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Effect of Black Cumin Ethanolic Extract Administration to Superoxide Dismutase and Malondialdehyde in Inhibiting Endothelial Dysfunction in Cigarette Exposed Rats

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OriginalArticle

Abstract

BACKGROUND: Oxidative stress caused by various components in cigarette smoke can induce endothelial dysfunction. Black cumin (*Nigella sativa*) has an oxidative stress inhibition capability by increasing antioxidant enzyme production and decreasing lipid peroxidation.

METHOD: This is an *in vivo* study with a post-test only design using Wistar rats as subjects. Rats were randomly assigned into five groups: negative control (NC) group without any treatment, positive control (PC) group exposed to cigarette smoke only, and treatment groups (T1, T2, and T3) which received exposure of cigarette smoke and administration of black cumin extract with doses of 0.3 g/kg BW/day (T1), 0.6 g/Kg BW/day (T2), and 1.2 g/kg BW/day (T3). After four weeks, samples were sacrificed with the aortas taken to measure superoxide dismutase (SOD) activity and malondialdehyde (MDA) level.

RESULTS: A significant reduction in SOD activity (p=0.022) was found between the NC and PC groups but not in MDA level (p=0.394). SOD activity increased significantly in T2 when compared to PC (p=0.007). MDA levels significantly increased at T1 (p=0.002), T2 (p=0.005), and T3 (p=0.006) when compared to PC.

CONCLUSION: Black cumin ethanolic extract increased SOD activity in Wistar rats exposed to cigarette smoke. However, no reduce of MDA level was observed.

Keywords: Endothelial Dysfunction; Cigarette Smoke; Black Cumin; Superoxide Dismutase; Malondialdehyde

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Introduction

Endothelial dysfunction is a predictor for the development of atherosclerosis and coronary artery disease (CAD).[1,2] Atherosclerosis is a disease of the blood vessel wall caused by the accumulation of lipids and fibrous tissue in the blood vessels, thus progressively narrowing the lumen of these vessels.[3] Since atherosclerosis is the leading cause of CAD, factors that influence atherosclerosis can be considered as predictors for the development of CAD.[4]

Law and Wald conducted a meta-analysis of the relationship between smoking and CAD in 2003, reporting an association between the number of cigarettes smoked per day and the increased relative risk of CAD. The study established smoking as a modifiable risk factor for CAD.[5] Cigarettes contain 7,000 types of chemicals that can narrow arteries and damage blood vessels, impeded by endothelial dysfunction.[6] Endothelial dysfunction can be caused by an imbalance between pro-oxidants, which are increased due to various components in cigarette

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smoke, and antioxidants in the body, such as the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). This imbalance is known as an oxidative stress state. Oxidative stress will instigate lipid peroxidation, damaging cells and producing malondialdehyde (MDA) products.[1,2,6]

The decrease in mortality due to cardiovascular disease in the Mediterranean population, compared to Northern European countries, is due to a diet rich in antioxidants.[7] Natural antioxidants are expected to be primary or secondary antioxidants. Primary antioxidants are antioxidants that prevent new radical compounds, such as SOD, CAT, and GPx, while secondary antioxidants are used to bind metals that act as pro-oxidants and scavenge free radicals.[8]

Black cumin (Nigella sativa) is a potent natural antioxidant native to Asia. Black cumin and its derivative components have radical scavenging potential and oxidative stress inhibition capability by increasing antioxidant enzymes.[9,10] Hence, we conducted this study to investigate the roles and mechanisms of black cumin ethanol extract in preventing endothelial dysfunction through superoxide dismutase activity and malondialdehyde production in cigarette exposed rat models.

Methods

Study design

This study was in vivo experimental post-test only design. The study was conducted in the animal laboratory of the Faculty of Veterinary Medicine of Airlangga University. The experimental protocol was approved by the Ethics Commission at Airlangga University, Indonesia

