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Administration of ethanol extract of galangal (*Alpinia Galanga*) on histopathology of male mouse (*Mus musculus*) lungs exposed to lead acetate

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Abstract

Objective: This research aims to assess the preventive effect of ethanol extract of red galangal (*Alpinia Galanga*) on type I pneumocyte cell necrosis and proliferation of type II pulmonary pneumocytes in male mice (*Mus musculus*) exposed to lead acetate-induced damage.

Method: A total of 25 male mice aged 2.5-3 months and weighing 25-30 g were divided into five groups. The negative control group (K-) received oral water without lead acetate exposure, while the positive control group (K+) received 20 mg/kg BW of lead acetate. The treatment groups P1, P2, and P3 were exposed to lead acetate at a dose of 20 mg/kg BW/day and received red galangal extract at doses of 200 mg/kg BW, 400 mg/kg BW, and 800 mg/kg BW, respectively. All treatment groups were administered lead and ethanol extract of galangal orally from days 4 to 24 at a rate of 0.2 ml/head.

Results: The Mann-Whitney U statistical test revealed a significant increase in type I pneumocyte cell necrosis and type II pneumocyte cell proliferation in the lungs of male mice *(Mus musculus*) exposed to lead acetate ($p<0.05$). Administration of ethanol extract of galangal after exposure to lead acetate significantly reduced type I pneumocyte cell necrosis and type II pneumocyte cell proliferation ($p<0.05$). The highest dose of galangal ethanol extract, 800 mg/kg BW, showed a significant decrease in type II pneumocyte cell proliferation ($p<0.05$).

Conclusion: This study concludes that red galangal extract has a preventive effect in reducing the damage to type I and type II pneumocytes in the lungs of male mice (*Mus musculus*) exposed to lead acetate.

Keywords: Red galangal extract; Lead acetate; Pneumocyte type I; Pneumocyte type II; Healthcare

1. Introduction

Lead is one of the byproducts of motor vehicle fuel combustion, which is one of the hazardous air pollutants due to its detrimental effects on the health of both animals and humans. Lead can enter the body through contaminated food, skin contact, and inhalation [1]. The concentration of lead residue in animal tissues depends on the route of entry and the duration of exposure to environmental pollutants from air, water, and plants [2]. Inhaled lead particles induce the most significant oxidative stress and pro-inflammatory response in the lungs because the lungs have a highly specific response to lead [3].

The proliferation of type II pneumocyte cells is the most sensitive indicator of alveolitis and also serves as a mechanism for repairing cell damage due to injury [4]. Inhalation of toxic substances such as lead, which can reach the alveoli, can damage most of the alveolar lining, especially type I pneumocyte cells and type II pneumocyte cells located in the interalveolar septum [5]. Type I pneumocytes, responsible for gas exchange, are also highly sensitive to injury from free

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radicals compared to type II pneumocytes. However, when type I pneumocytes are injured, type II cells differentiate into type I pneumocytes. Therefore, type II pneumocytes are also called progenitor cells that work by proliferating or multiplying to maintain damage [6].

Excessive proliferation can cause thickening of the interalveolar septum, leading to impaired lung function. To alleviate damage to pneumocyte cells, external antioxidants are needed to capture and neutralize free radicals. Red galangal (*Alpinia Galanga*) is known to contain antioxidants in the form of flavonoids, one of which is quercetin, which is the best flavonoid because it can reduce damage by scavenging free radicals and transition ions, thereby helping to reduce and prevent atherosclerosis, cancer, and chronic inflammation [7].

The flavonoid content in red galangal (*Alpinia Galanga*) can prevent the formation of reactive oxygen species (ROS) by oxidizing flavonoids with radicals and producing more stable and non-reactive radicals. Flavonoids stabilize ROS by reacting with reactive radical compounds, working to reduce free radical production due to the phenol group content as secondary metabolites. The more phenol groups, the more they can capture and stop the formation of ROS [7]. Based on the above description, researchers are interested in observing the effect of red galangal administration on the histopathological appearance of type I and type II pneumocyte cells in the lungs of male mice exposed to lead acetate.

2. Materials and Methods

The research conducted was a pure laboratory experimental study (true experimental) with a research design using a complete random approach/randomized posttest only control group design. The research was carried out at the Animal House of the Faculty of Veterinary Medicine, Universitas Airlangga. The preparation of ethanol extract of Galangal (*Alpinia Galanga*) was done at the Division of Basic Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga. Examination and preparation of histopathology were carried out at the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga.

