Effect of Black Seed (*Nigella Sativa*) Ethanol Extract on the Expression of Interleukin 6 (IL6) and Tumor Necrotic Factor (TNF-α) in Huvecs Model Exposed by Preeclamptic Plasma

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Abstract

**Background:** 10-15% of direct maternal death caused by preeclampsia and eclampsia. Almost 30% of maternal death in Indonesia caused by Hypertension in pregnancy Pathogenesis of preeclampsia characterized by increase of HIF-1α placenta expression in hypoxia and ischemia condition that leading producer of IL6 by endothelial cells, phagocyte cells, macrophage, mast cells and also fibroblast as an immunologic maladaptation response. The endothelial dysfunction also causing major producer of endhotelin-1 that induced production of IL-6 and TNFα as an inflammation response by activating Nfkβ. Black seed as known as black cumin (*Nigella sativa*) with Thymoquinone and thymol as the active substances is a potential in prevention and therapy for preeclampsia. Black seed act as anti-inflammatory scavenger and inhibit the activation of NF-kB and therefore reduces expression of IL-6 and TNFα and also the symptoms of preeclampsia.

**Objective:** To determine the effect of black seed ethanol extract on the expression of IL-6 and TNFα in Huvecs model exposed by preeclamptic plasma.

**Methods:** The trial study with post-test only control group design using Huvecs model exposed by preeclamptic plasma were randomly divided into six groups. The control group was Huvecs model exposed by normal pregnancy plasma. The Positive control group using Huvecs model exposed by preeclamptic plasma While the four treatment groups using Huvecs model exposed by preeclamptic plasma received treatment of ethanol extract of black seed with doses of 50, 100, 200, and 400 ppm. Then all the groups were moved to tissue culture well with gelatin covering cover glass inside incubated for 20 minutes Continued by examination of the expression of IL-6 and TNFα in endothelial cells using immunohistochemical staining.

**Results:** Data were analyzed using one way ANOVA test to compare the mean of TNFα and IL6 expression, and it showed significant influence of ethanol extract of black seed in various doses to decrease the expression of TNFα and IL6 expression of Huvecs model exposed by preeclampsia plasma. The optimal dose in both is 100 ppm.

**Conclusion:** The black seed ethanol extract administration decreases the expression of TNFα and IL6 expression in Huvecs model exposed by preeclampsia plasma.
Keywords: Ethanol Extract of Black Seed; Expression of TNFα; Expression IL6; Huvecs Model Preeclampsia

Introduction

Preeclampsia is still one of the complications in the mother during pregnancy. Although current advances in antenatal and neonatal care have grown rapidly, but preeclampsia is still the cause of the high rates of maternal and fetal morbidity and mortality. Preeclampsia is a pregnancy-related disorder that has a characteristic rise in blood pressure, significant proteinuria, with or without edema. According to the World Health Organization WHO generally preeclampsia complicates 2-10% of all pregnancies and the incidence is 7 times higher in developing countries (2.8% of all live births) [1-3].

Cytokines are mediators of dissolved polypeptides that maintain communication with leucocytes and other tissues and organs. Endothelial cells in addition to functioning as cytokine targets are also a source of cytokines. Endothelium is a major determinant in circulation, platelet adhesion and smooth muscle cell proliferation. In case of endothelial damage, the synthesis and biological activity of some vasodilators such as NO, prostacyclin and endothelium derived hyperpolarizing factor will decrease. Changes in the balance of endothelial cells will lead to an increase in superoxide that will cause oxidative stress. Cytokines other than those produced by the endothelium also activate the endothelium through the formation of thrombus and inflammation. In the formation of thrombus, cytokines induce the activity of procoagulant protein C and inhibit the destruction of fibrin. Some examples of cytokines that play a role in immunological reactions that occur in patients with preeclampsia, among others: Tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6) [3-6].