Experimental animals

The study samples were comprised of 45 adult male albino Wistar rats, aged eight weeks, weighing about 150 to 200 grams. Rats were fed uniformly through 511 HI-PRO-VITE ® (PT. Charoend Pokphand Indonesia), with maximal water composition of 13%; protein of 21-23%; fat of minimal 5%; fiber of maximal 5%; calcium of minimal 0.9%; phosphor of minimal 0.6%; aflatoxin of maximal 50 pbb; and total calories of 2,900-3,000 kkal/kg. Rats were randomly allocated to five research groups: the control group (NC and PC) and the treatment group (T1, T2, and T3). NC was the negative control group without any treatment (n = 9). The positive control (PC) was exposed to cigarette smoke without being given black cumin extract (n = 9). T1 was the treatment group exposed to cigarette smoke and black cumin extract at a dose of 0.3 g/kg BW/day (n = 9). T2 was the treatment group exposed to cigarette smoke and black cumin extract at a dose of 0.6 g/Kg BW/day (n = 9). T3 was the treatment group exposed to cigarette smoke and black cumin extract at a dose of 1.2 g/kg BW/day (n = 9).

Exposure to Cigarette Smoke

Cigarette smoke exposure was carried out by

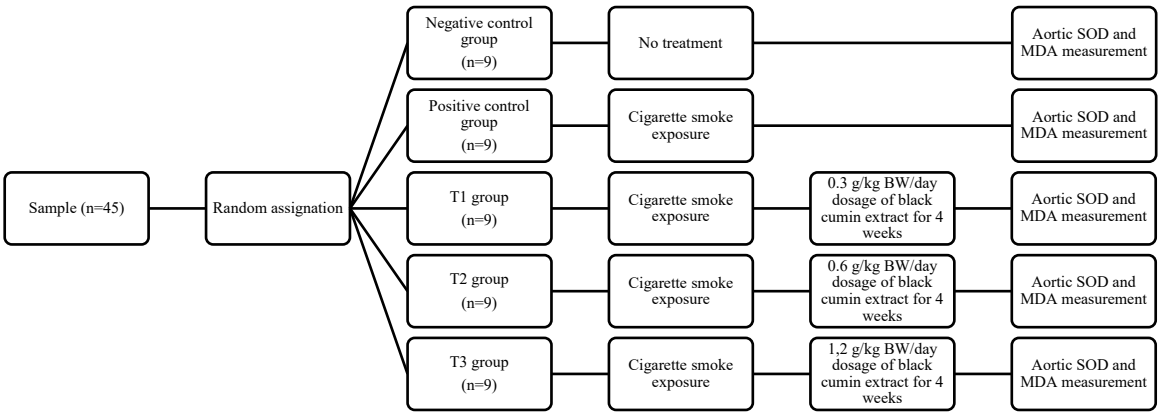

Fig. 1. Schematic graph of study protocol.

A total sample of 45 Wistar rats were randomly allocated into five groups as stated: 1) Negative control group (NC), receiving only standard feeding and ad libitum water; 2) Positive control group (PC), receiving standard feeding, ad libitum water, and 40 cigarettes smoke exposure; 3) Treatment group 1 (T1), receiving standard feeding, ad libitum water, 40 cigarettes smoke exposure, and 0,3g/kg BW/day of black cumin extract; 4) Treatment group 2 (T2), receiving standard feeding, ad libitum water, 40 cigarettes smoke exposure, and 0,6g/kg BW/day of black cumin extract; and 5) Treatment group 3 (T3), receiving standard feeding, ad libitum water, 40 cigarettes smoke exposure, and 1,2g/kg BW/day of black cumin extract. After 4 weeks of treatment, aorta samples were taken for SOD and MDA measurement. (Figure 1)

exposing rats to sidestream cigarette smoke from peristaltic pumps, smoke-producing rooms, and connected inhalation chambers through silicone tubes, according to a method modified from Ali et al.[10] and Jaldin et al.[11] The inhalation chamber consisted of an acrylic box with a volume of 95 x 80 x 65 cm. The inhalation chamber had six holes for air circulation measuring 2.5 cm. Eight cigarettes were ignited simultaneously and streamed to the inhalation chamber containing 10 rats through a duct. The smoke was then incubated in the box for 30 minutes. Kretek cigarettes without filter (brand Dji Sam Soe®; HM. Sampoerna, Indonesia) containing 39 mg tar and 2.3 mg nicotine were used in this study. The cigarette dose given was 40 cigarettes/day (8 cigarettes per 1 administration, 5 times a day). Cigarette exposure was carried out every day (280 cigarettes/week) for 4 weeks (subchronic).[10,11]

