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EFIKASI FLAVONOID PADA MULBERI MERAH TERHADAP PENURUNAN RADIKAL BEBAS DAN ALVEOLAR MAKROFAG AKIBAT PAPARAN ASAP ROKOK PADA TIKUS WISTAR

The Efficacy of Flavonoid in Red Mulberry on Reducing Free Radicals and Alveolar Macrophages Due to Cigarette Smoke Exposure in Wistar Rats

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ABSTRACT

Background. *Free radicals in cigarette smoke will hurt health when they enter to the respiratory tract. An excessive increase of free radicals in the body will cause oxidative stress. Free radicals are generated physiologically by the body's metabolism and can neutralize antioxidants in the body. An imbalance number of free radicals will result in cell damage and death. It has characterized by an increase in malondialdehyde levels in the blood and alveolar macrophages in the lung tissue. Giving red mulberry (Morus rubra) as an intake of antioxidants from outside the body can prevent adverse effects of cigarette smoke.* ***Objective.*** *This study analyses flavonoids' impact on red mulberry in reducing free radicals due to exposure to cigarette smoke by lowering levels of malondialdehyde and alveolar macrophages.* ***Method.*** *This research is experimental with a post-test control group design using male Wistar rats (Rattus novergicus) as experimental animals. Treatment of experimental animals through red mulberry per oral and exposure to cigarette smoke had conducted for 30 days. The parameters used were levels of malondialdehyde and alveolar macrophages in the lung tissue.* ***Results.*** *The research showed an increase in free radicals in the group exposed to cigarette smoke. Increasing intake of red mulberry can further reduce malondialdehyde levels and the number of alveolar macrophages ($p<0.05$).* ***Conclusions.*** *The antioxidants in red mulberry can reduce malondialdehyde levels in the blood and the number of alveolar macrophages in lung tissue due to exposure to cigarette smoke.*

Keywords: *alveolar macrophage, cigarette, flavonoids, malondialdehyde, red mulberry*

ABSTRAK

Latar Belakang. Radikal bebas yang banyak terkandung dalam asap rokok akan memberikan dampak negatif bagi kesehatan apabila masuk ke dalam saluran napas. Peningkatan radikal bebas dalam tubuh yang berlebihan akan menyebabkan terjadinya stres oksidatif. Secara fisiologis, radikal bebas dihasilkan dari metabolisme tubuh dan dapat dinetralisir oleh antioksidan dalam tubuh. Ketidakseimbangan jumlah radikal bebas akan mengakibatkan kerusakan dan kematian sel yang ditandai dengan peningkatan kadar malondialdehid dalam darah dan alveolar makrofag di jaringan paru. Pemberian flavonoid pada buah mulberi merah (*Morus rubra*) sebagai asupan antioksidan dari luar tubuh dapat mencegah dampak negatif dari paparan asap rokok. **Tujuan.** Penelitian ini bertujuan untuk menganalisis efek flavonoid pada mulberi merah dalam menurunkan radikal bebas akibat paparan asap rokok melalui penurunan kadar malondialdehid dan alveolar makrofag. **Metode.** Penelitian ini adalah eksperimental dengan *post-test control group design* menggunakan tikus wistar jantan (*Rattus novergicus*) sebagai hewan coba. Perlakuan pada hewan coba melalui pemberian mulberi merah dan paparan asap rokok dilakukan selama 30 hari. Parameter yang digunakan adalah kadar malondialdehid dan alveolar makrofag di jaringan paru. **Hasil.** Penelitian ini memperlihatkan adanya peningkatan radikal bebas pada kelompok yang

diberikan paparan asap rokok. Peningkatan pemberian asupan mulberi merah dapat menurunkan kadar malondialdehid dan jumlah alveolar makrofag (p<0,05). **Kesimpulan.** Antioksidan yang terkandung pada mulberi merah dapat menurunkan kadar malondialdehid dalam darah dan jumlah alveolar makrofag di jaringan paru akibat paparan asap rokok.

