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Black Cumin Inhibits Pro-Atherogenic Changes and Reduces Aortic Intima Media Thickness in Rats with Sub-Chronical Cigarette Smoke Exposure

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ABSTRACT

The study aims to explore the association between black cumin extract supplementation to endothelial dysfunction markers and aortic Intima Media Thickness (IMT) in rats with sub-chronical cigarette smoke exposure. Fifty Wistar rats (*Rattus norvegicus*) were classified into five kind of groups: negative control group (NC); positive control group (PC), which was shown up to 40 cigarettes/day for about 4 weeks; and three groups shown up to cigarette smoke and it has administration of extract of black cumin ethanolic for about four weeks but in different amount of doses: 0.3 g/kg/day (T1); 0.6 g/kg/day (T2); and 1.2 g/kg/day (T3). After getting intervented, aorta was examined to measure the level of vascular cell adhesion molecule-1 (VCAM-1), endothelial nitric oxide synthase (eNOS), and also IMT. The dominant active substances of black cumin are linoleic acid (71.31%), palmitate acid (15.86%), stearate (4.69%), octadicadienal (3.8%), beta monolinolein (1.8%), and thymoquinone (0.28%). eNOS level significantly increased in T1 compared to PC with p=0.001. A significant descending in VCAM-1 was found in T2 (p=0.014) and T3 (p=0.044) compared to PC. Aortic IMT decreased significantly in T1 (p=0.009), T2 (p=0.000), and also T3 (p=0.043) than the PC. Increased eNOS, decreased VCAM-1 and IMT following black cumin extract supplementation showed that black cumin may prevent endothelial dysfunction.

Keywords: Black cumin, Cigarette smoke, Endothelial dysfunction,VCAM, eNOS

Introduction

Endothelial dysfunction is a condition closely related to numerous progressions of cardiovascular diseases.[1] Endothelial dysfunction initiates atherosclerosis development, making it suitable for an early predictor of severity and mortality of atherosclerosis complications.[2] Since atherosclerosis is a major cause of coronary artery disease (CAD), endothelial dysfunction considerably becomes a predictor of CAD.[3,4] An essential factor that contributes to endothelial dysfunction is smoking. The mechanisms by which cigarette smoke exposure could trigger atherosclerosis are becoming challenging. Cigarette has more than 5000 different chemical products that causes pathological imbalance between pro-oxidant and antioxidant level, pro-inflammatory and also anti-inflammatory state, as good as pro-thrombotic and anti-thrombotic state. These imbalances are consistent with endothelial dysfunction. [5-10] The key to endothelial dysfunction pathomechanism centers on the role of Endothelial Nitric Oxide Synthase (eNOS) and it is known as an enzyme found in endothelial cells that contributes to Nitric Oxide (NO) production.[11-12] Upregulation of eNOS expression and it has a vital role in protecting endothelium via endothelium-dependent relaxation.[13-16] Meanwhile, oxidative stress is known to become the cause of decreased eNOS production.[17] Decreasing eNOS could trigger inflammation and coagulation process by the adhesion molecules' expression as like the cells of vascular adhesion molecule-1 (VCAM-1) on the cell surface for recruiting and attaching inflammatory cells. These molecules were stated to have involvements of smoking-related plaque of atherosclerosis.[11-18] VCAM-1 has such a significant factor in neointimal proliferation getting along with nicotine-induced arterial kind of injury.[19] Black cumin (*Nigella sativa*) is a natural antioxidant that has been proposed to inhibit endothelial dysfunction.[20-21] Black cumin and its derivative compounds has shown potentials in radical scavenging and inhibiting oxidative stress by increasing antioxidant enzyme products.[22] However, the underlying mechanisms regarding the mechanisms of black cumin inhibiting progression of endothelial dysfunction caused by cigarette smoke exposure have yet to be confirmed. This research was done on purpose to help explore the association of the igarette smoking toward eNOS and VCAM-1, it shows in the change of aortic IMT. This research also studied the effects of black cumin administration toward the change of eNOS levels, VCAM-1 expression, and IMT.

