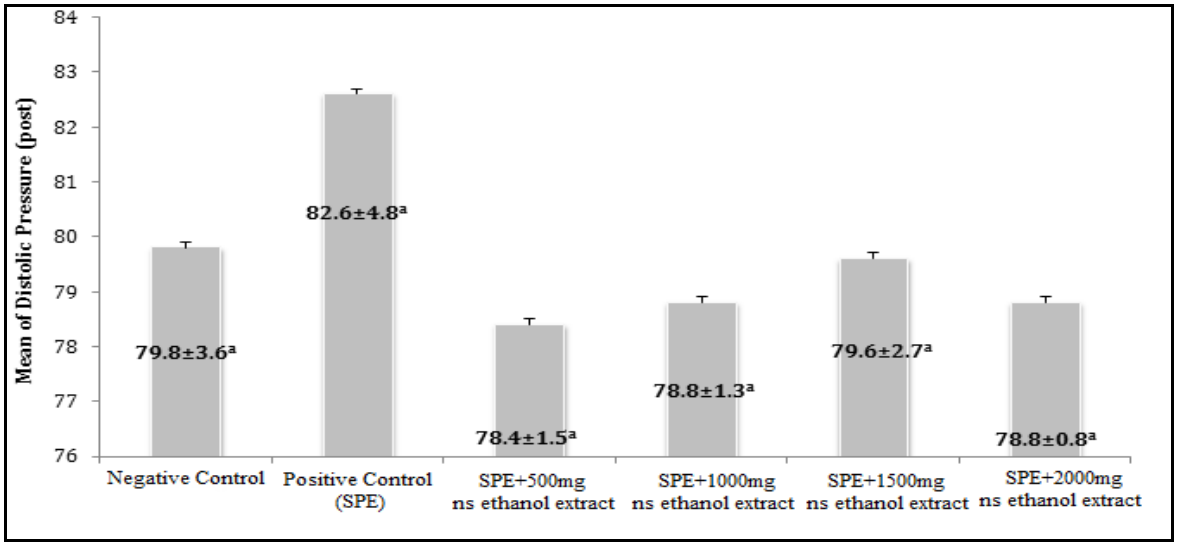

Figure 2. Average value of diastolic pressure on the six observation groups

Figure 2 showed that there were no significant differences of diastolic pressure average value of the four observation groups. It can be concluded that various doses *Nigella sativa* ethanol extract had the same effect on diastolic pressure in mice with preeclampsia.

Effect of Nigella sativa on the urine protein

The result showed that there were significant differences in the average value of urine protein on the six observation groups. It was indicated by the p-value=0.001<α.