Black Cumin Extraction

Black cumin (Nigella sativa) extraction was adapted from Al-Saleh et al., Hadad et al., Velho-Pereira et al., and Koshak et al.[12–15] The black cumin seeds were mashed and subsequently extracted with ethanol solvent using the maceration method with constant stirring at a speed of 1200 rpm for 24 h. The resulting mixture was filtered and then repeatedly stirred. This step was repeated until day 6. The mixture on day 6 was then filtered and evaporated using a rotary evaporator until the volume is constant. Black cumin extract was given via feeding tubes with a volume of 1 ml for each rat every day during the 4 weeks of experiment.[10]

Aortic Tissue Superoxide Dismutase (SOD) Activity Measurement

SOD activity measurement was done using aorta samples from the rats to ensure that changes in these parameter can represent local pathology in the aorta. Determination of SOD activity was according to the spectrophotometric colorimetric test; SOD was measured using the water-soluble tetrazolium salt (WTS)-1 kit from Elabscience, Houston, USA (catalog no. E-BC-K020). Four wells, consisting of control well, blank control well, sample well, and blank sample well were prepared. A 20 μL enzyme working solution was added to each control and sample well, and 20 μL of the diluent enzyme was added to the blank well. The 200 μL substrate solution was mixed in all wells. The mixed solution was incubated for 20 minutes at 37 °C and the solution was read immediately using a microplate reader.

Aortic Tissue MDA Level Measurement

Lipid peroxidation activity in aorta was determined by measuring the levels of thiobarbituric reactive species expressed by malondialdehyde, using the spectrophotometry method. An MDA examination used a kit from Elabscience, Houston, USA (Catalog no. E-BC-K025); four tubes were prepared (a blank tube, standard tube, sample tube and control tube). The blank tube was filled with absolute ethanol (0.2 mL), the standard tube was filled with standard solution, and 0.2 ml of the sample was inserted into the sample tube and the control tube, respectively. All tubes were added with a clarificant solution of 0.2 mL. Next, reagent acid with a volume of 3 mL (previously the reagent was diluted with water with a ratio of 1.2: 34) was added to each tube, then 1 mL of chromogenic agent was added to all tubes. The chromogenic agent powder was previously dissolved in 60 mL of water at a temperature of 90–100 °C, then 60 mL of glacial acetic acid was added, mixed, and allowed to stand at room temperature. All the solutions were mixed, and the tubes were covered with plastic film, with a small puncture hole. The solution was incubated in a water bath at 95 °C for 40 minutes, then cooled under running water. The solution was centrifuged at 3100 g for 10 minutes, then the supernatant was removed. The results were centrifuged and mixed with distilled water, then the OD value was measured with a 1 cm diameter cuvette at an absorbance of 532 nm.

Statistical Analysis

The overall data obtained were compared for each group and analyzed statistically using the Software Statistical Product and Service Solution (SPSS) version 25. Shapiro–Wilk test aims to test whether the data obtained from each group has a normal distribution. An Independent t-test is performed to compare the mean value between two variables if the data distribution is normal. If the data distribution is not normal, the Mann–Whitney non-parametric test is used. One-way ANOVA test is done to compare the mean value of more than two variables if the data distribution is normal. If the data distribution is abnormal, the Kruskal–Wallis non-parametric test is used. Last, Post hoc analysis (Tukey HSD) can determine which group is significantly different from the ANOVA test results. Meanwhile, Mann–Whitney test is used for Kruskal–Wallis' post hoc analysis.

Results

SOD Activity

SOD activity in the aorta was measured in U/mg protein unit of measurement as results. Independent t-test resulted in the value of P = 0.022, indicating a significant difference between the NC and PC

groups (Table 1 and Figure 2). In treatment groups, Kruskal–Wallis test showed a p-value of 0.005, indicating a significant difference. Mann–Whitney analysis showed that SOD activity decreased but not significantly at T1 when compared to PC with p value of 0.566. SOD activity increased significantly in T2 when compared to PC, with p value of 0.007. SOD activity decreased but not significantly at T3 when compared to PC, with p value of 0.479. SOD activity at T1 was significantly different when compared to T2 with p value of 0.004 and not significantly different when compared to T3 with p value of 0.627. The SOD activity at T2 was significantly different when compared to T3 with p value of 0.005. Data is presented in Table 2 and Figure 3.

