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Effect of a black cumin (*Nigella sativa*) ethanol extract on placental angiotensin II type 1-receptor autoantibody (AT1-AA) serum levels and endothelin-1 (ET-1) expression in a preeclampsia mouse model



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المخلص

أهداف البحث: يؤثر تسمم الحمل على 3-8% من جميع حالات الحمل. ويعتبر ثيموكينون هو المركب الرئيس من الكمون الأسود (نيجيلا ساتيفا)، الذي قد يكون له تأثير علاجي محتمل على تسمم الحمل. يحلل هذا البحث تأثيرات مستخلصات الإيثانول من بذور الكمون الأسود على انجيوتنسين 2 النوع 1-من مستقبلات مستويات مصل الأجسام المضادة والتعبير عن اندوتلين المشيمة-1 عند تسمم الحمل في نموذج الفئران.

طرق البحث: يستخدم هذا التصميم البحثي نموذجاً تجريبياً لما بعد الاختبار على تصميم مجموعة التحكم مع 6 مجموعات من الفئران (سيطرة سلبية، مراقبة إيجابية، 500، 1000، 1500، 2000 مجم/كجم وزن الجسم/يومياً).

النتائج: أظهرت النتائج انخفاض في مستويات مصل انجيوتنسين 2 النوع 1-من مستقبلات مستويات مصل الأجسام المضادة والتعبير عن اندوتلين المشيمة-1 بواسطة زيادة جرعات الكمون الأسود، مع الجرعة المثلى 1000 مجم/كجم/يومياً.

الاستنتاجات: مستخلص الإيثانول من بذور الكمون الأسود يقلل من مستويات مصل انجيوتنسين 2 النوع 1-من مستقبلات مستويات مصل الأجسام المضادة ويقمع التعبير عن اندوتلين المشيمة-1 عند تسمم الحمل في نموذج الفئران.

الكلمات المفتاحية: انجيوتنسين 2 النوع 1-من مستقبلات مستويات مصل الأجسام المضادة؛ مضادات الأكسدة؛ الكمون الأسود؛ اندوتلين المشيمة-1؛ تسمم الحمل؛ الحمل

Abstract

Objectives: Preeclampsia affects 3%–8% of all pregnancies. Thymoquinone is the primary compound in black cumin (*Nigella sativa*) and may have potential therapeutic effects in preeclampsia. This research analyses the effects of a black cumin seed ethanol extract on angiotensin II type 1-receptor autoantibody (AT1-AA) serum levels and the expression of the endothelin-1 (ET-1) in the placenta in preeclampsia mouse model.

Methods: The research design utilizes a post-test only experimental model on a control group design with 6 mice groups (negative control; positive control; and 500, 1000, 1500, and 2000 mg/kg body weight/day).

Result: The results showed a decrease in serum AT1-AA levels and ET-1 expression in the placenta by increased doses of black cumin with an optimal dose of 1000 mg/kg/day.

Conclusions: Black cumin seed ethanol extract reduces AT1-AA serum levels and represses ET-1 expression in the placenta in a preeclampsia mouse model.

Keywords: Angiotensin II type 1-receptor autoantibody (AT1-AA); Antioxidant; Black cumin; Endothelin-1 (ET-1) placenta; Preeclampsia; Pregnancy

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Introduction

Preeclampsia is a significant obstetric problem, of which the aetiology remains unclear. Preeclampsia affects 3–8% of all pregnancies, with a prevalence of 0.8% before 32 weeks of gestation.¹ The WHO (World Health Organization) reported that more than 60,000 maternal deaths occurring worldwide each year are caused by preeclampsia and 12% of infants will die within the first month.