Materials and Methods

Animals

This study applied for about 50 male Wistar rats (*Rattus norvegicus*), have ages of eight weeks, and the body average of weights of 150-200 grams. The rats from the experimental animal farm and their health has been examined at the Malang city agricultural service, in the field of animal husbandry and animal health. The rats were put inside the cages at room temperature in range between 24°C to 30°C, with a 12-h light/12-h cycle of dark, under made ventilation. Those rats were given food for a standard balanced diet of rodent and also given water was put ad libitum. This study of animal experiment was set by the approval of the Institutional Animal Care and Use Committee of Universitas Airlangga (UNAIR), Surabaya, Indonesia (approval no: 2.KE.184.10.2019). it was done in strict related to the standasrds of internationally-accepted of the Guide for the Care and Use of Laboratory Animals of the Health National Institute.

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Experimental design and groups

This study used a randomized post-test-just headed to the design of controlled group. Rats were randomly put and classified into 5 groups. Negative control (NC) group (n=9) contained rats with no interventions, while the positive control (PC) (n=9) group contained rats that were given smoke exposure. Other groups are treatment groups that are given cigarette smoke exposure and 0.3 g/kg/day (T1 group), 0.6 g/kg/day (T2 group), and 1.2 g/kg/day (T3 group) of black cumin extract. These groups also contained 9 rats, respectively. The animals were sacrificed and analyzed for VCAM-1 expression, eNOS level, and IMT of the aorta after 28 days of consecutive experiments.[21]

Cigarette smoke exposure

Rats were exposed to sidestream smoke of cigarette produced by a peristaltic pump and then transferred into a smoke exposure chamber. Smoke exposure chamber was made of acrylic with a volume of 95 x 80 x 65 cm that has a capacity of 9 rats. Cigarettes were lit simultaneously and transferred to an inhalation chamber, and then the smoke was incubated in the box for 30 minutes. Cigarette smoke dosage used was 40 cigarettes daily (8 amout of cigarettes per cycle and repeated 5 times for a day). The exposure was carried out every day for 4 weeks.[23,24] We used cigarettes without filter that contains tar in 39 mg and also nicotine in 2.3 mg based on the manufacturer label (Dji Sam Soe®, HM. Sampoerna, Indonesia).

Black cumin extraction

Black cumin seeds were dried, mashed, and then extracted using the maceration method. Maceration was done by constant stirring at 1200 rpm for 24 hours with ethanol as solvent. Those processes were repeated for six days. Lastly, the resulting mixture was filtered and evaporated until reaching constant volume. Thymoquinone levels and other extract contents were measured by Gas Chromatography-Mass Spectrometry (GC-MS) and also High-Performance Liquid Chromatography (HPLC). The extract was given to the rats using a gastric tube according to the dose mentioned before.

eNOS levels measurement

Measurements of eNOS levels were conducted using ELISA method by the kit's manufacturer protocol (*elabscience* E-EL-R0367). Aortic tissue was settled up to be the solution. Then, 100 µL of the solution sample was introduced to antibody of well-coated primary anti-eNOS and incubated overnight at 4^{o} C using a shaker. The mixture from the well was washed using 400 µL of wash solution. Secondary antibody anti-eNOS with the volume of 100 µL was given more to each well, having incubated for about 2 hours at temperature of room using a shaker, then washed again. The next step was addition of substrate with the volume of 100 µL each well and given incubation for about 30 minutes. Lastly, 50 µL of stop solution was given more into each well. The samples were counted at a wavelength of 450 nm using an ELISA reader.

VCAM-1 immunohistochemistry

Aortic tissue was prepared and deparaffinized following fixation. Anti-VCAM-1 primary antibody (*Santacruz biotech* SC-13160) was given up to and given incubation for about 30 minutes. Phosphate buffer saline (PBS) was used for the washing process. After washing, secondary antibody was given and incubation for about 30 minutes at the temperature of room, then washed. *Streptavidin-Hoseradish Peroxidase* (SA-HRP) was given and incubated for about 10 minutes at temperature of room, getting washed. Next steps were staining process using Chromogen DAB (3,3-diaminobenzidine tetrahydrochloride) and also counterstained with the certain amount of Hematoxylin-eosin) and dried. Expression of VCAM-1 in tunica intima and tunica media were observed under a light microscope with 400x magnification. VCAM-1 expression measurements were assessed semi-quantitatively by system of immunoreactivity scoring (IRS) (Table 1).

