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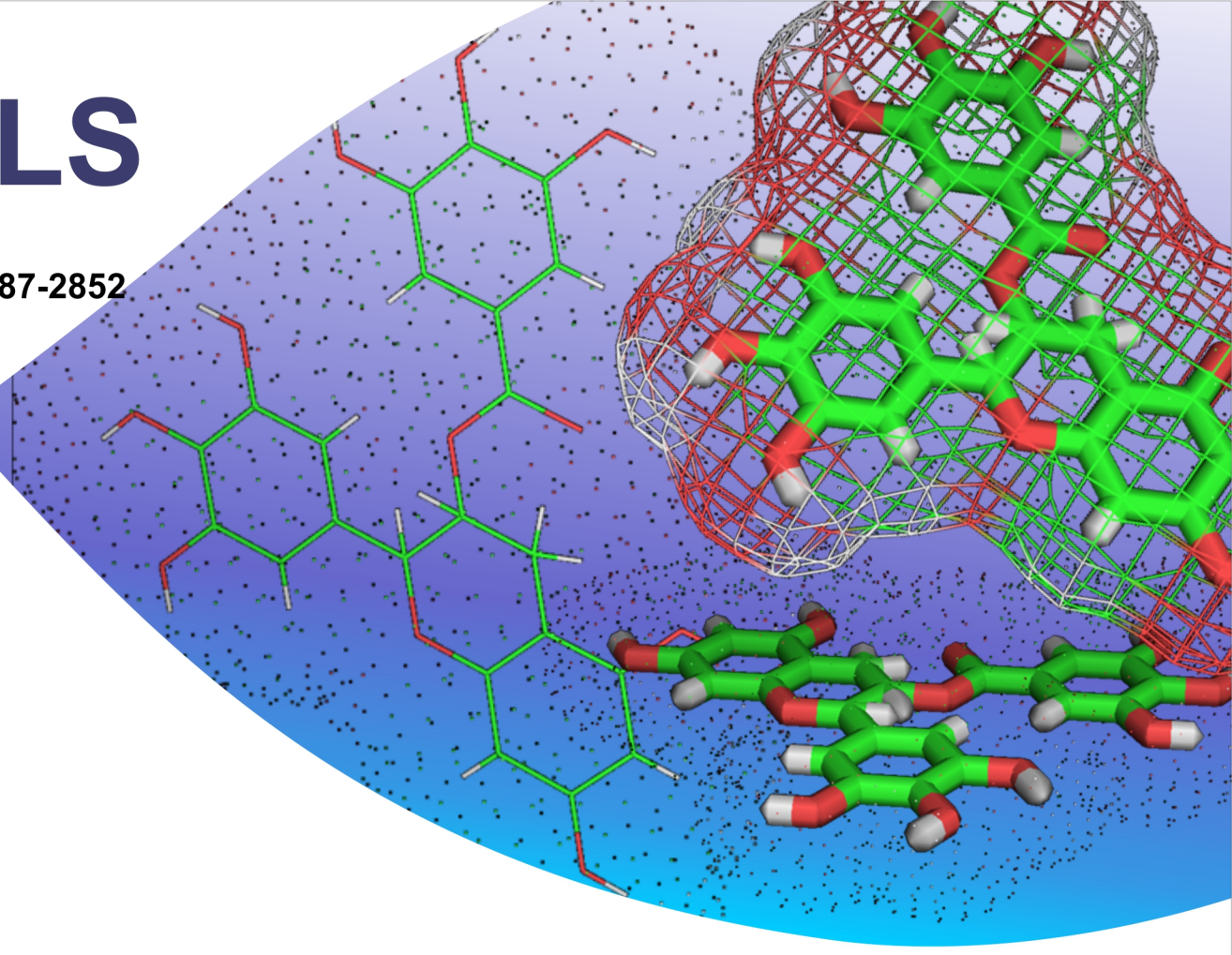

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Elephantopus scaber L. Ethanolic Leaves Extract Modulates IL-2 Production and T-Lymphocyte Activation in Pulmonary Fibrosis Mice Model

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Abstract

Pulmonary fibrosis is a chronic disease characterized by progressive connective tissue deposition that replaces healthy lung tissue. This study aimed to investigate the effect of *Elephantopus scaber* L. Ethanol Extract (ESEE) treatment on the relative number of IL-2 cytokine and lymphocyte activation in bleomycin (BLM)-induced pulmonary fibrosis mice model. Fifty-six male BALB/c mice were divided into seven treatment groups: N (normal); V or vehicle (corn oil); PF or Pulmonary Fibrosis (BLM 2 mg.kg^{-1}); Dex (Dexamethasone 3 mg.kg^{-1} + BLM); D1-D3 (ESEE at doses of 0.0504, 0.1008, and 0.2016 mg.kg^{-1} BW + BLM). ESEE, dexamethasone, and corn oil were administered orally, followed by intraperitoneal bleomycin injection daily for 14 days. Mice were dissected on days 7 and 14, and spleens were isolated to analyze cell populations expressing CD4$^+$IL-2$^+$, CD8$^+$IL-2$^+$, CD4$^+$CD62L$^+$, and CD8$^+$CD62L$^+$. The results showed that bleomycin injection could increase the relative number of IL-2 and decrease the relative number of naive T cells compared to normal mice. ESEE treatment significantly reduced the relative number of IL-2, thus decreasing naive T cell activation after one week of bleomycin injection compared to the mice model. In contrast, the increased IL-2 production led to the increasing naive T cell activation after two weeks of bleomycin injection. Therefore, ESEE treatment has the potential to maintain homeostasis through modulation of IL-2 production and T-lymphocyte activation in the pulmonary fibrosis mice model.

Keywords: *Elephantopus scaber*, IL-2, lymphocytes, mice, pulmonary fibrosis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease characterized by the formation of tissue wounds in the lungs and progressive dyspnea, which can lead to death. The mechanism is progressive connective tissue deposition in the lung interstitials and wound formation, replacing healthy lung tissue [1]. There are an estimated 3.2 million cases of IPF globally and 1.22 million new cases each year, according to the World IPF Joint Association. IPF cases in Indonesia reached 6.26-7.73% of the 1 million population in 2017 and are predicted to continue to increase [2]. The long-term effects of COVID-19 virus infection may increase the prevalence of IPF [3].

CD4 and CD8 T cells play an essential role in pulmonary fibrosis. Pulmonary fibrosis risk factors include environmental exposures, genetic variants, and epigenetic alterations. Epigenetic modifications are mainly caused by cigarette smoking and aging. Aging lungs have significantly fewer naive CD4 and CD8 T cells than memory T cells, so fewer naive T cells can be converted into functional memory T cells. This suggests a decline in the immune system [4]. Both naive T cells and T cells require IL-2 for activation and proliferation [5]. IL-2 increases Treg, inhibits CD4$^+$ T cell accumulation, attenuates CD8$^+$ T cell infiltration, and reduces the expression of proinflammatory cytokines and fibrosis in the lungs of mice [6].

Tapak Liman (*Elephantopus scaber* L.) from the Asteraceae family is widely used as an herbal medicine. Bioactive compounds contained in this plant include flavonoids, steroids, triterpenoids, sesquiterpene lactone, and anthraquinone [7]. *Tapak liman* also contains lupeol, lupeol acetate, stigmasterol, deoxyelephantopin (DET), isodeoxyelephantopin (IDET), epifrielinol, triacontane-1-ol, dotriacontane-1-ol, polyphenol luteolin-7, and other glucoside groups. Tapak liman has been used as an anti-inflammatory, antioxidant, laxative, blood enhancer, fever reducer, and phlegm remover [8]. IDET compounds in *E. scaber* acts as anti-inflammatory agents because they inhibit several cytokines through the downregulation of transcription factors [9].

Bleomycin injection as lung fibrosis modeling in mice for seven days causes excessive inflammatory infiltration of the lungs, then fibroblast activation, extracellular matrix deposition, and fibrosis will occur on day 14 [10].

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Administering the drug together with bleomycin injection is modeling a preventive measure to prevent fibrosis in the lungs. When extracts or drugs are administered together with bleomycin administration, these extracts or drugs are expected to have anti-inflammatory effects against pulmonary fibrosis disease [11]. However, the research about the effect of *E. scaber* L. on the pulmonary fibrosis mice model is still limited. Therefore, this study aimed to investigate the relative number of IL-2 produced by CD4 T cells ($CD4^+IL\text{-}2^+$), IL-2 produced by CD8 T cells ($CD8^+IL\text{-}2^+$), naive CD4 T cells ($CD4^+CD62L^+$), and naive CD8 T cells ($CD8^+CD62L^+$) in bleomycin-induced pulmonary fibrosis mice model.

MATERIAL AND METHOD

Experimental animals

This study used 56 male BALB/c mice aged six weeks (weight 25-30 g) and divided into seven treatment groups (n=4). Mice were obtained from the Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia. The mice had active health conditions, smooth hair, and no other physical defects. Mice were fed with standard food and water ad libitum. All mice were acclimatized for 14 days before the beginning of the study. The experimental procedures were approved by the Research Ethics Committee, University of Brawijaya, Indonesia (Approval no. 182-KEP-UB-2023).

Plant extract preparation

The leaf powder of *E. Scaber* was collected from the UPT Materia Medica, Batu, Indonesia. The plants were identified and confirmed with specimen number (067/656/102.20/2023). Plant extraction was carried out by maceration. The powdered samples from *E. Scaber* leaves were soaked in absolute ethanol solvent at 1:10 (W/V). The ethanol extract was then filtered with Whatman filter paper. Then, the filtered extracts were evaporated with a vacuum pump evaporator at 78°C until it was in the form of a paste. The ethanol extract of *E. scaber* leaves will be dissolved in corn oil (Mazola, ACH Food Companies Inc., US) and given daily for 14 days by oral gavages.

Bleomycin preparation

Bleomycin sulfate (MedChemExpress LLC, US) 10 mg was dissolved in 1 mL phosphate-buffered saline (PBS) and divided into propylene tubes containing 200 mL. Each tube was added 7.8 mL of PBS until the total volume was 8 mL. Injection was performed intraperitoneally daily for two weeks at 2 $mg.kg^{-1}$ BW [12].

Bleomycin injection and E. scaber extract treatment

The mice were randomly divided into seven experimental groups (n=4). The treatment group consisted of control and extract treatment (Table 1). The *E. scaber* leaf extract was dissolved in corn oil solvent based on the variation of each dose from IC_{50} value conversion in vitro: 0.0504 $mg.kg^{-1}$ BW, 0.1008 $mg.kg^{-1}$ BW, and 0.2016 $mg.kg^{-1}$ BW. Corn oil and *E. scaber* extract were administered daily by oral gavages for 7 and 14 days. Dexamethasone, as a control drug, was also dissolved in corn oil at a dose of 3 $mg.kg^{-1}$ [13]. Administration was done by oral gavage with a total volume of 0.3 mL. Bleomycin 2 $mg.kg^{-1}$ was injected intraperitoneally along with extract treatment.

Table 1. Experimental groups (n=4)

Group	BLM (2 $mg.kg^{-1}$)	Corn Oil	Dex (3 $mg.kg^{-1}$ BW)	ES extract ($mg.kg^{-1}$ BW)
N	-	-	-	-
V	-	+	-	-
PF	+	-	-	-
DEX	+	+	+	-
D1	+	+	-	0.0504
D2	+	+	-	0.1008
D3	+	+	-	0.2016

Notes: N = normal, V = vehicle, PF = pulmonary fibrosis, BLM = bleomycin, Dex = dexamethasone, ES = *Elephantopus scaber*, D1 = low dose, D2 = medium dose, D3 = high dose.

Lymphocyte isolation

Mice were dissected on day 7 and day 14. Lymphocytes were isolated from the spleen and washed with PBS 3 times. The organs were then crushed clockwise with the base of a syringe until homogeneous in a petri dish containing 1 mL of PBS. A total of 4 mL PBS was added to the petri dish, and then the homogenate was put into a 15 mL propylene tube. The homogenate was then centrifuged at 2500 rpm for 5 minutes at 10°C. The centrifuged supernatant was discarded, and the pellet was resuspended with 1 mL PBS. The cell suspension was taken as much as 50 µL and put into a 1.5 mL microtube according to the antibody staining label [5].

Flow cytometry analysis

For extracellular antibody staining, 50 µL cell suspension was added with 50 µL FITC anti-mouse CD4 (clone GK 1.5), PE anti-mouse CD8 (clone 53-6.7), and PE anti-mouse CD62L (clone MEL-14) monoclonal antibody solutions that were purchased from BioLegend (San Diego, CA) and incubated for 20 min at 4°C. After that, 400

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µL of PBS was added to the solution, transferred into a cuvette, and analyzed using flow cytometry.

For intracellular staining, the cell suspension was added with 50 µL of fixation buffer (eBioscience™, Thermo Fisher Scientific, USA) and incubated again for 20 min at 4°C. Next, the suspension was added 500 µL permeabilization buffer (PB) 1X (eBioscience™, Thermo Fisher Scientific, USA), homogenized and centrifuged at 2500 rpm and 10°C for 5 min. The supernatant was discarded, and the pellet was added with 50 µL of PE/Cy5-conjugated rat anti-mouse IL-2 antibody (clone MQ1-17H12) solution and incubated at 4ºC in the dark for 20 min. The solution was added with 400 µL of PBS, transferred into a cuvette, and flow cytometry analysis was performed [5]. Flow cytometry analysis was conducted using a flow cytometer (BD Biosciences FACSCalibur™, US) and a computer installed with the BD CellQuest Pro™ software.

Statistical analysis

Data were analyzed using Two-Way ANOVA along with the Duncan test after tested for normality and homogeneity of the data. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS version 25.0 for Windows (IBM Inc., US).

RESULT AND DISCUSSION

The Relative Number of IL-2 Produced by $CD4^+$ T Cells

Based on the flow cytometry analysis, there was a significant difference ($p<0.05$) in the relative number of $CD4^+IL\text{-}2^+$ in the Pulmonary Fibrosis (PF) model mice group compared to the normal group (N) after one week and two weeks of bleomycin injection. Meanwhile, there was an increase in the relative number of IL-2 in mice in the vehicle group (V) after two weeks of corn oil administration compared to N, and significantly different. There should be no significant difference between normal and vehicle groups.

Administration of low dose ESEE or D1 (0.0504 $mg.kg^{-1}$ BW), medium dose or D2 (0.1008 $mg.kg^{-1}$ BW), and dexamethasone after one week of bleomycin injection can reduce the relative number of IL-2 and is significantly different compared to fibrosis model mice. Meanwhile, ESEE D3 (0.2016 $mg.kg^{-1}$ BW) is not significantly different from PF. After two weeks of bleomycin injection, only D1 ESEE can increase the relative number of IL-2, which is significantly different from PF. The relative number of IL-2 in the D2 and D3 groups was not significantly different ($p>0.05$) with PF (Fig. 1).

Cytokine IL-2, under normal conditions, acts as a growth factor because it is needed by lymphocytes for proliferation and differentiation into effector cells [14]. When inflammation occurs, the number of activated T cells will increase because they produce excessive IL-2, which will bind to the IL-2R of the T cells themselves so that they increase in large numbers [15]. IL-2 produced by CD4 T cells promotes rapid and strong lung inflammation through NK cells and CD8 T cells [16]. Intraperitoneal injection of bleomycin can increase the relative number of IL-2 produced by CD4 T cells [17].

Corn oil has been widely used in various drug development studies as a vehicle for water-insoluble agents, one of which is water-insoluble ESEE. Administration of corn oil to rats did not affect the immune system. However, administration of corn oil to mice causes activation of the immune response, specifically through the production of digestion-related cytokines/chemokines. It explains the spike in IL-2 in the V group mice [18].

The increase in the number of IL-2 due to ESEE administration is caused by flavonoids and saponins [5]. Both compounds are known to be immunomodulators, so flavonoids and saponins can increase the proliferation of immune cells. Flavonoids can stimulate an increase in IL-2 by regulating the MAPK pathway [5]. ESEE administration is known to reduce the relative number of IL-2 so that the extract can suppress inflammation after the first week of bleomycin injection. Meanwhile, after the second week of bleomycin injection, the relative number of IL-2 increased compared to the pulmonary fibrosis mice model, which can inhibit the early fibrosis stages [6].

Dexamethasone is a glucocorticosteroid class drug used to control inflammation in pulmonary fibrosis. It can inhibit T cell proliferation, induce apoptosis, and inhibit IL-2 production [19]. It explains the decrease in IL-2 in the first week of drug control compared to the lung fibrosis model mice. However, in the second week, dexamethasone increased the number of IL-2, which could be due to the low dose of the drug, which was 3 $mg.kg^{-1}$ and given orally [13]. The low dose (D1) of ESEE can significantly increase the relative number of IL-2 cytokines after the second week of bleomycin injection, thus increasing CD4 T cell proliferation.