2.1. Materials

The experimental unit in this study used 25 healthy white male mice (Mus musculus), Balb/C strain, with an average weight of 25-30 grams and aged 2.5-3 months. The equipment used included mouse cages, feeding and drinking containers, digital scales, syringes, sonde, gloves. Equipment for lung histopathology collection included surgical scissors, surgical knives, forceps, organ containers, microscope, glass slides, cover slips, water bath, staining jar. Equipment used for galangal extraction included knives, knife holders, blenders, containers, filter paper, cloth, rotary evaporator.

The materials used in this study were mouse feed, ethanol (Merck, Indonesia) (Catalogue Number: 1060092500), lead acetate suspension pro analysis (Merck, Indonesia) (Catalogue Number: 1073750250), 10% Neutral Buffered Formalin, 70% alcohol, xylene, liquid paraffin, Haematoxylin Eosin (HE) dye.

2.2. Methods

2.2.1. Animals

The mice were randomized into five groups, each group containing six mice. The mice were acclimatized for seven days. The cages were lined with sawdust and maintained at suitable temperature, humidity, and light conditions. Food was provided twice a day ad libitum, and water bottles were filled daily. The cages, feeding containers, and drinking containers were cleaned twice a week. The weight and health of the mice were monitored daily.

2.2.2. Preparation of Lead Acetate

Lead acetate powder was dissolved in distilled water and administered orally to the mice at a dose of 20 mg/kg BW along with ethanol extract of Galangal (*Alpinia Galanga*).

2.2.3. Preparation of Ethanol Extract of Galangal

One kilogram of Galangal rhizomes was washed thoroughly with running water and then thinly sliced. Drying was done directly under sunlight with a black cloth cover for three days. After the drying process, the rhizomes were ground using a blender and sieved using a 25-mesh sieve. Fine powder of Galangal rhizomes (500 grams) was extracted using 1000 ml of pro analysis ethanol solution for the maceration process. The ethanol used was 75% concentration. Maceration was carried out for 2x24 hours at room temperature with stirring every 6 hours. Filtration with filter cloth was done at

the end of the maceration process. The filtrate was then evaporated using a Buchi evaporator to evaporate the ethanol solvent, resulting in a concentrated extract. The ethanol extract of Galangal rhizomes was stored at 0-5°C.

2.2.4. Treatment

This study consisted of five groups, namely: Group (K-): mice only given CMC-Na, starting on day 1 for 24 days. Group (K+): mice given lead acetate dissolved in aquadest at a dose of 20 mg/kg BW. Group (P1): mice given ethanol extract of Galangal at a dose of 200 mg/kg BW and lead acetate at a dose of 20 mg/kg BW. Group (P2): mice given ethanol extract of Galangal at a dose of 400 mg/kg BW and lead acetate at a dose of 20 mg/kg BW. Group (P3): mice given ethanol extract of Galangal at a dose of 800 mg/kg BW and lead acetate at a dose of 20 mg/kg BW. All treatment groups with lead and ethanol extract of Galangal were given from day 4 to day 24 at a rate of 0.2 ml/head orally.

2.2.5. Histopathology Preparation

After 24 days of treatment, on day 25, all mice (*Mus musculus*) were sacrificed by cervical dislocation. The abdominal part of the mice was dissected using surgical equipment to retrieve the lungs. The retrieved lungs were stored in containers filled with 10% formalin solution. The formalin-fixed lungs were embedded in paraffin, stained with Hematoxylin Eosin (HE), and examined under a microscope at 400x magnification.

2.2.6. Data Collection

Assessment of histopathological variables of male mouse lungs (*Mus musculus*) was done by scoring method. Histopathological assessment was performed on five different fields for each variable in one preparation. The assessed variables were necrosis of type I pneumocyte cells and proliferation of type II pneumocyte cells in the alveolar septa. Scoring was done using a Nikon Ei microscope with Optilab Plus camera at 400x magnification and Optilab Viewer image processing software.

2.2.7. Data Analysis

Histopathological data analysis was performed using the Kruskal-Wallis test followed by the Mann-Whitney U test to determine the comparison of differences among treatment groups. The scoring scale for necrosis of type I pneumocyte cells and proliferation of type II pneumocyte cells referred to the scoring system according to Hansel and Barnes [11] in Table 1.