IMT measurement

Thoracic aorta samples were getting fixed in 10% formaldehyde for 24 hours. Samples were processed and put in paraffin then cut at a 6-8 µm of thickness.

Table 1: Immunoreactivity Scoring System (IRS)

Score for percentage of cells staining	Score for intensity of staining
0 = no stained cells	0 = no reaction
1 = <10% cells are stained	1 = mild intensity of staining
2 = 10-50% cells are stained	2 = moderate intensity of staining
3 = 51-80% cells are stained	3 = heavy intensity of staining
4 = >80% cells are stained	

The cut tissues then were mounted in object-glass for hematoxylin-eosin staining. Aortic IMT was observed and measured using a light microscope. The of intima and also aorta media layer thickness was measured. Every sample was getting randomly getting measured in micrometer (µm) from five unsimilar kind of locations with a magnification of 400x using light microscope.

Statistical Analysis

Results were displayed as (1) means ± standard deviations (SD) for distributed data normally; (2) medians that has upper and lower value for data distributed abnormally. The consideration the normality was resulted by test of Shapiro-Wilk. An Independent t-test is performed to help compare the value of mean of the two variables if the it the distribution is normal and Mann–Whitney test is used as an alternative test if the data abnormally distributed. Data with more than two variables and normally distributed is analyzed using One-way ANOVA test. If its distribution becomes abnormal, the test of Kruskal–Wallis is going to apply Last, Tukey HSD post hoc analysis can help determine which group is knowingly different from the results of ANOVA test and Mann–Whitney test for Kruskal–Wallis' post hoc analysis. The mean or median difference was said to become more significant when the *p*-value is known to be less than 0.05 ($p < 0.05$). All analysis was done by having SPSS version 25.0 (IBM Corp, Chicago, USA).

Results and Discussion

HPLC and GC-MS results of black cumin extraction

This study identified the substances contained in Indonesian native black cumin through HPLC and GC-MS. HPLC results revealed thymoquinone content of 0.226 ± 0.008%. Furthermore, other contents and its concentration were also discovered through GC-MS as shown in Table 2. Statistical analysis of eNOS measurement yielded a p value = 0.000 ($p < 0.05$), it shows that it has a significant decrease of eNOS level in the PC group (54,83 ± 8,31) compared to NC group (101,22 ± 11,80). Observation of aorta's immunohistochemistry showed the increasing of VCAM-1 expression in PC groups indicated by a browner color. Exposure to smoke of cigarette also influences the histological aorta structure. Vacuolization and disorganization of smooth muscle cells are seen in tunica media (figure 1). However, semi-quantitative calculations using IRS resulted in a non-significant increase of VCAM-1 expression in PC group (10,33 ± 3,74) compared to NC group (7,67 ± 2,92) (p=0.111). Another variable measured was IMT of the aorta. IMT in PC group (88,39 ± 2,52) increased significantly compared to NC group (58,98 ± 13,61) (p=0.000). Data comparison was presented in Table 3.

eNOS level after black cumin extract administration

After 28 days of following experiments, the median of eNOS level in PC was 53.51, T1 was 111.52, T2 was 55.66, and T3 was 57.72. The comparative analysis demonstrated that there was a statistically difference of these groups (p=0.003). Post-hoc test result resulted that eNOS level significantly going up in T1 compared to PC with p=0.001, whereas it increased but not significantly in T2 (p=0.122) and T3 (p=0.171). There was a significant difference in eNOS level in T1 than T2 (p=0.030) and T3 (p=0.019). There was not any significant difference between T2 and T3 (p=0.895). Table 4 presents the data of eNOS level in each group.

