The effect of Java Plum Fruit (Zyzygium cuminii) extract on leucocyte and lung histopathology of mouse exposed cigarette smoke

A A S A Sukmaningsih1,3, N M R Suarni1, I Wiratmini1, C N Primiani2, N W Sudatri1

1Department of Biology, Faculty of Mathematics and Natural Sciences, Udayana University, Badung 80361, Bali, Indonesia
2Biology Education Study Program, Faculty of teacher training and education, Univeritas PGRI Madiun, East Java, Indonesia

Corresponding author: sukmaningsih@unud.ac.id

Abstract. Java Plum fruit can decrease free radical activities. The overwhelmed production of free radicals will reduce the immunity system in the body. This research aims to study the effects of java plum fruit extracts on leukocytes and histopathology of mouse lungs that have been exposed to cigarette smoke for 30 days. Frozen-dried java plum fruit is macerated with water. The mice are divided into Control groups (K-). The mice of the control group were given only water orally. (K+) are mice that are exposed to commercial cigarette smoke once a day. (RJ) were the mice exposed to cigarette smoke once a day as java plum fruit extract orally of 180 mg/kg body weight. (RF) were the mice exposed to cigarette smoke given with java plum fruit extract with 180 mg/kg of body weight that is applied on its filter. The results showed a significantly lower number of leukocytes \( p < 0.05 \) in the K-, RF, and RJ groups compared to K+. Meanwhile, there was a significant increase in the number of lung pathological cells \( p < 0.05 \) in the K-, RF, and RJ compared to the control group (K-). The results conclude that java plum fruit can reduce free radicals in animals affected by inflammation due to cigarette smoke particles in the lungs.

1. Introduction

Smoking is one of the activities that increase free radicals [1]. Free radicals will reduce antioxidant defenses and cause oxidative stress [2]. Cigarette smoke is an aerosol formed from droplets of smoke particles and has a complex composition without water and nicotine content which creates Tar [3]. Tar contains free radicals \( 10^{17} \) spin \(^{-1} \) [4] which are formed on fine particles and ultrafine particles of cigarette smoke. Cigarette smoke nanoparticles are inhaled into the lungs, diffuse into the blood vessels, and translocate to other organs. It quickly reacts with target cells, inhibits phagocytosis, and causes local and systemic inflammatory effects [5]. The presence of an inflammatory response also forms oxidative stress that will affect the integrity and function of proteins, DNA, and lipid peroxidation [6], including the immune system's function [7]. Oxidative stress causes a decrease in lymphocyte cell function. This causes the immune system to decrease and the body is susceptible to disease. Lymphocytes play an essential role in specific immune responses that function to eliminate various antigens in the body [8]. The immune response causes an increase in pro-inflammatory cells. Cells involved in the immune response are neutrophils, macrophages, lymphocytes and B lymphocytes. Cigarette smoke particles that are inhaled through the respiratory system will cause changes in morphology and histological structure in respiratory tissues and organs, namely the lungs, including the bronchi, bronchioles, and alveoli. Observations of the immune response evoked by cigarette smoke particles in lung histological tissue were carried out on the bronchioles and alveoli. Some of the pathohistological findings were emphysema and fibrotic granuloma [9].

The respiratory organ, which is the main inhalation route for cigarette smoke particles, can cause physiological and immunological responses. This response causes changes in the morphology and histologic structure of the lungs. The main structure of the lungs is the bronchi, with their branches into bronchioles and alveoli. A pulmonary artery and lymphatic vessels accompany each bronchus. In the presence of cellular and immune responses caused by cigarette smoke particles, the histologic...
changes are related to inflammation. Under normal circumstances, a collection of alveoli connected to the bronchial tubes will form a strong structure and keep the airways open. In emphysema, the alveoli walls are damaged so that the bronchioles lose their supporting structure [9]. Lymphocytes and lung cells exposed to cigarette smoke are also sources of free radicals that cause immune responses and form oxidative stress and cause lung histological abnormalities.

Java plum fruit which has a purple-black color when ripe, is known to contain various antioxidant compounds. Cyanidin, which is known to form complex compounds with several transition java plum fruit extract applied to cigarette filters, could also degrade/reduce free radicals of cigarette smoke [10] and reduce MDA levels as free radical markers in mice [11].

In this study, a freeze-dried java plum fruit extract was tested, which was applied to a cigarette filter as a free radical scavenger of cigarette smoke on the immune system, including the total number of leukocytes and lung histological abnormalities.

2. Materials and methods
2.1. Preparation
A ripe java plum (S. cumini) fruit was collected from Denpasar. The ripe fruit was washed and separated from its seed. The fruit was dried by a freeze-drying process for 72 hours and grounded to obtain a powder sample. The powder was extracted in water by stirring it and then centrifuged at 10 min. The results were residues and supernatants, and the residues were extracted again. The supernatants were filtered and dried by a freeze dryer [10]. Application of java plum fruit extracts on cigarettes was made by applying the frozen, dried extract to cigarette filters.