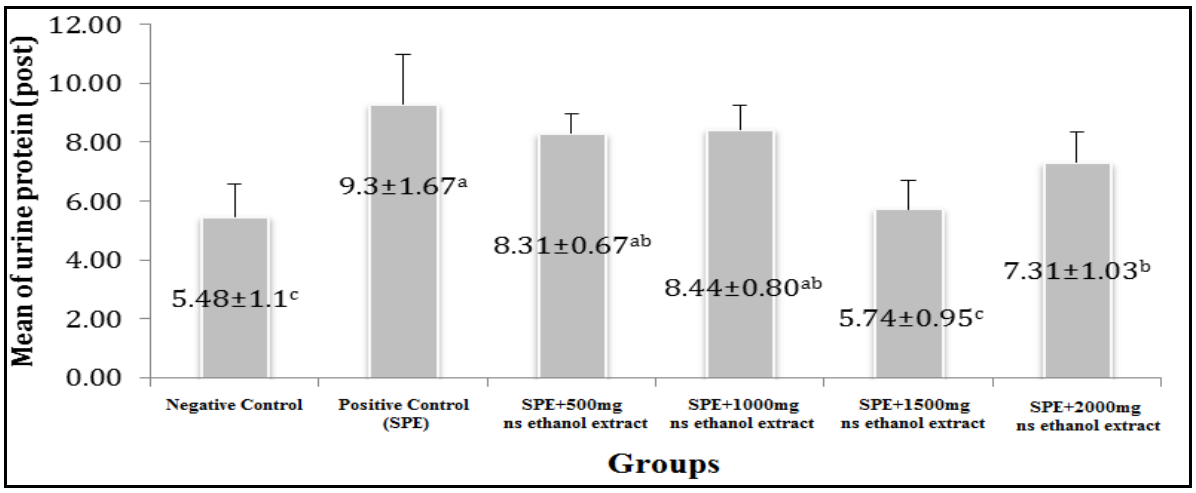

Figure 3. average value of urine protein on the six observation groups

Figure 3 showed that the average value of urine protein decreased as the doses of *Nigella sativa* ethanol extract increased. The group with a the lowest average value of urine protein was the 1500mg. (5.74±0.95) *Nigella sativa* ethanol extract and the negative control group (healthy mice) (5.48±1.1). It can be concluded that 1500mg *Nigella sativa* ethanol extract was the most optimum dose in lowering urine protein of mice with preeclampsia. Results also showed that administration of *Nigella sativa* ethanol extract in various doses significantly influenced the decreased urine protein of mice with preeclampsia.

Effect of Nigella sativa on the ET-1 level

The result showed that there were significant differences in the average value of ET-1 level on the six observation groups.

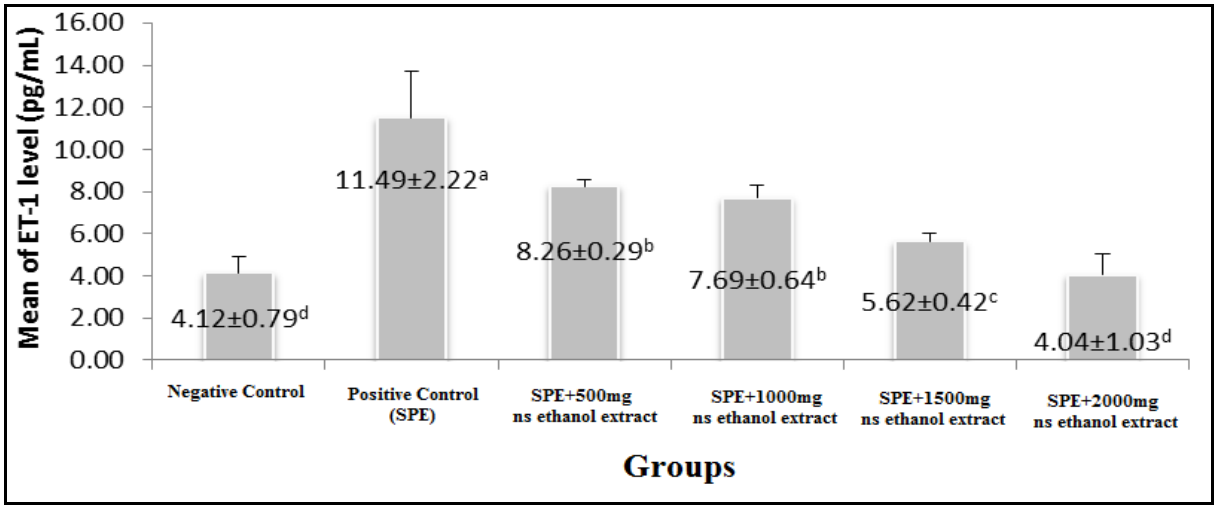

Figure 4. Average value of ET-1 level on the six observation groups

Figure 4 showed that the average value of ET-1 level decreased as the doses of *Nigella sativa* ethanol extract increased. The group with the lowest average value of ET-1 level was the 2000mg (4.04±1.03) *Nigella sativa* ethanol extract and the negative control group (healthy mice) (4.12±0.79). It can be concluded that 2000mg *Nigella sativa* ethanol extract was the most optimum dose in lowering ET-1 level of mice with preeclampsia. Results also showed that administration of *Nigella sativa* ethanol extract in various doses significantly influenced the decreased ET-1 level of mice with preeclampsia.

