

Effect of essential oil of black cumin (*Nigella sativa*) on hematological profiles and total cholesterol levels of Wistar rats exposed by cigarette smoke

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ABSTRACT

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Cigarette smoke contains toxic substances such as carbon monoxide, lead, cadmium, tar and hydrogen cyanide which can trigger oxidative stress and cause erythrocyte membrane damage and hemoglobin oxidation. In addition, it also contains nicotine which can increase the total cholesterol levels. Black cumin containing thymoquinone has been known for its antioxidant and anticholesterol activities. This study aimed to investigate the effect of black cumin extract on hematological profiles and total cholesterol levels of Wistar rats exposed by cigarette smoke. It was an experimental study with randomized posttest only control group design. Twenty Wistar rats (*Rattus norvegicus*) that divided into four groups were used in this study. The normal control group (N) was provided with standard feed, the negative control group (C) was exposed to the cigarette smoke with two pieces of cigarettes/day for 14 days, the treated groups were given black cumin extract 200mg/kg (T1) and 400mg/kg (T2) and exposed by cigarette smoke two pieces of cigarettes/day for 14 days. On day 15, blood samples from the rats were taken through the sinus orbitalis and then the erythrocyte, the hemoglobin and the total cholesterol levels were examined. Data were analyzed using the one-way ANOVA test and continued by the post-hoc test. The results showed the number of erythrocytes and hemoglobin levels of the T2 group was significantly higher than those of the C group ($p < 0.05$). Although, total cholesterol levels of the T2 group was lower than that of other groups, however it was not significantly different ($p > 0.05$). In conclusion, the administration of black cumin extract at 400mg/kg significantly increases the erythrocytes numbers and the hemoglobin levels in Wistar rats exposed by cigarette smoke.

ABSTRAK

Asap rokok mengandung senyawa toksik seperti karbon monoksida, timbal, kadmium, tar, dan hydrogen sianida yang dapat memicu stress oksidatif dan menyebabkan kerusakan membran eritrosit dan oksidasi hemoglobin. Selain itu, asap rokok juga mengandung nikotin yang dapat meningkatkan kadar kolesterol total. Jinten hitam yang mengandung timokuinon dikenal memiliki aktivitas anti oksidan dan anti kolesterol. Tujuan penelitian ini adalah mengkaji efek pemberian ekstrak jinten hitam terhadap gambaran hematologi dan kadar kolesterol total tikus putih Wistar yang terpapar asap rokok. Penelitian ini merupakan penelitian eksperimental dengan rancangan *randomized posttest only control group* menggunakan 20 ekor tikus yang dibagi menjadi empat kelompok. Kelompok control tikus normal (N) diberi pakan standar, kelompok control negatif (C) dipapar asap rokok dua batang/hari selama 14 hari, kelompok perlakuan dengan ekstrak jinten hitam 200 mg/kg (T1) dan 400 mg/kg (T2) dan dipapar asap rokok dua batang/hari selama 14 hari. Pada hari ke 15, sampel darah tikus diambil melalui sinus orbit alis untuk diukur jumlah eritrosit, kadar hemoglobin dan kolesterol totalnya. Data dianalisis menggunakan ANAVA satu jalan dilanjutkan dengan uji *post hoc*. Hasil penelitian menunjukkan jumlah eritrosit dan kadar hemoglobin kelompok T2 lebih tinggi secara nyata dibandingkan dengan kelompok C atau control negatif ($p < 0,05$). Meskipun kadar kolesterol total kelompok T2 lebih rendah dibandingkan kelompok lainnya, akan tetapi tidak bermakna secara nyata ($p > 0,05$). Dapat disimpulkan, pemberian ekstrak jinten hitam 400 mg/kg meningkatkan jumlah eritrosit dan kadar hemoglobin secara nyata pada tikus Wistar yang dipapar asap rokok.

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INTRODUCTION

Cigarette is being a problem throughout the world. In 2014, it was reported that as many as 650 million people consumed cigarettes. In Indonesia, cigarette consumption ranks third in the world. Cigarette not only affected the health of active smokers, but also the passive smokers in Indonesia.¹ In 2020, World Health Organization (WHO) estimated around six million people die from diseases caused by cigarette consumption, annually.²

Cigarette is dangerous to health due to it contains toxic substances such as carbon monoxide (CO), tar, nicotine and lead (Pb) which harmful to human body.³ These toxic substances can trigger oxidative stress due to free radicals produced. A radical molecule is a molecule that consisted by unpaired electrons and in an active conditions. These reactive molecules can damage cellular macromolecules such as proteins, lipids, carbohydrates and nucleic acids by giving rise to oxidative stress and increasing lipid peroxidase (LPO) activity.