2.2. Animal
This research has obtained an Ethical Clearance Number: 106/UN4.2.9./PT.01.04/2020, from the Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali. This study used a completely randomized design with a sample of 25 male mice aged 2-3 months, and weighing 25-30 grams, divided into four groups at random, each with five individuals—the control groups (K-). The mice of the control group were given only water orally. (K+) were the mice exposed to commercial cigarette smoke once daily for each. (RJ) were the mice exposed to cigarette smoke once daily given java plum fruit extract orally of 180 mg/kg body weight. (RF) were the mice exposed to cigarette smoke given with java plum fruit extract with 180 mg/kg body weight that is applied on its filter.

2.3. exposed cigarette smoke to mice
The mice were placed in a ventilated glass smoking room and exposed to cigarette smoke every day, at a rate of 1 stick per animal for 30 days. Fumigation is carried out using a Smoking Pump with a suction speed of 0.23 m/s, streamed through the glass smoking room [11].

2.4. Surgery on Experimental animal
Mice were injected with ketamine on the 31st day at a dose of 80-100 mg/kg and xylazine at 10 mg/kg. Blood and lung organs are collected. Examination of blood samples: Blood is taken through the heart's ventricles and put into a vacutainer tube that already contains EDTA solution, then the leukocytes are examined using a Hematology Analyzer.

2.5. The procedure of histological preparations
Histological preparations were made using the paraffin method. The processes include fixation, dehydration, clearing, infiltration, embedding, cutting, and staining. The fixative solution used was formalin buffer. Dehydration from the tissues must be removed by approximately 70% to 95% alcohols. The clearing is done by the removal of the alcohol with the embedding medium (paraffin). The clearing agent is xylene. Infiltration is infiltrated of the tissue with the paraffin. The embedding process of tissues must be oriented correctly in the block of paraffin. Once the tissues have been
embedded, they must be cut into sections by a microtome. The stain used was Hematoxylin-Eosin. Histological observations using a microscope connected to the Opti Lab [12].

2.6. Data analysis

The observed variable was the number of leukocytes, including lymphocytes, monocytes, eosinophils, neutrophils and basophils. Variables observed in the lungs include alveolar dilation, hemorrhage, necrosis, type II pneumocytes, and inflammatory cell infiltration. The data obtained from this study were analyzed using the Analysis of Variance (ANOVA) using the SPSS version 24 program.

3. Results and Discussions

The result from analysis data using the One Way ANOVA is presented in table 1 and table 2. Based on the data in table 1 that the number of monocytes, lymphocytes, and neutrophils in this study showed significant difference ($p < 0.05$).

Table 1. Mean average number of Leucocyte of Mouse exposed to cigarette Smoke after administration of java plum fruit extracts.

| Treatment | Monocyte  | Eosinophil | Neutrophil  | Basophil | Lymhocyte |
|-----------|-----------|------------|-------------|----------|-----------|
| K +       | 8.80±0.84a| 5.00±0.00a | 53.40±0.00a | 0±0.00a  | 56.00a    |
| K -       | 6.40±0.55b| 1.40±0.55b | 45.40±0.00b | 0±0.00b  | 46.00b    |
| RF        | 7.60±0.55b| 1.60±0.55b | 46.20±0.00b | 0±0.00b  | 43.40b    |
| RJ        | 4.20±0.44d| 1.80±0.45b | 53.20±0.00b | 0±0.00a  | 38.60d    |

Note: different letters behind the value in the same column indicate a very significant difference at the 5% error level ($p \leq 0.05$).

This research is about free radicals from cigarette smoke particles and free radical decay efforts by antioxidants from the java plum fruit extract. Cigarette smoke particles vary in many sizes. The body's defense system and immune system will respond to foreign particles or objects. In this case, the body's defense system consists of several stages or lines. In the first line, the particles will be managed by the morphological and anatomical structure of the respiratory tract in the nose and trachea, through the epithelium, cilia, cartilage, mucus secrets produced by goblet cells or Clara cells. When the first line fails to overcome this particle, the antigen, fine particles, or ultrafine particles will be deposited in the lungs. Particles of cigarette smoke from the lungs will diffuse into the blood vessels and be translocated to other organs in the body. The second line of immune response to cigarette smoke particles in the lungs will be carried out by the innate immune system, namely phagocytosis and inflammation. The process of phagocytosis is mainly carried out by macrophages, neutrophils, eosinophils, basophils, and monocytes.

