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THE PROTECTIVE EFFECT OF *HIBISCUS SABDARIFFA* ON RAT'S LUNG DAMAGE DUE CIGARETTE SMOKE EXPOSURE

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ABSTRACT

Background: Cigarette smoke consists of many free radicals that can reduce antioxidants in the body and further trigger oxidative stress. The state of oxidative stress can be minimized through antioxidant supplementation. *Hibiscus sabdariffa* (Rosella) is a herbal plant reported to be rich in antioxidants. **Objective**: To investigate the protective effect of Rosella extract on the microstructure of the lung and plasma Malondialdehyde (MDA) levels of rats exposed to cigarette smoke. **Methods**: This research is an experimental study with a post-test-only group design. A total of 36 male Sprague Dawley rats were randomly divided into six groups. Group K was given standard food and free access to water. Group K1, K2, P1, P2, and P3 were exposed to 4 cigarettes/day for 30 consecutive days. Before cigarette smoke exposure, each group received treatment with 1 mL saline (K1), vitamin E 0.2 g/kg b.w. (K2), and infused Rosella 0.25 g/kg b.w. (P1), 0.5 g/kg b.w. (P2), and 1 g/kg b.w. (P3). Plasma MDA levels were measured by the TBARS method. Statistical analysis was performed with one way ANOVA test and continued with a post hoc test. **Results**: Circulated MDA levels of groups K, K1, K2, P1, P2, and P3 were 1.84 ± 0.18 nmol/mL, 9.57 ± 0.27 nmol/mL, 2.24 ± 0.10 nmol/mL, 4.93 ± 0.31 nmol/mL 3.85 ± 0.55 nmol/mL, and 2.62 ± 0.37 nmol/mL respectively. Cigarette smoke exposure in group K showed significantly higher MDA levels (K versus K1; $p < 0.001$). The administration of rosella infusion (P1, P2, P3) and or vitamin E supplementation (K2) can significantly suppress the plasma MDA levels due to cigarette smoke exposure ($p < 0.05$). The administration of Rosella flower infusion 1 g/kg b.w. has an antioxidant effect similar to vitamin E supplementation 0.2 g/kg b.w. ($p = 0.268$). **Conclusion:** The administration of Rosella flower infusion could prevent lung damage from oxidative stress induced by cigarette smoke exposure.
***Keywords**: Cigarette, Hibiscus sabdariffa, MDA, Oxidative stress.*

INTRODUCTION

Cigarette smoke contains hazardous compounds for health and can cause various diseases, such as heart disease, stroke, and cancer[1,2]. Some harmful compounds in the cigarette are tar, nicotine, nitrosamines, carbon monoxide, polynuclear aromatic Hydrocarbon (PAH) compounds, phenols, carbonyls, chlorine dioxins, furans, hydrogen cyanide, acetone, and carcinogenic compounds[3]. Cigarette smoke also contains various free radicals, which can reduce the body's antioxidants and trigger oxidative stress. Free radicals present in cigarette smoke are Reactive Oxygen Species (ROS), Hydrogen peroxide, and hydroxyl radicals[4]. These compounds will react in the body and create products such as Malondialdehyde (MDA), 4-hydroxynenal, pentane, and ethane that have high destructive power against cells in the body[5].

To reduce the level of free radicals in the body, it is necessary to increase the body's antioxidant levels. The supplementation of herbal medicines, vitamins, foods, and beverages that contain antioxidants is one way to decrease the oxidant levels in the body. For example, Rosella or *Hibiscus sabdariffa* is a herbal plant that is reported to have many antioxidant constituents[6]. Rosella contains vitamins, minerals, and bioactive components such as organic acids, phytosterols, and polyphenols. The petals of rosella flowers are known to have antioxidants in the form of anthocyanin pigments, a class of flavonoid compounds. The main flavonoids in Rosella are Gossypetin, Hibiscetine, Luteolin, and Quercetin[7-9]. Rosella's flavonoid compounds and phenolic acids provide health benefits, including anti-inflammatory, antioxidant, antihypertensive, hypolipidemic, antidiabetic, antimicrobial, and anti-carcinogenic[10-12].

Considering the potential antioxidant content in rosella, we are interested in further investigating the effect of rosella flower extract on the lungs of Wistar rats induced by oxidative stress through repeated exposure to cigarette smoke. The severity of oxidative

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stress was determined by quantifying the concentration of plasma Malondialdehyde (MDA) resulting from lipid oxidation. The preventive effect of Rosella extract on the microscopic conditions of rat lungs was also evaluated.