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Table 2: Indonesian native black cumin substance contents and its concentration through GC-MS

Substance	Concentration
Linoleic acid	71.31%
Palmitate acid	15.86%
Stearate	4.69%
Octadicadienal	3.8%
Beta-monlinolein	1.8%
Thymoquinone	0.28%

VCAM-1 expression after black cumin extract administration

VCAM-1 expressions were taken observed toward aortic tunica intima and tunica media. Weak VCAM-1 expressions were observed toward T1, T2, and T3 groups, with the weakest observed on T2 group compared to PC group (Figure 2). The medians of the VCAM-1 expression's IRS in all groups were: 10.00 (PC), 8.00 (T1), 6.00 (T2), and 5.00 (T3). A significant difference was revealed of these groups based on comparative analysis (p=0.015). Result of Post-hoc analysis indicated a decrease in VCAM-1 IRS but not statistically significant in T1 group (p=0.116) compared to PC. A significant decrease was revealed in T2 group (p=0.014) and T3 group (p=0.044) compared to PC group. IRS of VCAM-1 in T1 group was significantly different when it is compared to the T2 group (p=0.039), but it was not when compared to T3 group (p=0.228).

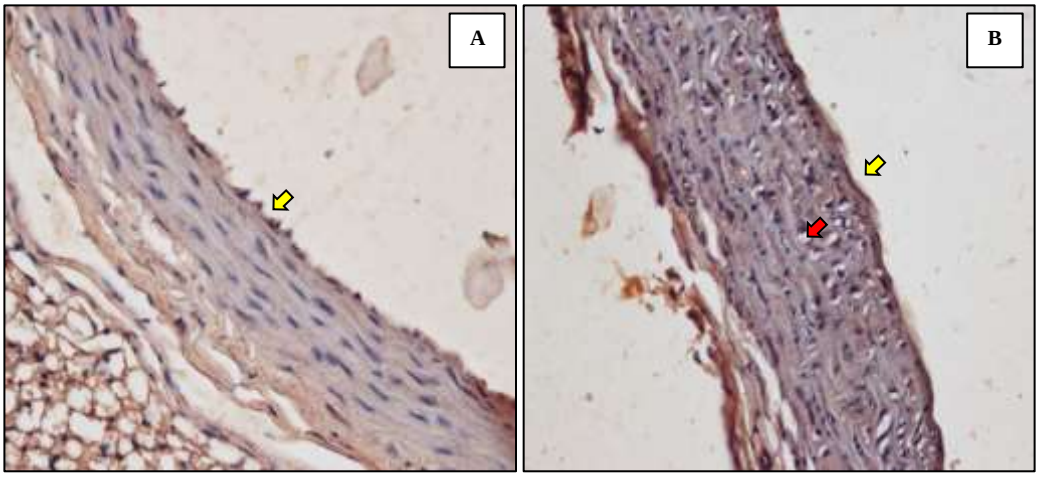

Figure 1: Expression of VCAM-1 in tunica intima and media of the aorta (yellow arrow). There was an increase in expression in PC group (picture A) compared to NC group (picture B). Exposure to cigarette smoke also affects the histological structure of the aorta. Vacuolization and disorganization of smooth muscle cells are seen in tunica media (red arrow).

Table 3: Value of eNOS, VCAM-1, and IMT in NC and PC group

Group	n	**eNOS** (pg/mL)		**VCAM-1** (IRS)		**IMT** (μm)	
		$\bar{x} \pm SD$	Min-Max	$\bar{x} \pm SD$	Min-Max	$\bar{x} \pm SD$	Min-Max
NC	9	101.22 ± 11.80^{a}	83.46-118.85	7.67 ± 2.92^{a}	4.00-11.00	58.98 ± 13.61^{a}	35.03-79.92
PC	9	54.83 ± 8.31^{b}	47.19-69.70	10.33 ± 3.74^{a}	5.00-16.00	88.39 ± 2.52^{b}	83.72-91.87
p-value		p< 0,05*		p> 0,05		p< 0,05*	

* significant at α=0.05 (Independent t-test)
[ab] different superscript shows significant differences between groups