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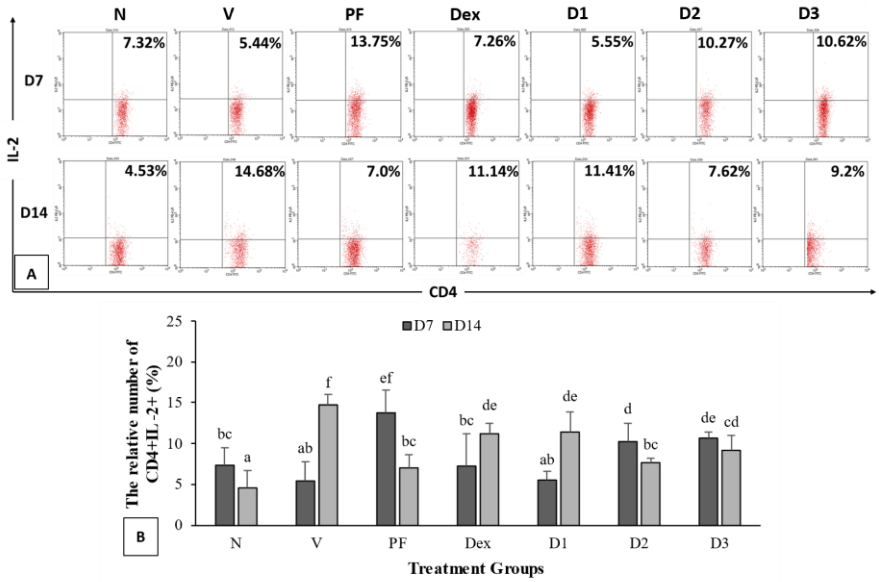

Figure 1. Flow cytometry analysis results of IL-2 produced by $CD4^+$ T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of $CD4^+IL\text{-}2^+$. (B) The bar chart showed the relative number of $CD4^+IL\text{-}2^+$ represented in mean ± SD of four mice in each group. Different notations indicate a significant difference based on the Duncan HSD Test ($p<0.05$). N = normal control; V = corn oil; PF = bleomycin 2 $mg.kg^{-1}$; DEX = bleomycin + dexamethasone 3 $mg.kg^{-1}$; D1 = bleomycin + ES 0.0504 $mg.kg^{-1}$ BW; D2 = bleomycin + ES 0.1008 $mg.kg^{-1}$ BW; D3 = bleomycin + ES 0.2016 $mg.kg^{-1}$ BW.

The Relative Number of IL-2 Produced by $CD8^+$ T Cells

Based on the flow cytometry analysis, there was a significant difference ($p<0.05$) in the relative number of $CD8^+IL\text{-}2^+$ in the Pulmonary Fibrosis (PF) model mice group compared to the normal mice group after one week and two weeks of bleomycin injection. Similar to $CD4^+IL\text{-}2^+$, in vehicle (corn oil) model, there was an increase in the relative number of IL-2 after two weeks of treatment, which an immune response to corn oil administration could cause. The administration of all doses of ESEE after one week of bleomycin injection can reduce the relative number of $CD8^+IL\text{-}2^+$ compared to PF. However, only low doses and dexamethasone were significantly different. After two weeks of bleomycin injection, only the low dose could increase the relative number of IL-2 but not significantly compared to lung fibrosis model mice, while dexamethasone significantly increased the relative number of IL-2 (Fig. 2).

During inflammation, naive CD8 T cells undergo clonal expansion and differentiate into cytotoxic effector T cells (CTLs) due to increasing their numbers from paracrine or autocrine signaling from IL-2. These cells will attack the pathogen directly by causing cytotoxicity [20]. The high relative number of IL-2 cytokines produced by CD8 T cells can induce these cells to perform direct elimination via perforin and granzyme and secretion of proinflammatory cytokines [21]. It indicates that bleomycin induction can increase the relative number of IL-2 cytokines by CD8 T cells, which helps lymphocyte activation and proliferation.

The decrease in the relative number of IL-2 produced by CD8 T cells in the ESEE treatment group may be caused by suppression of the immune system to prevent excessive inflammation after one week of bleomycin injection [10]. Meanwhile, the increase in the relative number of IL-2 in the second week of bleomycin injection may also be due to the activity of ESEE in increasing the proliferation of immune cells. In addition to flavonoids and saponins, ESEE also contains stigmasterol compounds that can differentiate $CD4^+$ T cells into Th1 and Th2 cells, where Th1 cells will produce IL-2 and induce the proliferation of CD8 T cells [5]. The decreased number of $CD8^+IL\text{-}2^+$ in dexamethasone-treated mice (Dex) compared to lung fibrosis model mice (PF) after the first week of bleomycin injection can be caused by the decrease in CD8 T cells due to the immunosuppressant effect of dexamethasone [19]. Low-dose (D1) ESEE can significantly increase the relative number of cytokine IL-2 in the second week, thereby enhancing CD8 T cell proliferation.

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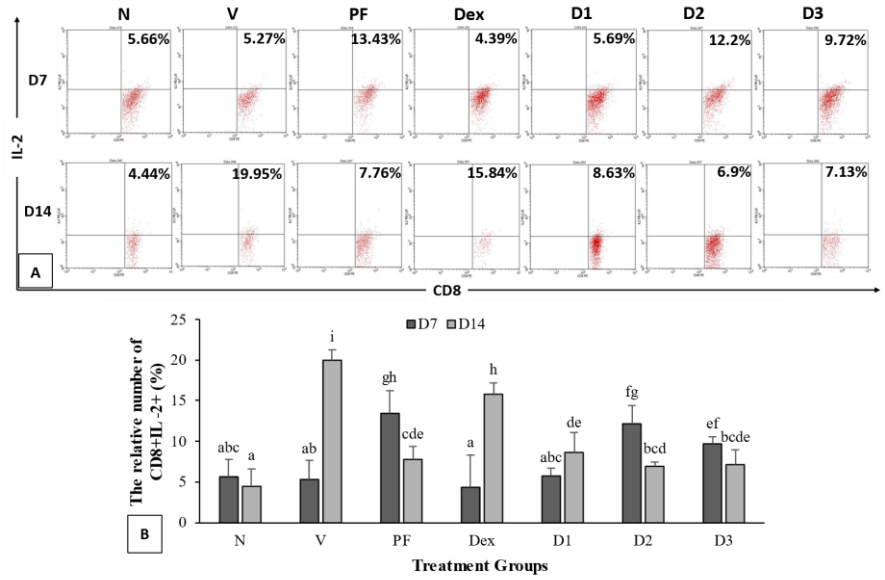

Figure 2. Flow cytometry analysis results of IL-2 produced by $CD8^+$ T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of $CD8^+IL\text{-}2^+$. (B) The bar chart showed the relative number of $CD8^+IL\text{-}2^+$ represented in mean ± SD of four mice in each group. Different notations indicate a significant difference based on the Duncan HSD Test ($p<0.05$). N = normal control; V = corn oil; PF = bleomycin 2 $mg.kg^{-1}$; DEX = bleomycin + dexamethasone 3 $mg.kg^{-1}$; D1 = bleomycin + ES 0.0504 $mg.kg^{-1}$ BW; D2 = bleomycin + ES 0.1008 $mg.kg^{-1}$ BW; D3 = bleomycin + ES 0.2016 $mg.kg^{-1}$ BW.

During the primary immune response, IL-2 enhances the proliferation and effector function of CD4 T cells. IL-2 secreted by CD4 T cells is also required by CD8 T cells for expansion and survival. Effector T cells can differentiate into Th1 and Th2 cells, while they are inhibited from differentiating into Th17 cells by IL-2. The presence of IL-2 during the priming phase of CD4 or CD8 T cell differentiation also helps develop long-lived memory cells [22]. However, IL-2 also suppresses the immune system by inducing regulatory T cells. Therefore, IL-2 is known to have a dual role as both an immunostimulant and an immunosuppressant in response to foreign antigens, thereby maintaining T-cell population homeostasis [23].

The Relative Number of Naive CD4 and Naive CD8 T Cells

Based on the flow cytometry analysis, there was no significant difference ($p>0.05$) in the relative number of naive CD4 T cells ($CD4^+CD62L^+$) and naive CD8 T cells ($CD8^+CD62L^+$) in the lung fibrosis mice model (PF) compared to the normal mice group (N) after one week of bleomycin injection. The profile of activated T cells ($CD4^+CD62L^-$ and $CD8^+CD62L^-$) in normal and vehicle groups was much higher than naive T cells, where the number of naive T cells should be abundant because they were not injected with antigen. After two weeks of injection, there was a decrease in the relative number of naive T cells, but also not significant (Fig. 3 and Fig. 4). The pulmonary fibrosis model (PF) group of mice should have a lower percentage of naive T cells because of the activation of lymphocytes due to exposure to foreign antigens in the form of bleomycin in the body of mice.

Administration of low-dose ESEE (D1) can inhibit the activation of naive CD4 T cells. In contrast, all doses inhibit the activation of naive CD8 T cells after the first week of bleomycin injection, both significantly compared to PF. However, the medium dose (D2) increased CD4 T cell activation while D1 and D2 increased naive CD8 T cell activation, but neither was significantly different from PF. Compared to other treatment groups, Dexamethasone stimulated lymphocyte activation after one week and two weeks of bleomycin injection.

Naive T cells are defined as T cells that have never been exposed to antigens [15]. The CD62L molecule is a marker for naive T cells expressed on the cell surface, with a function for T cells homing to secondary splenic organs and interacting with ligands expressed on high endothelial venules (HEV) [24]. Naive T cells exposed to foreign antigens will be activated and differentiate into effector T cells and memory T

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cells, thus losing the surface molecule CD62L. When individuals experience illness, the relative number of naive CD4 or CD8 T cells will decrease due to cell activation [15,25]. It proves that many CD4 and CD8 T cells are activated in response to bleomycin injection [26]. The high activation of lymphocytes after the first week of bleomycin injection in normal mice (N) can be caused by exposure to bacteria or fungi in mice from the environment in the animal room so that CD4 T cells will be activated and differentiate towards Th2 cells in response to infection [27].

According to Djati *et al.*, low lymphocyte activation after ESEE administration can be caused by the content of lupeol and flavonoids in ESEE, known as anti-inflammatory [8]. As a result, lymphocyte activation will be suppressed so that the relative number of naive T cells is high. Only D2 ESEE can increase CD4 T cell activation after the second week of bleomycin injection, while D1 and D2 ESEE increase CD8 T cell activation insignificantly. This is probably because herbal extracts can stimulate or suppress the immune system. The higher the dose of extract, the more negative feedback, so the relative number of naive T cells was greater. Negative feedback is an immune system mechanism in maintaining homeostasis where IL-2 produced by CD4 T cells will trigger the differentiation of regulatory T cells that express FOXP3. Treg cells will actively inhibit T cell activation and proliferation [28].

The low relative number of IL-2 in the previous analysis also led to low lymphocyte activation. In addition, the high relative number of naive CD4 T cells can also be caused by increased proliferation and differentiation of hematopoietic cells that will become lymphocytes [29]. The high activation of lymphocytes in the group of dexamethasone-treated mice (Dex) is due to the high relative number of IL-2 in the previous analysis, thus stimulating the differentiation of naive CD4 T cells into regulatory T cells that will suppress the immune system [30].

Administration of ethanol extract of *Elephantopus scaber* L. leaves after the first week of bleomycin injection can reduce IL-2 production by CD4 and CD8 T cells while increasing IL-2 production after the second week of bleomycin injection. Although not significant, it is in line with the inhibition of lymphocyte activation in the first week and an increase in lymphocyte activation after the second week of treatment. The results of this study indicate that ESEE has potential as an immunomodulator in lung fibrosis models through the regulation of immune system homeostasis.

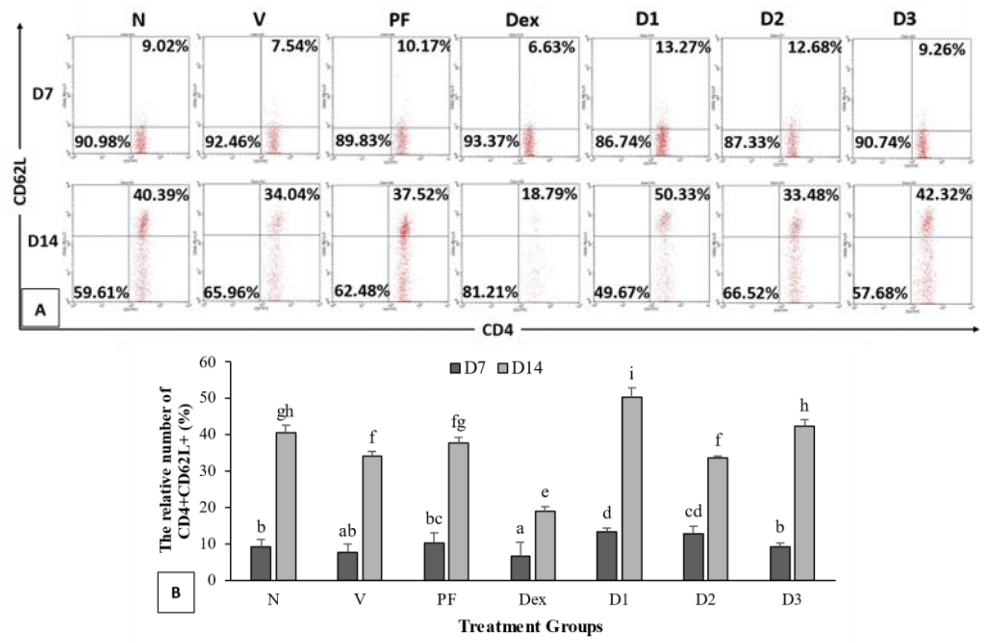

Figure 3. Flow cytometry analysis results of CD4¬ naive T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of CD4+CD62L+. (B) The bar chart showed the relative number of $CD4^{+}CD62L^{+}$ represented in mean ± SD of four mice in each group. Different notations indicate a significant difference based on the Duncan HSD Test ($p<0.05$). N = normal control; V = corn oil; PF = bleomycin 2 $mg.kg^{-1}$; DEX = bleomycin + dexamethasone 3 $mg.kg^{-1}$; D1 = bleomycin + ES 0.0504 $mg.kg^{-1}$ BW; D2 = bleomycin + ES 0.1008 $mg.kg^{-1}$ BW; D3 = bleomycin + ES 0.2016 $mg.kg^{-1}$ BW.

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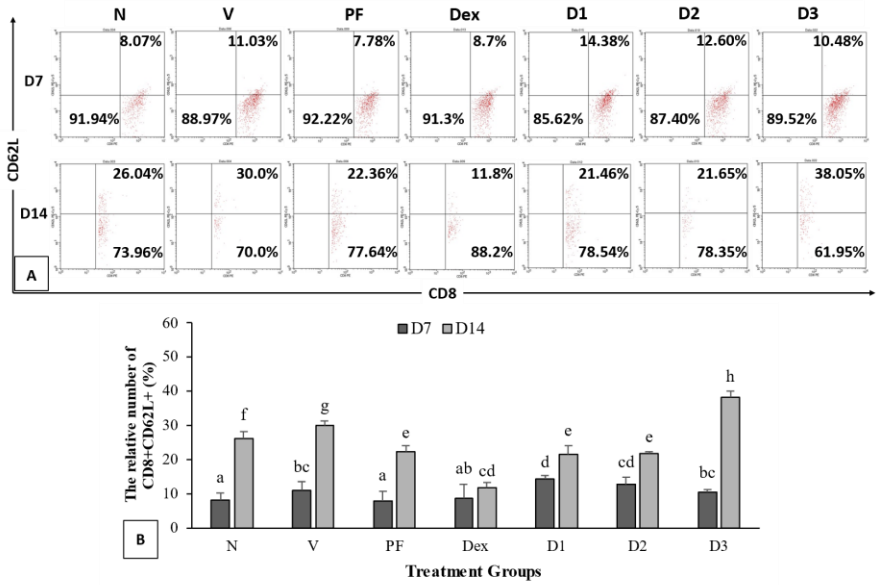

Figure 4. Flow cytometry analysis results of CD48 naive T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and E. scaber ethanol extract treatment. (A) Dot plot diagrams showed the relative number of CD8+CD62L+. (B) The bar chart showed the relative number of CD8+CD62L+ represented in mean ± SD of 4 mice in each group. Different notations indicate a significant difference based on the Duncan HSD Test ($p<0.05$). N = normal control; V = corn oil; PF = bleomycin 2 $mg.kg^{-1}$; DEX = bleomycin + dexamethasone 3 $mg.kg^{-1}$; D1 = bleomycin + ES 0.0504 $mg.kg^{-1}$ BW; D2 = bleomycin + ES 0.1008 $mg.kg^{-1}$ BW; D3 = bleomycin + ES 0.2016 $mg.kg^{-1}$ BW.