The results of the statistical analysis of leukocytes in table 1 showed significant differences in the number of monocytes, eosinophils, neutrophils and lymphocytes ($p < 0.05$), especially between the K- and K+ animal groups. Cigarette smoke-free radicals cause disturbances in antioxidant activity balance in the body, such as SOD Glutathione and catalase. It obstructs energy flow, so it is suspected that there is an accumulation of electrons and superoxide and hydrogen peroxide, which forms a chain reaction and causes an inflammatory response in the lungs, initiated by alveolar macrophages and airway epithelial cells. Macrophages have the potential to produce pro-inflammatory mediators. Alveolar macrophages and lung epithelial cells will form a defense when cigarette smoke particles enter mice's bodies [13]. One of the characteristics of inflammatory response in this study is the presence of inflammatory cell infiltration, which is generally dominated by neutrophils. Using java plum fruit extract in cigarette filters on RF or oral administration of java plum fruit to mice exposed to RJ cigarette smoke showed significantly lower leukocyte yields ($p < 0.05$).
Table 2. Mean average number of lung histopathology of Mouse exposed to cigarette Smoke after administration of java plum fruit extracts.

| Histopathology of Lung ( % number) | Inflammation Cell infiltration | Hemorrhage | congestion | Necrosis | Pneumocyte II |
|----------------------------------|--------------------------------|------------|------------|----------|---------------|
| K -                              | 14.50 ± 2.83 a                 | 5.16 ± 2.37 a | 11.87 ± 2.94 a | 11.00 ± 2.73 a | 22.84 ± 1.72 a |
| K +                              | 32.62 ± 3.35 b                 | 18.96 ± 3.09 b | 17.75 ± 8.11 a | 45.00 ± 2.00 b  | 20.76 ± 4.12 a  |
| RF                               | 32.37 ± 4.91 b                 | 18.64 ± 1.83 b | 18.18 ± 7.67 a | 38.60 ± 2.60 c  | 21.08 ± 1.76 a  |
| RJ                               | 24.31 ± 4.89 c                 | 10.46 ± 1.63 c | 10.87 ± 2.49 a  | 17.60 ± 1.34 d  | 20.76 ± 1.18 a  |

Note: different letters behind the value in the same column indicate a very significant difference at the 5% error level (p<0.05).

Based on the results of the study, there was an increase in the number of inflammatory infiltration cells, hemorrhagic, and necrotic infiltrates in mice exposed to cigarette smoke (K+) compared to the control group (K-) significantly (p < 0.05). The RF and RJ groups treated with java plum fruit extract showed a significant decrease in the number of inflammatories infiltrates, hemorrhagic, and necrosis (p < 0.05) compared to K+. K- which has the smallest amount of histological damage in the lungs (Figure 1).

Inflammation that occurs due to exposure to cigarette smoke has an impact on lung organ damage. Free radicals that cause an immune response also affect other cells or tissues. In this case, as free radicals with unpaired electrons have highly reactive properties, this property is a stable electron that will always be in a paired position. Because it has no electron pairs, free radicals will always find a partner as soon as possible to become stable. This is done by taking nearby electrons so that other compounds will also become radicals and form chain radicals. If not controlled by endogenous antioxidant systems such as SOD, glutathione, catalase, and others, free radicals will cause oxidative stress. This oxidative stress can cause the process of lipid peroxidation in cell membranes organelles and functions will be disrupted. In addition to causing lipid peroxidation, free radicals can also affect double-strand DNA damage and oxidation of protein structure and function. This can cause apoptosis, necrosis, or excessive proliferation of cells in the lungs, causing various diseases such as cancer, pulmonary obstruction and many more.

This study is related to the variables in type II pneumocyte cell necrosis, or hemorrhage. Damage to lung organs causes the increase of immune response in removing foreign substances and particles. This causes infection and inflammation in the lungs [14, 15] stated that particles that enter the alveolus cause an inflammatory response. During the inflammatory process, activation and phagocytosis by leukocytes release ROS, a series of processes resulting in macrophages and neutrophils in the alveolus. Type 2 pneumocyte cells are responsible for the production and secretion of surfactants and are responsible for the elasticity of the lungs [14, 15]. When type 2 pneumocytes are damaged, they can replicate and replace type 1 pneumocytes. The proliferation of type 2 pneumocytes is an indicator of alveoli damage [16]. Type 2 pneumocyte cells have a high sensitivity to foreign substances that enter the lungs. The proliferation of type 2 pneumocytes is a response to trauma in the lungs [17].

