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NEUTROPHIL AND LYMPHOCYTES OF RAT PUPS OF THE DOES EXPOSED TO CIGARETTE SMOKE TREATED WITH ETHANOL EXTRACT OF KEBAR GRASS (*Biophytum petersianum* Klotzsch)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Kebar grass contains vitamin A and vitamin E, which are antioxidant compounds that are thought to improve the blood picture of rats that are disturbed due to the exposure of the free radicals from cigarette smoke. The purpose of this study was to determine the neutrophil and lymphocyte profiles of rat pups of the does which treated with the ethanol extract of *kebar* grass (*Bhiophitum petersianum* Klotzsch) due to the exposure toward the cigarette smoke. This study used an experimental method by using a Completely Randomized Design (CRD) which consisted of 4 (four) treatment groups and 3 (three) replications. After the treatment, all groups of does were mated and the pups that were born would be taken 3 in each group as a replication, and then their blood would be taken to analyze the neutrophils and lymphocytes. The data which successfully obtained were analyzed by analysis of variance (ANOVA). The results showed that there was a change in the neutrophil and lymphocyte profiles of rat pups from does which were treated with ethanol extract of *kebar* grass due to the exposure toward cigarette smoke, in which there was an increase in the percentage of neutrophils and lymphocytes ($P<0.05$), where an increase in the percentage of neutrophils to normal direction while the increase in the percentage of lymphocytes occurred continuously. The increase in lymphocytes is strongly suspected to be due to the administration of ethanol extract of *kebar* grass which contains flavonoids, resulting in the proliferation of lymphocytes as antibody products.

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1. INTRODUCTION

Blood is a fluid that functions as a means of transportation to carry nutrients, metabolic substances and oxygen that is needed by body tissues and as body defense found in all living things. Blood consists of fluids in the form of plasma and solids in the form of cells, namely erythrocytes, leukocytes and platelets.

Exposure to cigarette smoke can cause free radical activity since it contains high concentrations of oxidant molecules. When inhaling the cigarette smoke, oxidant molecules enter and undergo oxidation by donating its electrons to the body's oxygen molecules. Oxygen molecules will turn into super oxidants and other derivative molecules that are reactive, or also known as ROS (Reactive Oxygen Species) [1]. Excessive ROS productions potentially cause damage in various places in the body [2]. In the case of exposure to cigarette smoke, it will have damaging impact toward the organ and respiratory tract resulting in inflammation which leads into an increase toward the amount of leukocytes [3]. The increase in ROS causes an imbalance of oxygen and antioxidants in the body which causes cell damage [4]. Oxidative stress is characterized by an increase in oxidant radicals and triggers an inflammatory reaction resulting in an increase in the amount of absolute leukocytes but also an increase in the amount of neutrophils, lymphocytes and monocytes compared to non-smokers [5].

In order to reduce the negative impact caused by cigarette smoke, substances that can act as antioxidants as well as anti-inflammatory are needed [6], such as those found in *kebar* grass, a traditional medicine from Papua. *Kebar* grass contains vitamin A and vitamin E which are antioxidant compounds, and it is considered to improve the blood picture of rats which are hampered due to the exposure toward the free radicals from cigarette smoke. Vitamin E can inhibit oxidation reactions by binding vitamin E radicals to become free vitamin E which functions as an antioxidant [7].

The infiltration of free radicals from cigarette smoke causes an unbalanced state and it is even fathomed that the effect will be hereditary to the children if they are in the mothers' womb. Therefore, the use of *kebar* grass extract is predicted to be able to neutralize and balance the number of free radicals in the mother's blood as well as in the child in the womb. The existence of vitamin E in *kebar* grass is also thought to increase fertility in women [8]. Vitamin E works to prevent miscarriage, maintain the health of the uterine wall, placenta and child in the womb. This can be useful for pregnant women to maintain their pregnancy until birth. However, women who smoke or are exposed to the cigarette smoke can pass on physiological abnormalities to their children, especially in blood, so it is necessary to conduct a study to analyze the blood picture, especially neutrophils and lymphocytes in the first offspring of brood-stock exposed to cigarette smoke treated with the ethanol extract of *kebar* grass.