METHOD

Preparation of Rosella (*Hibiscus sabdariffa*) extract

The extraction of the Rosella flower was performed by the method of infundation. Rosella infusion 10% (w/v) is made by carefully weighing 10 grams of dried rosella flower powder and mixing it with 100 mL of aquadest. The mixture was then heated for 10 minutes at a temperature of 85 ^{o}C with occasional stirring. Afterward, the heated infusion was seized using a flannel, and the volume was fixed to 100 mL. The Rosella infusion stocks of 10% (w/v) were used to make stocks of 2.5% (w/v) and 5% (w/v). The prepared infusion of Rosella was given to rats with a volume of 10 mL/kg b.w.

Animal and treatments

This research is an experimental study with a post-test-only group design carried out at the Satmoko private laboratory, the Akurat private laboratory, and the animal laboratory in the Faculty of Medicine, Universitas Diponegoro. The research was conducted from July to September 2020. A total of 36 male healthy Sprague Dawley rats aged 2–3 months and weighing 150–200 grams were used in this study. All animals were selected by purposive random sampling and acclimatized for seven days in a care cage of 45 x 35 x 12 cm^3, maintained on a 12-hour light/dark schedule and supplied with food and water via *ad libitum* for 30 days. Rats were divided into six groups: three control groups (K0, K1, and K2) and three test groups (P1, P2, and P3). In the control normal group (K0), animals served as a reference for normal values. All groups except K0 were exposed to four cigarettes smoke every evening for 30 consecutive days. Every morning for 30 days, rats in group K2 were given 200 mg/kg b.w. of vitamin E. The three test groups (P1, P2, and P3) were given Roselle infusion of graded dosage 0.25 g/kg b.w. (P1), 0.5 g/kg b.w. (P2), and 1 g/kg b.w. (P3). In contrast, the cigarette smoke group (K1) was given a saline solution.

Specimen collection and histological analysis

Thirty days after the treatment, 3 mL of blood samples were taken from the plexus vena retro-orbital and put into a vacutainer tube containing EDTA. Animals were anesthetized by Ether inhalation and sacrificed through cervical dislocation. Following the termination, the lung was taken out carefully and placed immediately in a container containing 10% formalin buffer and preservative. Histological lung specimens were prepared by longitudinal cuts and stained with hematoxylin-eosin. Lung specimens were observed under a light microscope in five different fields of view, with magnifications of 400x. Each field of view was scored based on the scoring parameters, including Inflammation, Erythema, and Necrosis[13]. The scoring criterion can be seen in Table 1.

Table 1. Scoring of lung microstructure examination.

Criteria	Details	Score
Inflammation	No inflammatory cells found	0
	Inflammatory cells found in <10% of fields of view	1
	Inflammatory cells found in 10-30 % of fields of view	2
	Inflammatory cells found in> 30 % of fields of view	3
Erythema	No Eritrosite cells found	0
	Eritrosite cells found in 10-30 % of fields of view	1
	Eritrosite cells are found in 31-50 % of fields of view	2
	Eritrosite cells found in>50 % of fields of view	3
Necrosis	No necrosis found	0
	Necrosis found in 10-30 % of fields of view	1
	Necrosis found in 31-50 % of fields of view	2
	Necrosis found in>50 % of fields of view	3

Analysis of circulating MDA level

Rat blood was taken through a retro-orbital vein plexus and put into a vacutainer tube containing EDTA, and then centrifuged for 15 minutes at a speed of 3000 rpm at a tabletop centrifuge. The plasma was subjected to MDA analysis using the Thiobarbituric Acid Reactive Substance (TBARS) assay method. A total of 50 µL of blood plasma was put into a test tube containing a solution of 750 µL Phosphoric acid and 250 µL of 40 mM TBA solution. Afterward, 450 µL of aquades were added to the mixture, incubated at 100 ^{o}C for 60 minutes, and cooled with an ice bath for 15 minutes. The mixture was centrifuged for 15 minutes at 3000 rpm, and the absorbance was read using a spectrophotometer at 532 nm.

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Statistical analysis

Statistical analyses were performed using SPSS version 21. Data distribution was evaluated using the Sapphiro-Wilk. One Way ANOVA was performed to assess the difference between groups and continued with the Games-Howel posthoc test.