Discussion

General View of Mice with Preeclampsia

Mice with preeclampsia are characterized by hypertension and elevated levels of urinary protein. The basic characteristic is homogeneous in accordance with the inclusion and exclusion criteria in Table 1.

A hundred female mice (*Mus musculus*), within reproductive age and between 20-30 mg, were mated with 50 male mice of the same type at a ratio of 2: 1. Vaginal plug observation method was used to determine mice pregnancy. Vaginal plug observation resulted in 45 out of 100 positive female mice and it was set as day zero of pregnancy, then increased weight and distention were observed onward. Weight gain and abdominal enlargement are significant on 10^{th} day of gestation [33]. After that, 30 mice were randomly picked as experimental animals. Experimental animals were injected with 0.1 cc per day of intraperitoneal serum from severe preeclampsia patients and normal serum in 10^{th} and 11^{th} days of gestation. Blood pressure and urine protein were measured on 15^{th} (pre) and 20^{th} (post) gestation, then termination was performed on the 20^{th} gestation day [20, 21, 22].

Table 1 showed data of pregnant mice injected by 0.1 cc intraperitoneal serum from patients with severe preeclampsia on 10^{th} and 11^{th} days of gestation. Mice that were injected with normal patient's intraperitoneal serum were used as a negative control, whereas mice that were injected with severe preeclampsia patient's intraperitoneal serum were used as preeclampsia mice model, in which the systolic blood pressure ≥ 140 mmHg. Meanwhile, mice urine protein is said to be high when it reaches more than 10 mg/dl. Mice were divided into 6 groups: the negative control group, the positive control group, the black cumin seeds (*Nigella sativa*) ethanol extract treatment groups with doses of 500 mg/kg/day, 1000 mg/kg/day, 1500 mg/kg/day and 2000 mg/kg/day for 5 days (15^{th}-19^{th} of gestation days). The termination was done on the 20^{th}gestation day and the serum was taken. Evaluation of blood pressure and urine protein results as a characteristic of preeclampsia in mice models was in accordance with inclusion and exclusion in each group.

Table 1. Primary Characteristics of Preeclampsia Mice

Characteristic	K(-)	K(+)	KP I	KP II	KP III	KP IV
Group	5	5	5	5	5	5
Initial Weight	25.8±0.84	26.4±1.14	26.6±41.6	27.4±0.5	27±0.9	26.4±1.6
Final Weight	39±4.3	40.2±3.27	41.6±3.9	41.8±2.1	42±3.9	39±1.4
Systolic pressure (pre)	118.4±7.27	174.6±8.62	174.4±13.7	160.2±9.1	166±7.7	163.6±11.3
Diastolic pressure (pre)	78.8±1.3	96.2±9.6	79.4±0.8	77.8±3.9	79±0.9	79.2±0.7
Urine Protein (pre)	4.89±1.36	12.2±0.85	12.8±1.3	14.3±0.7	12.4±1.1	11.8±2

Description: Characteristics of the preeclampsia mice model by hypertension and high urine protein levels. K (-): negative control, K(+): positive control (injected Maternal serum severe preeclampsia (SPE)), KPI-IV: injected SPE+ Ethanol Extract of Nigella sativa (500, 1000, 1500, 2000 mg/kg/day)

Effect of black cumin ethanol extract in ET-1, blood pressure, and proteinuria on preeclampsia mice model

This study found significant differences between the average values of Endothelin 1 (ET-1) on mice with preeclampsia. It proved the assumption that black cumin seeds (*Nigella sativa*) ethanol extract can reduce the ET-1 level in preeclampsia mice's plasma. These results are consistent with in vivo studies before on 40 asthma pigs with Thymoquinone (TQ) treatments in the low and high doses. Results of this study showed the levels of ET-1 in lung tissue of groups injected with TQ in low doses (20 μM) when compared with control group. It was clear that TQ, the main content of black cumin seeds (*Nigella sativa*) can reduce the ET-1 level [37]. Other studies also reported that the extract of black cumin seeds (*Nigella sativa*) can lower blood pressure significantly [34] .