Another visible damage is the widening of the alveolus. Alveolar dilation is chronic and progressive, so that it can be an indication that a person has symptoms of chronic obstructive pulmonary disease [5]. Generally, dilation of the alveoli is a feature of emphysema. Emphysema is damage to the lungs that causes damage to the air sacs and loss of elasticity. Cigarette smoke is one of the causes of loss of elasticity in the alveoli. Patients with emphysema have a larger lung volume because the carbon dioxide that should be released is trapped in the alveoli. The body does not get enough oxygen, causing shortness of breath [18]. It is known that java plum fruit contains various antioxidants of anthocyanin compounds: Cyanidin-3,5-O-diglucoside, Delphinidin-3-O-glucoside Malvidin-3,5-O-diglucoside, Cyanidin-3-O-glucoside, Peonidin-3-O-glucoside, Cyanidin -3-Opentoside, Pelargonidin-3-O-glucoside, Malvidin-3-O-(t-611-O-coumaroyl)-glucoside, 5-O-glucoside [11].
These compounds have potent antioxidant activity because it consists of two aromatic rings. Both aromatic rings are bound to phenolic hydroxyl, methyl group, or oxygen. These groups can release or accept electrons from free radicals to reduce the reactivity of free radicals in forming lipid peroxidation or chain reactions through a mechanism of single electron transfer, hydrogen atom donation, as well as metal chelation. The Cyanidin-3,5-O-diglucoside of java plum fruit can act as a chelator of Fe²⁺, Co²⁺, Ni²⁺ (11). Previous studies have shown that java plum extract applied to cigarette filters can reduce free radicals of cigarette smoke [19]. This shows that java plum fruit extract can reduce the inflammatory process from free radicals of cigarette smoke in the immune system. Reduction of the inflammatory process will reduce the number of pathological cells in such as: inflammatory infiltration, and hemorrhagic.

Figure 1. mouse lung microscopy. (A) K - (B) K+ (C) RF (D) RJ (E) JW
(a) necrosis, (b) inflammatory inflammation, (c) congesti, (d) hemorrhage). Stain:
(b) Hematoxylin – eosin. Magnification: 400X
4. Conclusion
This study concludes that cigarette smoke affects total leukocyte count due to free radicals and inflammation. Antioxidants in java plum can react and neutralize free radicals so that the provision of java plum fruit can reduce the total leukocyte count and lung pathological cell count.

Acknowledgments
The author would like to thank the Ministry of Education, Culture, Research and Technology, which has provided funding through the Higher Education Applied Research Grant (PUU), with contract no: B/20-159/UN14.4A/PT.01.05/2020 on 10 March 2020 and Udayana University which has provided laboratory facilities.

References
[1] Baker R 2006 Prog. Energy and Combust. Sci 32 375-385
[2] Forkink M, Smeitink J M, Brock R, Willems P H G M, and Koopman W J H 2010 Biochim Biophys Acta 1797
[3] Angelis N, Propodis K, Zarogoulidis P, Spyrather D, Kioumis I, Papaiwannou A, and Pitsiouw G 2014 Journal Thorac. Dis. 6: 4-9
[4] Valvanidis A, Vlachogianis T and Fiotakis K 2009 International Journal of Enviromental Research and Public Health. 6(2) : 445-462.
[5] Suryadinata R V 2018 Jurnal Sains. 2(1) : 37-324
[6] Chari M G & Colagar A H 2011 Journal of Men's Health, 8(1), 43–49.
[7] Yoshida T and Tuder R M 2007 Physiol Rev. 87: 1047–1082.
[8] Baratawidjaya 2002 Balai Penerbit Fakultas Kedokteran Universitas Indonesia. Jakarta.
[9] Chen H W, Su S F, Chien CT, Lin W H, Yu S L, Chou C C, Chen J J W, Yang P C 2006. FASEB J. 20: 1732–1741.
[10] Sukmaningsih A A S A, Permana S, Santjojo D J D, Wardoyo A Y P and Sumitro S B. 2018 Rasayan J Chem. 11(3):1193–203.
[11] Sukmaningsih A A S A, Suarni N M R, Wiratmini N I, Setyawati I, Sudatri N W, Pangestiningisih, T W 2021 J. Phys.: Conf. Ser. 1751 012057
[12] Muntiha, M. 2001 Balai Penelitian Veteriner. Bogor
[13] Holgate S, Lackie P, Wilson S, Roche W and Davies D 2000. Am J Respir Crit Care Med. 162.
[14] Sun Y, Ito S, Nishio N, Tanaka Y, Chen N and Isebe K I 2014 Toxicology Letters. 229(2): 384-392.
[15] Mitchell R N, Kumar V, Abbas A K, and Fausto N 2009 Flavour Fragr Journal. 25(29) : 291-312 (2009),
[16] Junqueira L C and Carneiro J 2009 Edisi 10. EGC. Jakarta
[17] Honda T, Ota H, Yamazaki Y, Yoshizawa A, Fujimoto K, and Sone S 2003 Respir Med. 97(1): 80-85.
[18] Wynimko, J.C. 2016 Taylor and Francis Group. 1(1) : 1-9.
[19] Sukmaningsih A A S A, Permana S, Santjojo D J D, Wardoyo A Y P and Sumitro S B 2019 AIP Conference Proceedings 2155 020015