RESULTS

Effect of *Hibiscus sabdariffa* infusion on circulated MDA levels

Figure 1. shows circulated MDA levels of all study groups presented as mean with a standard deviation of a minimal two measurements. The normal control group (K) exhibits the lowest circulated MDA level (1.84 ± 0.18 nmol/mL). The MDA level is the highest (9.57 ± 0.27 nmol/mL) in the group that received cigarette smoke exposure (K1). Lower levels of MDA are observed in all intervention groups, vitamin E (K2), Rosella 0.25 g/kg b.w. (P1), Rosella 0.5 g/kg b.w. (P2), and Rosella 1 g/kg b.w. (P3). ANOVA test confirmed a significant difference between groups ($p < 0.001$). *Post-hoc* Games-Howell analysis showed MDA level of each antioxidant intervention group of K2, P1, P2, and P3 were significantly lower ($p < 0.001$) than the cigarette-smoke group (K1), implying that antioxidant intervention reduced the oxidative stress level of rats exposed to cigarette smoke. The intervention group P3 (Rosella 1g/kg b.w.) exhibited the most potent effect in lowering MDA levels, similar to the vitamin E intervention group ($p = 0.268$).

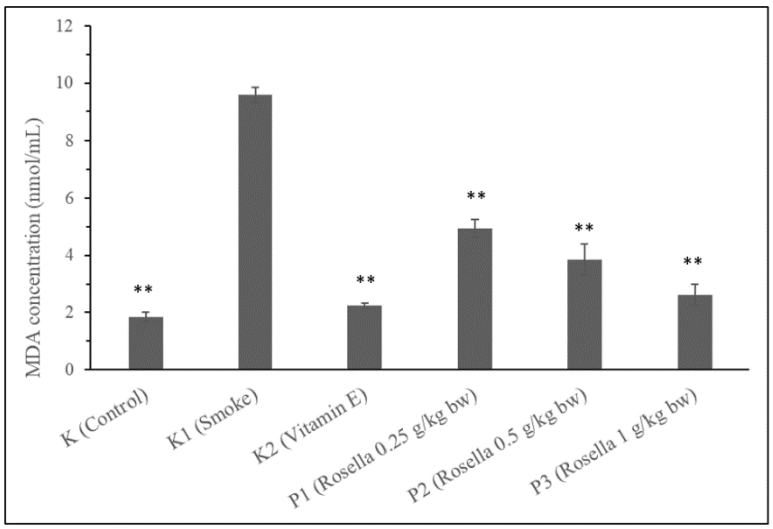

Figure 1. Effect of the different treatments on lipid peroxidation. Smoke inhalation induced a significant elevation in lipid peroxidation measured as circulating malondialdehyde (MDA). Rosella treatment significantly reduced the MDA level in a dosage-dependent manner. The highest concentration of Rosella exerts similar results with Vitamin C treatment.

Histopathological examination

Figure 2 shows the microstructure of the lung of all groups. Rats in the control group (K0) showed no inflammatory cells, while eritosite cells and signs of necrosis were found. Exposure to cigarette smoke (K1) caused inflammation and the appearance of eritosite cells and necrosis. Antioxidant supplementation in the form of vitamin E (K2) could prevent inflammation and necrosis of the lung structure. Rosella supplementation (1 g/kg b.w.) seems to lessen lung damage, as observed in milder inflammation, lower eritosite cells, and lower levels of necrosis.

Table 2. shows the scoring of lung damage based on the severity of inflammation, erythema, and necrosis. It was evident that cigarette smoke exposure in the K1 group caused severe lung damage compared to the healthy control rats (K0). All lung damage parameters in the K1 group were significantly higher than those of K0. Supplementation of vitamin E before cigarette smoke exposure in group K2 provides a protective effect against lung damage. The score for inflammation, erythema, and necrosis almost return to normal, as seen in the healthy control group (K0). A similar trend was also observed in groups treated by Rosella (P1, P2, and P3). Rosella protects against lung damage from cigarette smoke exposure in a dosage-dependent manner.