Table 4: Value of eNOS, VCAM-1, and IMT in PC, T1, T2, and T3 group

Group	n	**eNOS** (pg/mL)		**VCAM-1** (IRS)		**IMT** (μm)	
		Median	Min-Max	Median	Min-Max	$\bar{x} \pm SD$	Min-Max
PC	9	53.51^{a}	47.19-69.70	10.00^{a}	5.00 -16.00	88.39 ± 2.52^{a}	83.72-91.87
T1	9	111.52^{b}	62.93-249.70	8.00^{ab}	5.00 - 11.00	73.34 ± 10.30^{b}	59.22-94.15
T2	9	55.66^{a}	48.84-118.85	6.00^{c}	4.00 - 7.00	63.88 ± 8.49^{b}	45.91-74.56
T3	9	57.72^{a}	47.56-128.55	5.00^{bc}	4.00 - 11.00	69.26 ± 17.35^{b}	47.37-99.89
p-value		P < 0.05*		P < 0.05*		P < 0.05**	

* significant at α=0.05 (Kruskal-Wallis test)
** significant at α=0.05 (Brown-Forsythe test)
[abc] different superscript shows significant differences between groups (post-hoc analysis: Mann-Whitney test for eNOS and VCAM-1; Games-Howell for IMT)

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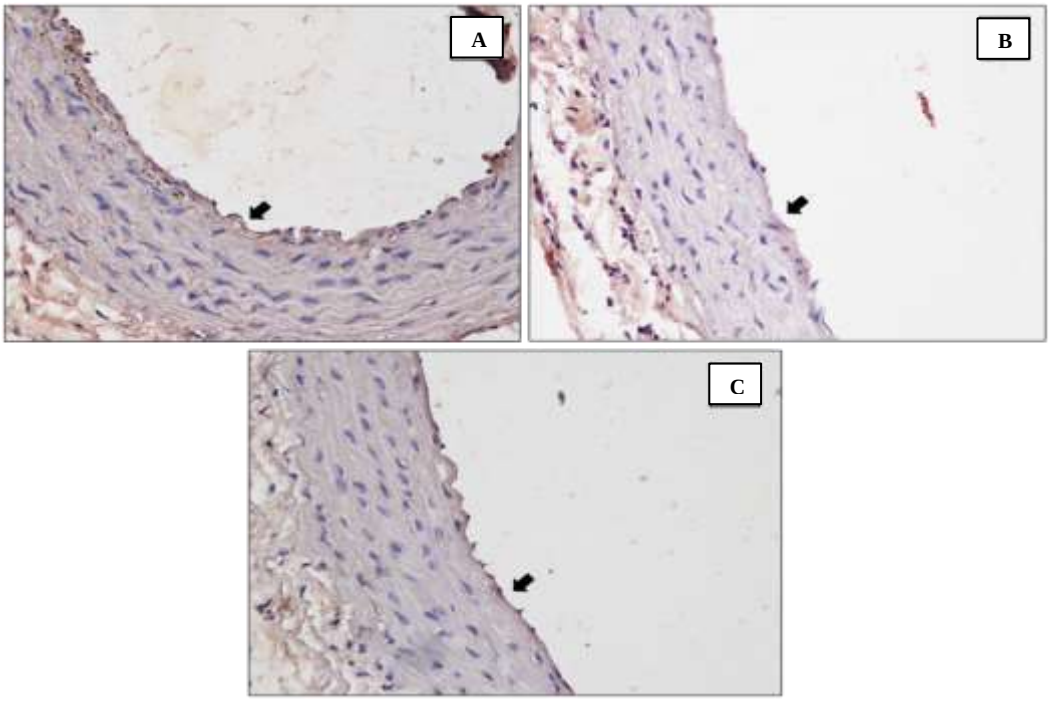

Figure 2: Weak VCAM-1 expressions (black arrow) were observed on (A) T1, (B) T2, and (C) T3 groups, with the weakest observed on T2 group.