Kata kunci: alveolar makrofag, rokok, flavonoid, malondialdehid, mulberi merah

INTRODUCTION

Cigarettes have become a significant problem around the world. It has estimated that cigarettes account for six million deaths due to smoking each year. Although there has been a decrease in smokers' prevalence globally, the number of people who smoke has increased.[1] Therefore, many countries continue to reduce the number of smokers even though they have not achieved satisfactory results. The increase in the number of active smokers will not only increase the number of diseases caused by smoking, but it will directly improve the health costs.[2,3] The increasing number of active smokers is more common in developing countries than in developed countries.[4] The adult age group in developed countries such as the US has shown a decline in numbers.[5] The opposite happens in developing countries where the number of active smokers is increasing, such as India, Saudi Arabia, and Indonesia.[6,7,8]

Cigarette smoke that enters the respiratory tract continuously will cause an increase in airways diseases such as Chronic Obstructive Pulmonary Disease (COPD), asthma, chronic bronchitis, lung infections, and lung cancer.[9] Various harmful substances in cigarette smoke will trigger free radicals, which disrupt the body's physiological processes. Free radicals can come from both inside and outside the body. Normally, free radicals are usually formed by metabolic waste and neutralized by enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT).[10] The increase in free radicals from the body due to exposure to cigarette smoke entering the respiratory tract will result in an excessive number of free radicals.[11]

It will cause an imbalance between the number of free radicals and antioxidants in the body, resulting in oxidative stress and triggering lipid peroxidation reactions. This reaction will result in cell damage and death.[12] Prevention can do by taking antioxidants from outside the body to help neutralize free radicals. Antioxidants given orally are not enzymatic antioxidants because enzymatic antioxidants result from metabolism. Thus, they cannot obtain from the outside.[13] It can identify the increase in cell damage caused by free radicals increasing malondialdehyde levels in the blood. The malondialdehyde parameter is one of the end products of lipid peroxidation. It not includes as free radicals.[14] In addition, an increase in free radicals can also cause cell death and lung tissue damage. It will stimulate macrophages' alveolar movement as one of the primary defences in body tissues to carry out the phagocytosis process.[15]

Various antioxidants that can be consuming as non-enzymatic antioxidants are flavonoids, vitamin C, vitamin E, and carotenoids.[16] Flavonoid-type antioxidants often reduce free radicals because they have a broad spectrum of health-enhancing effects as antioxidants, anti-inflammatory, and anti-carcinogenic.[17] Red mulberry fruit (*Morus rubra*) is high in flavonoids and often consumed by people. This study aims to determine the effect of giving red mulberry in reducing free radicals due to the exposure to cigarette smoke through levels of malondialdehyde and alveolar macrophages as parameters.

METHODS

This study is an experimental study (Randomized Controlled Trial) with a post-test control group design. Male Wistar rats (*Rattus novergicus*) used as research samples for 30 days. This study divides the experimental animal group into a negative control group, a positive control group, and three treatment groups. Each treatment group contains six experimental animals (Federer's Formula).[18] This research has been approved by Komite Etik Penelitian Kesehatan Universitas Surabaya (No: 136/KE/VI/2020).

The male Wistar rat (*Rattus novergicus*) weighs ±200 g in normal conditions has never been the object of research. Meanwhile, the exclusion criteria were the experimental animals who were sick or died during the study. The maintenance and care of experimental animals during the study was regarding on the 3R principle (replacement, reduction, and refinement).[18]

Red mulberry fruit (*Morus rubra*) obtained from Mojokerto will be sorted and cleaned to get the fruit in good condition. Next, the juice-making process conducted using a blender and a filter to separate the pulp's juice. Red mulberry intake to experimental animals to adjust the flavonoid dose in red mulberry to the daily nutritional adequacy rate. The flavonoids of the red mulberry obtained will be measured (±38 mg/g). Then, the flavonoids will convert from adults' daily needs (±190 mg/kg) to the daily intake dose of Wistar rats 200 g (±240 mg/tail) by using table Laurence and Bacharach. The red mulberry was given to experimental animals by oral sonde. The amount of red mulberry to be given to the treatment group was 3.25 g, 6.5 g, and 13 g.