2. METHODOLOGY

This study was conducted from August to December 2021 by using an experimental method; with the engagement of a completely randomized design (CRD) which consisted of four treatments and each was repeated three times. The treatment studied was the difference in the dose of ethanol extract of *kebar* grass given to the doe models, with the following stages of research:

Preparation Phase: Twelve does were divided into four groups and then acclimatized for a week. Group (-): negative control group, they are does that were not exposed to cigarette smoke and ethanol extract of *kebar* grass; group (+): positive control group, this group are does which were exposed to cigarette smoke for 28 days, the group (0.067): the does group exposed to cigarette smoke for 28 days then they were given the ethanol extract of *kebar* grass at a dose of 0.067 mg/dose/day for 28 days , and group (0.135): this does group was exposed to cigarette smoke for 28 days and then the ethanol extract of *kebar* grass was given at a dose of 0.13 5 mg/dose/day for 28 days .

Cigarette Smoke Exposure Stage: Does that had been placed in animal cages were transferred to a *smoking chamber* and then exposed to cigarette smoke. *Smoking chamber* is a box in which there is a tested barrier to separate the experimental animal from the burning end of the cigarette, so that the experimental animal can be directly exposed to the smoke. The treatment box has a hole which is functioned to insert a hose containing cigarette smoke which is first accommodated in a vacuum. The exposure to 10 cigarettes of cigarette smoke per day toward the does was carried out at 09.00 and 15.00 WIT [9,10] for 28 days after the exposure process for 28 days.

Kebar Grass Extraction Stage: *Kebar* grass is taken as much as 1 kg and dried up, and then it was mashed using a blender. After obtaining the *kebar* grass powder, then it was followed by the extraction

process using the maceration method. The procedure is as follows:

1) Weighed as much as 250g of *kebar* grass powder and put it in an Erlenmeyer.
2) After that, 1 liter of 70% ethanol was added and settling for 24 hours.
3) After 24 hours, it should be filtered using Whatman 0.2 filter paper to obtain a liquid extract of *kebar* grass. The extraction residue was repeated 3 times.
4) The liquid extract from the *kebar* grass that has been obtained was then concentrated with a rotary evaporator.
5) The results of the concentration would obtain a concentrated ethanol extract of *kebar* grass.

The Dosing Stage of the Ethanol Extract of *Kebar* Grass: The ethanol extract of *kebar* grass was administered toward the does that had been exposed to cigarette smoke. The first dose was 0.0675mg/doe/day, and the second dose was 0.135mg/doe/day.

Parent Stage Mating: After treatment, all groups of does were mated in a ratio of 1:1 (one buck and one doe). 3 rat pups will be taken from each group as a replication. Subsequently, the rat pups' blood would be taken to analyze the neutrophils and lymphocytes.

Preparation Stage of Pups Blood Smear: Blood smears were made by taking blood from the tails of the pups. Then, the blood would be dripped on the first object glass in a horizontal position. Another object glass was placed on the blood part with a position which forming an angle of 45^{o}, consequently the blood would spread along the line of contact between the two object glasses. Following that, the object glass was pushed forward quickly until a thin blood smear was formed on the object glass. After that, it was dripped with Giemsa coloring to cover the surface of the blood smear for 45 minutes. It was then washed with running water at an inclined position and left to dry.

Stages of Observation of Neutrophils and Lymphocytes: Observations of neutrophils and lymphocytes (leukocytes) from the blood smear preparations were carried out using a microscope with 1000x magnification. The preparations were then dripped with immersion oil to clear up the observations. Then, the types of neutrophils and lymphocytes (leukocytes) were calculated with the following formula:

$$calculation\ of\ leukocytes\ type\ (\%) = \frac{\text{amount of a type of leukocytes}}{\text{100 leukocytes cells}} \text{ x } 100$$

The number of neutrophils and lymphocytes (leukocytes) counted was 100 leukocytes for each preparation. The hundred leukocytes were grouped based on differences in size, color, number and cytoplasmic granulation, chromatin shape and nucleus into five groups, they are: neutrophils and lymphocytes. The calculation results are expressed in percent (%).