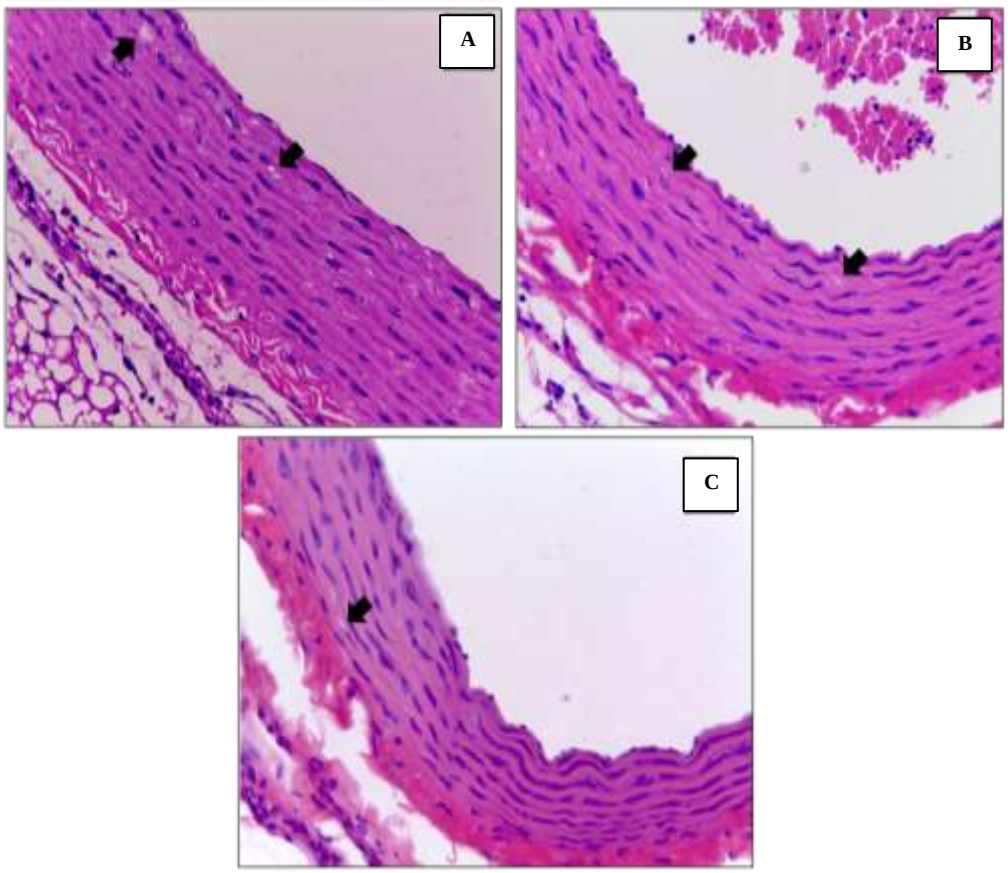

Figure 3: Microscopic observation of aortic structure. Aorta in group (A) T1, (B) T2, and (C) T3 showed an improvement in aortic structure marked by regular smooth muscle cell organization and lessening vacuolization (black arrow) in tunica media.

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T2 group was also not different significantly when it is compared to T3 group (p=0.892). VCAM-1 IRS data is presented in table 4.

Effect of black cumin extract administration in aorta structure and IMT

Improvement in aortic structure was found in group T1, T2, and T3, marked by regular smooth muscle cell arrangement and reduced vacuolization in the tunica media (figure 3). Results of IMT measurement were 88,39 ± 2,52 in PC group, 73,34 ± 10,30 in T1 group, 63,88 ± 8,49 in T2 group, and 69,26 ± 17,35 in T3 group. Comparison test indicated significant differences of these groups (p=0.001). Post-hoc test showed aortic IMT decreased significantly in T1 (p=0.009), T2 (p=0.000), and T3 (p=0.043) compared to PC group. IMT in T1 group was not importantly different compared to T2 (p=0.188) and T3 (p=0.928). Furthermore, IMT in T2 group was also not significantly shown different when it is compared to T3 (p=0.836). Those results are summarized in the table 4. This study identified the substances contained in Indonesian-native black cumin. The dominant active substances revealed through GC-MS linoleic acid (71.31%), palmitate acid (15.86%), stearate (4.69%), octadicadienal (3.8%), beta monolinolein (1.8%), and thymoquinone (0.28%). This composition is distinctive from Iranian or Indian native black cumin. Iranian-origin black cumin linoleic acid of 49.93%, thymoquinone of 8.26%, *p-cymene* of 8.19%, ascorbic acid of 8,07%, and oleic acid of 2,86%. Meanwhile, Indian origin black cumin contained linoleic acid of 61.85%, oleic acid of 18.97%, ascorbic acid of 6.81%, and thymoquinone of 0,72%. [25] These results suggested that due to their distinct contents, Indonesian native black cumin may provide different biological and pharmacological effects to the body. If we know, it becomes the only current research that studied about the effects of Indonesian-native black cumin supplementation, with its distinct contents, to altered endothelial dysfunction parameters induced by sub-chronical cigarette smoke exposure.