MDA Levels

MDA levels in the aorta were measured in nmol/mg protein as the unit of measurement. An Independent t-test resulted in the value of p=0.394, indicating that there was no significant difference between the NC and PC groups (Table 3 and Figure 4). In treatment groups, one-way ANOVA test resulted in a p-value of 0.002, which indicates a significant difference. Tukey's Post Hoc test showed aortic MDA levels significantly increased at T1 (P = 0.002), T2 (P = 0.005), and T3 (P = 0.006) when compared to PC. MDA levels at T1 were not significantly different when compared to T2 and T3 with p-values of 0.994 and 0.988, respectively. Moreover, MDA levels were not significantly different in T2 when compared to T3 with p value of 1.000. Data are presented in Table 4 and Figure 5.

Discussion

SOD activity can be used to determine antioxidant status in tissue. SOD is a family of enzymes that

Table 1. SOD activity in control groups (U/mg protein).

Groups	n	SOD activity		p
		$\bar{x} \pm SD$	Min–Max	
Negative control (NC)	9	15.1 ± 5.52^{a}	6.80-24.2	<0.05*
Positive control (PC)	9	8.77 ± 5.05^{b}	2.12-14.9	

* significant at $\alpha = 0.05$

[ab] different notation shows a significant difference between groups

SOD activity within the aorta was measured by colorimetric method in U/mg protein as unit of measurement. Independent t-test result revealed p of 0.022, implying a significant difference between NC and PC group.

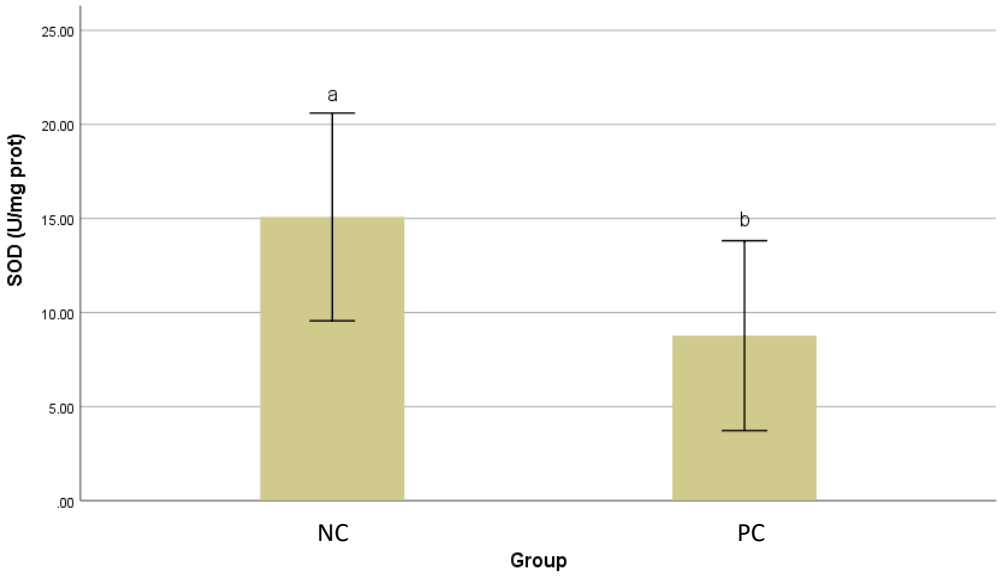

Fig. 2. SOD activity value after exposure to cigarette smoke.

Positive control group receiving exposure of cigarette smoke was shown to have a decreased rate of mean SOD activity. SOD activity signifies antioxidant status in tissue. This study showed that SOD activity was lower in the PC group compared to the NC group; suggesting exposure to cigarette smoke for at least 4 weeks significantly reduced SOD activity.

Table 2. Mean SOD activity (U/mg protein) PC, T1, T2, and T3.

Groups	n	SOD activity		p
		Median	Min–Max	
PC	9	10.6[a]	2.12–14.9	<0.05*
T1	9	5.52[a]	1.19–12.7	
T2	9	25.7[b]	2.12–37.4	
T3	9	5.95[a]	<0.20-18.3	

* significant at α = 0.05 (Kruskal–Wallis test)

[ab] different notation shows a significant difference between groups (Mann–Whitney U-test)

Kruskal-Wallis test revealed p value of 0.005, implying significant differences among the treatment groups. Mann–Whitney analysis showed that SOD activity decreased but not significantly at T1 when compared to PC with p value of 0.566. SOD activity increased significantly in T2 when compared to PC, with p value of 0.007. SOD activity decreased but not significantly at T3 when compared to PC, with p value of 0.479. SOD activity at T1 was significantly different when compared to T2 with p value of 0.004 and not significantly different when compared to T3 with p value of 0.627. The SOD activity at T2 was significantly different when compared to T3 with p value of 0.005