Data Analysis Stages: The results obtained were analyzed by analysis *of variance (ANOVA) and then continued with Duncan's test at a* significant level of = 0.05 by using SAS software.

3. RESULTS AND DISCUSSION

The results showed that the mean percentage of the neutrophil and lymphocyte cells of the rat pups underwent changes, which is presented in Table 1. The results of the analysis by using the ANOVA test showed that the neutrophils, eosinophils, lymphocytes and monocytes of the first offspring from parents which were exposed to cigarette smoke and then treated with the ethanol extract of *kebar* grass at a dose of 0.067 mg and 0.135 mg experienced significant changes ($p< 0.05$). Meanwhile, basophils, which are one of the leukocyte differentiation cells in this study, were not found in the blood circulation. This was presumably because basophil cells do not act as macrophages in an inflammatory response that occurred in the treatment, so it did not affect the activity that occurred.

The results of the calculation of the mean percentage of neutrophil percentage of the rat pups from the normal doe group was 51.33%; positive control was 32 .67%; and the group of rat pups from the doe which were treated with the ethanol extract of *kebar* grass successively: at a dose of 0.067 mg was 30.33% and at a dose of 0.135 mg was 37.33%. The result of ANOVA showed a significant difference ($p<0.05$) between the positive control and the dose of 0.067 mg compared to the negative control, but the dose of 0.135 mg was not significantly different from that of the negative control. This means that the exposure to the cigarette smoke toward the does could affect the first offspring because there was a decrease in neutrophils in the positive control, but after the does were treated with the ethanol extract of *kebar* grass it can also affect the first offspring, which was characterized by an increase in neutrophils which approaching the negative control.

The result of the calculation of the mean percentage of the lymphocytes of the rat pups from the normal doe group was 28.00%; positive control was 65.33%; and the group of rat pups from the does which were

Table 1. Mean of neutrophils and leukocytes of rat pups from does which were treated with the ethanol extract *kebar* grass due to the exposure to cigarette smoke

Leukocyte Differentiation (%)	Treatment Results			
	(-)	(+)	0.067	0.13 5
Neutrophil	51.33±0.03[a]	32.67±0.03[b]	30.33±0.06[b]	37.33±0.03[ab]
Lymphocytes	28.00±2.01[d]	65.33±3.07[a]	42.00±0.01[c]	53.67±0.08[b]

Description: Numbers followed by different letters in the same row are significantly different (P < 0.05). (-): negative control group, are the rat pups from does which were not exposed toward the cigarette smoke and the ethanol extract of kebar grass, (+) : positive control group, are the rat pups from does which were exposed to cigarette smoke for 28 days, (0.067): group of rat pups from does which were exposed to cigarette smoke for 28 days and then treated with the ethanol extract of kebar grass at a dose 0.067mg/doe/day for 28 days, and (0.135): a group of rat pups from female parents which were exposed to cigarette smoke for 28 days and then treated with the ethanol extract of kebar grass at a dose 0.135 mg/doe/day for 28 days

given the ethanol extract of *kebar* grass successively: at a dose of 0.067 mg was 42.00% and at a dose of 0.135 mg was 53.67%. The results of the ANOVA showed a significant difference ($p<0.05$) between the positive control compared to the 0.067 mg dose and the 0.135 mg dose, but the 0.135mg dose was not significantly different from the negative control ($p>0.05$) . This means that the exposure to cigarette smoke toward the does could affect the first offspring with an increase in lymphocytes, and it still increased after being treated with the ethanol extract of *kebar* grass. The phenomenon of the increase in lymphocytes was suggested to be due to the effect of cigarette smoke as being toxic in the body of the rat pups.