Oxidative stress, inflammation, genetics and immunology disorders are evident processes that underlie the pathogenesis of preeclampsia. These conditions can lead to ischaemia or hypoxia in the placenta.² Hypoxia Inducible Factor 1 α (HIF-1 α), soluble Flt-1 (sFlt-1), angiotensin II type 1-receptor autoantibody (AT1-AA), and placental growth factor (PIGF) are factors known to be involved in preeclampsia. Increasing these factors causes endothelial dysfunction, reduced Nitric Oxide (NO) bioavailability and increased Reactive Oxygen Species (ROS) and Endothelin (ET-1), which leads to changes in renal function, increased Total Peripheral Resistance (TPR), and eventually to hypertension.³

Efforts to prevent the development of preeclampsia with various supplements have been tried and resulted in failures or limited success. Currently, the termination of pregnancy is considered the most appropriate treatment for preeclampsia. Black cumin (*Nigella sativa*) is known to have therapeutic benefits, including anti-inflammatory, antioxidant and anti-hypertensive effects. Black cumin seed, which is also an antioxidant, can act as a radical scavenger that will protect organs from oxidative damage caused by various free radicals.⁴ Thymoquinone in black cumin functions as an anti-inflammatory by inhibiting pro-inflammatory cytokines and the transcription factor Nuclear Factor Kappa Beta (NF- κ B).⁵

The antioxidants in black cumin are expected to improve hypoxic conditions in the placenta and promote anti-inflammatory effects to prevent endothelial dysfunction and oxidative stress in preeclampsia; this effect would result in improved markers for endothelial dysfunction in preeclampsia, such as increased ET-1 expression and a decrease in AT1-AA serum levels. Therefore, we performed this study to examine the effects of a black cumin seed ethanol extract on placental AT1-AA serum levels and ET-1 expression in a preeclampsia mouse model.

Materials and Methods

The research design used experiments with a post-test only control group design. This study used 30 pregnant mice that were randomly assigned to six groups. The negative control group included pregnant mice intraperitoneally injected with normal serum from pregnant women. The positive control group was pregnant mice intraperitoneally injected with serum from women with preeclampsia without administration of black cumin ethanol extracts. The treatment groups were pregnant mice intraperitoneally injected with serum from women with severe preeclampsia and administered black cumin extract at various dosages of 500, 1000, 1500, and 2000 mg/kg body weight/day.

Extraction from black cumin seeds

Cleaned black cumin seeds were dried and placed in the oven at 40–60 °C and then mechanically powdered. Seed powder (100 g) was poured into a 1000 ml Erlenmeyer, soaked with ethanol to a final volume of 900 ml, homogenized, and left overnight. The top layer of the ethanol, which included the active substances, was filtrated and placed in a 1 L evaporation flask. Then, the ethanol containing the active substances was allowed to separate in the evaporation flask. The ethanol flow was allowed to persist until it stopped dripping at approximately \pm 900 ml. The resulting extraction was placed in a bottle and stored in a freezer.⁶

Procedures for making the preeclampsia mouse model

Blood serum samples were obtained from women who have severe preeclampsia at gestational ages \geq 20 weeks-old and were diagnosed according to the criteria of the National High Blood Pressure Education by an experienced obstetrician. Blood samples were drawn from the cubital vein, maintained at room temperature for 12 h, and the centrifuged at 6000 rpm for 10 min to collect the serum from the blood. Serum was stored at -40 °C until it was time to inject the experimental mice.

Serum (0.1 cc) was injected intraperitoneally into the pregnant mice in the lower left quadrant of the abdomen twice on gestational days 10 and 11 with a 1 cc syringe with a 27G needle. The needle was inserted in parallel to the legs at a 30° angle. Blood pressure measurements were performed using a CODA™ (Kent Scientific, USA) and protein urine measurement with a QuantiChrom™ (BioAssay Systems, USA) protein assay kit on gestation day 15 to ensure the preeclampsia mouse model.⁷

The preeclampsia mice were orally administered black cumin ethanol extracts at different dosages (500, 1000, 1500, and 2000 mg/kg body weight/day) each day from gestation day 15 until 19. Cervical dislocation was used to collect the serum and placenta. Then, the mice were buried properly to avoid environmental contamination.^{7,8}

Measurement of serum AT1-AA levels

Mice blood was taken directly from the right heart using a 1 cc syringe. Blood samples were maintained for 12 h and the centrifuged at 3000 rpm for 10 min. The blood serum was stored in an Eppendorf at -40 °C until use. Serum AT1-AA levels were measured using an ELISA kit (My Biosource, Mouse Angiotensin 2 Receptor Type 1 autoantibodies ELISA kit No. MBS 731 268). The data were analysed by one-way ANOVA with the SPSS 19.0 software.