ET-1 reduction by black cumin seeds (*Nigella Sativa*) extracts and its potency as an antioxidant has been proven to be capable against several reactive oxygen species (ROS). The capability can suppress the formation of peroxynitrite (ONOO), which will further reduce the occurrence of endothelial dysfunction. It is characterized by a decrease in ET-1, which will cause smooth muscle relaxation and lower blood pressure [17]. Other studies also reported the inhibition of nitric oxide synthesis in pregnant mice will lead to increased ET-1 level [35, 36.] Black cumin (*Nigella Sativa*) potential as an antioxidant and anti-inflammatory can reduces levels of inflammatory cytokines serum, which will influence the decreased production of ET-1 by endothelial cells of preeclampsia mice model.

ET-1 level influenced the urine protein directly and significantly. A decrease of the ET-1 level resulted in a decrease of the urine protein, due to the administration of Nigella sativa ethanol extract in preeclampsia mice model. Similarly, the ET-1 level also influenced the systolic blood pressure directly and significantly. A decreased of ET-1 level resulted in a decrease of systolic blood pressure, due to the administration of Nigella sativa ethanol extract in preeclampsia mice model.

Conclusion

It can be concluded that the average value of the ET-1 level, systolic pressure, and urine protein were significantly different in each sample, but diastolic pressure did not provide a significant difference in each sample. In addition, treatment of black cumin (*Nigella sativa*) ethanol extract was proven to have the ability to reduce the ET-1 level and lower urine protein. The 2000mg/kg/d dose of *Nigella sativa* ethanol extract was the optimum dose to reduce the ET-1 level, systolic pressure and urine protein in preeclampsia mice model.

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References

1. Amaral LM., Cunningham MW, Cornelius DC, LaMarca B., Preeclampsia: Long-term consequences for vascular health, Vasc. Health Risk Manag., 2015; 11: 403–415. doi:10.2147/VHRM.S64798
2. Amash A, Holcberg G, Sapir O, Huleihel M., Placental Secretion of Interleukin-1 and Interleukin-1 Receptor Antagonist in Preeclampsia: Effect of Magnesium Sulfate, J. Interf. Cytokine Res., 2012; 32: 432–441. doi:10.1089/jir.2012.0013
3. George EM, Granger, JP., Endothelin: key mediator of hypertension in preeclampsia, Am. J. Hypertens., 2011; 24: 964–969. doi:10.1038/ajh.2011.99
4. Gössl M, Lerman A., Endothelin: beyond a vasoconstrictor, Circulation., 2006; 113: 1156–8. doi:10.1161/CIRCULATIONAHA.105.609271
5. Hladunewich M, Karumanchi, SA, Lafayette R., Pathophysiology of the clinical manifestations of preeclampsia, Clin. J. Am. Soc. Nephrol., 2007; 2: 543–9. doi:10.2215/CJN.03761106
6. Lindheimer MD, Taler SJ, Cunningham FG., Hypertension in pregnancy, J. Am. Soc. Hypertens., 2008; 2: 484–94. doi:10.1016/j.jash.2008.10.001
7. Prochazka M., Procházková J, Lubuský M, Pilka R, Úlehlová J, Michalec I, Polak P, Kacerovsky M, Slavik L., Markers of Endothelial Activation in Preeclampsia, Clin Lab., 2015; 9: 1–8.