Experimental animals were exposed to cigarette smoke using a smoking chamber (CO levels ±50 PPM). The measurement of malondialdehyde levels (µg/ml) had carried out using spectrometry, while the alveolar macrophage measurement had conducted by making histology. The lung tissue obtained will be processed histo-technique and carried out with hematoxylin-eosin (HE) staining. All tissue specimens had fixed with 10 percent formalin solution and a histological incision. Hematoxyline and eosin (HE) staining performed after transverse incisions of six microns had made with a rotary microtome.[3] The mean number of alveolar macrophages calculated as many as five fields of view on each preparation.

This research was conducted for 30 days by dividing the rats into five groups. The first group was the negative control group, where the experimental animal did not receive any treatment; they only received daily intake (30 g/day). The second group was the positive control group, where the experimental animals' received daily information and exposure to tobacco smoke of 2 rods per day. In contrast, the three treatment groups were the groups that received exposure to tobacco smoke of 2 rods per day, daily intake, and different amounts of mulberry fruit (3.25 g, 6.5 g, and 13 g).

The research results obtained ratio data in the form of malondialdehyde and alveolar macrophage levels in each group. Data analysis was performed using the ANOVA test and continued with the least significance difference (LSD) test using SPSS version 22 to see any differences between groups.

RESULTS

The observations results of the mean of malondialdehyde levels in each experimental animal group are the negative control group (I); positive control group (II); treatment groups (III, IV, V) with exposure to cigarette smoke and with an oral dose of *Morus rubra* fruit 3 g, 6 g, and 12 g. Based on table 1, the mean of each treatment group, i.e., group I of 1.99±0.40, group II of 3.13±0.61, group III of 2.40±0.44, group IV of 2.28±0.31 and group V of 2.15±0.27.

Table 1. The Mean Value of Malondialdehyde Levels Post Treatment (µg/ml)

	Groups	Mean±SD
I	Negative control group, only food intake	1.99±0.49
II	Positive control group, food intake and exposure to cigarette smoke	3.13±0.61
III	The treatment group was exposed to cigarette smoke and intake of red mulberry (3 g)	2.40±0.44
IV	The treatment group was exposed to cigarette smoke and intake of red mulberry (6 g)	2.28±0.31
V	The treatment group was exposed to cigarette smoke and intake of red mulberry (12 g)	2.15±0.27

The observations results of the mean number of alveolar macrophages (Figure 1) in each experimental animal group are the negative control group (I); positive control group (II); treatment groups (III, IV, V) with exposure to cigarette smoke and with an oral dose of *Morus rubra* fruit 3 g, 6 g, and 12 g.