CONCLUSION

Administration of *Elephantopus scaber* L. leaves ethanol extract at a dose of 0.0504 $mg.kg^{-1}$ BW decreased IL-2 production, thereby decreasing naive T cell activation after one week of bleomycin injection. At the same time, the same dose also increased IL-2 production, thereby increasing naive T cell activation after two weeks of bleomycin injection. These results suggest that ESEE treatment could modulate immune system homeostasis in the pulmonary fibrosis mice model. Studies related to the bioactive compounds and mechanisms of ESEE that play a role in pulmonary fibrosis therapy can be carried out in the future to determine their effectiveness in treating pulmonary fibrosis.

Acknowledgement

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Tapak Liman (*Elephantopus scaber* L.) Leaves Ethanol Extract Improves the Production of IL-6 and IL-17 Cytokines in Mice with Bleomycin-induced Pulmonary Fibrosis

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Abstract

IL-6 and IL-17 are pro-inflammatory and pro-fibrotic cytokines that increase pulmonary fibrosis due to lung alveolar epithelial cell damage. Tapak liman leaves (*Elephantopus scaber* L.) have anti-inflammatory and anti-asthmatic properties. This study aimed to investigate the effects of the *Elephantopus scaber* L. ethanol extract (ESEE) on IL-6 and IL-17 produced by $CD4^+$ and $CD8^+$ in the bleomycin-induced pulmonary fibrosis mice model. Fifty-six male BALB/c mice will be divided into seven groups consisting of healthy mice (N), vehicle mice (V), pulmonary fibrosis (PF), Dexamethasone (DEX) as a drug control, and three doses of ESEE (0.0504, 0.1008, and 0.2016 $mg.kg^{-1}$ BW). ESEE will be administered orally, followed by intraperitoneal bleomycin injection for 14 days. Mice are then dissected on days 7 and 14, and the spleen will be isolated for analysis of the expression of IL-6 and IL-17. The results showed that ESEE effectively reduced levels of IL-6 and IL-17 cytokines produced by $CD4^+$ and $CD8^+$ T cells, and doses three of ESEE (0.2016 $mg.kg^{-1}$ BW) (0.2016 $mg.kg^{-1}$ BW) showed the most effective reduction activity than the Dexamethasone group. The treatment was proven to reduce the expression of IL-6 and IL-17 in mice with a model of pulmonary fibrosis.

Keywords: bleomycin, *Elephantopus scaber* L., IL-6, IL-17, pulmonary fibrosis.

INTRODUCTION

Fibrosis is a condition or process characterized by excessive accumulation of fibrous connective tissue [1]. Pulmonary fibrosis is one of the diseases that attacks the lungs with marked scar tissue formation without clear cause [2]. Pulmonary fibrosis first occurs due to damage to the base or edges of the lung alveolar epithelium, which will continuously cause migration, proliferation, and differentiation of fibroblasts into active myofibroblasts and causes the secretion of extracellular matrix (ECM) uncontrollably and in large quantities [3].

The number of pulmonary fibrosis incidents is estimated to range between 2-30 cases per 100,000 people, with a case prevalence ranging from 10-60 cases per 100,000 people [4,5,6]. In contrast, pulmonary fibrosis in Indonesia is known to be part of acute respiratory distress syndrome (ARDS). The latest data indicates that 20% of patients with ARDS cases can develop progressive pulmonary fibrosis [7].

Another characteristic of pulmonary fibrosis that is often found is inflammation, which is characterized by high expression of several pro-fibrotic cytokines and mediators such as interleukin-6 (IL-6), Transforming Growth Factor-beta (TGF-β), and interleukin-17 (IL-17), which play an essential role in the process of wound repair, migration, and fibroblast differentiation [8,9]. Over-expression of IL-6 and IL-17 in the lung epithelium correlates with an increased risk of pulmonary fibrosis [1]. The lack of clarity regarding the leading cause of this disease means that developing appropriate drugs to treat this disease is still an unresolved challenge. So far, the conventional therapy most often used to treat pulmonary fibrosis is by administering anti-fibrotic drugs such as pirfenidone, which has many side effects such as nausea, vomiting, dizziness, indigestion, anorexia, and photosensitivity [10]. Then, previous research explained that administration of dexamethasone was able to reduce lung fibrosis in mice induced by bleomycin via the Smad3, Transforming Growth Factor-beta (TGF-β), and JAK-STAT pathway [11].

Tapak liman (*E. scaber* L.) is a wild grass plant commonly found in Central America, South America, Europe, Asia, Australia, and Africa [12]. Previous research shows that ethanol extract from Tapak Liman leaves can stimulate wound healing activity in mice characterized by reduced chronic inflammatory cells, reduced swelling, and increased collagenation [13]. However, there has

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been no research so far explaining the potential anti-fibrosis effect of the *E. scaber* leaves. Therefore, this study aimed to explore and provide additional information regarding the anti-fibrotic potential of the ethanol extract of tapak liman leaves as a therapeutic agent in pulmonary fibrosis.

MATERIAL AND METHOD

Experimental Research Design

This research has received approval from the Brawijaya University Research Ethics Commission (No.182-KEP-UB-2023). The research used an experimental design with a completely randomized design (CRD) (56 mice with two-time series). Mice were acclimatized for 14 days and then randomly divided into seven groups (n = 4) (Table 1).

Table 1. Research group design

Treatment group	Type of treatment	
	Oral Administration for 7 days and 14 days	**Intraperitoneal Administration Bleomycin (mg.kg^{-1} BW)**
Healthy mice (N)	-	-
Vehicle mice (V)	Corn oil (0.3 mL)	-
Pulmonary fibrosis mice model (PF)	-	
Drug control (DEX)	Dexamethasone (3 mg.kg^{-1} BW)	2
D1	Ethanol extract of *E. scaber* leaves (0.0504 mg.kg^{-1} BW)	2
D2	Ethanol extract of *E. scaber* leaves (0.1008 mg.kg^{-1} BW)	2
D3	Ethanol extract of *E. scaber* leaves (0.2016 mg.kg^{-1} BW)	2

Plant Material and Extraction

The leaves of the *E. scaber* were purchased and identified by UPT. Laboratorium Herbal Materia Medica Batu (specimen number 067/656/102.20/2023). The leaves of the plant were dried and ground to a fine powder. A total of 100 grams of powdered samples were then macerated with 1.000 mL of 96% ethanol (1:10, w/v) for 24 h at room temperature while stirring several times. The sample was filtered using Whatman No.1 filter paper and then concentrated using a vacuum pump evaporator at 70°C until it formed a paste. The obtained extract was stored at 3°C in the refrigerator.

Experimental Animals

The current study used male strain BALB/c mice (*Mus musculus*) aged 6-7 weeks, btained from the Faculty of Pharmacy, Airlangga University, Surabaya. The inclusion criteria for mice were body weight (BW) between 25-30 g, health, and no physical defects.

Induction of Pulmonary Fibrosis

Bleomycin (MedChemExpress LLC, US) was induced in PF, DEX, D1, D2, and D3 groups (Table 1). A total of 10 grams of bleomycin was dissolved in 1 mL of Phosphate Buffer Saline (PBS) and separated into five propylene tubes with 200 µL each. Then, 7.8 mL of PBS was added to each propylene tube. Each mice was injected with 2 mg.kg^{-1} BW dissolved bleomycin daily for two weeks [14]. Bleomycin was injected via the intraperitoneal route [15].

Administration of *E. scaber* Leaves Ethanol Extract Dexamethasone

Elephantopus scaber ethanol extract (ESEE) leaves were dissolved in corn oil (Mazola, ACH Food Companies Inc., US) with different doses where the dose is obtained based on the IC50 value (Table 1). Dexamethasone was used as a control drug at a dose of 3 mg.kg^{-1} BW (DEX) where this dose was determined based on previous research as the best dose for using dexamethasone is (3 mg.kg^{-1}) [16] and dissolved in corn oil (Mazola, ACH Food Companies Inc., US). *Elephantopus scaber* and dexamethasone were administered orally to the mice for seven and 14 days [11].

Lymphocyte Isolation

Mice were sacrificed to isolate the spleen. The spleen was washed three times using PBS and then crushed using the syringe base clockwise in a petri dish containing 1 mL of Phosphate Buffer Saline (PBS). 4 mL of Phosphate Buffer Saline (PBS) was added to the petri dish and transferred into a 15 mL propylene tube. The homogenate was then centrifuged at 2.500 rpm for 5 min at 10°C. The supernatant was discarded, and the pellet was resuspended with 1 mL Phosphate Buffer Saline (PBS). Then, 50 µL of the cell suspension was transferred into a 1.5 mL microtube and subjected to antibody staining [17].

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Antibody Staining and Flow cytometry Analysis

Antibody staining was done in extracellular and intracellular staining. For extracellular staining, 50 µL of cell suspension was added with 50 µL of specific extracellular antibody solution (FITC anti-mouse CD4 and PE anti-mouse CD8) (Biolegend, California, USA) and incubated at 4°C for 20 minutes in a dark room in the ice box.

The cell suspension was added with 50 µL intracellular (IC) fixation buffers (eBioscienceTM, Thermo Fisher Scientific, USA) for intracellular staining and incubated at 4°C for 20 min in a dark room on an ice box. The cell suspension was added with 400 µL permeabilization buffer (eBioscienceTM, Thermo Fisher Scientific, USA) (10 times dilution) and homogenized. Samples were centrifuged at 2.500 rpm, 10°C, for 5 minutes. Pellets were added with 50 µL of specific intracellular antibody solution PE/Cy5-conjugated anti-mouse IL-17 (Biolegend, California, USA) and PE/Cy5-conjugated anti-mouse IL-6 (Biolegend, California, USA) and incubated again at 4°C for 20 minutes in the dark on the ice box.

After incubation, 400 µL of PBS was added to the sample and transferred into a flow cytometry (FCM) cuvette to analyze the data using flow cytometry [17]. Data was then analyzed using BD CellQuest Pro™ Software connected to a BD FACS Calibur™.

Data Analysis

The percentage (%) of each parameter was tabulated and then analyzed statistically using the IBM SPSS Statistics program version 26 for Windows by testing the normality and homogeneity of the data. Data was analyzed parametrically using Two Way ANOVA (analysis of variance) with $P < 0.05$ and then continued with the Duncan test for post hoc test.

RESULT AND DISCUSSION

Production of IL-6 by $CD4^+$ ($CD4^+IL\text{-}6^+$)

The use of bleomycin to induce a fibrosis response in mice has been carried out in several previous studies. The induction of bleomycin in mice has been shown to cause increased IL-6 expression in the lungs. However, increased IL-6 expression was not observed in normal or vehicular mice [18]. The relative number of $CD4^+IL\text{-}6^+$ cells in (PF) mice increased significantly after seven days after bleomycin induction compared to healthy mice without any treatment. Results were assessed on days 7 and 14 as a time variation to see differences in results.

Treatment of three groups with Tapak Liman extract (UPT. Materia Medica Batu, East Java, Indonesia) for two weeks significantly reduced the relative number of $CD4^+IL\text{-}6^+$. The group (D1-D3) at 14 days had results that were not significantly different from (PF) with (D3) (0.2016 $mg.kg^{-1}$ BW), which is an effective dose to reduce the rate of inflammation by (6.05%). The most effective dose group of Tapak Liman treatment on day 7 was only (D1), compared to the pulmonary fibrosis (PF) group. The longer the administration of Tapak Liman treatment, the more the relative amount of IL-6 will be reduced in mice model pulmonary fibrosis (Fig. 1).

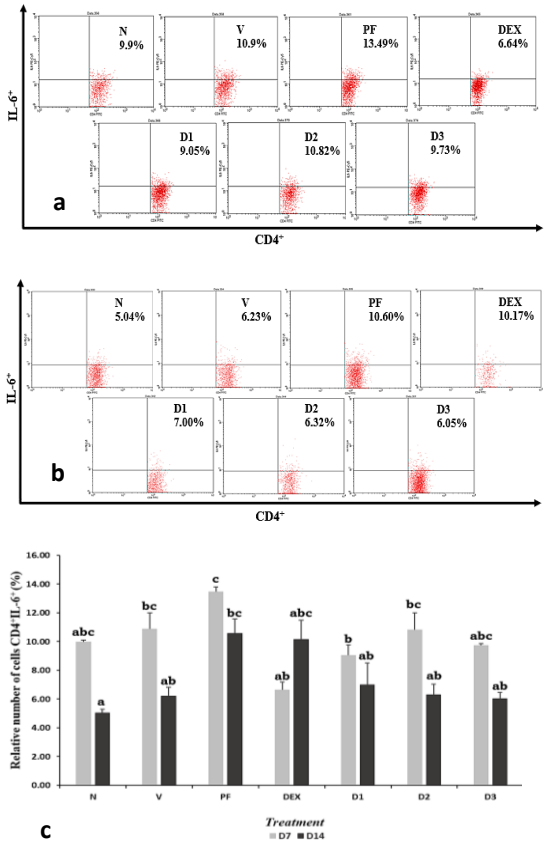

Figure 1. Relative number of $CD4^+IL\text{-}6^+$: (a) Dot plot diagrams showed the percentage of $CD4^+IL\text{-}6^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of CD4+IL-6+ cells for each 14-day treatment group; and (c) The representative bar showed the relative number of $CD4^+IL\text{-}6^+$ cells for each treatment group. *Healthy mice (N); Vehicle mice (V); Pulmonary fibrosis mice model (PF); Drug control (DEX); D1 (ESEE); D2 (ESEE); D3 (ESEE).

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Therapy with *E. scaber* ethanol extract (ESEE) leaves showed decreased $CD4^+IL\text{-}6^+$ expression in all treatment groups (Fig. 1c). This shows that the treatment of *E. scaber* ethanol extract (ESEE) leaves can affect the development process of pulmonary fibrosis. The results were significant for the vehicle and drug groups compared to the control treatment (Fig. 1c). Then, the Dexamethasone group (DEX) did not show to improve the state of fibrosis significantly.

Interleukin-6 (IL-6) is a cytokine that plays a role in many biological processes, such as inflammation and immune responses, which activate the immune response [1]. Apart from these functions, IL-6 is a cytokine that acts as a modulator of the inflammatory process and wound healing process. The wound-healing process involving IL-6 will begin when IL-6 binds to its receptor, namely sIL-6R, on the surface of neutrophils and is released into the wound [1]. This shows that in pulmonary fibrosis conditions, a response mechanism from the immune system causes an increase in the relative amount of IL-6 cytokines secreted by $CD4^+$ T cells.

IL-6 has a pro-inflammatory effect that can contribute to collagen deposition and the development of fibrosis. IL-6 will be produced at the site of inflammation and regulate the acute phase response with its receptor (sIL-6Rα) to determine the transition of acute to chronic inflammation by changing the nature of the leukocyte infiltrate and providing a stimulatory effect on T cells and B cells so that it can support the chronic inflammatory response [19].

Production of IL-6 by $CD8^+$ ($CD8^+IL\text{-}6^+$)

The $CD8^+$ T cells are known to release cytokine IL-6, which helps with cytotoxicity in inflamed areas of the body [17]. The presence of large numbers of $CD8^+$ T cells indicates a strong immune response against foreign pathogens or antigens. The $CD8^+$ cells are a type of cytotoxic T cell that plays a role in recognizing and destroying infected cells. This reflects the body's efforts to fight infection. The immune system responds to the presence of antigens by producing antibodies and activating T cells, such as $CD8^+$ cells, to fight off pathogens or foreign substances [13].

Several studies showed increased $CD8^+$ T cell expression in pulmonary fibrosis patients or mice injected with bleomycin [20]. In the second week, the highest production of the proinflammatory cytokine interleukin-6 (IL-6) secreted by $CD8^+$ T cells in bleomycin-induced mice was found in the pulmonary fibrosis mice model (PF) group. The relative number of $CD8^+IL\text{-}6^+$ in the pulmonary fibrosis mice model (PF) increased significantly ($p < 0.05$) after one week and two weeks post-bleomycin induction compared to healthy mice (Fig. 2).

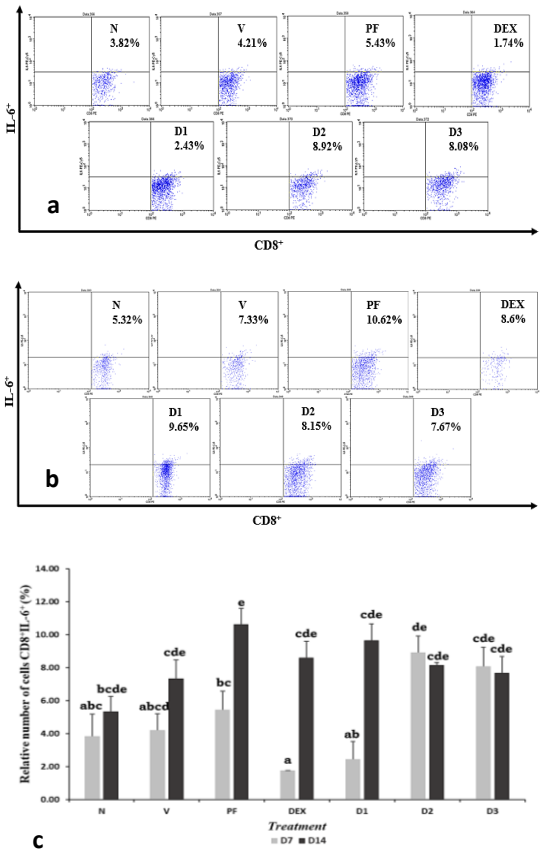

Figure 2. Relative number of $CD8^+IL\text{-}6^+$: (a) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}6^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}6^+$ cells for each 14-day treatment group; and (c) The representative bar showed the relative number of $CD8^+IL\text{-}6^+$ cells for each treatment group. *Healthy mice (N); Vehicle mice (V); Pulmonary fibrosis mice model (PF); Drug control (DEX); D1 (ESEE); D2 (ESEE); D3 (ESEE).