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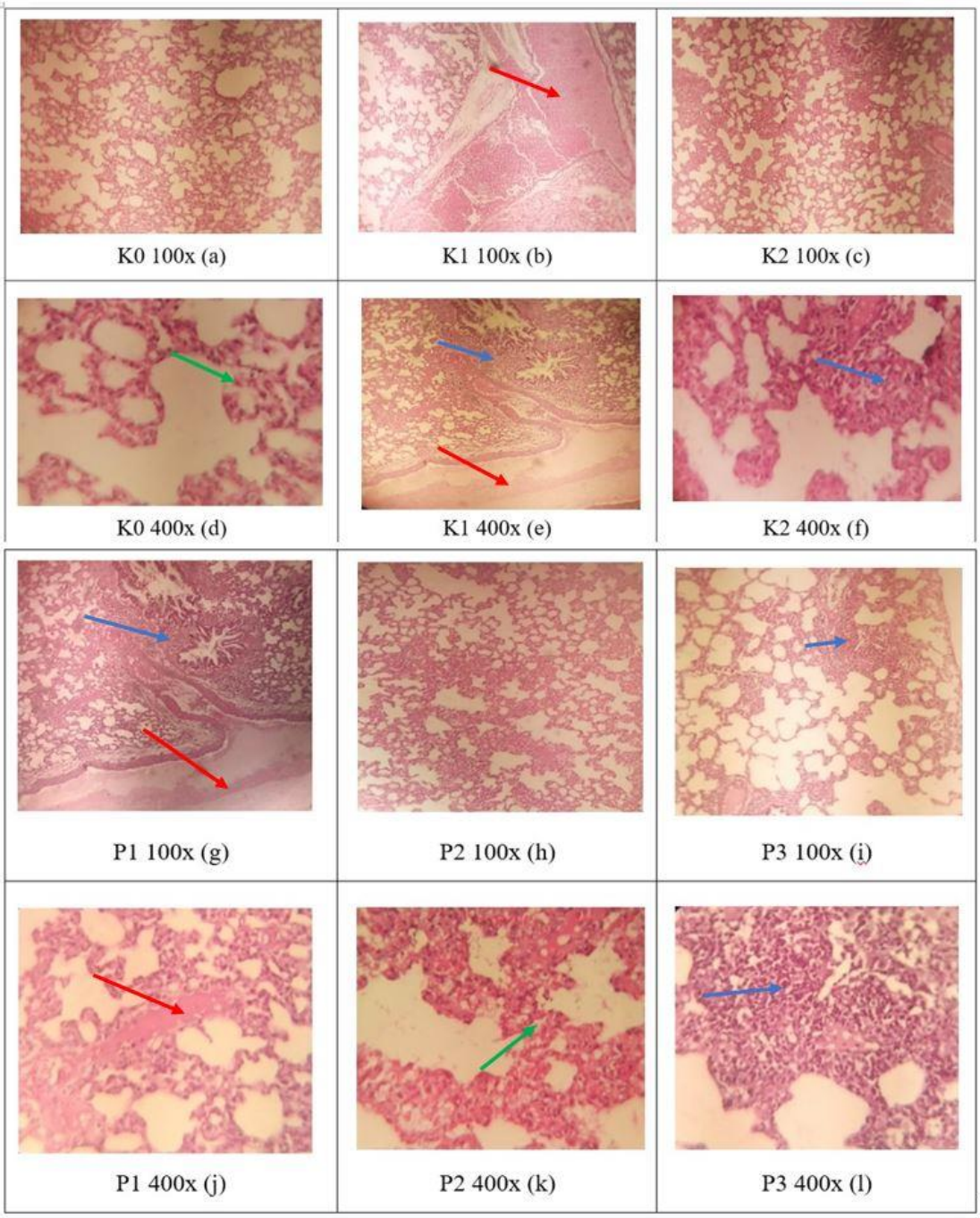

Figure 2. Photomicrographs of rat lung structure stained with hematoxylin-eosin (HE) and viewed with 100x and 400x magnification. (a,d) Control group K0 showed the normal structure of the lung without inflammatory cell eritrosite and necrosis found; (b,e) smoke group K1 showed inflammatory cells found in 10-30 % of fields of view, eritrosite cells found in>50 % of fields of view, and necrosis found in>50 % of fields of view; (c,f) vitamin E group K2 showed the normal structure of the lung without inflammatory cell, eritrosite and necrosis were found; (g,j) treatment group P1 showed Inflammatory cells found in 10-30 % of fields of view, eritrosite cells found in>50 % of fields of view and necrosis found in>50 % of fields of view; (h,k) treatment group P2 showed Inflammatory cells found in 10-30 % of fields of view, eritrosite cells found in 31-50 % of fields of view and Necrosis found in 31-50 % of fields of view; (i.l) treatment group P3 showed inflammatory cells found in <10% of fields of view, eritrosite cells found in 10-30 % of fields of view and necrosis found in 10-30 % of fields of view. (Blue arrow: inflammatory cells, red arrow: Eritrosite cells, green arrow: necrosis cell)

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Table 2. Scoring of lung examination and result of Kruskal Wallis test for all test groups.