Measurement of ET-1 expression in the placenta

Mice placentas were placed into 10% formalin for at least 24 h. The placentas were cut using a microtome into 3–5 micron thick pieces, placed on an object glass, and inspected with an immunohistochemistry (IHC) method. For the staining process, deparaffinization was performed by heating the samples at 60 °C for 60 min. Then, the samples were immersed sequentially in xylol, absolute ethanol, and 90%,

80%, and 70% ethanol for each 5 min and then washed with sterile distilled water 3 times for 5 min. The antigen was retrieved using citrate buffer and immunohistochemical staining was performed with ET-1 primary antibodies from Abcam, USA; then the samples were incubated for 18 h. Next, the placenta specimens were incubated again using secondary antibody and Streptavidin-Horseradish Peroxidase (SA-HRP) for approximately 30 min. Chromagen Diaminobenzidine (DAB) was applied, the specimens were counterstained with Mayer's haematoxylin, mounted with Entellan, and observed under an Olympus Type CX21 microscope ($M = 1000\times$) in 20 fields of view. The placental ET-1 expression was considered positive if there was a brown colour in the vascular endothelial cell cytoplasm. The mean expression of the ET-1 in the placenta was calculated for each field of view. The data were analysed by one-way ANOVA with the SPSS 19.0 software.

Results

A preeclampsia mouse model was obtained by injecting mice with serum from pregnant women with severe preeclampsia at gestational days 10 and 11; the mouse model was confirmed by measuring the blood pressure and protein urine to identify mice showing indications of hypertension and proteinuria. Mice with preeclampsia displayed a significant increase in serum AT1-AA level as well as increased placental ET-1 expression compared to the normal pregnant mice.

Effect of black cumin seed ethanol extract on AT1-AA serum levels

The histogram (Figure 1) shows that the preeclampsia mice that were not treated with the black cumin seed had a higher AT1-AA serum levels compared to the preeclampsia mice model treated with the black cumin seed extracts. A significant decrease in AT1-AA serum levels was observed in the preeclampsia mice administered black cumin seed at 1000, 1500, and 2000 mg/kg body weight/day doses. Black

cumin seed extracts at the 500 mg/kg body weight/day does did not appear to significantly decrease AT1-AA serum level in the preeclampsia mouse model (see Figures 2 and 3).

The data showed that the serum levels of AT1-AA in the treated group at the 1000 mg/kg body weight/day dose did not differ significantly from the mean in the normal pregnant mice group. This means that the optimum dose of black cumin seed ethanol extract to reduce the serum AT1-AA levels in the preeclampsia mouse model was 1000 mg/kg body weight/day (see Table 1).

Effect of black cumin seed ethanol extract on placental ET-1 expression

Placental ET-1 expression was significantly different in the normal pregnant mice compared to the preeclampsia mouse model. This indicates that the injection of serum from women with severe preeclampsia into the mice can cause a significant increase in placental ET-1 expression (Table 2).

Treatment with black cumin seed extraction at various dosages showed significant differences in placental ET-1 expression in the preeclampsia mouse model. We observed that treatment with black cumin seed extracts at the 1000, 1500 and 2000 mg/kg body weight/day doses showed no significant differences in placental ET-1 expression between the treated mice and the normal pregnant mice. However, it differed significantly from the preeclampsia mice without treatment, indicating that the optimum dose of black cumin seed ethanol extract was 1000 mg/kg body weight/day because it was the minimum dose that could reduce placental ET-1 expression and did not significantly affect placental ET-1 expression in the normal pregnant mice.