Smoking of cigarette becomes a well-established risk factor in the development of atherosclerosis, its mechanisms tend to be closely connected to upcoming stress of oxidative. It can be convinced that smoking can make stress of oxidative increased in some kind of ways, taking the direct damage by some radical species and also inflammatory kind of response made by smoking of cigarette. The making of oxidative stress and also species of reactive oxygen because of the smoke of cigarette get presumed on purpose to help increasing the expression of VCAM-1, whereas eNOS level will decrease. Based on some prior research by Yang *et al.*,[26] an increase of expression's VCAM-1 inside the arteries of rat after getting exposed to smoke of cigarette for about 7 days observed.[26] Another research revealed that smokers already elevated VCAM-1 concentrations and also compromised status of eNOS.[27]

Decreased the bioavailability of NO is known as a central mechanism in the endothelial dysfunction's pathophysiology. He *et* al,[28] indicated that exposure to smoke of cigarette in cell culture of endothelial is able to help reduce the eNOS genes and also proteins expression, showing the dysfunction of endothelial-cell.[28] Moreover to help decreasing eNOS at the level of gene, Pini *et al*,[29] indicated that exposure to secondhand smoke can know to help reduce eNOS at levels of protein. levels of eNOS tend to get decreased inside the guinea pigs' aorta after exposed to cigarettes for about 8 weeks.[29] An eNOS decreased and alsolevel of NO are going to increase tone of vascular, adhesion molecules' expression is getting increased, and can help giving the cascade of coagulation and also inflammation.[30]

VCAM-1 is known to be expressed in cells of vascular endothelial, and also VCAM-1 expression tends to give promote the leukocytes adhesion of the cells of endothelial. VCAM-1 can help accelerating the adherent leukocytes' migration with the surface of endothelial and also tends to promotes the proliferation of vascular smooth cells of muscle; so, VCAM-1 can take such an essential role as a molecule of pro-atherogenic.[31] The VCAM-1 expression inside the cells of endothelial needs some stimulus which is known to be in high levels of lipid, specifically for the low-density lipoprotein (LDL). An also increase inside the oxidized LDL in the endothelium is going to be phagocytosed by macrophages. Macrophages' recruitment can take the role of VCAM-1.[32] Mu *et* al,[33] had shown this hypothesis through the indicated result: VCAM-1 expression was positively related to the triglyceride, total cholesterol and also levels of LDL while VCAM-1 and high-density lipoprotein (HDL) gives a negative connection.[33] Cigarette smoking exposure underlies the dysfunction of endothelial by reducing level of eNOS and adding the expression of VCAM-1.[34] Exposure to smoke of cigarette can influence the aorta's histological structure. Several studies showed that no changes at the tunica intima were observed, but there were disorganizations seen in tunica media.[35,36] Vacuolization can be known as the cytotoxic processes' complications inside the cells and also preclinical atherosclerosis' earlier marker. Chemical components inside the smokes of cigarette are going to make stress of oxidative characterized by permanent vacuolization in the cells. In the phenotyping of microscopic, vacuolization can help the vascular smooth cells of muscle has different sizes and also hapes, thus making them are easy to be disorganized and brings into the atherosclerosis.[37] Black cumin seeds have more than 100 chemical substances.[38] Thymoquinone and its derivates are considered bioactive component of black cumin.[20,21,39,40] The escalation of eNOS level is thought to be caused by thymoquinone due to its antioxidant effect (strong radical scavenging).[41] The increased superoxide can disrupt *tetrahydrobiopterin* (BH4) cofactor, thus eNOS cannot be formed.[42] Radical scavenging effect of thymoquinone contributes to NO forming on blood vessel by inhibiting eNOS activity interference.[43] The effect of increased eNOS as a result of black cumin is also dosage dependent. This study showed that significant eNOS increase is found in group T1, whereas its increase weakened in group T2 and T3. A research done by Rahma, *et* al.[44] also indicated the same result where black cumin extract administered in pre-eclampsia rats with the dose of 0.5-1.5 grams enhanced eNOS level significantly, but eNOS level decreased with the dose of 2 grams.[44] Thymoquinone owns antioxidant effect at low dose, while conversely, thymoquinone at high dose can possess pro-oxidant effect. This phenomenon can occur because thymoquinon can undergo redox cycle forming semiquinon and resulting superoxide.[45] Reduction in VCAM-1 expression after administration of black cumin is thought to relate to increased eNOS level. This study showed a reduction of VCAM-1 expression in group T2 and T3 significantly, but did not show significant differences between both groups. This result is similar to a study conducted by Abbasnezhad *et* al.[46] showing black cumin administration in diabetic rats for 6 weeks could lower VCAM-1 gene expression, independent of dosage.[46] Thymoquinon in black cumin can lower VCAM-1 expression by inhibiting pro-inflammatory cytokines as like the *tumor necrosis factor* (TNF)-α, *interleukin* (IL)-1α, IL-6, IL-8, and also enhancement of NO production.[47,48] In this study, we found improvement in aortic structure and lower IMT after black cumin extract administration for 4 weeks.