The development of medication in recent years began to be interested in developing drugs from herbs, one of which is Nigella sativa. Nigella sativa (of the family ranunculaceae) is a plant that is synonymous with Nigella critical and is commonly called or commonly known as Habbatussaudah, Al-Habbah Al Sawda, Habbet El-Baraka, Camoun Aswad, Schuniz and Khodria. In Pakistan India, and Sri Lanka are known as Kalvanji, Kalunji, Kalonji, Azmut, Gurat, Aoif and Aosetta. In English, the plant is known as black seed, black cumin, black caraway, cinnamomum flower, nutmeg flower and love-in-a-mist. Black cumin plants contain flavonoid compounds are efficacious as anti-inflammatory, in addition black cumin plants are also efficacious as a stimulant, carminative, emenagogue, galactogena and diaforetik. This plant usually grows in Europe, Middle East and West Asia. Black cumin grows on the semi-arid ground state. Black cumin flower is also marked by the existence of nectar. Black cumin seeds are small with a weight of 1-5 mg dark gray or black with a wrinkled skin surface. The fruit is a large and bulging capsule consisting of 3-7 follicles into one, where each of these follicles contains several seeds. Black cumin seeds pointed sharply like the shape of sesame seeds, hard, and bubblier. It has a distinctive odor like spices and is rather spicy, which will get sharper the smell after chewing [7-9].

Nigella sativa is a simple spice used in food that appears to be a very well renowned seed, and is used for a large variety of medicinal purposes as well as general wellbeing and longevity. Medicinal usage of these seeds mostly centers around diarrhea and abdominal pain, Dyslipidemia, asthma and coughs, headache, dysentery, renal calculi, infections, obesity, back pain, hypertension, and dermatological problems Nigella sativa seeds are sometimes eaten alongside honey or simply used to spice food products such as breads and even in these food products it is considered medicinally since the recommended dosages are low, with the crushed seed powder (no further processing required) being recommended at 1-2g (Unani Pharmacopoeia of India), 1-3g (Ayurvedic Pharmacopoeia of India), or 0.5-4g (Siddha Pharmacopoeia of India). Nigella sativa (Black Cumin or Kalonji) appears to be medicinally active in fairly low doses, and does not appear to require any particular extraction processes of sources to have medicinal properties. As such, it is a prime candidate for being a functional food product (something that can be eaten in the diet for benefits exceeding normal nourishment) [7-9].

In some studies, the active ingredients of black cumin, Thymoquinone, can function as anti-inflammatory by inhibiting the Enzyme Cyclooxygenase (COX) and Lipoxygenase (LOX) and inhibit transcription factor NF-kB and TNF-α. Shahraki, (2016) in his study concluded that there were cytotoxic and apoptotic effects of black cumin extract of various doses (50, 100, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 ppm) on ACHN and GP-293 cultures which were cultured Human Adenocarcinoma Cell line. In addition, black cumin also has efficacy as a potent enough antioxidant and lowering blood pressure. Thymoquinone, Dithymoquinone, and Thymol contained in black cumin seed oil may decrease free radicals and may act as antihypertensives [6-11].

Methods

The trial study of Effect of Black Seed (Nigella sativa) Ethanol Extract on The Expression of Interleukin 6 (IL6) and Tumor Necrotic Factor A (TNF-α) in Huvecs Model Exposed by Preeclamptic Plasma. Biomark Applic: BMAP-105.
histochemical staining. This research held in Biomedics Laboratory Brawijaya University and the samples was taken from pregnant women that having delivery in Saiful Anwar Hospital Malang, East Java at July until September 2016. In this study using 6 research groups, then the number of replications required in each group from Al rasyid equation is at least 4 so the total number of samples to 24 samples. The endothelial sample came from umbilicus taken from newborn baby from Caesar delivery, with inclusion criteria healthy mother Hb > 10 gr/dl, normal pregnancy, caesarean delivery, baby umbilical from normal pregnancy. And the exclusion criteria without any NSAID or corticosteroid medication during or before pregnancy. Testing of IL-6 expression and TNF-α expression by using 2 control groups (KK and K+) and 4 levels of black cumin extract dose (Nigella sativa) was performed using ANOVA. There are two assumptions underlying ANOVA, namely assumptions of normality and homogeneity of varieties. Normality assumption test is done by using Saphiro-Wilk test. And continued with closeness relationship test between measured variables (interval scale) and its correlation between various giving of black cumin extract (Nigella sativa) dose 50ppm, 100ppm, 200ppm, 400ppm. If the data is normally distributed then the correlation test used is Pearson correlation test.