The relative number of $CD8^+IL\text{-}6^+$ after treatment with ESEE for 14 days was able to decrease the production of IL-6 by $CD8^+$ compared to the PF group with no significantly different results, especially at (D3) doses that effectively reduced the relative number of $CD8^+IL\text{-}6^+$. However, there was no significant difference between each dose. The vehicle mice had significant results from healthy treatment and reduced level of inflammation with lower results compared to the (PF) group (Fig. 2).

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Relative number of IL-17 by CD4⁺ (CD4⁺IL-17⁺)

Bleomycin can be used as an inducer of pulmonary fibrosis, and the number of pro-inflammatory cytokine IL-17 expressed by CD4⁺ T cells and cytokine IL-17 is also known to play a significant role in inflammation in pulmonary fibrosis. The (PF) group had higher relative CD4⁺IL-17⁺ cell counts than the healthy groups within one week or two weeks of treatment, with insignificant results. Under healthy conditions, the organism's body systems generally do not respond to foreign antigens, so the amount of cytokine IL-17 does not increase [21]. The production of pro-inflammatory cytokine IL-17 secreted by CD4⁺ T cells in bleomycin-induced mice was highest in the sick group in the second week (Fig. 3).

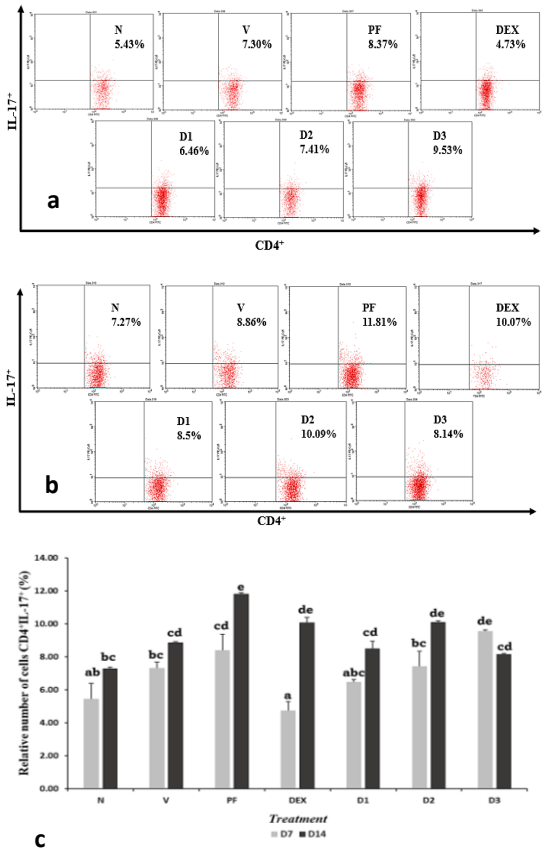

Figure 3. Relative number of CD4⁺IL-17⁺: (a) Dot plot diagrams showed the percentage of CD4⁺IL-17⁺ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of CD4⁺IL-17⁺ cells for each 14-day treatment group; and (c) The representative bar showed the relative number of CD4⁺IL-17⁺ cells for each treatment group. *Healthy mice (N); Vehicle mice (V); Pulmonary fibrosis mice model (PF); Drug control (DEX); D1 (ESEE); D2 (ESEE); D3 (ESEE).

The treatment of *E. Scaber* ethanol extract (ESEE) leaves for two weeks reduced the production of IL-17 by CD4⁺ cells. The D3 group reduced CD4⁺IL-17⁺ more effectively than the other doses. The dexamethasone group (DEX) had a lower relative number of CD4⁺IL-17⁺ cells than the fibrosis group in the second week, which was not significantly different. However, when compared to the *Tapak Liman* dose group, the drug group was known to have significant results with the (D2) group in the second-week post-injection with bleomycin. Vehicle treatment (V) between the first and second weeks did not differ significantly from healthy treatment. So, corn oil did not affect the dosage group of the extract.

Interleukin-17 (IL-17) is a cytokine produced from the differentiation process of CD4⁺ T cells and CD8⁺ T-cells through T-helper 17 (Th17) and Tc17, which are proinflammatory cytokines. In pulmonary fibrosis, this cytokine will trigger inflammation in the form of excessive IL-17 expression (e.g., IL-17A) caused by bleomycin induction, which can increase the number of proinflammatory cytokines and cause the attachment of some inflammatory cells to the alveolar surface and form pulmonary fibrosis [22].

Relative number of IL-17 by CD8⁺ (CD8⁺IL-17⁺)

Induction with bleomycin may lead to increased expression of Interleukin-17 (IL-17). This is because IL-17 is one component that acts as a defense system of the immune system when foreign antigens are present. Bleomycin compounds can also be toxic, which can cause an inflammatory response in certain parts of organs such as the lungs, which can cause pulmonary fibrosis as a side effect of using these drugs [23].

In the one-week treatment, the (PF) group had the highest relative number of CD8+IL-17+ compared to the healthy group. During the two weeks of treatment, (the PF) group showed a significant decrease in the relative number of CD8+IL-17+ and was much lower than the healthy group, with results that did not differ significantly (Fig. 4). It may indicate that immune regulation occurs in these mice with the process of resistance to foreign antigens carried out in the first week after induction with bleomycin.

In addition, CD4 T cells are known to have a more significant or dominant number of cells in the body of an organism experiencing inflammation compared to a large number of

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CD8 T cells. These events can be caused by stimulants from exogenous factors, foreign substances, or agents originating from outside the body, such as bacteria and viruses [24].

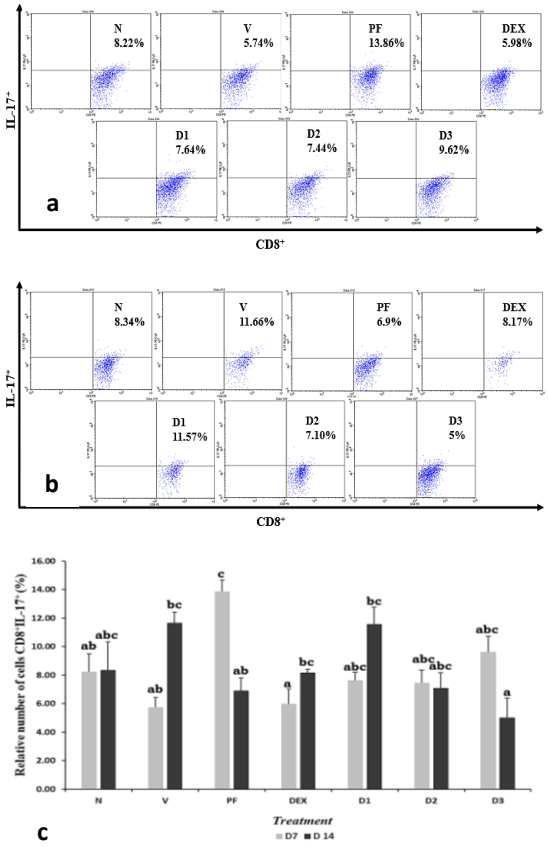

Figure 4. Relative number of $CD8^+IL\text{-}17^+$: (a) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}17^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}17^+$ cells for each 14-day treatment group; and (c) The representative bar showed the relative number of $CD8^+IL\text{-}17^+$ cells for each treatment group. *Healthy mice (N); Vehicle mice (V); Pulmonary fibrosis mice model (PF); Drug control (DEX); D1 (ESEE); D2 (ESEE); D3 (ESEE).

The (PF) group should have a relatively higher cell count than the healthy group, vehicle, drug administration, and treatment using ESEE due to increased IL-17 levels in pulmonary fibrosis conditions. However, in this case, it has not happened. It could be due to fibrosis not reaching the chronic phase, being in the early stages, or transitioning from the exudative phase to the early proliferative phase during the two weeks of treatment after being induced with bleomycin. The relative number of cytokine IL-17 can peak in the development of mid-stage pulmonary fibrosis pain [25].

The two-week treatment with *E. scaber* L. ethanol extract (ESEE) leaves resulted in low $CD8^+IL\text{-}17^+$ cell production at dose (D3) with a relative number of $CD8^+IL\text{-}17^+$ cells (5%) compared to the (PF) group (6.9%). Then, the drug groups had results similar to the healthy and vehicle groups in the second week.

The results obtained from the *E. scaber* dose treatment align with previous research, which stated that the bleomycin compound can cause inflammation, which causes pulmonary fibrosis [26]. So, there will be more pro-inflammatory cytokines such as IL-17, whose production can be inhibited, or the relative number of cells reduced using ESEE. Interleukin-17 (IL-17) is a type of inflammatory cytokine produced by $CD8^+$ T cells, which can stimulate T cell proliferation and play a significant role in the initial inflammatory response [22].

Disorders of any IL-17 can cause the development of pulmonary fibrosis. IL-17A is the first and most common IL-17 cytokine group produced by Th17-like cells, innate immune system, and non-hematopoietic cells. IL-17 has the primary function of inducing fibroblast activity through several mechanisms, such as inflammatory response, tissue repair, or wound healing, as well as epithelial-mesenchymal transition (EMT) [27].

$CD8^+$ T cells can recognize antigens presented by MHC class I molecules that can be found in cells that have nuclei, which will easily monitor cells if there are signs of infection. These cells will be activated into effector T cells after encountering antigens on the surface of the Antigen Presenting Cell (APC) by receiving *second signaling* so that cytokines, such as interleukin-17 (IL-17), can be secreted by $CD4^+$ T cells and $CD8^+$ T cells [17].

CONCLUSION

Ethanol extract from tapak liman leaves (*E. scaber* L.) effectively reduced cytokine levels of IL-6 and IL-17 cytokines secreted by $CD4^+$ and $CD8^+$ T cells in Balb/C mice with pulmonary fibrosis. D3 group (0.2016 $mg.kg^{-1}$ BW) was the most effective dose to decrease cytokines IL-6 and IL-17 mice with bleomycin-induced pulmonary fibrosis compared to other treatment dose groups, namely D2 and D3.

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Effect of Curcumin *(Curcuma xanthorriza)* and Red Ginger *(Zingiber officinale* var. *rubrum)* Ethanol Extract on Improvement of Mice Sperm Quality Exposed by Monosodium Glutamate

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Abstract

Temulawak (*Curcuma xanthorriza*) and red ginger (*Zingiber officinale* var. rubrum Theilade) contain antioxidant compounds that play an important role in inhibiting the negative effects of the excessive administration of free radicals. Excessive free radicals affect the spermatogenesis process. Which results in decreased sperm quality. This research determined the effect of administering ethanol extract of ginger and red ginger on the sperm quality of mice exposed to MSG. This study used 25 male mice (*Mus musculus*), aged 2.5 – 3 months and weighing 25 – 30 g, which were randomly divided into five groups: P0 (control), P1 MSG 4 mg.g^{-1} bw, P2 MSG 4 mg.g^{-1} bw and *C. xanthorriza* extract 0.2 mg.g^{-1} bw, P3 MSG 4 mg.g^{-1} bw and *Z. officinale* extract 0.4 mg.g^{-1} bw, P4 4 mg.g^{-1} bw, and a combination of *C. xanthorriza* extract 0.1 mg.g^{-1} bw and *Z. officinale* extract 0.2 mg.g^{-1} bw, MSG, and all extracts were administered orally for 30 days. On the 31st day, the mice were dissected, and epididymis was collected for sperm quality analysis, such as motility, viability, abnormality, and spermatozoa concentration. Subsequently, the sperm quality data were analyzed using One-way ANOVA through the SPSS 16.0 program for Windows ($P<0.05$), followed by Tukey's Honestly Significant Difference (HSD) test. The addition of both single and combination from *C. xanthorriza* and *Z. officinale* can ameliorate motility, viability, and spermatozoa compared with the group that was only given by MSG. Thus, adding temulawak (*C. xanthorriza*) and red ginger (*Z. officinale*) ethanolic extract can also ameliorate the mice's sperm quality.

Keywords: *C. xanthorriza*, MSG, sperm quality, *Z. officinale*

INTRODUCTION

Monosodium Glutamate (MSG) is a chemical substance classified as a derivate of glutamic acid. This chemical is often used as an additive in food to enhance the umami taste. Recommended MSG consumption for Asian countries ranges from 1.2 to 1.7 g daily. However, MSG consumption in Indonesia has increased to 4.32 g per day [1]. This increase in MSG consumption can trigger physiological disturbances in the body, particularly within the reproductive system. Excessive MSG consumption can lead to the formation of Reactive Oxygen Species (ROS) in the testes and brain [2].

The mechanism by which MSG can cause damage to the testes and brain is mediated by glutamate receptors, including N-Methyl-D-Aspartate (NMDA), Metabotropic Glutamate (mGLU), and kainate (ka) receptors. Glutamate diffuses through the calcium ions (Ca^{2+}). This rise in Ca^{2+} within mitochondria leads to ROS formation, particularly in the Tricarboxylic Acid (TCA) [3]. The produced ROS can cause cell damage due to oxidative stress. The damage to cells within the testicular tissue can lead to morphological alterations in spermatozoa, an imbalanced testosterone secretion by Leydig cells, and disrupted spermatogenesis [4].

Glutamate Toxicity also directly affects the Hypothalamic-pituitary-Gonadal (HPG) Axis, leading to changes in reproductive homeostasis [5]. The HPG axis plays a crucial role in hormone secretion, particularly Gonadotrophin-releasing Hormone (GnRH) secretion. The ROS produced due to glutamate metabolism can trigger a decrease in the secretion of Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH), both of which are essential in the process of spermatogenesis [6].

Sperm quality is vital to reproductive health, particularly in assessing individual fertility levels [7]. Parameters used in sperm quality analysis include motility, viability, abnormalities, and spermatozoa concentration. Treatment of Monosodium Glutamate (MSG) at a dose of 4 mg.g^{-1} body weight MSG to Rattus norvegicus for 30 days also leads to reduced sperm quality, decreased testosterone hormone levels, and progression of spermatogenic cell damage. One critical factor that can influence sperm quality is nutrition. Nutrition rich in antioxidants can reduce oxidative stress levels in sperm and increase sperm quality [7].

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Curcumin, a major component of *C. xanthorriza* that acts as an antioxidant. A previous study has demonstrated that adding 0.2 $mg.g^{-1}$ bw Curcumin to *Mus musculus* that exposed to MSG can improve the mice's sperm quality. Another plant known for its antioxidant properties is red ginger *Zingiber officinale*, which contains gingerol, gingerdiol, and gingerdione as antioxidants [8]. The *Z. officinale* can increase sperm production by acting as a suppressor of oxidative damage, thereby promoting cell development [9]. The oral addition of ethanol extract of *Z. officinale* to *Rattus norvegicus* for 26 days has been shown to improve sperm motility and viability [10]. Previous studies also indicate that adding *Z. officinale* extract at a dose of 0.05 $mg.g^{-1}$ body weight can increase sperm quality parameters and the number of spermatogenic cells in *Mus musculus* [11].

Despite the *fact that C. xanthorriza* and *Z. officinale* are widely known as sources of antioxidants, research on the effect of adding ethanol extract of *C. xanthorriza* and *Z. officinale*, both individually and in combination, in the field of reproduction, particularly on sperm quality in MSG-exposed mice, remains limited. The aim of this research was to determine the effect of administering ethanol extract of ginger and red ginger on the sperm quality of mice exposed to MSG.

MATERIAL AND METHOD

Ethical approval and Research design

This research procedure has earned an agreement from the Animal Care and Use Committee of Brawijaya University, number: 004-KEP-UB-2023. Twenty-five male mice (*Mus musculus*) aged 2.5 – 3 months and weighing 25 – 30 g were randomly assigned to five treatment groups for a duration of 30 days (Table 1).

Table 1. Group of Mice (n= 5 per group)

Group Code	MSG ($mg.g^{-1}$ bw)	CX extract ($mg.g^{-1}$ bw)	ZO extract ($mg.g^{-1}$ bw)
P0*	-	-	-
P1	4	-	-
P2	4	0.2	-
P3	4	-	0.4
P4	4	0.1	0.2

Notes: P0 (control) received a standard diet of pellets and *ad libitum* water, MSG = Monosodium Glutamate, CX = *Curcuma xanthorriza*, ZO = *Zingiber officinale*, bw = body weight.