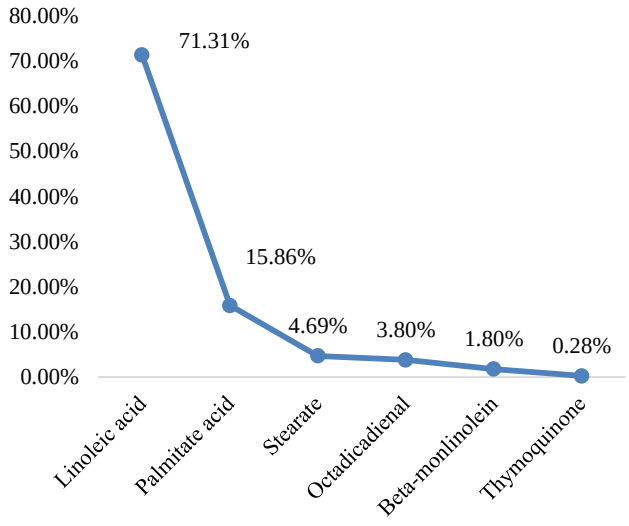

Figure 4: The GCMS Chromatogram

A significant reduction in IMT after black cumin administration was also found in a previous study by Al-Naqeep *et al.*[50] in hypercholesterolemic rabbits for 8 weeks and a study by Cüce *et al.*[51] in diabetic rats for 4 weeks.[50,51] This decrease in IMT is thought to be the effect of a decrease in VCAM-1 after black cumin administration. It becomes knowingly consistent with a study by Luengas *et* al.[52] revealed a positive and also significant influence between VCAM-1 and IMT in patients toward disease of coronary heart.[52] Every research has the limitations which tend to emerge in the realization of the study, makes challenges, and thus, have to be highlighted. First, this study had the limitations of having small samples number that tend to increase the error likelihood and also the imprecision. Second, results from the model of animal used not to translate and turn into some replications in the model of human. Third, critical difference is the IMT, it becomes generally lower in the rats. Another consideration to make is that VCAM-1 expression is strongly related to lipid profile in the blood vessel. Further study is required to establish the connection between lipid profile and VCAM-1 expression. These elements may have impacts on results interpretation. Thus, the result and findings have to be translated through the context of the limitations that this study has. However, this study may be of value due to its strengths in high statistical power and also each groups' homogeneity, enabling a justful comparison of the periods and the groups.

Conclusion

Black cumin extract administration can inhibit endothelial dysfunction caused by cigarette smoke exposure and furthermore, decrease the risk of atherosclerosis. High eNOS level and low VCAM-1 expression are observed using the proper black cumin administration. Aortic IMT shows the established CVD risk factors' level and its decrease is observed by seeing the VCAM-1 expression's decreased.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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