Those free radicals content may change cell membrane structure of the erythrocyte that lead to lysis which cause a decrease in the number of erythrocytes followed by a decrease in the hemoglobin levels. In addition, the tar content in cigarettes may damage the spinal cord thus disrupt the formation of red blood cells.⁴ Carbon monoxide easily binds with hemoglobin thus become carboxy-hemoglobin. Other chemicals in cigarette can interfere the heme-biosynthesis thus reduce the hemoglobin levels in blood.⁵

Besides causing abnormalities in erythrocytes and hemoglobin, cigarette smoke may also causing an increased in the total cholesterol levels. The entered nicotine within the body would trigger the release of catecholamine, cortisol, and growth hormones. This hormone activated the adenyl cyclase in adipose tissue, thereby increasing lipolysis and releasing free fatty acids into the plasma. This, eventually, causes change

in lipid profile such as increased the levels of triglycerides and the very low-density lipoproteins (VLDL).⁶ High blood cholesterol levels can have a negative impact on health, including increases the risk of atherosclerosis, hypertension, and coronary heart disease.⁷

Alternative medicine using natural products has been practiced for a long time to cure various health problems. Various medicinal plants have been used traditionally to treat various illness. *Nigella sativa L.*, well known as black cumin or black seed, is one of natural medicine used in various regions in the world for wide range of illness. Black cumin is a shrub originating from the Mediterranean region and spread to India and Pakistan. However, this plant spreads to Southeast Asia including in Indonesia.^{8,9}

Black cumin contains major active substances well known as thymoquinone, thymohydroquinone, and thymol which exhibited various biological activities. *In vitro* study revealed that *N. sativa* epicotyl suspension cultures have a high antioxidant activity,¹⁰ where as in *in vivo* study on mice showed that black cumin oil has anti immunotoxic activity caused by DMBA (dimethylbenzanthracene) induction.¹¹ In addition, dietary supplementation of *N. sativa* powdered seeds was reported ameliorate the serum lipid profile in rats.¹²

In this study, the effects of black cumin (*N. sativa*) extract on erythrocytes and hemoglobin and total cholesterol levels in Wistar rats exposed by cigarette smoke. Two weeks of cigarette smoke exposure was applied and 2 doses of the black cumin extract were used according to the previous studies.^{13,14}

MATERIAL AND METHODS

Essential oil of *N. sativa* preparation

Black cumin seeds were purchased from a local grocery store CV Almanar HerbaFit in Yogyakarta. Extraction of essential oil of the black cumin was carried out using a steam distillation method. Five hundred g of black cumin seed sample was taken in a distillation

flask and 1000mL of water was added. The condenser and the collection vessel were installed. The distillation flask was heated and distillate was collected in the conical flask. This distillate had two layers, one dense layer and other less dense layer. This was then separated using a separating funnel. The less dense upper layer was the black cumin oil. Then the black cumin seeds oil was separated from water by 96% ethanol.

Animal treatments

It was an experimental study using posttest only control group design. The protocol of the study was approved by the Bioethics Commission of Faculty of Medicine, Universitas Sultan Agung Semarang. The study was conducted in The Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. Twenty four male Wistar rats (*Rattus norvegicus*) aged 8-12 weeks with 150-200 g body weight were used in this study. The rats were maintained for seven days under standard laboratory condition at room temperature in a 12 h light-dark cycle with free access to rat feeds and water *ad libitum*. Following after adaptation, the rats were then randomly divided into four groups with six rats in each group. The normal control group (N) was provided with standard feed, the negative control group (C) was exposed to the cigarette smoke with two pieces of cigarettes/day for 14 days, the treated groups were given black cumin oils 200 mg/kg (T1) and 400 mg/kg (T2) and exposed by cigarette smoke two pieces of cigarettes/day for 14 days. The cigarette smoke exposure was conducted 30 min before administration of black cumin oils.

Erythrocytes and hemoglobin analysis

On the day 15, rats blood samples were taken from *sinus orbital cantus* for

erythrocyte counts, hemoglobin levels, and total cholesterol level examinations. The erythrocytes count and hemoglobin levels were analyzed by Sysmex poH-100i haematology analyzer. Erythrocytes were reported in total numbers (10^6 cells/L) and hemoglobin levels were reported as proportion of mg/dL.

Total cholesterol analysis

The total cholesterol levels were determined using CHOD-PAP method. The CHOD-PAP reagent was added in three tubes marked as Blank, Standard and Test, each 1000 μ L. Then 10 μ L of distilled water was added in Blank, 10 μ L of standard (200mg/dL) in Standard and 10 μ L serum in Test. The solutions were mixed properly and incubated at 37°C for 10 min. The serum total cholesterol levels was measured using a spectrophotometer (Thermo-Genesys 10S UV) at 546 nm against reagent blank and reported in mg/dL.