Sperm Quality Analysis

On the 31st day, *Mus musculus* were euthanized by cervical dislocation, and epididymal organs were isolated. The caudal epididymal was placed in an object glass containing 1.5 mL PBS solution at 37°C. Sperm quality was assessed based on various parameters, including motility, viability, abnormalities, and spermatozoa concentration [12], using a microscope at magnifications of 100x and 400x.

Sperm Motility

The 10 µL of semen was collected and placed onto an object glass. Sperm motility analysis was conducted by observing progressively moving spermatozoa under a microscope at 100x magnification in five fields of view. The progression of spermatozoa was categorized into several classifications [13].

Viability and Abnormality Spermatozoa

The 10 µL of semen was aspirated using a micropipette and placed onto an object glass. Subsequently, a stain containing 1% Eosin and 5% Negrosin was added. The percentage of spermatozoa viability and abnormalities were observed under a light microscope at a magnification of 400x, following the formula [1] :

$$Viability\ (\%) = \frac{number\ of\ live\ spermatozoa}{total\ number\ of\ spermatozoa}\ x\ 100\%$$

$$Abnormality\ (\%) = \frac{number\ of\ live\ spermatozoa}{total\ number\ of\ spermatozoa}\ x\ 100\%$$

Spermatozoa Concentrations

The 10 µL of semen was collected and mixed with fixative (physiological NaCl) in a 1:10 ratio, resulting in a total of 100 µL. The two solutions were homogenized, and then 10 µL of the mixture was loaded into a Neubauer Haemocytometer. Spermatozoa concentration was determined in triplicated under a microscope at a magnification of 400X, employing the formula as provided [3]:

$$SC = n \times k \times FP \times 10^4$$

Description:
SC : Spermatozoa concentration
n : the number of spermatozoa
10^4 : volume of Haemocytometer counting chamber
FP : dilution factor (10)
K : number of small squares

Statistical analysis

The data about sperm quality, encompassing motility, viability, abnormalities, and spermatozoa concentration, were statistically analyzed by One-way ANOVA with the SPSS 16.0 software program for Windows ($p<0.05$). It was then followed by the Tukey Honestly Significant Difference (HSD) post-hoc test.

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RESULT AND DISCUSSION

Data on sperm quality results are contained in Table 2. Parameters of sperm quality were used, including motility (Fig. 1), viability, abnormality, and spermatozoa concentrations.

Table 2. The effect of MSG and extract *C. xanthorriza* as well as *Z. officinale* on quality sperm of *Mus musculus*

Sperm quality ±SD				
Group	**SM (%)**	**SV (%)**	**SA (%)**	**SC (10^6.mL^{-1})**
P0	58 ± 3.49^a	84 ± 1.10^a	26 ± 1.51^a	9 ± 2.7^a
P1	48 ± 2.31^a	62 ± 4.96^a	45 ± 12.3^c	12 ± 3.0^a
P2	51 ± 8.58^a	70 ± 11.5^a	35 ± 4.56^b	21 ± 6.5^c
P3	73 ± 4.99^c	81 ± 12.2^a	25 ± 4.81^a	18 ± 4.0^b
P4	71 ± 10.3^b	78 ± 9.41^a	27 ± 4.62^a	15 ± 3.4^a

Notes: Different letters within the same column show a statistically significant ($p \leq 0.05$). Group P1 that was exposed to MSG had effects on sperm quality. In contrast, groups P3 and P4 with extract of *C. xanthorriza* and red ginger have improved quality of sperm although exposed to MSG.

SM (%) = Percentage of sperm motility
SV (%) = Percentage of sperm viability
SA (%) = Percentage of sperm abnormality
SC (10^6.mL^{-1}) = spermatozoa concentrations

P0 = control
P1 = 4 mg.g^{-1} bw MSG
P2 = 4 mg.g^{-1} bw MSG + 0.2 mg.g^{-1} bw *C. xanthorriza*
P3 = 4 mg.g^{-1} bw MSG + 0.4 mg.g^{-1} bw *Z. officinale*
P4 = 4 mg.g^{-1} bw MSG + 0.1 mg.g^{-1} bw *C. xanthorriza* + 0.2 mg.g^{-1} bw *Z. officinale*

Sperm Motility

The results of this study reveal that the group exposed to a dose of 4mg.g^{-1} body weight of MSG (P1) exhibited a motility percentage of 48.66±2.31% (Table 2). This result aligns with prior research, which demonstrated a significant reduction in sperm motility in *Mus musculus* following the addition of 4 mg.g^{-1} body weight MSG for 30 days [19]. There is a decrease of 10% compared to the group control (P0), which was not exposed to MSG (Table 2). Furthermore, the addition of MSG at a dose of 4 mg.g^{-1} bw for 21 days was found to elevate testicular Malondialdehyde (MDA) production, serving as a biomarker for lipid peroxidation. This increase in MDA production points to oxidative stress due to the generation of augmented Reactive Oxygen Species (ROS) and decreased antioxidant levels in the body after MSG exposure [14].

The decline in sperm motility caused by oxidative stress is closely associated with the bioavailability of energy within spermatozoa. Consequently, ATP production, essential for bioenergy in spermatozoa, decreases due to cellular dysfunction, which can result in apoptosis or necrosis of cells [15]. The redominate reduction in sperm motility is linked to mitochondrial dysfunction in spermatozoa, leading to increased ROS and decreased ATP production [16].

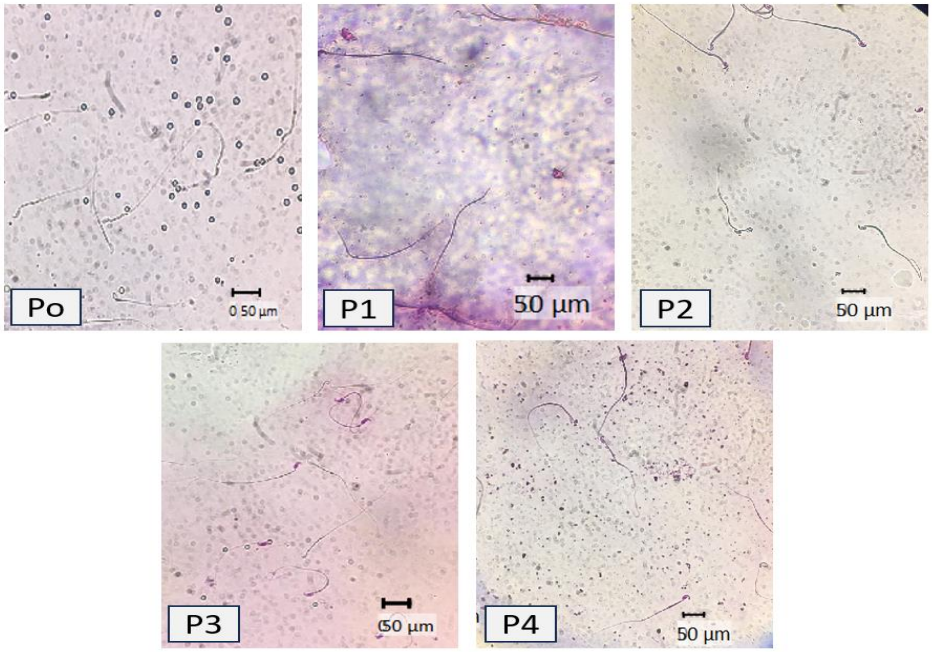

Figure 1. Sperm motility in each treatment; P0 was only given feed, P1 was given MSG treatment, P2 was given *C. Xanthorria* extract treatment with MSG exposure, P3 was given red ginger extract treatment with MSG exposure, and P4 group were given a combination of *C. xanthorriza* and red ginger extract treatment with MSG exposure.

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The addition of *Z. officinale* extracts at a dose of 0.4 $mg.g^{-1}$ bw results in increased sperm motility, with a percentage group P3 of 73±4.99%. Prior research mentioned that providing ethanol extract of red ginger for 26 days to *Rattus norvegicus* re-increase sperm motility [18]. The *Z. officinale* extract also contains phytochemicals that can modulate androgenic activity, particularly by elevating testosterone levels [18]. The antioxidative and chemoprotective compounds within *Z. officinale* increase the activity of superoxide Dismutase (SOD) and catalase (CAT) in the testes, thereby safeguarding against the free radical that triggers DNA damage and ultimately improve sperm quality in mice [12].

The combination of *C. xanthorriza* and *Z. officinale* extracts yields an increase in sperm motility, with a percentage of 71±10.3%. The curcumin content within *C. xanthorriza* acts as a scavenger of free radicals, preventing ROS formation. The phytochemical content in *Z. officinale* modulates androgenic activity, optimizing spermatogenesis through increased testosterone levels. Protective agents found in *Z. officinale* include gingerol and shogaol. The addition of *Z. officinale* extract can maintain the Hypothalamic-pituitary-Gonadol (HPG) axis and modulate steroidgenesis [19].

Sperm Viability

The addition of MSG at a dose of 4 $mg.g^{-1}$ body weight for 30 days led to a reduction in sperm viability in the P1 group by 22% compared to the control (P0) group (Table 2). The increased production of Reactive Oxygen Species (ROS) from MSG consumption by the body elevates Ca^{2+} production within the mitochondria. Ca^{2+} serves as a primary regulator of various cellular [20]. Accumulation of Ca^{2+} can increase ROS levels, impacting spermatozoa's fluidity and membrane integrity [21]. Previous research by Firstiantono *et al.* [3] demonstrated that adding MSG at a dose of 0.4 $mg.g^{-1}$ body weight for 30 days significantly reduced sperm viability compared to the control group.

The Spermatozoa membrane contains numerous polyunsaturated fatty acids (PUFA) susceptible to ROS. An imbalance in ATP production can lead to axonemal damage, resulting in decreased sperm viability [22]. Adding *C. xanthorriza* and *Z. officinale* extracts can increase sperm viability with percentages of 70±11.5% and 81±12.2%, respectively, compared to the MSG treatment. The combined addition of *C. xanthorriza* and *Z. officinale* extracts also increases sperm viability percentage of 81±12.2%. The curcumin content within *C. xanthorriza* extract protects the spermatozoa membrane against the reactivity of free radicals. Curcumin is a scavenger of free radicals, thus preserving the fluidity and integrity of the spermatozoa membrane.

Previous research has indicated that curcumin can elevate testosterone and Lutenizing Hormone (LH) levels in MSG-exposed rats [23]. The extract of *Z. officinale* contains active phenolic compounds such as gingerol, shogaol, zingerol, and gingerdiol, all exhibiting antioxidant activity. Prior studies have shown that adding ethanol extract of *Z. officinale* for 26 days can increase sperm viability in rats [24]. Supplementation with turmeric and rats with a high blood pressure diet can increase testosterone levels and sperm quality [24].

Sperm Abnormality

Based on the result of this study, it is evident that abnormalities in spermatozoa were observed across all treatment groups. The addition of MSG at a dose of 4 $mg.g^{-1}$ body weight resulted in the highest percentage of spermatozoa abnormalities, reaching 45±12.3% compared to the control group (P0) (Table 2).

Exposure to MSG can increase antioxidant activity, such as glutathione (GSH) [25]. The High production of Reactive Oxygen Species (ROS) due to MSG exposure can impact the plasma membrane of spermatozoa, which contains a significant amount of phospholipids and unsaturated fatty acids. These components are particularly vulnerable to ROS, especially the highly reactive hydroxyl radical. The hydroxyl radical can induce lipid peroxidation, damaging the fatty acid chains and forming toxic products to spermatozoa and ethane (C_2H_6) [26]. Such products can trigger morphological damage to spermatozoa.

This study's results reveal the presence of spermatozoa abnormalities, such as those lacking heads and coiled tails (Fig. 2). Oxidative stress conditions can also impact changes in the morphology of spermatozoa heads [27]. The addition of ethanol extracts of *C. xanthorriza* and *Z. Officinale* can decrease abnormalities in spermatozoa exposed to MSG, with respective percentages of 4.56% and 4.81% (Table 2).

The combined addition of *C. xanthorriza* and *Z. officinale* extracts resulted in a percentage of spermatozoa abnormalities at 27±4.62%,

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compared to the MSG treatment group (P1). The Curcumin content within *C. xanthorriza* plays a role in inhibiting lipid peroxidation by absorbing free radicals and stimulating endogenous antioxidant activities, such as superoxide Dismutase (SOD) and catalase (CAT) [28]. This protective mechanism is attributed to curcumin and gingerol in *Z. officinale*, which exhibits antioxidant activity. Previous studies have demonstrated that adding 6-gingerol isolated from *Z. officinale* resulted in the lowest percentage of spermatozoa abnormalities among treatment groups and improved testicular function [29]. Other bioactive compounds found in *Z. officinale* possess antioxidant activity and contribute to protecting DNA in spermatozoa [30].

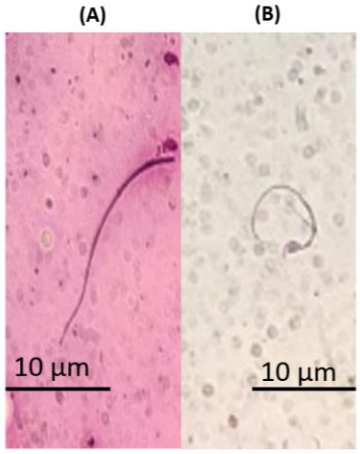

Figure 2. Morphology sperm abnormality correct the number of abnormality (400X). (A) Detache head, (B) Sperm coiled tail abnomality found in this study.

Spermatozoa Concentration

The results of this study demonstrate that the highest spermatozoa concentration was observed in the group treated with *C. xanthorriza* extract at a dose of 17.27 cell.mL^{-1}, and this deferred significantly from the group exposed solely to MSG at a dose of 4 mg.g^{-1} body weight over 30 days (Table 1). Previous research has indicated that *C. xanthorriza* extract is capable of increasing the spermatozoa concentration in *Mus musculus* exposed to MSG [31].

The addition of MSG can lead to decreased levels of Luteinizing Hormone (LH) and testosterone. These hormones play pivotal roles in maintaining the normal function of the testes and spermatogenesis [32]. Elevated production of Reactive Oxygen Species (ROS) can trigger lipid peroxidation, leading to membrane and DNA damage in spermatozoa, resulting in cellular apoptosis or necrosis. This progression of cell death consequently leads to a decline in spermatozoa concentration [33].

CONCLUSION

The addition of *C. xanthorriza* extract at a dose of 0.2 mg.g^{-1} body weight, and *Z. Officinale* extract at a dose of 0.4 mg.g^{-1} body weight, as well as their combination, proves to be effective in increasing the quality of spermatozoa exposed to MSG. There is a need for further research regarding the use of Curcumin (*Curcuma xanthorriza*) and red ginger on infertile organisms who suffer from reproductive disorders. It is necessary to carry out a histopathological analysis of the liver and kidneys to determine the toxicity caused by MSG.

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Preventive Effect of Antioxidant From Purple Rosella Yoghurt on Duodenum and Colon Histopathology of *Rattus norvegicus* Exposed to Rhodamine B And Saccharin

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Abstract

Rodhamine B and Saccharin are often misused by food producers as food additive, which, if their use is not monitored, can produce free radicals that cause oxidative stress conditions in the body, leading to dangerous damage to the body. The addition of purple rosella in yoghurt can increase the functional value of the yoghurt, namely increasing the antioxidant content. The purpose of this study was to determine the benefits of giving purple rosella yoghurt in preventing damage to the duodenum and colon of (*Rattus norvegicus)* caused by Rodhamine B and Saccharin. This study used Completely Randomized Design (CRD) with 28 male *R. novergicus*, *Wistar* strain in aged 8-12 weeks. Group K was not given any treatment; R was given Rodhamine B, S was given Saccharin, KRS was given Rodhamine B and Saccharin, YR was given Rodhamine B and yoghurt, YS was given Saccharin and yogurt, YRS was given Rodhamine B, Saccharin and yogurt. The dose of Rodhamine B was 22.5 $mg.kg^{-1}$, Saccharin was 157.77 $mg.kg^{-1}$, and the volume of yoghurt was 1 $mL.head^{-1}$. They were administered orally with a gastric tube for 14 days. The concentration of purple rosella extract added to yoghurt was 15% (v/v). Parameters were observed in histopathology of the duodenum and colon with the scoring method. Data analysis was done with the Kruskal-Wallis test followed by the Mann-Whitney test. The KRS group showed the heaviest damage, namely necrosis, villi erosion, and inflammatory cell infiltration of >75% of the total visual field in the duodenum and colon. The YR, YS, and YRS groups showed significant changes compared to the R, S, and KRS groups, namely necrosis, villous erosion, and inflammatory cell infiltration, which appeared to be 25-50% of the total visual field. However, this did not match the condition of group K, which appeared to be <25% of the total visual field. The provision of purple rosella yoghurt can prevent 50% damage to the duodenum and colon of *Rattus novergicus* due to Rodhamine B and Saccharin.