Discussion

Our study confirmed that AT1-AA serum levels significantly increased significant increase ($p < 0.000$) in pregnant mice injected with severe preeclampsia serum compared with normal pregnant mice. This shows that the intraperitoneal

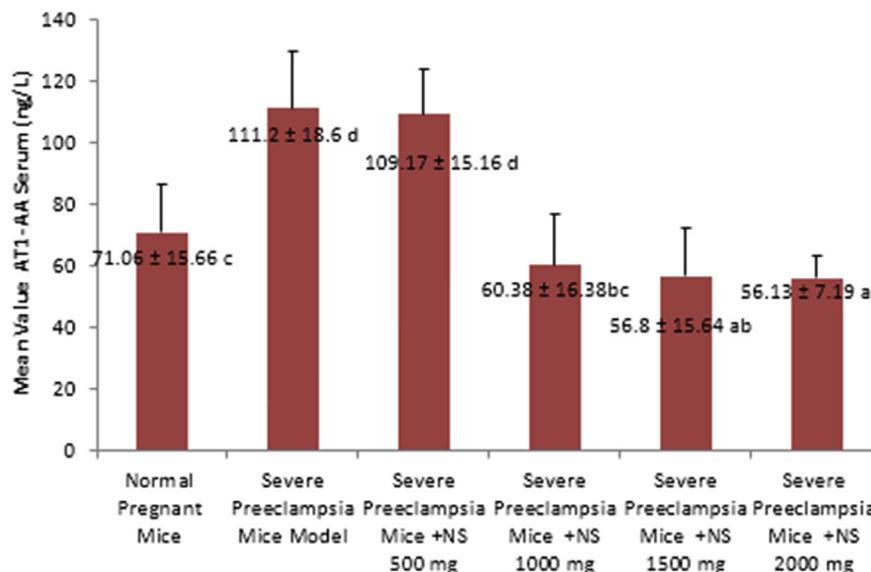
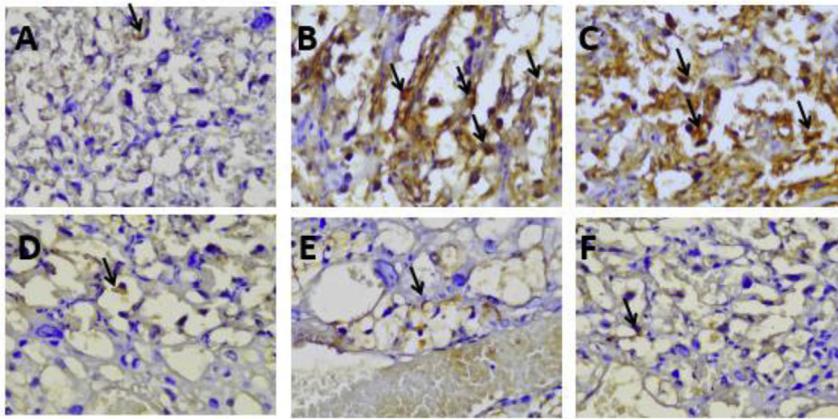


Figure 1: Mean AT1-AA serum levels (ng/L).



Note: A: Negative control, B: Positive control, C: 500 mg, D: 1000 mg, E: 1500 mg, F: 2000 mg

Figure 2: The expression of ET-1 in the cytoplasm of placental vascular endothelial cells (magnification of 1000×).

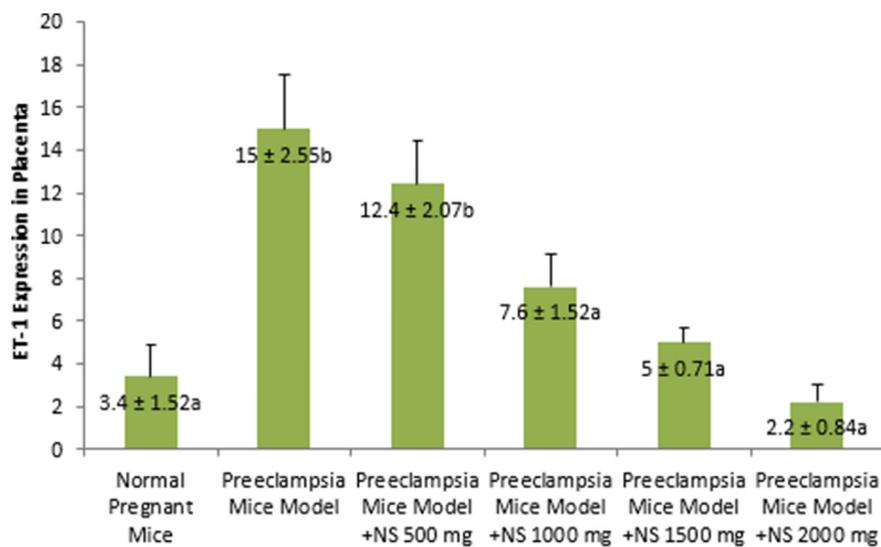


Figure 3: Histogram of mean placental ET-1 expression.

injection of severe preeclampsia serum may cause a significant increase in AT1-AA serum levels in a preeclampsia mouse model.