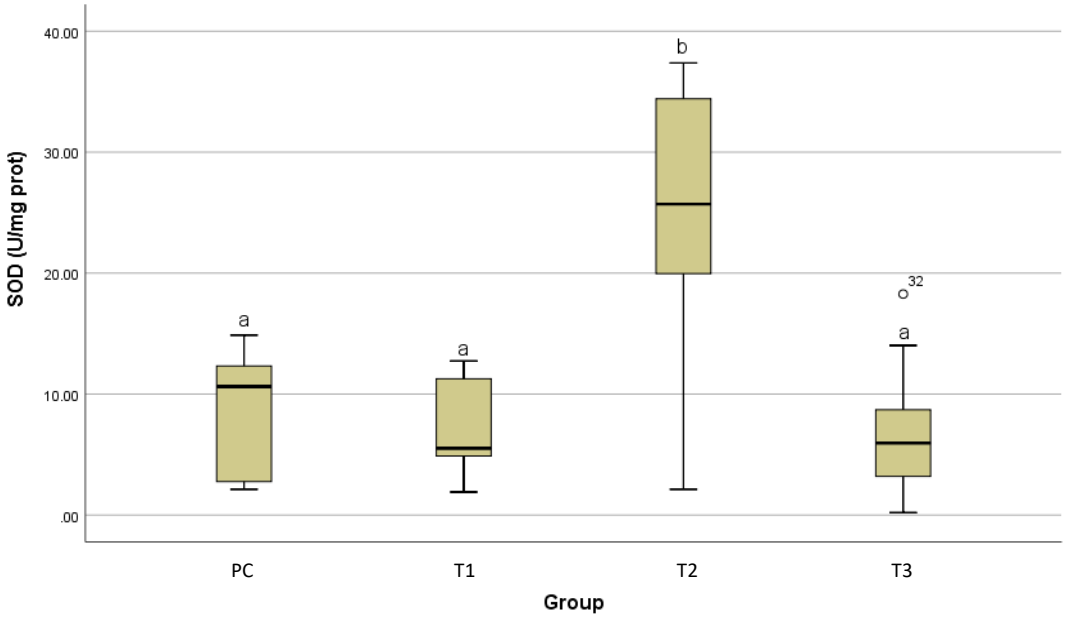

Fig. 3. SOD activity after administration of black cumin.

The administration of 0.6 g/kg BW/day dosage of black cumin extract as demonstrated in T2 group showed a significant increase of SOD activity within the aortas of rat samples exposed to cigarette smoke compared to groups receiving doses of 0.3 g/kg BW/day and 1.2 g/kg BW/day. These results suggest that black cumin has an effect of increasing SOD activity at low doses, but that the effect is reduced at higher doses.

Table 3. MDA levels in control groups (nmol/mg protein).

Groups	n	MDA		P
		$\bar{x} \pm SD$	Min–Max	
Negative control (NC)	9	25.9 ± 4.22[a]	16.9–31.1	>0.05*
Positive control (PC)	9	29.1 ± 10.3[a]	17.7–45.2	

* significant at α = 0.05

MDA levels within the aorta was measured by colometric method in nmol/mg as unit of measurement. Independent t-test revealed p value of 0.394, implying insignificant differences within PC and NC.