Results

TNFα Expression Observation Result on Huvec Preeclampsia Model Given Ethanol Black Cumin Extract (Nigella Sativa).

TNFα expression in each sample was done quantitative manual assessment by observing 20 different field of view with 1000X magnification on the sample. TNF expression α is expressed by calculating the average number of tissue cell cytoplasm stained with chromagen DAB (brown color) per view field of immunohistochemical preparation using TNFα monoclonal antibodies. Then the calculation results obtained in the form of mean or average. Based on the results of observation in the control group (KK) obtained lighter coloration picture with very minimal brown colored cells when compared with the positive control group (KP) which appears dominant brown cell density. In the treatment group, the number of brown tissue cells gradually decreased slightly in the ethanol extract group of Nigella sativa dose 50 ppm (P1), 100 ppm (P2), 200 ppm (P3) and 400 ppm (P4) compared to the control group. (Figure1)

Figure 1: TNFα expression observation result description: A. Control group, B. Positive control, C. Treatment group dose 50 ppm, D. Treatment group dose 100 ppm, E. Treatment group dose 200 ppm and F. Treatment group dose 400 ppm. Arrow: TNF expressed α Cell.

IL6 Expression Observation on Huvec Preeclampsia Model Given Black Cumin Ethanol Extract Based on the results of observation in the control group (KK) obtained a lighter coloration picture with very minimal brown colored cells when compared with the positive control group (KP) which appears dominant brown cell density. In the treatment group, the number of brown tissue cells gradually decreased slightly in the ethanol extract group of Nigella sativa dose 50 ppm (P1), 100 ppm (P2), 200 ppm (P3) and 400 ppm (P4) compared to the control group. (Figure2)

Figure 2: IL6 expression observation result description: A. Control group, B. Positive control, C. Treatment group dose 50 ppm, D. Treatment group dose 100 ppm, E. Treatment group dose 200 ppm and F. Treatment group dose 400 ppm. Arrow: IL6 expressed cells.
In variable expression of IL-6 and TNFα obtained p-value more than α = 0.05 (p > 0.05). This suggests that the normality assumption of the IL-6 expression variable has been met, similarly with TNF-α expression. Assay of homogeneity assumptions was done using Levene test, that expression of IL-6 and TNFα obtained p-value greater than α = 0.05 (p > 0.05) indicating that assumption homogeneity of IL-6 expression range has been fulfilled, so does with TNF-α expression. Based on the results of the analysis using ANOVA, p-value was 0.000, smaller than α = 0.05 (p < 0.05). So, from this test can be concluded that there is significant effect of giving black cumin extract (Nigella sativa) to expression of IL-6 and TNFα (Table 1, Table 2).

Table 1: Comparison of IL6 Expression with ANOVA and Tukey 5%.

| Perkaya   | Mean ± SD | p-value |
|-----------|-----------|---------|
| KK        | 4.5 ± 1.73 |         |
| K+        | 16.25 ± 1.71 | 0.000   |
| P1        | 9.0 ± 0.82  |         |
| P2        | 4.75 ± 1.25  |         |
| P3        | 3.0 ± 0.82   |         |
| P4        | 2.25 ± 0.96   |         |

Table 2: Comparison of TNF-α Expression with ANOVA and Tukey 5%.

| Perkaya   | Mean ± SD | p-value |
|-----------|-----------|---------|
| KK        | 3.50 ± 1.28 |         |
| K+        | 13.75 ± 1.26 |         |
| P1        | 10.5 ± 1.29  |         |
| P2        | 5.75 ± 0.96  |         |
| P3        | 3.50 ± 0.96  |         |
| P4        | 2.25 ± 0.96  |         |