In addition to placental ischaemia being a stimulus for AT1-AA production, an increase in tumour necrosis factor- α (TNF- α) is also associated with the increased

production of AT1-AA.⁹ Page et al. and Tosun et al. reported that TNF- α levels are higher in women with preeclampsia compared to normal pregnant women.¹⁰ The study also proved that administration of TNF- α to pregnant mice resulted in the production of AT1-AA. It may be possible that immune mechanisms were induced at

Table 1: Mean AT1-AA serum values (ng/L).

Variable	Mean ± SD	p-value
Normal mice	71.06 ± 15.66 ^c	0.000
Preeclampsia mouse model	111.20 ± 18.60 ^d	
Preeclampsia mouse model + NS 500 mg	109.17 ± 15.16 ^d	
Preeclampsia mouse model + NS 1000 mg	60.38 ± 16.38 ^{bc}	
Preeclampsia mouse model + NS 1500 mg	56.8 ± 15.64 ^{ab}	
Preeclampsia mouse model + NS 2000 mg	56.13 ± 7.190 ^a	

The different letter of superscript indicates a significant different between treatment.

Table 2: Mean expression of placental ET-1.

Variable	Mean ± SD	p-value
Normal mice	3.4 ± 1.52 ^a	0.000
Preeclampsia mouse model	15.0 ± 2.55 ^b	
Preeclampsia mouse model + NS 500 mg	12.4 ± 2.07 ^a	
Preeclampsia mouse model + NS 1000 mg	7.6 ± 1.52 ^a	
Preeclampsia mouse model + NS 1500 mg	5.0 ± 0.71 ^a	
Preeclampsia mouse model + NS 2000 mg	2.2 ± 0.84 ^a	

The different letter of superscript indicates a significant different between treatment.

the time of placental ischaemia resulting in the production of AT1-AA.¹¹ This is consistent with a study by Granger et al. in 2006, who surgically manipulated the mouse uterus to create an Reduce Uterine Perfusion Pressure (RUPP) model to determine whether placental blood flow can lead to preeclampsia features. Of course, with a decrease in uterine perfusion, the mice showed signs of preeclampsia, such as hypertension; proteinuria; increased sFlt-1, tumour necrosis factor- α (TNF- α), and production of endothelin; and endothelial dysfunction. AT1-AA was also detected in this mouse model, while unmanipulated pregnant mice had undetected levels of AT1-AA.¹²

Hypertension and AT1-AA are detected in the circulation of pregnant mice who received low doses of TNF- α during pregnancy. Non-pregnant animals did not show any similar symptoms to the tested pregnant animals, indicating that adequate placental perfusion is required for a healthy pregnancy and that decreased perfusion can cause an inflammatory response that can lead to the production of autoantibodies. The development of AT1-AA in the reduced uterine perfusion pressure mouse models that received low doses of TNF- α demonstrates the important relationship between RAS regulation and the health of the mother during pregnancy.¹³

In 2010, Siddiqui et al. showed that AT1-AA was detected in more than 95% of 37 women with preeclampsia, and that the bioactivity of these autoantibodies correlated significantly with the severity of the disease, especially at the protein urine level. AT1-AA was also detected in normotensive pregnant women, but less than 30% of those women had detectable autoantibodies, which is five times less than in the group with preeclampsia.¹⁴

This research also showed a significant difference between placental ET-1 expression in the pregnant mice injected with the severe preeclampsia serum (3.4 ± 1.52) compared to the normal pregnant mice (15.0 ± 2.55) ($p < 0.000$). This proved that the administration of intraperitoneal injection of the serum from women with severe preeclampsia can increase the placental ET-1 expression in pregnant mice.