Keywords: colon, duodenum, Rodhamine B, Saccharin, yoghurt.

INTRODUCTION

In food industries these days, more and more food producers are using food additives in food and beverage processing for various purposes. If food additives are not used in accordance with the dosage in the long term, it will cause harm to health and may cause diseases. The dosage of food additives needs to be better monitored. Even though it has been banned, synthetic dyes such as Rodhamine B and artificial sweeteners such as Saccharin are still sold in the market. Rodhamine B is often misused to color sausages, corned beef, and sauces. Rodhamine B is typically used in the textile industry, paper industry, and fabric dyes [1,2]. The impact of repeatedly consuming Rodhamine B in large quantities can lead to body precipitation, causing digestive tract irritation [3]. Apart from artificial coloring, artificial sweeteners such as Saccharin are often used because they taste 400 times sweeter than sucrose. Saccharin is usually found in products that use sweeteners, such as ice cream. Excessive consumption of Saccharin can cause side effects, such as diarrhea, allergies, hypertension, and bladder cancer [4].

Rodhamine B and Saccharin are xenobiotic substances that can produce free radicals as side products. Free radicals will induce the formation of Reactive Oxygen Species (ROS) and oxidative stress. Free radicals that are formed will be distributed throughout the body, including the duodenum and colon [2]. The best way to protect the body from oxidative stress exposure is to strengthen or consume foods that contain antioxidants. Along with technological developments, there has been an increase in the popularity of foods like yoghurt [5].

Purple rosella extract can be added to improve the quality of the yoghurt. Purple rosella (*Hibiscus sabdariffa* L.) contains anthocyanins with high antioxidant activity. Anthocyanin can act as an antioxidant to maintain and protect the

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body from free radical effects [6]. Anthocyanins can capture and counteract free radicals (scavengers) by donating one hydrogen atom of the phenolic hydroxyl groups when reacting with free radicals. Anthocyanins have a conjugated double bond structure capable of destroying and counteracting free radicals, as well as preying on various types of reactive oxygen-derived free radicals, such as hydroxyl (OH), peroxyl (ROO), and single oxygen (O2) group [7]. Therefore, this research was conducted to determine the benefits of giving yoghurt with the addition of 15% purple rosella, as a source of antioxidants, in preventing damage that occurs to the duodenum and colon of *Rattus norvegicus* using the toxicity model of Rodhamine B and Saccharin.

MATERIAL AND METHOD

Experimental Research Design and Sample Preparation

This research used a Completely Randomized Design (CRD) with a sample of 28 male *Rattus norvegicus*, Wistar strain, aged 8 to 12 weeks, body weight 150-200 g, obtained from Iwan Farm Pakisaji, Malang City. The research used experimental animals, which have received an Ethical Clearance from the Ethics Commission of Universitas Brawijaya (No. 079-KEP UB-2022). Animals were acclimated for seven days to adapt to the condition and environment through animal cages of the Experimental Animal Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya. BR-1 feeding and drinking water were given ad libitum. Each treatment group, consisting of four individuals of *Rattus norvegicus*, has been shown in Table 1.

Table 1. Group treatment of *Rattus norvegicus*

Groups	Rodhamine B ($mg.kg^{-1}BW^{-1}$)	Saccharin ($mg.kg^{-1}BW^{-1}$)	purple rosella yoghurt ($rat.day^{-1}$)
K	-	-	-
R	22.5	-	-
S	-	157.77	-
KRS	22.5	157.77	-
YR	22.5	-	1 mL
YS	-	157.77	1 mL
YRS	22.5	157.77	1 mL

The administration of Rodhamine B, Saccharin, and purple rosella yoghurt to *Rattus norvegicus* was carried out using the gavage method, namely orally using a gastric probe for 14 days. Purple rosella yoghurt was administered four hours before Rodhamine B and Saccharin administration. Rodhamine B and Saccharin were obtained from Duta Wijaya Labware, Malang City.

Purple rosella yoghurt is made at the Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Universitas Brawijaya. The purple roselle (*Hibiscus sabdariffa* L.) used in making yoghurt is obtained from local plantations in Kediri City. Dried purple rosella flower petals were ground with a blender to a powder, sifted through a 60 mesh sieve, dissolved in distilled water in a ratio of 1 : 5 (g/v), pasteurized using the Low-Temperature Long Time (LTLT) technique at 63℃ for 30 minutes, filtered with filter paper to separate liquid and sediment, the liquid that has been obtained can be stored in a refrigerator at 4℃ [5].

The first stage of making purple rosella yoghurt is making a yoghurt starter; as much as 50 mL of milk is pasteurized using the High-Temperature Short Time (HTST) technique at 72℃ for 15 seconds. Milk is obtained from KUD Batu Jeding Kulon, Batu City. Pasteurized milk is added with freeze-dried Yogourmet® starter powder, which contains *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus* as much as 0.5% (g/v), homogenized using a stir bar, incubated at 45℃ incubator until pH reaches 4.4 - 4.5 (± 4 hours incubation). The second step is to make yoghurt, 150 mL of milk is pasteurized using the HTST technique at 72℃ for 15 seconds, added with 3% (v/v) yoghurt starter, homogenized using a stir bar, and incubated at 45℃ until the pH reaches 4.5 - 5 (± 2-3 hours of incubation) [8]. The third step is to make purple rosella yoghurt. As much as 15% (v/v) of purple rosella liquid was added to 100 mL of yoghurt and homogenized using a blender. Purple rosella yoghurt can be stored in the refrigerator at 4℃ [5].

Data Collection and Analysis

Rattus norvegicus was euthanized using the cervical os dislocation technique on the 15th day of the study treatment to take samples of the duodenum and colon. The duodenum and colon were washed using 0.9% NaCl to remove residual blood and put into an organ pot containing 10% BNF as an organ fixation fluid. Histopathological preparations were made for Hematoxylin Eosin (HE) staining. Histopathological preparations of the duodenum and colon were observed under a light microscope with magnification of 100x and 400x. Images were taken using the Optilab digital microscope camera and the Optilab Viewer application. The histopathological changes were

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scored in 5 visual fields based on the scoring criteria shown in Table 2.

Table 2. Scoring Criteria of Duodenum and Colon [9] with modification

Damage Percentage	Score
Lesion <25% total field of view	1
Lesion 25-50% total field of view	2
Lesion 50-75% total field of view	3
Lesion >75% total field of view	4

The data obtained was analyzed using the Kruskal-Wallis Test, followed by the Mann-Whitney Test. The analysis was assisted by the application of the Statistical Product and Service Solution (SPSS) program for Windows Version 26.

RESULT AND DISCUSSION

Histopathology of *R. norvegicus* Duodenum

The observed duodenum histopathology was the presence or absence of lesions, including necrosis, villous erosion, and inflammatory cell infiltration. No lesions were detected in the duodenum of group K. Meanwhile, groups R, S, and KRS showed lesions, i.e. necrosis, villous erosion, and a quite heavy infiltration of inflammatory cells. In the YR and YRS groups, lesions were not as severe as those in the R, S, and KRS groups. Even close to the condition of group K (Fig. 1).

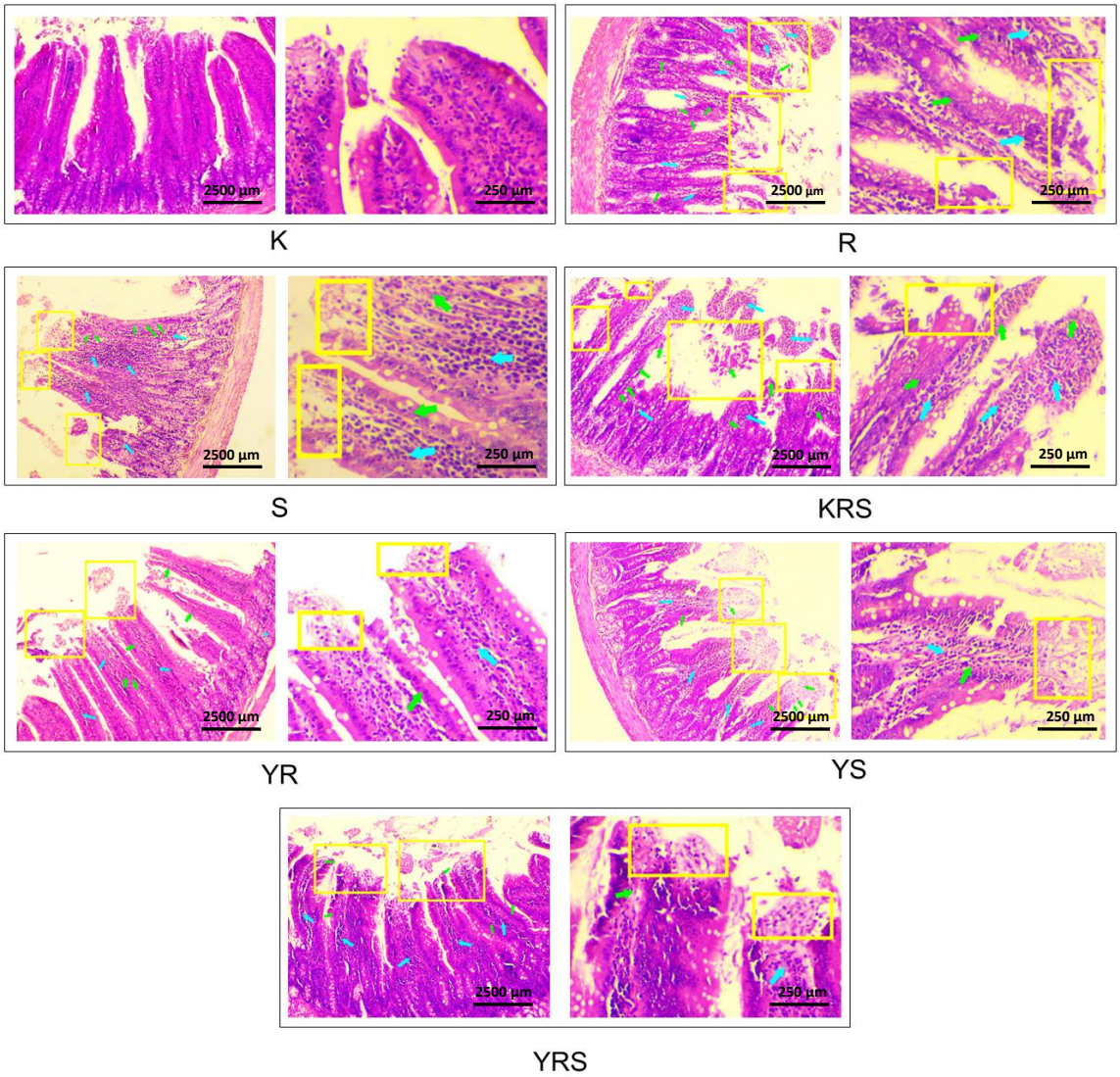

Figure 1. Histopathology of *Rattus norvegicus* Duodenum in All Treatment Groups Using Hematoxylin Eosin (HE) Staining. Magnification 100x (left), 400x (right)
Necrosis
Villi erosion
inflammatory cell infiltration

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The statistical test results showed significant differences between the R, S, KRS groups and the K, YR, YS, and YRS groups. The K group was not significantly different from the YR, YS, and YRS groups (Table 3). The necrosis in the YR (1.75 ± 0.58) group was better than in the R group (2.75 ± 0.50); necrosis in the YS group (1.50 ± 0.58) was better than in the S group (2.75 ± 0.50); and necrosis in the YRS group (1.50 ± 0.58) than KRS group (3.50 ± 0.58). Villous erosion in the YR group (1.50 ± 0.50) was better than R group (3.25 ± 0.50); villous erosion in the YS group (1.50 ± 0.58) was better than in the S group (2.75 ± 0.50); and villious erosion in YRS group (1.50 ± 0.58) better than in KRS group (3.50 ± 0.58). Inflammatory cell infiltration in YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); inflammatory cell infiltration in YS group (1.50 ± 0.58) better than S group (3.00 ± 0.82); and inflammatory cell infiltration in YRS group (1.75 ± 0.50) better than in KRS group (3.75 ± 0.50). This shows that giving purple rosella yoghurt prevent 50% damage to the duodenum due to exposure to free radicals caused by Rodhamine B and Saccharin.

Table 3. Duodenum Histopathological Scoring Average

Groups	Lesion Average ± SD		
	Necrosis	Villi Erosion	Inflammatory Cell Infiltration
K	1.25 ± 0.50[a]	1.25 ± 0.50[a]	1.25 ± 0.50[a]
R	2.75 ± 0.50[b]	3.25 ± 0.50[b]	3.25 ± 0.50[b]
S	2.75 ± 0.50[b]	2.75 ± 0.50[b]	3,00 ± 0.82[b]
KRS	3.50 ± 0.58[b]	3.50 ± 0.58[b]	3.75 ± 0.50[b]
YR	1.75 ± 0.58[a]	1.50 ± 0.50[a]	1.50 ± 0.58[a]
YS	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.50 ± 0.58[a]
YRS	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.75 ± 0.50[a]

Notes: Results are shown as mean values ± SD with different letter superscripts showed a significant difference ($p<0.05$)

K = no treatment
R = Rodhamine B 22.5 mg.kg^{-1}BW^{-1}
S = Saccharin 157.77 mg.kg^{-1}BW^{-1}
KRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1}
YR = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YS = Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}

The duodenum as a part of the small intestines, a place for digestion and absorption of food, which is susceptible to damage caused by oxidative stress. One of the changes caused by free radicals due to exposure to Rodhamine B and Saccharin is a change in the properties of cell membranes and cytoplasmic membranes of cell organelles, such as lysosomes and mitochondria. The most critical component in the cell membrane contains Polyunsaturated Fatty Acids (PUFA), which are very susceptible to free radical attack and cause the cell membrane to become brittle [10]. Free radicals will damage the structure of the cell membrane. It allows free radicals to enter the cytoplasm and damage the cell nucleus, which causes necrosis, as in the treatment group [11].

Damaged cells will release chemical compounds to signal inflammatory cells by the circulatory system to the damaged site. This condition is called inflammatory cell infiltration. Villi erosion and necrosis can trigger an increase in inflammatory cells that move from vessels to the damaged site [12,13]. Damage in the toxification process occurs due to the reactions between toxic substances and molecules in the body. The appearance of inflammatory cells is a form of the body's defense against foreign antigens. Inflammatory cells provide rapid and strong body defense against pathogens [10].

The human body needs antioxidants as the main compounds of free radicals scavengers to reduce the adverse effects of free radicals. Antioxidants release an electron to free radicals, inhibiting free radical activity [6]. The anthocyanin in purple rosella yoghurt has a conjugated double bond structure that destroy and ward off free radicals, as well as prey on various types of free radicals derived from reactive oxygen, such as hydroxyl (OH), peroxyl (ROO), and single oxygen (O_2) [14,15]. Therefore, the treatment groups that was given purple rosella yoghurt had a mild damaging effect on the duodenum. It has antioxidant compounds that can capture free radicals [16]. However, in this study, damage prevention was not optimal, because the concentration of purple rosella extract added to the yoghurt was not adequate.

Histopathology of *R. norvegicus* Colon

Observed colon histopathology is the lesions, including mucosal epithelial erosion, loss of goblet cells, and inflammatory cell infiltration. There were no lesions detected in the colon of group K. Group R, S, and KRS showed lesions, namely erosion of the mucosal epithelium, loss of goblet cells, and infiltration of inflammatory cells, which is quite heavy. In the YR, YS, and YRS groups, lesions were not as severe as those in the R, S, and KRS groups. Even almost close to the conditions of group K (Fig. 2).