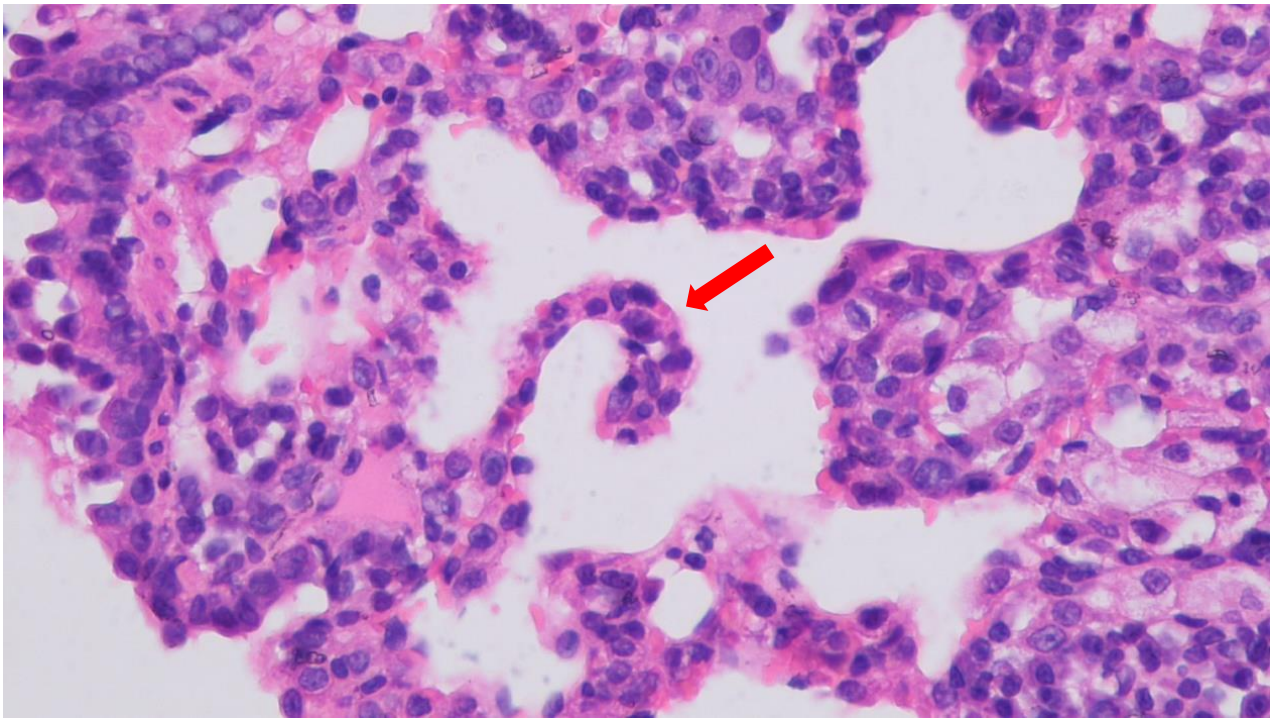

Figure 1. The Histology of Alveolar Macrophages in the Positive Control Group (Red Arrow)

Table 2 shows that the mean of each treatment group, i.e., group I is 5.36±0.52, group II is 32.08±2.09, group III is 22.52±1.50, group IV is 17.58±1.10 and group V is 10.88±1.43.

Table 2. The Mean Number of Alveolar Macrophages Post Treatment

	Groups	Mean±SD
I	Negative control group, only food intake	5.36±0.52
II	Positive control group, food intake and exposure to cigarette smoke	32.08±2.09
III	The treatment group was exposed to cigarette smoke and intake of red mulberry (3 g)	22.52±1.50
IV	The treatment group was exposed to cigarette smoke and intake of red mulberry (6 g)	17.58±1.10
V	The treatment group was exposed to cigarette smoke and intake of red mulberry (12 g)	10.88±1.43

Table 3 shows the normality and homogeneity test results of malondialdehyde levels. It offers a *p*-value more significant than 0.05. So, the data obtained has normality ($p>0.05$) and is homogeneous ($p=0.717$). ANOVA test will conduct to determine the differences between groups, and the *p*-value has obtained 0.006.

Table 3. ANOVA Test Results on Research Variable

Groups	Malondialdehyde Levels	Alveolar Macrophages
I		
II		
III	0.006	0.000
IV		
V		

Based on table 3, the results of the normality and homogeneity test for alveolar macrophage show a *p*-value more significant than 0.05, so that the data obtained has normality ($p>0.05$) and is homogeneous ($p=0.085$). ANOVA test has determined the differences between groups, and the *p*-value has obtained 0.000 ($p<0.05$).