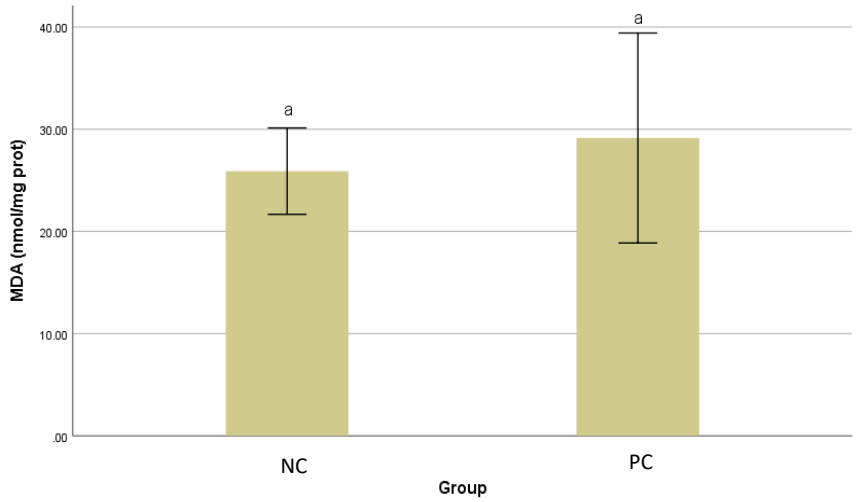

Fig. 4. MDA levels after exposure to cigarette smoke
MDA levels in this study did not differ significantly between negative and positive controls. In this study, cigarette smoke exposure was done in 4 weeks. It is presumed that the damage caused by oxidative stress had not occurred at the fourth week of exposure.

Table 4. MDA (nmol/mg protein) in positive control (PC) and treatment groups (T1, T2, and T3).

Kelompok	n	MDA		p
		$\bar{x} \pm SD$	Min–Max	
PC	9	29.1 ± 10.3^{a}	17.7–45.2	<0.05*
T1	9	56.1 ± 20.9^{b}	26.1–88.6	
T2	9	54.2 ± 12.3^{b}	39.4–80.2	
T3	9	53.6 ± 16.9^{b}	30.2–78.6	

*significant at $\alpha = 0.05$ (One-way ANOVA)
[ab] different notation shows a significant difference between groups (Post Hoc Turkey)
One-way ANOVA test resulted in a p-value of 0.002, which indicates a significant difference. Tukey's Post Hoc test showed aortic MDA levels significantly increased at T1 ($p = 0.002$), T2 ($p = 0.005$), and T3 ($p = 0.006$) when compared to PC. MDA levels at T1 were not significantly different when compared to T2 and T3 with p-values of 0.994 and 0.988, respectively. MDA levels were also not significantly different in T2 when compared to T3 with p value of 1.

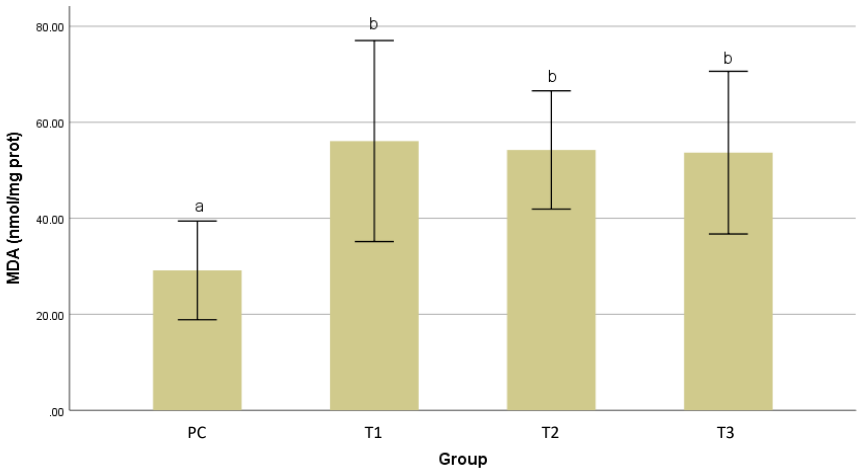

Fig. 5. MDA levels after black cumin administration.
The high MDA levels in this study, while unexpected, were presumably due to high content of linoleic acid in the black cumin extract.

catalyzes the conversion of free radicals O_2^- to H_2O_2, which is then converted to H_2O by GPx or catalase.[7] Cigarette smoke produces free radicals such as O_2^-, OH and H_2O_2 through endogenous processes via NADPH oxidase, xanthine oxidase, and mitochondria. This excessive accumulation of free radicals can, in turn, deactivate the antioxidant enzymes.[7,16,17] This study showed that SOD activity was lower in the PC group compared to the NC group; exposure to cigarette smoke for at least 23 days can significantly reduce SOD activity. Multiple research has shown similar results to this study.[18–20] Pasupathi et al. found that the antioxidant activity of SOD was decreased in smokers compared to non-smokers.[21] Manafa et al. found that the more the number of cigarettes (more than 10 cigarettes/day) and duration of smoking (>10 years), the more SOD activity decrease was observed.[22]