The effect of the exposure to cigarette smoke toward the does that are passed on to the first offspring is considered to be due to the toxic entry of cigarette smoke of 10 cigarettes per day in the doe which lead into high toxic accumulation and being able to be hereditary through the nutritional pathway from the doe to the embryo, resulting in disruption of metabolism of the embryo. After birth, the rat pups experienced a stressful condition that affects neutrophils and lymphocytes. The entry of toxic compounds from cigarette smoke for a long time can interfere with metabolism, causing chronic stress on cells and tissues in both the doe and the pup. Chronic stress can increase cortisol to a constant. Cortisol can cause an increase in neutrophils [11]. The possibility of *kebar* grass engagement which contains antioxidants is able to repair cells and tissues which were damaged by chronic stress from the toxins of cigarette smoke. The mechanism of action of antioxidants is to inactivate the chain reaction of lipid peroxidation by preventing the formation of peroxide radicals that can cause cell and tissue damage. This phenomenon shows the performance of *kebar* grass which is able to increase the percentage of neutrophils to be stable so that it can protect cells and tissues. Medicastore [12] states that neutrophils help protect the body against bacterial and fungal infections and digest foreign materials from the remnants of inflammation.

According to research by Ali *et al.,* [13] the percentage of neutrophils is one of the parameters used to diagnose cases of acute appendicitis. The treatment with *kebar* grass which contains vitamins A, E and flavonoids toward the does exposed to cigarette smoke can affect the first offspring (rat pups). Vitamin A plays a role in cell formation, as a strong antioxidant and able to ward off free radical attacks on cells, while vitamin E in *kebar* grass is able to maintain cell health [8]. In addition, the administration of the ethanol extract of *kebar* grass can increase the accumulation of flavonoids in *kebar* grass so that it is suggested to be able to overcome the inhibition of cell metabolism. This is in line with Pavlovic *et al.,* [7] which stated that flavonoids were able to inhibit the induction of apoptosis.

The performance of lymphocytes in this study was fluctuating, where the rat pups from does which were only exposed toward cigarette smoke experienced an increase in lymphocytes. In contrast to the rat pups from the does which were exposed to cigarette smoke and then treated with the ethanol extract of *kebar* grass at a dose of 0.067mg, there was a decrease, but in the rat pups from the does which were expose to cigarette smoke and then treated with the ethanol extract of *kebar* grass at a dose of 0.135mg, there was an increase. The increase in lymphocytes in the treatment of ethanol extract of *kebar* grass at a dose of 0.135 mg is considered to be the trigger of an increase in lymphocytes which play a role in destroying cancer cells and forming antibodies. According to Medicastore [12] T lymphocytes function as protection against virus infections and devastate some cancer cells, while B lymphocytes play a role in the formation of antibodies. The inclusion of the ethanol extract of *kebar* grass which containing flavonoids is suggested to be able to overcome cancer by inactivating carcinogens. The mechanism of action of flavonoids in overcoming cancer is by inactivating

carcinogens, cell cycle inhibition, and inducing apoptosis [7].

According to Verstraeten *et al*., [14] Flavonoid compounds can protect cell membranes from oxidative stress caused by free radicals by increasing membrane fluidity and preventing the entry of molecules that can affect membrane integrity. In addition, flavonoids are considered to be able to react with reactive compounds and inhibit the activity of these reactive compounds, hence they do not react and do not damage cell membranes and prevent lipid peroxidation. Flavonoids which are also antioxidants can neutralize or devastate free radicals by interacting directly with oxidants or free radicals, preventing the formation of reactive oxygen species, converting reactive oxygen to be less toxic and repairing cell and the damage of tissue that occurs [15]. The action of flavonoids as antioxidants in the blood helps to reduce the differentiation of eosinophil leukocytes, but has not been able to reduce lymphocytes. This is suggested to be due to the occurrence of high cell and tissue damage which is caused by the toxic effects of cigarette smoke. The phenomenon of the increase of lymphocytes is thought to be due to the administration of the ethanol extract of *kebar* grass which plays a role in triggering an increase of lymphocytes as antibody products. The content of tannins in *kebar* grass is examined to be able to increase antibodies. This is in line with Velayutham *et al*., [16] which states that tannins are polyphenolic compounds which can reduce fat oxidative stress, as well as able to bind and precipitate proteins which later can be functioned as antibodies. Sarawar *et al*., [17] reported that tannins can cause a significant reduction (up to 23%) of protein and amino acid digestibility of rats.