doi:10.7754/Clin.Lab.2014.140521
8. Yinon Y, Kingdom JCP, Odutayo A, Moineddin R, Drewlo S, Lai V, Cherney DZ, Hladunewich MA., Vascular dysfunction in women with a history of preeclampsia and intrauterine growth restriction: insights into future vascular risk, Circulation, 2010; 122: 1846–53. doi:10.1161/CIRCULATIONAHA.110.948455
9. Young BC, Levine RJ, Karumanchi SA,. Pathogenesis of preeclampsia, Annu Rev. Pathol., 2010; 5: 173–92. doi:10.1146/annurev-pathol-121808-102149
10. Murphy SR, LaMarca B, Cockrell K, Arany M, Granger JP., L-arginine supplementation abolishes the blood pressure and endothelin response to chronic increases in plasma sFlt-1 in pregnant rats, Am. J. Physiol. Regul. Integr. Comp. Physiol., 2012; 302: R259–63. doi:10.1152/ajpregu.00319.2011
11. Singh A., Endothelin: Link between the Primary Placental Causes and the Secondary Systemic Endothelial Dysfunction in Pathophysiology of Pre-Eclampsia, Obstet. Gynecol. Int J., 2015; 2: 2–5. doi:10.15406/ogij.2015.02.00042
12. Watanabe K, Mori T, Iwasaki A, Kimura C, Matsushita H, Shinohara K, Wakatsuki A., Increased oxygen free radical production during pregnancy may impair vascular reactivity in preeclamptic women, Hypertens. Res., 2013; 36: 356–60. doi:10.1038/hr.2012.208
13. Dehkordi FR, Kamkhah AF., Antihypertensive effect of Nigella sativa seed extract in patients with mild hypertension, Fundam. Clin. Pharmacol., 2008; 22: 447–52. doi:10.1111/j.1472-8206.2008.00607.x
14. Hassanien MFR, Assiri AMA, Alzohairy AM, Oraby HF., Health-promoting value and food applications of black cumin essential oil: an overview, J. Food Sci. Technol., 2015; 52: 6136–6142. doi:10.1007/s13197-015-1785-4
15. Leong XF, Rais MM, Jaarin K., *Nigella sativa* and Its Protective Role in Oxidative Stress and Hypertension, Evid. Based. Complement. Alternat. Med., 2013: 120732. doi:10.1155/2013/120732
16. Najmi A, Nasiruddin M, Khan RA, Haque SF., Indigenous herbal product Nigella sativa proved effective as an antihypertensive in metabolic syndrome, Asian J. Pharm. Clin. Res., 2013; 6: 61–64.
17. Sethi G, Ahn KS, Aggarwal BB., Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis, Mol. Cancer Res., 2008; 6: 1059–70. doi:10.1158/1541-7786.MCR-07-2088
18. Mazouchian H, Mirzaei B, Ebrahimi S, Bonyadi MR, Keyhanmanesh R., The Effect of Thymoquinone, the main Constituent of *Nigella sativa*, on Endothelin level of Ovalbumin Sensitized Guinea Pigs, Scholars Research Library, 2013; 4(4): 105–108.
19. Karumanchi SA, Stillman IE., In vivo rat model of preeclampsia, Methods Mol Med., 2006; 122: 393–399.
20. Kalkunte S, Boij R, Norris W, Friedman J, Lai Z, Kurtis J, Lim KH, Padbury JF, Matthiesen L, Sharma S., Sera from Preeclampsia Patients Elicit Symptoms of Human Disease in Mice and Provide a Basis for an in Vitro Predictive Assay, Am. J. Pathol., 2010; 177: 2387–2398. doi:10.2353/ajpath.2010.100475
21. Mccarthy FP, Kingdom JC, Kenny LC, Walsh SK., Animal models of preeclampsia ; uses and limitations, Placenta, 2011;32,: 413–419. doi:10.1016/j.placenta.2011.03.010
22. Wicaksono BA, Candra S, Baktiyani W, Fitri LE., Intraperitoneal Injection of High Tumor Necrosis Factor (TNF- α) Serum Increase Soluble Fms-like Tyrosine Kinase 1 (sFlt-1) and Blood Pressure of Pregnant Mice, 2015;5(1).
23. Kalkunte SS, Neubeck S, Norris WE, Cheng SB, Kostadinov S, vu-Hoang A, vo_Eggeling F, Shaikh Z, Padbury J, Berg G, Olofsson A, Markert UR, Sharma S., Transthyretin is dysregulated in preeclampsia, and its native form prevents the onset of disease in a preclinical mouse model, Am. J. Pathol., 2013; 183: 1425–1436. doi:10.1016/j.ajpath.2013.07.022
24. Fitri LE, Syahroni YA, Siti CWB., Injection of Serum Containing High TNF- α Lowers VEGF Concentration and Glomerulus Nephrin Expression of Pregnant Mice, Jurnal Kedokteran Brawijaya, 2015; 29: 10–13.
25. Tao X, Fan F, Hoffmann V, Longo NS, Lipsky PE., Therapeutic impact of the ethyl acetate extract of Tripterygium wilfordii Hook F on nephritis in NZB/W F1 mice, Arthritis Res. Ther., 2006; 8: R24. doi:10.1186/ar1879
26. Levine RJ, Lam C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA., Soluble Endoglin and Other Circulating Antiangiogenic Factors in Preeclampsia, N. Engl. J. Med., 2006; 355(10):992-1005
27. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, Hicks MJ, Ramin SM, Kellem RE, Xia Y,

Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice, Nat. Med., 2008; 14: 855–862. doi:10.1038/nm.1856

28. Alam MM, Yasmin M, Nessa J, Ahsan CR, Atcc K., Antibacterial activity of chloroform and ethanol extracts of black cumin seeds (*Nigella sativa*) against multi-drug resistant human pathogens under laboratory conditions, 2010; 4: 1901–1905. doi:10.5897/JMPR10.324
29. Rifai, M. Disertasi Wisnu Barlianto : Ekstrak Etanol Jinten Hitam pada Model Mencit Asma sebagai Modulasi Sistim Imun, 2011: 3–4.
30. Hosseinzadeh H, Tafaghodi M, Mosavi MJ, Taghiabadi E., Effect of Aqueous and Ethanolic Extracts of Nigella sativa Seeds on Milk Production in Rats, JAMS J., 2013.
31. Meziti A, Meziti H, Boudiaf K, Mustapha B, Bouriche H., Polyphenolic Profile and Antioxidant Activities of Nigella Sativa Seed Extracts In Vitro and In Vivo, 2012: 24–32.
32. Hoskin BT., Berd-5-6, Acupunct. Meridian Stud., 1962; 6: 18–23. doi:10.1016/j.jams.2012.07.019
33. Cencig S, Coltel N, Truyens C, Carlier Y., Fertility , Gestation Outcome and Parasite Congenital Transmissibility in Mice Infected with TcI , TcII and TcVI Genotypes of Trypanosoma cruzi, 2013; 7. doi:10.1371/journal.pntd.0002271
34. Sultan MT, Butt MS, Karim R., Iqbal SZ, Ahmad S, Zia-Ul-Haq M, Aliberti L, Ahmad AN, De Feo V, Effect of *Nigella sativa* fixed and essential oils on antioxidant status, hepatic enzymes, and immunity in streptozotocin induced diabetes mellitus, BMC Complement. Altern. Med., 2014; 14: 193. doi:10.1186/1472-6882-14-193
35. Csiszar A, Wang M, Lakatta EG, Ungvari Z, Santos-parker JR, Larocca TJ., Inflammation and endothelial dysfunction during aging : role of NF- κ B Inflammation and endothelial dysfunction during aging : role of NF- κ B, 2015: doi:10.1152/japplphysiol.90470.2008
36. Kohan, DE, Rossi NF, Inscho EW, Pollock DM., Regulation of blood pressure and salt homeostasis by endothelin, Physiol. Rev., 2011; 91: 1–77. doi:10.1152/physrev.00060.2009

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