MDA is one of the primary products from lipid degradation by free radicals through a process called lipid peroxidation. MDA levels have a positive correlation with the number of damaged cells in the tissue. Thus, MDA levels can be used to predict the number of dead cells and tissue damage. MDA has been used as a marker to determine tissue damage due to oxidative stress in various clinical situations, including in cardiovascular diseases.[23] In this study, MDA levels did not differ significantly between negative and positive controls. This result contradicts several studies showing a significant increase in MDA levels, but a longer period of exposure to cigarette smoke in those studies should be considered. Cigarette smoke exposure was done for 8 weeks,[24] 12 weeks,[19,25] and 30 weeks,[20] whereas in this study, cigarette smoke exposure was carried out for 4 weeks. Research by Huang et al. also showed that four weeks of exposure did not increase MDA levels, presumably because the damage caused by oxidative stress had not occurred at the fourth week of exposure.[26] In addition, the research sample used in the above studies was systemic blood serum, which had a greater volume compared to the aortic tissue used in this study, so the baseline MDA value would be different and affect the treatment results. This is supported by research which found that with the same treatment, MDA levels in blood serum were higher when compared to MDA levels in the aortic tissue.[27,28]

This study also demonstrated that SOD activity increased after the administration of black cumin. These results are consistent with published studies. Al-seeni et al. stated that the provision of black cumin orally for 4 weeks increased the activity of SOD in rat models with hepatotoxicity.[29] Other studies showed increased SOD activity after oral administration of black cumin to mice with diabetes for 30 days[30] and mice with fatigue induced models for 21 days.[31] Besides, those studies showed an increase in SOD in the third to fourth week after administration of black cumin. The results in this study indicate that SOD activity increased significantly in the T1, T2, and T3 groups, with the most significant increase in T2. SOD activity in this study increased most significantly at a dose of 0.6 g/kg BW of black cumin and decreased again at a dose of 1.2 g/kg BW of black cumin. These results suggest that black cumin has an effect of increasing SOD activity at low doses but that the effect is reduced at higher doses of black cumin. High doses of thymoquinone in black cumin can cause pro-oxidant conditions in cells. An in vitro study showed that thymoquinone administration causes cytotoxic and genotoxic effects through the production of ROS. The toxic effect will decrease along with the decrease in the concentration of thymoquinone.[32–34] Thymoquinone has an antioxidant effect when given in low doses but is pro-oxidant at high doses.[32]

The administration of black cumin in this study did not reduce the MDA levels as expected. The result is inconsistent with several previous animal studies. Administration of black cumin ethanol extract for 30 days at 0.3 g/kg BW/day was reported to significantly reduce MDA in streptozotocin-induced diabetic rats.[35] Hydroalcoholic extract of black cumin at 0.4 g/kg BW/day given for 42 days can reduce MDA levels in diabetic rats hippocampus tissue.[36] Black cumin powder at 7.5 g/kg BW/day for 60 days can significantly reduce MDA in hyperlipidemic rabbits.[37] The high MDA levels in this study are presumably caused by the high content of linoleic acid in black cumin extract. Linoleic acid is a fatty acid that is prone to lipid peroxidation. Among the end product of linoleic acid lipid peroxidation are MDA and ketones.[38–40] Increased levels of MDA in the body due to intake of linoleic acid are very dependent on the omega-6/omega-3 ratio in the diet. One study showed that an omega-6/omega-3 diet with a ratio of 8.8:1 can increase the serum MDA levels of patients with osteoarthritis.[41] This study, unfortunately, did not measure the omega-6/omega-3 content in the black cumin extract.

Conclusion

This study showed that ethanolic extract black cumin could increase SOD activity in Wistar rats which were exposed previously to cigarette smoke. However, it is not able to decrease MDA levels. This study only

measured the antioxidant potential of black cumin in increasing SOD. To better understand the mechanism of the antioxidant effect of black cumin in inhibiting endothelial dysfunction, further research is needed to assess its ability in radical scavenging and its effect on other antioxidant enzymes such as GPx and catalase.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interests.

Authors Contribution

A.M., S.A., P.B.S., S.I.G.R: Study conception and design. A.M., H.H.O.: Acquisition of data. H.H.O., H.P.M., R.D.A.: Analysis and interpretation of data. A.M., H.H.O., H.P.M., R.D.A.: Drafting of manuscript. S.A., P.B.S., S.I.G.R.: Critical revision.

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