Table 1 Assessment of histopathological images of mouse lungs (Hansel and Barnes [11])

Lesion Form	Scoring	Information
Type I Pneumocyte Cell Necrosis	0	No necrosis
	1	Low < 25% field of view
	2	Medium 25-50% field of view
	3	High 50-75% field of view
Type II Pneumocyte Cell Proliferation	0	No proliferation
	1	Low < 25% field of view
	2	Medium 25-50% field of view
	3	High 50-75% field of view

3. Results

The scoring results obtained were then averaged and analyzed using the SPSS for Windows 23 program. The results were tested using the Kruskal-Wallis statistical test followed by the Mann-Whitney U test with a significance level of 0.05. In the Kruskal-Wallis statistical test, a significant difference was found ($p<0.05$), the test was continued with the Mann-Whitney U test.

Table 2 Scores of Type II Pneumocyte Cell Proliferation and Type I Pneumocyte Cell Necrosis of Male Mice (*Mus Musculus*) Given Galangal Ethanol Extract and Lead Acetate

Group (Treatment)	Type I Pneumocyte Cell Necrosis (Mean Rank ± SD)	Type II Pneumocyte Cell Proliferation (Mean Rank ± SD)
K-	$0.80^{a} \pm 0.41$	$0.38^{a} \pm 0.16$
K+	$2.78^{b} \pm 0.79$	$2.28^{e} \pm 0.42$
P1	$1.24^{a} \pm 0.54$	$1.68^{cd} \pm 0.19$
P2	$1.16^{a} \pm 0.21$	$1.38^{bc} \pm 0.27$
P3	$1.14^{a} \pm 0.33$	$1.06^{b} \pm 0.20$

Superscript (^{abc}) that differ in the same column indicate significant differences ($p<0.05$). Group (K-): CMC-Na, (K+): lead acetate dose of 20 mg/kg BW, (P1): ethanol extract of Galangal dose of 200 mg/kg BW and lead acetate dose of 20 mg/kg BW, (P2): ethanol extract of Galangal dose of 400 mg/kg BW and lead acetate dose of 20 mg/kg BW, and (P3): ethanol extract of Galangal dose of 800 mg/kg BW and lead acetate dose of 20 mg/kg BW.

3.1. Necrosis of Type I Pneumocyte Cell

The Mann-Whitney U statistical test for necrosis of type I pneumocyte cells in the lungs of male mice (*Mus musculus*) showed a significant increase in the group exposed to lead acetate ($p<0.05$). Administration of ethanol extract of Galangal (*Alpinia Galanga*) after exposure to lead acetate can significantly reduce necrosis of type I pneumocyte cells ($p<0.05$). There was no significant difference in the necrosis of type I pneumocyte cells in the groups with varying doses (groups P1, P2, P3) of ethanol extract of Galangal (*Alpinia Galanga*) administration (Table 1).