4. CONCLUSION

Based on the research, it was concluded that there was an increase in the percentage of neutrophils and lymphocytes of rat pups from does which exposed to cigarette smoke treated with the ethanol extract of *kebar* grass. The increase of the percentage of neutrophils leads into normal, while the increase of lymphocytes occurred continuously. The phenomenon of the increase of lymphocytes is suggested occurred due to the administration of the ethanol extract *kebar* grass which plays a role in triggering an increase in lymphocytes as antibody products.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Faux Sp, Tail T, Thorre D, Xu Y, Breheny D, M Gaca. The Role of Oxidative Stress In ThenBiological Response of Lung Epithelial Cell to Cigarette Smoke. Biomarker Journal. 2009;14(1).
2. Lee DZ, Chung JM, Chung K, MG Kang. Reactive Oxygen Species (ROS) Modulate AMPA Receptor Phosphorylation and Cell-Surface Localization in Concert With Pain-Related Behavior. Pain Journal. 2012;153(9).
3. Prihandari R, Muniroh L. Watermelon Juice Lowers Neutrophils in Wistar Strain Bucks which Exposed to Cigarette Smoke. Jurnal Media Gizi Indonesia. 2016;11(2).
4. Rahman I, IM Adock. Oxidative and Redox Regulation of Lung Inflammation. ERS Journal. 2006;28(1):11-13
5. Lavi S, Prasad A, Yang EH, Mathew V, Simari RD, CS Rihal. Smoking Isasociated With Epicardialcoronary Endothelial Dysfunctionand Elevated While Blod Celcount in Patients With Chest Painand Early Coronary Artery Disease. AHA Journal. 2007;15(26):21-27.
6. Prakasa AB. The Effect of Honey toward the Amount of Total Leukocyte due to the Exposure of Cigarette Smoke. Majority Journal. 2015;4(7):8-11
7. Pavlovic V, Cekic S, Rankovic G, N. Stoiljkovic. Antioxidant and Prooxidant Effect of Ascorccbic Acid. *Acta Medica Medanae*. 2005;(44):65-69.
8. Unitly AJA, C Inara. Potensi Rumput Kebar (*Biophytum petersianum* Klotzsch) Dalam Meningkatkan Kinerja Reproduksi Prosiding seminar nasional: Pengembangan Pulau-Pulau Kecil. 2011;229-223.
9. Unitly AJA. The Potency of *Kebar* Grass Extract (Biophytum petersianum Klotzsch) on the Reproductive Function of Bucks which Exposed to Cigarette Smoke. Dissertation. Veterinary Faculty. Postgraduate Program, Bogor Agricultural University. Bogor; 2013.
10. Unitly AJA, Kusumorini N, Agungpriyono S, Satyaningtijas AS, A Boediono. The Changes in the Quality of Sperm and the Number of Spermatogenic Cells in Bucks Which Exposed to Cigarette Smoke. ISSN : 1978-225X. Jurnal Kedokteran Hewan. 2014;8(2):116-119.

11. Guyton AC, JE Hall. Textbook of Medical Physiology.11.Philadelphia Elsevier; 2006.
12. Medicastore. Biologi Darah. Modern Management and Public Health. 2011;1-4.
13. Ali S, Shah O, Wani N, Shah M, Ahmed L, S Mallik. Role of Total Leukocyte count, Neutrophil Percentage, C - reactive protein and Ultrasonography in diagnosis of acute appendicitis. The Internet Journal of Surgery. 2010;23 (2). ISSN: 1528-8242.
14. Verstraeten SV, Oteiza PI, CG Fraga. Membrane effects of cocoa procyanidins in liposomes and Jurkat T cells. Biol Res. 2004;37:293-300.
15. Sizer F, E Whitney. Nutrition Concept and Controversies. Thomson Learning Library of Congres Cataloging; 2000.
16. Velayutham R, Sankaradoss N, KF Ahamed. Protective effect of tannins from Ficus racemosa in hypercholesterolemia and diabetes induced vascular tissue damage in rats. Asian Pac J Trop Med. 2012;5(5):367-73. DOI: 10.1016/S1995-7645(12)60061-3.
17. Sarawar GG, Wu Xiao C, KA Cockell. Impact of antinutritional factors in food proteins on the digestibility of protein and the bioavailability of amino acids and on protein quality. Br J Nut. 2012;108(2):315-32. DOI: 10.1017/S0007114512002371.