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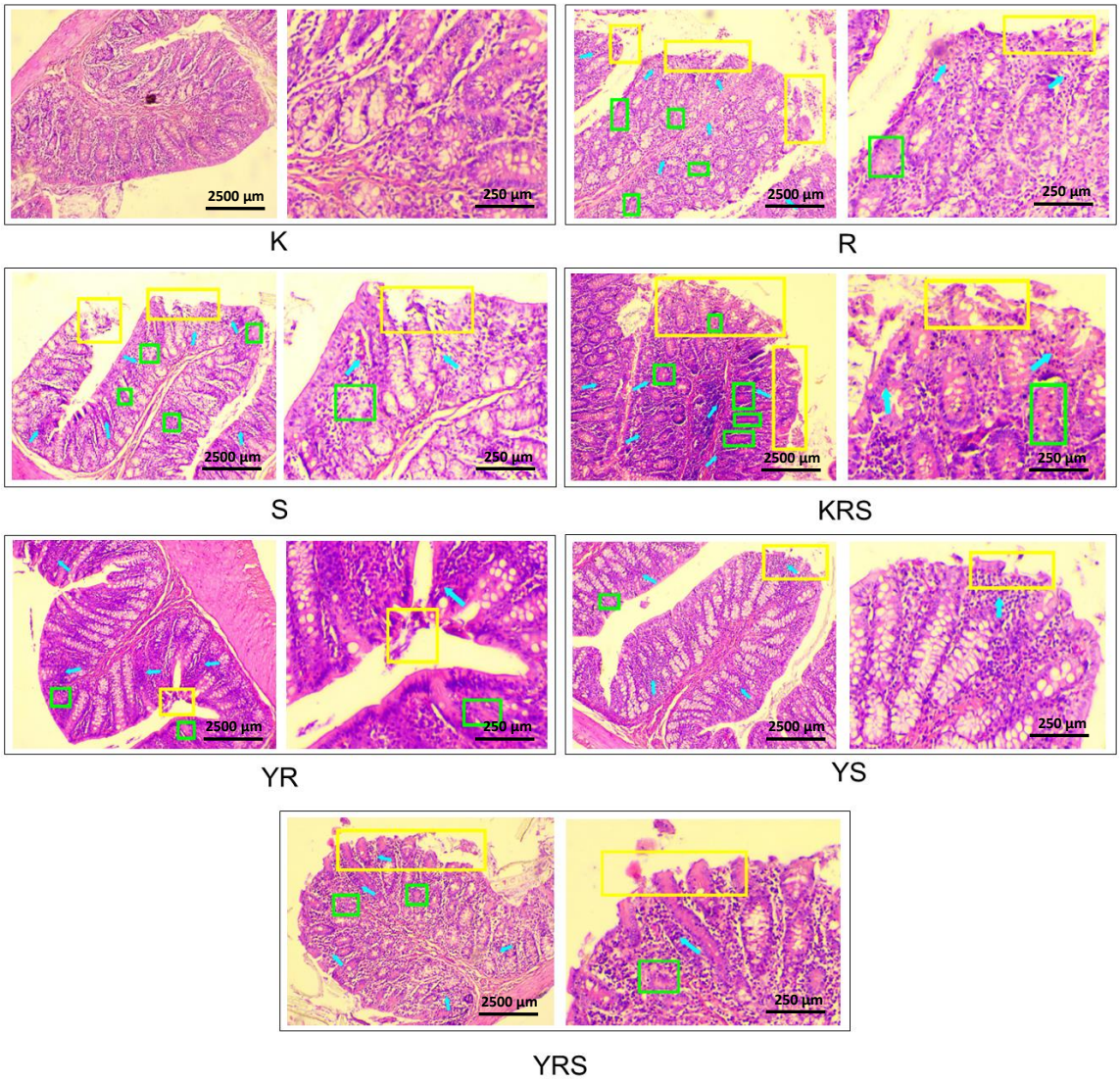

Figure 2. Histopathology of *Rattus norvegicus* Colon in All Treatment Groups Using Hematoxylin Eosin (HE) Staining. Magnification 100x (left), 400x (right) colon and superscripted.
lost of Goblet cells
Mucosal epithelial erosion
inflammatory cell infiltration

The statistical test results showed significant differences between the R, S, KRS groups and the K, YR, YS, and YRS groups (Table 4). The K group was not significantly different from the YR, YS, and YRS groups. Table 4 showed that erosion of the mucosal epithelium in the YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); erosion of the mucosal epithelium in YS group (1.25 ± 0.50) better than in S group (2.75 ± 0.50); and erosion of the mucosal epithelium in YRS group (1.75 ± 0.50) better than in KRS group (3.50 ± 0.58). The loss of goblet cells in YR group (1.50 ± 0.58) better than in R group (3.25 ± 0.50); the loss of goblet cells in YS group (1.50 ± 0.58) better than in S group (2.75 ± 0.50); and the loss of goblet cells in YRS group (1.75 ± 0.50) better than in KRS group (3.50 ± 0.58). Inflammatory cell infiltration in YR group (1.75 ± 0.50) better than in R group (3.25 ± 0.50); inflammatory cell infiltration in YS group (1.50 ± 0.58) better than in S group (2.75 ± 0.50); and inflammatory cell infiltration in YRS group (1.75 ± 0.50) better than in KRS group (3.75 ± 0.50). This generally shows that giving purple rosella yoghurt is able to prevent damage to the colon due to exposure to free radicals caused by Rodhamine B and Saccharin.

Cells in the colonic mucosa are more susceptible to damage caused by the induction of oxygen radicals and through direct contact with

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toxic substances. The colon is the organ that exposed longest to toxic substances in the feces and is easily damaged due to potent oxidizing agents [17]. The loss of PUFA causes damage to the structure of the cell membrane, thereby affecting the permeability and function of the cell membrane. It is known that PUFA is most sensitive to free radicals and will form lipid peroxide chain reactions, causing cell membranes to lose integrity and rupture. Cell damage can occur if the damage continues to affect the lysosomal membrane, causing hydrolytic enzymes to be released and damage other organelles [18].

Table 4. Colon Histopathological Scoring Average

Groups	Lesion Average ± SD		
	Mucosal Epithelial Erosion	Loss of Goblet Cells	Inflammatory Cell Infiltration
K	1.25 ± 0.50[a]	1.25 ± 0.50[a]	1.25 ± 0.50[a]
R	3.25 ± 0.50[b]	3.25 ± 0.50[b]	3.25 ± 0.50[b]
S	2.75 ± 0.50[b]	2.75 ± 0.50[b]	2.75 ± 0.50[b]
KRS	3.50 ± 0.58[b]	3.50 ± 0.58[b]	3.75 ± 0.50[b]
YR	1.50 ± 0.58[a]	1.50 ± 0.58[a]	1.75 ± 0.50[a]
YS	1.25 ± 0.50[a]	1.50 ± 0.58[a]	1.50 ± 0.58[a]
YRS	1.75 ± 0.50[a]	1.75 ± 0.50[a]	1.75 ± 0.50[a]

Notes: Results are shown as mean values ± SD with different letter superscripts showed a significant difference ($p<0.05$)

K = no treatment
R = Rodhamine B 22.5 mg.kg^{-1}BW^{-1}
S = Saccharin 157.77 mg.kg^{-1}BW^{-1}
KRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1}
YR = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YS = Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}
YRS = Rodhamine B 22.5 mg.kg^{-1}BW^{-1} + Saccharin 157.77 mg.kg^{-1}BW^{-1} + Purple Rosella Yoghurt 1 mL each rat.day^{-1}

The increase of free radicals caused by exposure to Rodhamine B and Saccharin can cause damage to epithelial cells and interfere with the absorption of nutrients. Lipid peroxidation is the process of converting unsaturated fatty acids into free radicals through the absorption of hydrogen. Erosion of the mucosal epithelium is one of the damages in the colon, in which the colon loses some of the epithelial cells in the mucosal lining [19]. Mucosal erosion causes an increase in epithelial permeability so that the normal flora damages the mucosa and causes an inflammatory response to occur. The inflammatory response can increase the amount of proinflammatory cytokines. An increase in the number of proinflammatory cytokines in areas experiencing inflammation is directly proportional to the rise in ROS, which will cause damage to colonic tissue [20].

Erosion of the colonic mucosal epithelium results in the loss of goblet cells. Goblet cells in the colon function as a barrier to the colonic mucosa by secreting mucin compounds. The barrier function of goblet cells prevents foreign antigens and pathogens from entering the mucosal tissue; therefore, colonic homeostasis can be maintained [21]. Goblet cells and secreted mucin prevent pathogens, which will cause inflammation from entering the mucosal tissue. If inflammation occurs, it indicates that the pathogen has succeeded in entering the mucosal tissue by destroying goblet cells [22].

The antioxidant content in purple rosella can suppress ROS by inhibiting the activity of the superoxide dismutase enzyme so that it can protect cells in the body from free radical attacks [23,24]. The anthocyanin compounds contained in purple rosella yoghurt are able to prevent damage to the *Rattus norvegicus* colon. Anthocyanin compounds will release a hydrogen atom (H+) to ROS, causing ROS to become stable compounds and unable to take atoms from cells, thus stopping the process of cell damage. This situation decreases oxidative stress in the tissue [9,25]. Therefore, reduced oxidative stress in the tissues will prevent lipid peroxidation [16], so the treatment group given purple rosella yoghurt can suppress damage to the duodenum and colon more than the treatment group without given purple rosella yoghurt, but not optimal.

CONCLUSION

Giving purple rosella yoghurt can prevent 50% damage to the duodenum and colon of *Rattus novergicus* due to exposure to free radicals from Rodhamine B and Saccharin. It is recommended that further research be carried out by increasing the concentration of purple rosella extract added to yoghurt by more than 15% (v/v).

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Histological and Biochemical Evaluation of the Protective Potential of Ascorbate and Alpha-Tocopherol against Cypermethrin-Induced Toxicity

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Abstract

The unrestricted and unsystematic use of cypermethrin pesticides has detrimental effects on the organs, ranging from short-term sickness to long-term effects. The ameliorating effect of alpha-tocopherol and ascorbate was investigated singly and in combination with cypermethrin-induced oxidative stress using murine models. Additionally, the livers and kidneys of rats were histologically evaluated. Twenty-five (25) adult male Wistar rats with an average weight of 190 g were allocated randomly into five groups consisting of five rats each. Group I consists of the unexposed control rats, while rats in groups II-V were the test group exposed to cypermethrin at standard doses of 10 $mg.kg^{-1}$ bw. While rats in group II were exposed and untreated, group III-V was administered with ascorbate (5000 $mg.kg^{-1}$ bw), alpha-tocopherol (3000 $mg.kg^{-1}$ bw), and co-administered with both vitamins at their standard doses, respectively. Regimen administration was by gavage for 28 days, and while the vitamins were administered daily, cypermethrin exposure was done twice a week. At the end of the experiment, rats were euthanized, and blood obtained via cardiac puncture was used for biochemical analysis, while the liver and kidneys excised were processed for histopathological evaluation. Results revealed elevated aspartate transferase (AST), alanine transferase (ALT), malondialdehyde (MDA), and creatinine levels. At the same time, a decrease in superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities was observed in the test group ($p<0.05$). Additionally, treatment with ascorbate and alpha-tocopherol co-administration reversed the biochemical parameters in the exposed rats. Conclusively, ascorbate and alpha-tocopherol ameliorate oxidative damage associated with cypermethrin exposure.

Keywords: Cypermethrin, Histopathology, Pesticides, Toxicity, Vitamins.

INTRODUCTION

Pesticides function by attracting, seducing, and then destroying pests and are commonly referred to as insecticides, fungicides, bactericides, herbicides, or rodenticides, which are chemical groups used to control weeds and populations of insects, fungi, or other harmful pests [1,2]. The uncontrolled usage of pesticides adversely impacts the environment and is also associated with a variety of health problems ranging from short-term illness to cancer. Reducing their use is difficult because intensive agriculture heavily relies on pesticides to maintain maximum yields [3]. Cypermethrin pesticides are broad-spectrum, less toxic, biodegradable, and highly effective synthetic pesticides used globally in agriculture, pest management, and households as mosquito repellents for about four decades [4,5]. Cypermethrin is based on pyrethrin, a natural compound extracted from Chrysanthemum plants, and is rapidly metabolized with metabolites that are easily excreted [6]. Although they do not severely bioaccumulate in humans, chronic exposure may result in poisoning symptoms or even death [4].

Cypermethrin's biological activity results from neuronal membrane depolarization, which allows more sodium ions to pass through voltage-gated sodium channels [7]. There is a paucity of information on the chronic health effects of repeated exposure to cypermethrin. However, epidemiological studies associated with environmental and occupational exposure have reported the presence of cypermethrin metabolites in urine [8]. Additionally, it has been established that cypermethrin is lipophilic and can be distributed to tissues with high lipid content, such as fat and nervous tissue, as well as the liver, kidney, and milk, with the liver being the most commonly affected target tissue because it is the primary site of metabolism for these chemicals as well as the first organ to be exposed to the chemical following cypermethrin absorption [9,10]. Hence, this study investigates the histopathological and biochemical changes in the studied organs of male rats sub-acutely exposed to cypermethrin, as well as the possible mitigating effects of ascorbate and alpha-tocopherol that could serve as a therapeutic relief in cypermethrin toxicity.

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MATERIAL AND METHOD

Experimental animals

Twenty-five (25) adult male Wistar rats purchased from the Animal Holding of the University of Medical Sciences Ondo State, Nigeria (UNIMED) were acclimatized for four weeks and fed with standard rat pellets obtained from Chikun farms and water ad-libitum. The rats were properly housed in a clean, well-ventilated space [11].

Experimental Design and Ethical approval

After four weeks of acclimatisation, the rats were divided into five groups, each with five rats, following the method of Adeniyi *et al.* [12]. The Ethical Committee of UNIMED approved the use of experimental animals and assigned the approval number NHREC/TR/UNIMED-HREC-Ondo St/22/06/21.

Specimen collection

After experimentation, all animals in the groups were euthanized, and blood samples were collected and transferred into a lithium heparinized anticoagulant bottle for biochemical analysis. The liver and kidneys were immediately excised, preserved, and processed for histopathological studies [13].

Histopathological and Biochemical studies

The prefixed liver and kidney samples were histologically processed as described by Moronkeji *et al.* [14]. Briefly, the tissues were dehydrated in graded alcohol solutions, followed by clearing in two changes of xylene, infiltrated in two changes of wax bath, and embedded using paraffin wax. The sections were hydrated and stained with Harris Haematoxylin for five minutes, rinsed in water, and differentiated with 1% acid alcohol for one minute. Sections were rinsed in water and blued in tap water for ten minutes, followed by counterstaining using 1% aqueous eosin, and dehydration in ascending grades of alcohol was ensured. The stained slides were cleared in two changes of xylene, mounted using dibutyl phthalate propylene xylene (DPX), and examined microscopically using x10 and x40 objective lenses. For the biochemical studies, the plasma from the euthanized rats was used to perform the liver and renal function tests described in the diagnostic kits obtained from Randox Laboratory, UK [14].

Oxidative Stress Assays

The supernatant obtained from each rat's homogenate samples of the liver and kidney was preserved at -70°C. For the assessment of superoxide dismutase (SOD), glutathione peroxidase (GPX), and Catalase activity (CAT), protocols from the ELISA kits obtained from Elabscience Biotechnology Inc USA were used. Additionally, Malondialdehyde (MDA) was spectrophotometrically analyzed as described by Oludare *et al.* [15].

Statistical analysis

The data obtained were analyzed using SPSS version 23. The chi-square and Fisher exact tests compared the categorical variables, and P-values < 0.05 were considered statistically significant.

RESULT AND DISCUSSION

Histopathological Findings

Liver of Rats

The histopathological findings in this study revealed that cypermethrin exposure had a deleterious impact on the livers of the exposed rats. While the unexposed control rats had normal liver histoarchitecture (Fig. 1a), the untreated cypermethrin-exposed rats had congested venules and sinusoidal spaces, as well as hepatic vacuolation and necrosis (Fig. 1b). Furthermore, when compared to the cypermethrin-exposed untreated rats, treatment with 5000 $mg.kg^{-1}$ bw of ascorbate showed reduced cytopathic changes typified by normal hepatocytes (Fig. 1c). In contrast, the alpha-tocopherol (3000 $mg.kg^{-1}$ bw) treated rats showed inflamed liver parenchyma (Fig. 1d). The cypermethrin-exposed rats treated with ascorbate and alpha-tocopherol at standard doses of 5000 $mg.kg^{-1}$ bw and 3000 $mg.kg^{-1}$ bw respectively had histoarchitecture similar to the unexposed control (Fig. 1e).

Kidney of Rats

The kidneys of the control rats showed no pathological lesions (Fig. 2a). In contrast, the cypermethrin-exposed untreated rats had a poor histoarchitectural structure characterized by periglomerular inflammation with degenerated renal tubules as well as congested and inflamed interstitial space (Fig. 2b).

The kidneys of the ascorbate-treated rats had a normal glomerulus and no congested or inflamed interstitial space (Fig. 2c), whereas the interstitial space of the alpha-tocopherol-treated rats was mildly inflamed (Fig. 2d). The co-administration with ascorbate and alpha-tocopherol revealed a normal glomerulus with a mildly congested and infiltrated interstitial space (Fig. 2e).