3.2. Proliferation of Type II Pneumocyte Cells

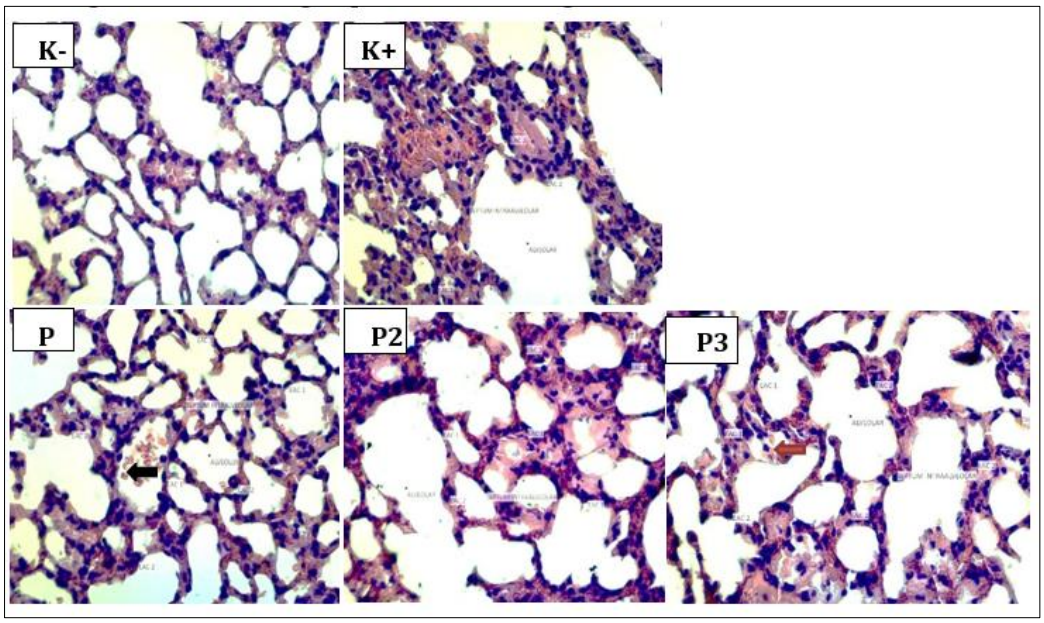

Figure 1 Necrosis of type I pneumocyte cells and proliferation of type II pneumocyte cells in the lungs of mice (*Mus musculus*) at 400x magnification with HE staining. Black arrows indicate proliferation of pneumocyte cells, while red arrows indicate necrosis. Group (K-): CMC-Na, (K+): lead acetate dose of 20 mg/kg BW, (P1): ethanol extract of Galangal dose of 200 mg/kg BW and lead acetate dose of 20 mg/kg BW, (P2): ethanol extract of Galangal dose of 400 mg/kg BW and lead acetate dose of 20 mg/kg BW, and (P3): ethanol extract of Galangal dose of 800 mg/kg BW and lead acetate dose of 20 mg/kg BW

The Mann-Whitney U statistical test for the score of proliferation of type II pneumocyte cells in the lungs of male mice (*Mus musculus*) showed a significant increase in the group exposed to lead acetate ($p<0.05$). Administration of ethanol extract of Galangal (*Alpinia Galanga*) after exposure to lead acetate can significantly reduce proliferation of type II pneumocyte cells ($p<0.05$). A significant decrease in proliferation of type II pneumocyte cells was found in the group

with the highest dose of ethanol extract of Galangal, namely 800 mg/kg BW (P3) (Table 1). Group P3 had the lowest mean among the treatment groups, indicating that the dose of group P3 was the most effective in providing preventive therapy. The pattern of changes in necrosis of type I pneumocyte cells and proliferation of type II pneumocyte cells in the lungs of mice in each group can be seen in Figure 1.

4. Discussion

Repeated exposure to inhaled lead is one of the causes of oxidative stress due to an imbalance between free radicals and endogenous antioxidants, which can trigger an increase in ROS [12]. This leads to injury to cells in the lungs, especially type I pneumocytes responsible for gas exchange during breathing and type II pneumocytes, which are progenitor cells for type I pneumocytes.

Lead entering the body binds to compounds in the body, such as Glutathione peroxidase (GSH-Px). Under normal conditions, GSH-Px acts as a cofactor in antioxidant defense against ROS and lipid hydroperoxide compounds, thereby neutralizing free radicals and reducing oxidative stress [13]. Lead can initiate the formation of complexes with GSH-Px, thus interfering with GSH-Px and resulting in increased oxidative stress [14]. This causes damage to lung cells, especially type I and type II pneumocytes.

At the molecular level, lead is involved in tissue injury mechanisms, including increased permeability and endothelial damage. To counteract this damage, type II pneumocytes proliferate and differentiate to replace damaged type I pneumocytes. In the lungs themselves, the effects of damage include pathological changes in lung parenchyma characterized by inflammation, surfactant production disorders, decreased pulmonary macrophage phagocytosis activity, and the appearance of fibrotic connective tissue [15]. Inflammation is the tissue or cell response when foreign substances induce an inflammatory response, which can result in cell death or necrosis, degeneration, proliferation, and thickening of the alveolar walls, thereby disrupting lung function [15].

Based on the histopathological observations of lung tissue, it was found that the group given ethanol extract of Galangal at a dose of 800 mg/kg BW, along with lead acetate, could maintain cells from damage compared to the positive control group. The damage to type I pneumocyte cells in the lungs, which were only given aquadest and lead acetate at 20 mg/kg BW, was greater than in the other treatment groups. Group (P1) with a dose of ethanol extract of Galangal at 200 mg/kg BW and lead acetate showed slightly less cell damage compared to the (K+) group. Group (P2) with a dose of ethanol extract of Galangal at 400 mg/kg BW and lead acetate also experienced reduced cell damage and almost matched group (P3). Group (P3) with a dose of ethanol extract of Galangal at 800 mg/kg BW and lead acetate showed a decrease in damage almost approaching that of group (K-).