Another study by Zhou et al. (2011) reported that IgG from women with preeclampsia, in contrast to IgG in normotensive pregnant women, was able to induce the expression of preproET-1 mRNA through the activation of the angiotensin II type 1 receptor in the placenta of pregnant rats.¹⁵ Several other cohort studies also have examined the levels of ET-1 in normal pregnancy and preeclampsia and found increases in ET-1 plasma levels in the preeclampsia group.¹⁶ Moreover, another study by Napolitano et al. found increased ET-1 expression in preeclampsia placentas compared to normal pregnancy placentas.¹⁷

Effect of black cumin seed ethanol extract on AT1-AA serum levels in the preeclampsia mice model

This study showed significant differences between AT1-AA serum levels in the preeclampsia mouse model without black cumin seed treatment (positive control) compared to the preeclampsia mouse model treated with black cumin seed ethanol extract with a p-value of 0.000 ($p < 0.05$). These results indicate that black cumin seed ethanol extract can lower AT1-AA serum levels in a mouse model of preeclampsia.

The mechanism of how black cumin seed ethanol extract can lower AT1-AA serum levels is not fully understood. Black cumin can affect oxidative stress and inflammatory processes simultaneously through the inhibition of NF- κ B.¹⁸ Thymoquinone inhibits the expression of the p65 subunit of NF- κ B and the p50 subunit *in vivo*, which binds to TNF- α promoter.¹⁹ TNF- α , IL-6, and various other cytokines are not only regulated by NF- κ B but also act as activators of NF- κ B that lead to the preservation of pro-inflammatory conditions.²⁰ In contrast, ROS is known to cause oxidative stress, which is important in preeclampsia and plays an important role in both the upstream and downstream NF- κ B pathway.²¹ Therefore, black cumin may disrupt these interactions through suppression of NF- κ B, thus playing an important role through its antioxidant and anti-inflammatory activities. The decrease in NF- κ B and ROS due to TNF- α production also decreases other pro-inflammatory factors, such as IL6, resulting in decreased production of AT1-AA.

Effect of black cumin ethanol extract in placental ET-1 expression in the preeclampsia mouse model

This study showed a significant difference between the placental ET-1 expression in the preeclampsia mouse model without black cumin treatment (positive control) compared to the group treated with black cumin seed ethanol extract with a p-value 0.000 ($p < 0.05$). These results indicate that the black cumin seed ethanol extract can decrease placental ET-1 expression in a mouse model of preeclampsia.

This is consistent with the results of *in vivo* studies by Ozlam et al. in 2011 using a sepsis mouse model. The sepsis mouse model group treated with black cumin showed lower ET-1 serum levels than the group that was not treated with black cumin.²² Another study also showed similar results in a study on 40 pigs that were made to have asthma and then administered black cumin extract. Administration of black cumin significantly reduces ET-1 levels in lung tissue compared to the positive controls that had asthma.²³

The ET-1 reduction mechanism by black cumin presumably occurs through its active component Thymoquinone, which can prevent organ damage by free radicals by preventing the formation of ROS.²⁴

Thymoquinone also suppresses NF- κ B activation. Suppression of NF- κ B activation is associated with the inhibition of activation, phosphorylation, and degradation of protein kinase B (I κ B α) and inhibition of degradation and translocation of p65. Thymoquinone inhibits p65 from binding to DNA. Thymoquinone's actions to inhibit the activation of NF- κ B inhibit the occurrence of endothelial dysfunction in preeclampsia,²⁵ resulting in a decrease in markers of endothelial dysfunction, such as ET-1.

Conclusions

1. Intraperitoneal injection of serum from women with severe preeclampsia into pregnant mice increased AT1-AA serum levels and increased the expression of ET-1 in the placenta.
2. Black cumin seed ethanol extract reduced AT1-AA serum levels and decreased placental Et-1 expression in a preeclampsia mouse model.

3. The optimal dose to decrease AT1-AA serum levels and increase placental ET-1 expression in a preeclampsia mouse model is 1000 mg/kg body weight/day.

Conflicts of interest

The authors have no conflict of interest to declare.

Authors' contributions

HR and MN conceived and designed the study, conducted research, provided research materials, and collected and organized the data. IWAY and R analysed and interpreted data. HR and IWAI wrote the initial and final drafts of the article and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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