Statistical analysis

Data was presented as mean \pm standard deviation (SD). The Saphiro Wilk and Levene tests were employed for data distribution and homogeneity. Considering the data obtained were normal and homogenous, the One Way ANOVA was employed, followed by the Post Hoc test. A p value <0.05 was considered as significant result.

RESULT

Normality test of the erythrocytes count, hemoglobin levels, and cholesterol levels data both using the Saphiro-Wilk test and the Lavene homogeneity test showed the p values > 0.05 (TABEL 1). It was indicated that data was normally distributed and had a homogeneous variance. Therefore, the One Way ANOVA followed by the Post Hoc test was employed.

TABLE 1. Normality and homogeneity of the erythrocytes count, hemoglobin levels, and cholesterol levels data

Group	Erythrocytes count		Hemoglobin levels		Cholesterol levels	
	Saphiro-Wilk test (p)	Lavene test (p)	Saphiro-Wilk (p)	Lavene test (p)	Saphiro-Wilk (p)	Lavene test (p)
N	0.413		0.525		0.123	
C	0.664	0.140	0.708	0.090	0.152	0.098
T1	0.877		0.727		0.688	
T2	0.115		0.468		0.961	

The erythrocytes count, hemoglobin levels, and total cholesterol levels of all groups are presented in TABLE 2. The erythrocytes count of C group was lower than that of N group (TABLE 2 and 3), although it was not significantly different ($p > 0.05$), whereas the Hb levels of C group was significantly higher than that of N group ($p < 0.05$). It was demonstrated, the cigarette exposure could decrease in the Hb levels but not in the erythrocytes count of rats. In addition, the total cholesterol levels of C group was higher than that of N group (TABLE 2 and 3), although it was not significantly different ($p > 0.05$). It was demonstrated, the cigarette exposure could not increase of the cholesterol levels of rats.

The erythrocytes count and Hb levels

of T2 group was significantly higher than those of N, C and T1 group ($p < 0.005$) as presented in TABLE 3 and 4. However, there was no significantly different in the erythrocytes count and Hb levels between T1 group and C group ($p > 0.05$). It was demonstrated that the effect of the black cumin oils on the erythrocytes count and Hb levels was observed at the dose of 400mg/kg BW.

Although the total cholesterol levels of T2 groups tended to lower compared to the N, C and T1 groups (TABLE 2), however they were not significantly different ($p > 0.05$). It was demonstrated that there was no effect of the black cumin oils on the total cholesterol levels of rats.

TABLE 2. The erythrocytes count, Hb levels, and total cholesterol levels (mean \pm SD) of rat serum of all groups

Group	Erythrocytes count (10^6 cell/ μ L)	Hb levels (mg/dL)	Total cholesterol levels (mg/dL)
N	9.10 \pm 0.55	17.46 \pm 0.74	74.69 \pm 5.47
C	8.52 \pm 0.83	14.94 \pm 0.40	82.20 \pm 13.66
T1	8.44 \pm 0.45	15.50 \pm 0.58	68.63 \pm 15.97
T2	10.27 \pm 0.32	18.80 \pm 0.21	64.21 \pm 6.80

Note. N: normal control (diet only); C: negative control (diet and smoking cessation); T1 (diet with smoking cessation and extract 200 mg/kg BW), T2 (diet with smoking cessation and extract 400 mg/kg BW).

TABLE 3. One-way ANOVA and post hoc analysis of erythrocytes count

Groups	ANOVA (p)	Post Hoc (p)			
		N	C	T1	T2
N	0.001*	-	0.122	0.082	0.005*
C		-	-	0.826	0.001*
T1		-	-	-	0.001*
T2		-	-	-	-

Note.N:normal control (diet only); C: negative control (diet and smoking cessation);T1 (diet with smoking cessation and extract 200 mg/kg BW), T2 (diet with smoking cessation and extract 400 mg/kg BW).

TABLE 4. One-way ANOVA and Post Hoc analysis of hemoglobin levels.

Groups	ANOVA (p)	Post Hoc (p)			
		N	C	T1	T2
N		-	0.001	0.001	0.001*
C	0.001*	-	-	0.110	0.001*
T1		-	-	-	0.001*
T2		-	-	-	-

Note.N:normal control (diet only); C: negative control (diet and smoking cessation);T1 (diet with smoking cessation and extract 200 mg/kg BW), T2 (diet with smoking cessation and extract 400 mg/kg BW).