Group (K+) experienced higher cell necrosis because lead acetate in alveolar epithelium can induce inflammatory mediators such as IL-1, IL-8, TNF-α, and secretion of other mediators such as prostaglandins and leukotrienes, which ultimately cause cell damage [16]. TNF-α release can trigger Polymorphonuclear (PMN) release, which can produce free radicals such as nitric oxide [17]. The inflammatory response due to TNF-α release can cause degeneration and necrosis of type I pneumocyte cells because type I pneumocytes are highly vulnerable to toxic substances such as lead acetate that have reached the alveoli [18]. Type I pneumocytes span a very large area of lung parenchyma and are responsible for gas exchange, but they do not have the ability to replicate. Therefore, in type I pneumocytes, groups (K+) and (P1) showed a high level of necrosis, while group (P2) showed decreased cell damage. Groups (P3) and (K-) showed minimal necrosis because the damage was minimal.

Red Galangal contains high levels of flavonoids, including quercetin, which is one of the best flavonoids found in Red Galangal. Quercetin is one of the flavonol compounds, a derivative of flavonoids that have a 3-hydroxyflavone framework. The presence of hydroxyl groups (-OH) in the quercetin compound structure gives quercetin many bioactivities, including antioxidant properties [19]. The mechanism of radical scavenging reactions can occur by donating hydrogen atoms present in hydroxyl groups, so compounds with hydroxyl groups have bioactivities as antioxidants, thus able to capture and prevent ROS production [7].

Based on the observation results of type II pneumocyte cell proliferation given treatment with lead acetate at a dose of 20 mg/kg BW, increased type II pneumocyte cell proliferation was found on the alveolar surface due to lead acetate exposure without the administration of Red Galangal ethanol extract, which is a factor in cell proliferation. The alveolar surface of mice given Red Galangal extract at doses of 200 mg/kg BW/day, 400 mg/kg BW/day, 800 mg/kg BW/day orally could reduce cell proliferation compared to the positive control group, which was only given lead acetate at a dose of 20 mg/kg BW. The proliferation of type II pneumocyte cells can be reduced in these treatments because

inflammation activity decreases, so cells do not need to proliferate to repair damage because continued proliferation will cause thickening and hyperplasia.

The administration of Red Galangal extract doses of 200 mg/kg BW/day, 400 mg/kg BW/day, 800 mg/kg BW/day can significantly reduce type I pneumocyte cell proliferation compared to the (K+) group. Group (P1) at a dose of 200 mg/kg BW/day did not significantly differ from P2 at a dose of 400 mg/kg BW/day but significantly differed from the negative control group (K-), indicating that groups (P1) and (P2) were less effective in reducing type II pneumocyte cell proliferation, proving that the administration of Red Galangal extract at doses of 200 mg/kg BW/day and 400 mg/kg BW/day was less effective in providing preventive effects. Groups (P2) and (P3) with doses of 400 mg/kg BW/day and 800 mg/kg BW/day did not significantly differ from group (K-), which means that the preventive effects on (P2) and (P3) successfully reduced type II pneumocyte cell proliferation and approached normal preparations. This indicates that the administration of Red Galangal extract at a dose of 400 mg/kg BW/day alone can match group (K-) without lead acetate and a dose of 800 mg/kg BW/day also provides the most maximal preventive effect.

Flavonoid compounds function as protease inhibitors. Lung tissue degradation occurs when lead acetate enters the body, triggering an inflammatory process that causes an imbalance between antiproteases and proteases present in alveolar tissue. With alveolar lumen dilation, lung tissue will undergo repair because these flavonoids will inhibit protease activity. As antioxidants in reducing the degree of lung damage, flavonoids can reduce the release of inflammatory cells such as alveolar macrophages and neutrophils, thereby reducing the proliferation of type II pneumocyte cells [20]. In addition, flavonoids can inhibit proinflammatory cytokines such as TNF-α, IL-6, and IL-1β [21].

5. Conclusion

The administration of Red Galangal ethanol extract (*Alpinia Galanga*) can reduce necrosis of type I pneumocyte cells and proliferation of type II pneumocyte cells in male mice (*Mus musculus*) from damage due to exposure to lead acetate. The administration of Red Galangal ethanol extract (*Alpinia Galanga*) can reduce the proliferation of type II pneumocyte cells in male mice (*Mus musculus*) from damage due to exposure to lead acetate.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by Faculty of Veterinary Medicine Animal Ethics Committee. The testes samples were collected from the animals after considerations in accordance to Faculty of Veterinary Medicine Animal Ethics Committee related to animal handling were observed to ensure no discomfort or pain to animal during sampling (No: 1.KE.118.10.2021)

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