Group	Mean ± SD				Median (Min-Max)			*p*
	I	**E**	**N**	**Total score**	**I**	**E**	**N**	
K0	0±0	0±0	0±0	0 [a]	0±0	0±0	0±0	*(0,000-0,006)
K1	1.93±0.25	2.9±0.31	2.9±0.31	2.57 [b]	2±(1-2)	3±(2-3)	3±(2-3)	
K2	0±0	0.13±0.35	0.13±0.35	0.08 [c]	0±(0-0)	0±(0-1)	0±(0-1)	
P1	1.83±0.38	2.83±0.38	2.83±0.38	2.49 [d]	2±(1-2)	3±(2-3)	3±(2-3)	
P2	1.57±0.5	0.38±0.5	1.57±0.5	1.17 [e]	2±(1-2)	2±(1-2)	2±(1-2)	
P3	0.87±0.35	0.87±0.35	1.17±0.38	0.97 [f]	1±(0-1)	1±(0-1)	1±(1-2)	

I: Inflammation; E: Erythema; N: Necrosis
abcdef = post hoc test. The score was significantly different at $p < 0.005$

DISCUSSION

This study's main outcome is that Rosella could protect against lung damage induced by repetitive cigarette smoke exposure. Cigarette smoke contains vast amounts of free radicals that can significantly elevate the oxidative level and eventually damage organs. It has been hypothesized that as many as 10^{14} free radical molecules are present in every smoke inhalation[14]. Considering the anatomical position and functional characteristics, the lungs are an organ that is vulnerable to damage caused by free radicals present in cigarette smoke. The present study shows that cigarette smoke exhibited damage to lung microstructure. Cigarette smoke could increase oxidative stress, induce local inflammation, and disturb the oxidant-antioxidant balance, eventually damaging the alveolar and lung parenchyma[15-17]. Cigarette smoke impairs the airway epithelial barrier, which may contribute to the pathogenesis of lung illnesses[18]. It also can disrupt the metabolism of surfactants by producing free radicals and increasing oxidative stress, which can increase TNF levels and other proinflammatory markers[19]. Furthermore, smoking could increase the formation of free radicals in smokers' phagocytic cells and decrease specific antioxidants.

The connective tissue spaces also include a small number of interstitial cells, including mast cells, fibroblasts, myofibroblasts, macrophages, and plasma cells. Inflammatory and repair processes are triggered by any adverse stimulus that causes lung tissue damage. If the effect is prolonged and severe, proinflammatory and profibrotic cytokines released by inflammatory cells, proliferating epithelial cells, and matrix substances slow down the healing process. This unchecked proliferation causes collagen deposition, fibroblast proliferation, and thickening of the pulmonary capillaries. Alveolar collapse and interstitial and intra-alveolar fibrosis emerge when it gets chronic[23]. Antioxidant and anti-inflammatory properties are seen in Hibiscus sabdariffa (HS) constituents. The effective reduction in TNF- (tumor necrosis factor) and IL-6 levels caused by TLR4 protein inhibition suppressed the production of inflammation[20].

The injury of free radicals in the body could occur directly and indirectly. Biomolecules that are often affected by free radicals' activity is lipid. High lipid peroxidation is evident in the state of oxidative stress. It has been reported by numerous studies that oxidative stress could elevate lipid peroxidation, which is usually reflected by the MDA level[21,22]. Our study demonstrated that rats exposed to cigarette smoke have significantly higher plasma MDA levels than rats in the healthy control group. This result is in line with a previous study that showed lipid peroxidation is higher among smokers, usually accompanied by depletion of total antioxidant capacity[23-25]. Importantly, this study confirmed that the high level of MDA in rats exposed to cigarette smoke could be normalized by Rosella supplementation. Our result matches previous studies that demonstrated the beneficial effect of different types of antioxidant supplementation on the MDA level[26].

Rosella is rich in antioxidants, which can elevate the total antioxidant capacity. It has been reported that most of the Rosella parts (flower, seed, and leaves), as water or ethanol extract, exhibit potent antioxidant capacity[10]. Rosella's strong antioxidant capacity is due to its amazing capability in scavenging effect on reactive oxygen and free radicals, inhibiting several

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pro-oxidant enzymes, inhibiting lipid peroxidation, and increasing the level of superoxide dismutase, catalase, and glutathione in the liver[27]. Additionally, rosella's high content of polyphenols, flavonoids, anthocyanin, and many other bioactive compounds exert therapeutic benefits as potent antioxidants, anti-inflammatory, antimicrobial, and anti-carcinogenic herbal.

In conclusion, our study demonstrated that Rosella is a potent antioxidant capable of protecting the lung from oxidative damage due to chronic cigarette smoke exposure.

ETHICAL APPROVAL

This study was reviewed and approved by The Ethics Commission of the Faculty of Medicine, Universitas Diponegoro, and Dokter Kariadi Hospital Semarang, Indonesia, with approval number: 107.2/EC/H/KEPK/FK-UNDIP/VII/2019. Animals were handled with the Guide for Care and the approval from the institution's Ethics Committee.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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