DISCUSSION

The effect of *N. sativa* oils on erythrocytes count and hemoglobin levels

No significantly decrease in the erythrocytes counts of rats after the cigarette smoke exposure (C) could occur due to lack of time and length of the exposure. In this study, the cigarette smoke exposure was only carried out for 14 days. Wulandari *et al.*¹³ reported that smoke exposure time significantly reduce the number of erythrocytes at 28 days. The study reported that 1 to 4 cigarettes/day can reduce the number of erythrocytes and hemoglobin levels in Wistar rats exposed to cigarette smoke.¹³ Reduction in the number of erythrocytes and hemoglobin can be caused by the presence of oxidants which causes lipid peroxidase in red blood cell membranes, then lead to lysis in red blood cell

membranes.^{15,16}

In this study, significantly different in the erythrocytes count of the rats was observed after administration of black cumin oils at dose of 400 mg/kg BW ($p < 0.05$) but not at dose of 200 mg/kg BW. This result is in accordance with previous study which reported that black cumin oils at dose of 400 mg/kg has protective effect to free radicals from cigarette smoke, whereas not at dose 200 mg/kg BW.¹⁷

Black cumin extract contains substances such as polyphenols, thymoquinone, dithymoquinone, thymohydroquinone and thymol which have high antioxidant activity against free radicals. Thymoquinone can protect the erythrocyte membrane from oxidants that enter the cell to prevent hemolysis in erythrocytes.¹⁸ A study conducted by Kanter *et al.*¹⁹ showed that black cumin

extract dose of 2 mL/kg BW for 30 days can protect blood including red blood cells from the toxic effects of cadmium.

To decrease the hemoglobin levels, cigarette smoke has the same mechanism as to decrease the number of erythrocytes, because hemoglobin is one of the components forming erythrocytes. This study shown that black cumin oils had an effect on increasing hemoglobin levels and protection from cigarette smoke free radicals. The administration of 400 mg/kgBW black cumin oils for 14 days showed a significant increase in hemoglobin levels, when compared to the C group.

Thymoquinone in black cumin extract can also inhibits the formation of hydroxyl radicals which oxidize the hemoglobin. In addition, thymoquinone as an antioxidant can prevent synthesis inhibition of hem.^{20,21} Baskoro *et al.*²² stated that the dose of black cumin extract that can significantly increase Hb levels is at 500 mg/kg body weight. Although it mentioned that the number of cigarettes and the exposure duration of cigarette smoke was 4 cigarettes/day and given for 21 days, which is greater dose and longer period than in this study.²²

The effect of *N. sativa* on total cholesterol levels

This study used black cumin oils as an exogenous antioxidant. It has been known to reduce total cholesterol levels by inhibiting the enzyme HMG CoA reductase.^{9,12} Meanwhile, smoking is known to increase the lipid profile of adult smokers over the age of 20 years.⁶ However, according to this study it was indicated that the cigarette smoke is not able to increase the total cholesterol levels in Wistar rats after 14 days smoke exposure period. The black cumin oils also could not significantly reduce the total cholesterol levels in Wistar rats. The inability of black cumin extract to reduce LDL cholesterol levels was also reported by Khairunnisa *et al.*²³ It was reported that given black cumin extract up to 2 g/kg BW still has not been able to reduce LDL cholesterol levels of rats that

was exposed to cigarette smoke.

Although in this study black cumin oils could not reduce the total cholesterol levels of rats exposed to cigarette smoke, but the effect of black cumin to reduce cholesterol levels was reported in previous studies. An *in vivo* study conducted by Kocyigit *et al.*¹² using a supplement of powder from black cumin at various doses of 100, 200, 400 and 600mg/kg BW can produce a significant decrease in cholesterol after the fourth week of treatment. While in this study, although the dose of black cumin oils used was the same as the Kocyigit's study, the length of time for administration was only 14 days.

Although the effects of black cumin on the cholesterol of animal study are not consistent. Studies conducted using human subjects showed the effectiveness of black cumin. For example, different preparations of black seed including seed powder (100 mg–20 g daily), seed oil (20–800 mg daily), thymoquinone (3.5–20 mg daily), and seed extract (methanolic extract especially), could reduce plasma levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides. But the effect on high-density lipoprotein cholesterol (HDL-C) was not significant. *Nigella sativa* and thymoquinone have been reported to be safe and well tolerated with no severe adverse effect.²⁴

Other meta-analysis study conducted by Sahebkar *et al.*²⁵ also shown a significant association between *N. sativa* supplementation and a reduction in total cholesterol. A greater effect of *N. sativa* seed oil versus seed powder was observed on serum total cholesterol and LDL-C levels. An increase in HDL-C levels was found only after *N. sativa* seed powder supplementation. *Nigella sativa* has a significant impact on plasma lipid concentrations, leading to lower total cholesterol, LDL-C, and TG levels. While the increased HDL-C was associated with *N. sativa* powder only. According to Sahebkar *et al.*²⁵ further RCTs are needed to explore the *N. sativa* benefits on cardiovascular outcomes.

CONCLUSION

In conclusion, the black cumin oils at dose of 400 mg/kg BW significantly increase the erythrocytes count and hemoglobin levels of rats after cigarette smoke exposure. Meanwhile, there is no effect of black cumin oils on total cholesterol levels of the rats.

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