Table 4. Least Significance Difference Test Results on Malondialdehyde Levels

Groups	I	II	III	IV	V
I	-	-	-	-	-
II	0.001	-	-	-	-
III	0.163	0.016	-	-	-
IV	0.316	0.006	0.679	-	-
V	0.574	0.002	0.390	0.652	-

The least significance difference (LSD) test used to compare between malondialdehyde groups (table 4). Group 2 showed a significant difference with each group (p<0.05). It showed that the group exposed to cigarette smoke could increase free radicals. Meanwhile, the other groups did not show any differences between each group (p>0.05).

Table 5. Least Significance Difference Test Results on Alveolar Macrophages

Groups	I	II	III	IV	V
I	-	-	-	-	-
II	0.000	-	-	-	-
III	0.000	0.000	-	-	-
IV	0.000	0.000	0.000	-	-
V	0.000	0.000	0.000	0.000	-

The least significance difference (LSD) test compared alveolar macrophage groups (table 5). Each group showed significant differences between the other groups (p<0.05).

DISCUSSION

Malondialdehyde levels and the number of alveolar macrophages in the lung tissue showed an increase in the positive control group compared to the negative control group. The malondialdehyde levels decreased gradually in the treatment group given red mulberry compared to the positive control group. The higher the amount of red mulberry, the lower the malondialdehyde levels and the number of alveolar macrophages. However, the decrease in malondialdehyde levels could not be the same as the negative control group. It showed that exposure to cigarette smoke that entered the body through the respiratory tract would increase free radicals in the blood.

Cigarette smoke contains various kinds of free radicals with a concentration of more than 10^6 molecules for each inhale. The types of free radicals in cigarettes are reactive oxygen species (ROS) and reactive nitrogen species (RNS).[19] All kinds of extremists, when entering the airway, will increase the immune response in the lung tissue. The free radicals entering the body will be considered foreign objects by the body to clean through the lung tissue's non-specific immune system, namely alveolar macrophages. Alveolar macrophages will carry out the phagocytosis process to reduce the free radicals that enter the lung tissue. It indicates that the increase in free radicals will directly follow an increasing number of alveolar macrophages in the lung tissue.[20]

Free radicals are standard products produced by cells through metabolic processes and are neutralized physiologically by enzymatic antioxidants in the body. The most reactive type of radical is the superoxide radical. The radicals can be neutralized by enzymatic antioxidants, i.e., superoxide dismutase (SOD), to be converted into hydrogen peroxidase (H_2O_2), which will then be neutralized again by enzymatic antioxidants, namely glutathione peroxidase (GSH-Px) and catalase (CAT) into water (H_2O) and oxygen (O_2).[21] Health problems will occur if there is a continuous increase in free radicals so that the antioxidants in the body are unable to neutralize all free radicals. It will result in oxidative stress, which triggers lipid peroxidase causing cell damage and death. The process of lipid peroxidase in cells will produce malondialdehyde. Thus, the decrease

or increase has dramatically influenced by free radicals in the body.[22]

Prevention can provide an intake of antioxidants from outside of the body to neutralize excess free radicals.[23] The flavonoid antioxidant found in red mulberry is the type of antioxidant that can protect the body from reactive oxygen species. Flavonoids will react directly with superoxide radicals and other types of extremists. The high reactivity of the hydroxyl group from the flavonoid antioxidants causes the free radicals that have bounded to become more stable and unreactive.[17]

This study has shown a decrease in malondialdehyde levels and alveolar macrophages due to the administration of red mulberry. Exposure to cigarette smoke shows an increase in free radicals and the number of alveolar macrophages that can trigger an inflammatory response and cell damage. The flavonoids containing in red mulberry fruit can provide effective prevention against exposure to cigarette smoke. The malondialdehyde level with flavonoid 13 g has the same effectiveness as the negative control group. Meanwhile, the alveolar macrophages show differences in each treatment group.

CONCLUSION

Red mulberry, which contains flavonoid antioxidants, can reduce free radicals from exposure to cigarette smoke that enters the airways by reducing malondialdehyde levels in the blood and the number of alveolar macrophages in lung tissue.

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