From Tukey 5% the highest expression of TNFα and IL-6 is on positive control, and provision of black cumin extract (Nigella sativa) with a dose of 50 ppm can significantly reduce the expression of IL-6 and TNFα. This is shown from the mean value of ± sd in the treatment group of Black Cumin Extract (Nigella sativa) with a lower dose of 50 ppm (P1) and contained different letters with positive control group (K +). Compared with control group (KK), it was shown that the mean value of ± sd in the treatment group of black cumin extract (Nigella sativa) at doses of 100, 200 and 400 ppm (P2, P3 and P4) contained the same letters as the control group (KK). This proves that the treatment of black cumin extract (Nigella sativa) with doses of 100, 200 and 400 µg / mL (P2, P3, and P4) can decrease the expression of IL-6 and TNFα to near normal condition.

To determine the effect of black cumin extract (Nigella sativa) on the expression of IL-6 and TNF-α expression can be tested by using regression analysis. Based on regression analysis of IL-6 expression, regression coefficient was obtained -0.0068 with p-value of 0.000. Coefficient of determination (R-square) equal to 83.62% indicated that giving of black cumin extract (Nigella sativa) able to influence changes of expression of IL-6 equal to 83.62%. Based on TNF-α expression regression analysis, regression coefficient was obtained -0.0058 with p-value of 0.000. The coefficient of determination (R-square) of 89.05% showed that giving of black cumin extract (Nigella sativa) could influence TNF-α expression change of 89.05%. (Table 3)

Discussion

Preeclampsia syndrome is a disorder that occurs through two stages of the first stage is preclinical and characterized by abnormalities in the process of remodeling the trophoblastic vascular in the uterine artery which will result in the occurrence of placental hypoxia. The second stage releases placental factors into the maternal circulation such as soluble Fms-Like Tyrosine Kinase1 (sFlt-1), Tumor Necrosis Factor Alpha (TNF-α) and IL-6, Angiotensin II Type 1 Receptor Antibodies (AT1-AA), and Thromboxan (TX) causes an inflammatory response and increased Endothelial Cell Activation.

The function of Endothelial Cells is to regulate vascular tone, prevent thrombosis, prevent Leucocyte attachment and regulate vascular blood vessels. Endothelial disorders such as hemodynamic stress, oxidative stress, or exposure to inflammatory cytokines and hypercholesterolemia, will turn out to be abnormal [12-15]. The condition of preeclampsia is a condition where endothelial dysfunction is caused by increased proinflammatory cytokines one of which is IL-6 and TNFα. In this study, there was an increase in IL-6 levels in cultures of HUVECs exposed to preeclamptic plasma. This is supported by the results of the Takacs study suggesting that IL-6 has been elevated in HUVECs cultures exposed to preeclamptic plasma [16,17].

Conclusion

Preeclampsia is a severe complication in pregnancy characterized by a broad maternal systemic inflammatory response with activation of the immune system, cytokines and adhesion molecules. Inflammatory cytokines as playing an important role are activators of endothelial vascular and an important mediator of endothelial dysfunction causing preeclampsia [17]. In some studies,
the active ingredients of black cumin, Thymoquinone, function as anti-inflammatory by inhibiting the enzyme Cyclooxygenase (COX) and Lipooxygenase (LOX) and inhibit transcription factor NF-kB and TNF-α [2]. In this study, black cumin in various doses can decrease proinflammatory cytokine such as TNFα and IL6 in huvec that exposed by preeclamptic plasma

**Limitation**

The limitation of this study is the need of further research on black cumin ethanol extract (*Nigella sativa*) in more extensive way, especially to test the toxicity in animal model so that it can be applied to humans and also the test in other pre-eclampsia models such as human monocyte cell cultures for the use of *Nigella sativa* ethanol extract for prevention and treatment of preeclampsia are required.

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