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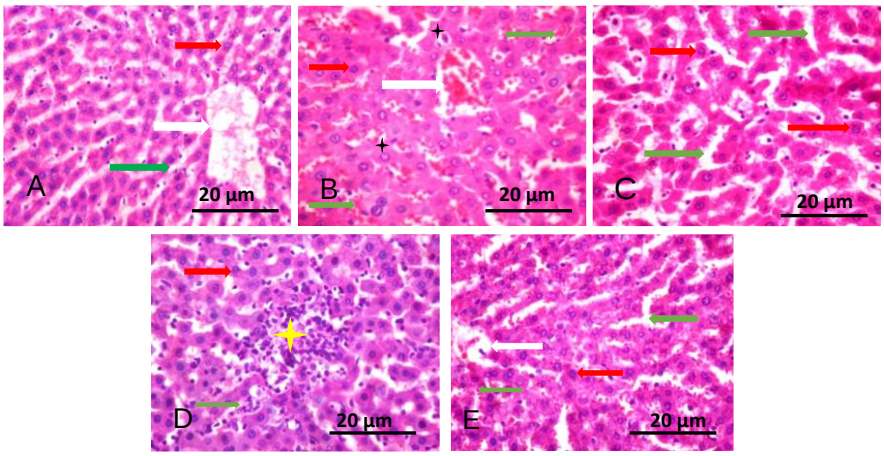

Figure 1. H and E-stained section of the liver of rats, with magnification of 400x

Description:

The central venules
Sinusoid
The hepatocytes
Vacuolation and necrosis
Mononuclear inflammatory cellular aggregate

A. Unexposed control rats: normal liver histoarchitecture devoid of lesions, the central venules, the sinusoid spaces and the hepatocytes appear normal.
B. Cypermethrin-exposed untreated rats: mildly congested central venules, vacuolation and necrosis is also evident in some of the hepatocytes while the morphology of other hepatocytes appears normal, the sinusoids are mildly dilated and congested.
C. Cypermethrin-exposed rats + 5000 mg.kg^{-1} bw ascorbate: hepatocytes with normal morphology, and mildly dilated sinusoid space evident.
D. Cypermethrin-exposed rats + 3000 mg.kg^{-1} bw alpha tocopherol: the liver parenchyma with focal area of mononuclear inflammatory cellular aggregate, the morphology of the hepatocytes appears normal, the sinusoids are dilated and mildly infiltrated by inflammatory cells.
E. Cypermethrin-exposed rats + 5000 mg.kg^{-1} bw ascorbate + 3000 mg.kg^{-1} bw alpha tocopherol: normal central venules, the morphology of the hepatocytes appears normal, the sinusoids appear normal and not infiltrated.

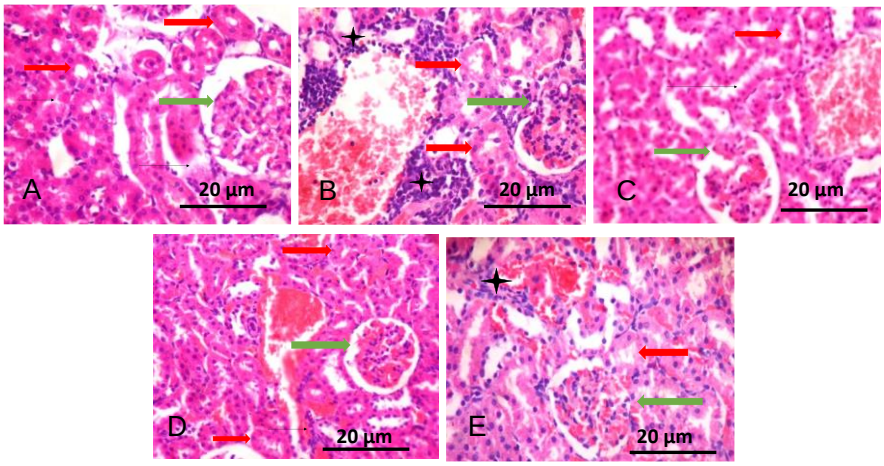

Figure 2. H and E-stained kidney sections with magnification of 400x

Description:

Normal glomeruli
Renal tubules
Mononuclear inflammatory cells
Interstium

A. Unexposed control rats: appeared normal, the renal cortex shows normal glomeruli, mesengial cells, and capsular spaces, with the renal tubules appearing normal, while the interstium are devoid of inflammation.
B. Cypermethrin exposed untreated rats: poor histoarchitecture (periglomerular inflammation with degenerated renal tubules); interstitium are congested and infiltrated with mononuclear inflammatory calls.
C. Cypermethrin-exposed rats + 5000 mg.kg^{-1} bw ascobate: the renal cortex consisting of a normal glomeruli, the renal tubules are normal, with the interstitium devoid of congestion or inflammation.
D. Cypermethrin-exposed rats + 3000 mg.kg^{-1} bw alpha-tocolpherol treated rats: moderate architecture with a normal glomerulus, the renal tubules are normal, while some regions of the interstitium are mildly inflammed.
E. Cypermethrin-exposed rats + 5000 mg.kg^{-1} bw ascorbate + 3000 mg.kg^{-1} bw alpha tocopherol: a normal glomeruli, the renal tubules and the interstitium are mildly congested and infiltrated with mononuclear inflammatory cells.

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Biochemical Findings

Aspartate Transferase and Alanine Transferase

The biochemical findings in this study revealed that cypermethrin-exposed untreated rats had elevated AST and ALT levels. Meanwhile, rats administered with ascorbate, alpha-tocopherol, or the combinative with both vitamins had reduced AST and ALT levels ($p<0.05$) (Table 1).

Creatinine

The creatinine values in the Cypermethrin-exposed, alpha-tocopherol, or co-administrative treatment groups were statistically insignificant ($p>0.05$). In contrast, rats treated with ascorbate had a significantly lower creatinine value ($p<0.05$) compared to the Cypermethrin-exposed untreated rats and other treatment groups (Table 2).

Oxidative stress markers in the liver samples

The oxidative stress parameters in the liver tissue homogenate samples revealed a disruption in the oxidant-antioxidant homeostasis as a result of cypermethrin exposure. While MDA levels were significantly elevated in the untreated cypermethrin-exposed rats, SOD, GPX, and CAT levels were significantly reduced ($p<0.05$). However, treatment with ascorbate, alpha-tocopherol, or co-administrative treatment with both vitamins alleviated the deleterious impact of cypermethrin by lowering MDA levels while increasing SOD, GPX, and CAT levels ($p<0.05$) (Table 3).

Oxidative Stress Markers in the Kidney Samples.

The oxidative stress parameters in the kidney homogenate samples revealed elevated MDA levels in the untreated cypermethrin-exposed rats, while SOD, GPX, and CAT values were significantly reduced ($p<0.05$). Furthermore, vitamin treatment mitigated the negative impact of cypermethrin by lowering MDA levels while increasing SOD, GPX, and CAT levels in the treated rats ($p<0.05$) (Table 4).

Table 1. Mean and standard deviation values of aspartate transferase and alanine transferase across all groups

Parameter(s)	Group 1	Group 2	Group 3	Group 4	Group 5
AST (mmol.L^{-1})	0.0752±0.046^{b}	0.1598±0.0228^{a}	0.1032±0.01994^{b}	0.1098±0.02168^{b}	0.0978±0.03339^{b}
ALT (mmol.L^{-1})	0.0628±0.057^{b}	0.3006±0.1144^{a}	0.1236±0.04403^{b}	0.114±0.05787^{b}	0.0674±0.04466^{b}

Notes: Group 1: control, Group 2: exposed untreated, group 3: ascorbate treated, group 4: alpha-tocopherol treated, group 5: ascorbate and alpha-tocopherol co-administered treated rats. a>b. ^{a} = (p = 0.000) ^{b} = (p = 0.012).

Table 2. Mean and standard deviation of Creatinine values across all groups

Parameter (s)	Group 1	Group 2	Group 3	Group 4	Group 5
Creatinine (µmol.L^{-1})	51.924±48.7243^{b}	95.48±8.31607^{a}	76.41±10.00598^{ab}	90.132±12.40513^{a}	89.626±19.41008^{a}

Notes: Group 1: control, Group 2: exposed untreated, group 3: ascorbate treated, group 4: alpha-tocopherol treated, group 5: ascorbate and alpha-tocopherol co-administered treated rats. a>ab>b. ^{a} = (p = 0.011), ^{ab} = (p = 0.019), ^{b} = (p = 0.027).

Table 3. Mean and standard deviation values of oxidative stress parameters in the liver homogenate samples across all groups

Parameter(s)	Group 1	Group 2	Group 3	Group 4	Group 5
MDA (µmol.L^{-1})	0.33±0.052^{c}	7.793±1.122^{a}	1.879±0.373^{b}	1.564±0.637^{b}	0.668±0.182^{c}
SOD (µmol.mL^{-1})	6.815±0.407^{b}	2.864±0.754^{d}	4.89±0.35^{c}	5.586±0.718^{c}	12.577±1.395^{a}
GPx (µmol.L^{-1})	84.741±2.782^{b}	45.589±2.612^{d}	56.683±3.02^{c}	58.33±2.536^{c}	113.735±8.892^{a}
CAT (µmol.L^{-1})	15.196±0.645^{b}	8.135±0.756^{d}	9.909±1.189^{cd}	11.48±1.225^{c}	28.168±4.065^{a}

Notes: Group 1: control, Group 2: exposed untreated, group 3: ascorbate treated, group 4: alpha-tocopherol treated, group 5: ascorbate and alpha-tocopherol co-administered treated rats a>b>c>cd>d. ^{a} = (p = 0.000), ^{b} = (p = 0.019), ^{c} = (p = 0.027), ^{cd} = (p = 0.032), ^{d} = (p = 0.042).

Table 4. Mean and standard deviation values of oxidative stress parameters in the kidney homogenate samples across all groups

Parameter(s)	Group 1	Group 2	Group 3	Group 4	Group 5
MDA (µmol.L^{-1})	0.211±0.051^{c}	5.315±0.765^{a}	0.905±0.339^{b}	0.867±0.164^{b}	0.664±0.354^{bc}
SOD (µmol.mL^{-1})	14.184±1.646^{a}	3.841±1.525^{d}	8.116±0.935^{c}	8.571±0.647^{c}	12.333±1.543^{b}
GPx (µmol.L^{-1})	64.522±6.737^{a}	21.767±1.2^{d}	30.638±2.729^{c}	32.448±2.229^{c}	46.322±2.143^{b}
CAT (µmol.L^{-1})	12.772±1.071^{a}	4.785±0.795^{c}	7.871±0.736^{b}	8.595±0.829^{b}	12.248±0.892^{a}

Notes: Group 1: control, Group 2: exposed untreated, group 3: ascorbate treated, group 4: alpha-tocopherol treated, group 5: ascorbate and alpha-tocopherol co-administered treated rats. a>b>bc>c>d. ^{a} = (p = 0.002), ^{b} = (p = 0.021), ^{bc} = (p = 0.029), ^{c} = (p = 0.035), ^{d} = (p = 0.045).

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DISCUSSION

Cypermethrin is a class II pyrethroid widely used in agriculture due to its efficacy. However, it is considered moderately toxic [16]. We investigated the histopathological, biochemical, and oxidative stress responses to cypermethrin exposure using murine models and the ameliorative potential of ascorbate and alpha-tocopherol when singly administered or in combination. The liver, being the most commonly targeted organ exposed to chemicals following absorption as well as the major site of metabolism, plays an integral role in the biotransformation and detoxification of chemicals [10]. This integral metabolic function played by the liver predisposes it to constantly coming into contact with these toxic chemicals, and long-term exposure could lead to derangement and predispose it to the development of several diseases.

The liver section of the unexposed control rats was normal, evidenced by an unaltered histoarchitecture with a non-congested central vein and hepatocytes with normal sinusoids, which is consistent with the findings of Soliman *et al.* [17]. Conversely, the cypermethrin-exposed untreated rats showed hepatocyte vacuolation and necrosis, additionally, the sinusoids were mildly dilated and congested [18].

The ascorbate-treated cypermethrin-exposed rats had better liver histoarchitecture than the untreated group, as evidenced by normal-appearing hepatocytes and sinusoidal spaces devoid of congestion or inflammation. In contrast, the alpha-tocopherol-treated group showed a focal area of mononuclear inflammatory cellular infiltration in the liver parenchyma with mildly inflamed sinusoidal space. Furthermore, the co-administered group had normal central veins and sinusoids devoid of inflammation and congestion.

Our findings align with Abdellatif *et al.* [19], who documented the beneficial impact of co-administration of ascorbate and alpha-tocopherol. Furthermore, as observed in our studies, cypermethrin can accumulate in the kidneys due to the lipophilic nature of the organ, resulting in severe renal damage such as tubular cell toxicity, inflammation, and nephrotoxicity [20]. While the kidney histoarchitecture of the unexposed control rats was devoid of lesions, the exposed untreated rats had poor kidney histoarchitecture as evidenced by periglomerular inflammation and degenerated renal tubule with congested and inflamed interstitial space [17].

The histoarchitecture of the ascorbate-treated rats was better relative to the cypermethrin-exposed untreated rats with a normal appearing glomerulus and interstitial space devoid of inflammation or congestion. Adikwu and Deo [18] previously reported on the hepatoprotective property of ascorbate, which has been linked to its antioxidant properties. The alpha-tocopherol treatment group had a moderate histoarchitecture, with some regions within the interstitial space inflamed. However, the renal tubules and the renal cortex appeared. The kidneys of the rats co-administered with the vitamins had a mildly congested and inflamed interstitial space, with the renal tubules and renal cortex appearing normal, which aligns with the observations of Abdellatif et al. [19].

When compared to the unexposed negative control rats, biochemical studies revealed elevated AST and ALT levels in the cypermethrin-exposed untreated group; however, single or co-administration of the vitamins reverted the enzyme activities in the rats, with the most significant reduction observed with the co-administration of ascorbate and alpha-tocopherol at standard doses of 5000 mg.kg-1 bw and 3000 mg.kg-1 bw. Studies by Mossa *et al.* [16] reported elevated liver AST and ALT in cypermethrin-exposed male mice, thus indicating the cytopathic effect of this type II cypermethrin. Additionally, elevated creatinine levels were observed in all exposed groups. However, vitamin administration reversed the creatinine values in the treatment group, with the most significant reduction observed in rats treated with ascorbate at 5000 mg.kg-1 bw, supporting ascorbate's mitigating potential in pyrethroid-induced toxicity [18,21].

The analysis of the oxidant-antioxidant levels in the liver homogenate samples from all groups revealed a derangement in the cypermethrin-exposed group. Significantly elevated MDA levels were observed in the untreated exposed rats, whereas co-administrative treatment with both vitamins reversed the MDA levels better than single administration. According to Chrustek *et al.* [6], cypermethrin exposure elevates MDA while decreasing antioxidant levels. A finding consistent with our findings confirms that cypermethrin can cause oxidative stress, corroborating the findings of Ullah et al. [22]. We observed that the SOD, GPX, and CAT values were better reversed in rats co-administered with the vitamins when compared to the other tests group (Table 3).

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The evaluation of oxidative stress parameters in kidney homogenate samples revealed elevated MDA levels in untreated exposed rats, with the most significant mitigating effect observed in rats co-administered with both vitamins. Furthermore, the GPX, SOD, and CAT values were elevated compared to the other test groups, supporting the beneficial effects of ascorbate and alpha-tocopherol previously reported [18].

CONCLUSION

Ascorbate and alpha-tocopherol co-administration mitigates cypermethrin-induced toxicity better. It also ameliorates the cytopathic effects on liver and kidney histoarchitecture and aids in reversing oxidant-antioxidant disruption in rats that are exposed to cypermethrin.

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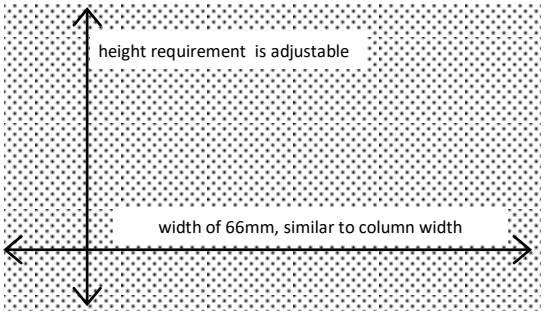

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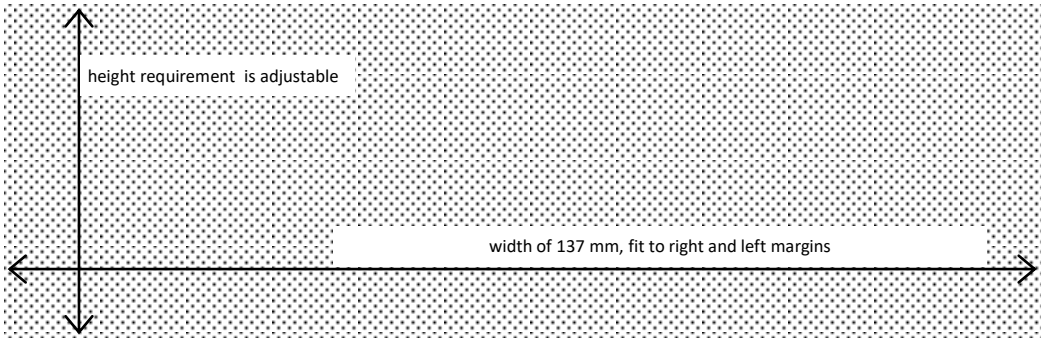

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ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

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