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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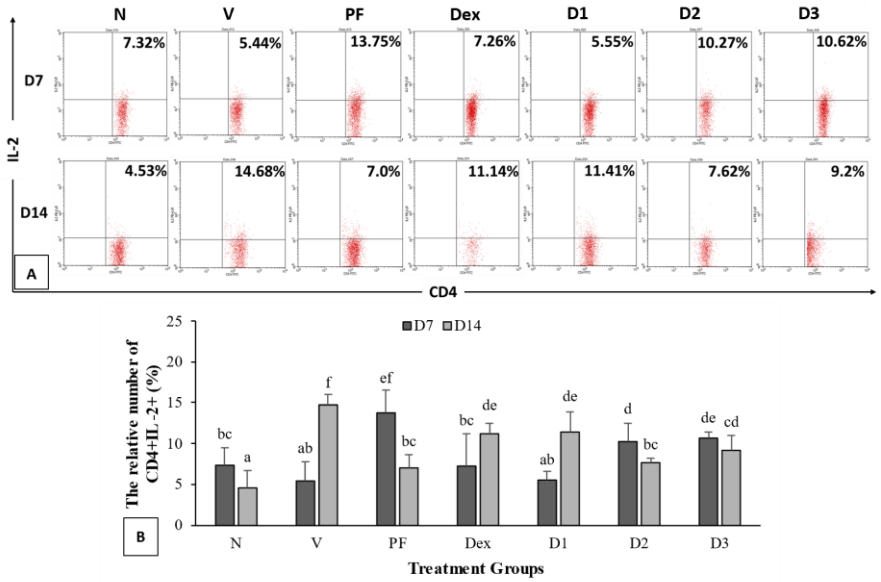

Figure 1. Flow cytometry analysis results of IL-2 produced by $CD4^+$ T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of $CD4^+IL\text{-}2^+$. (B) The bar chart showed the relative 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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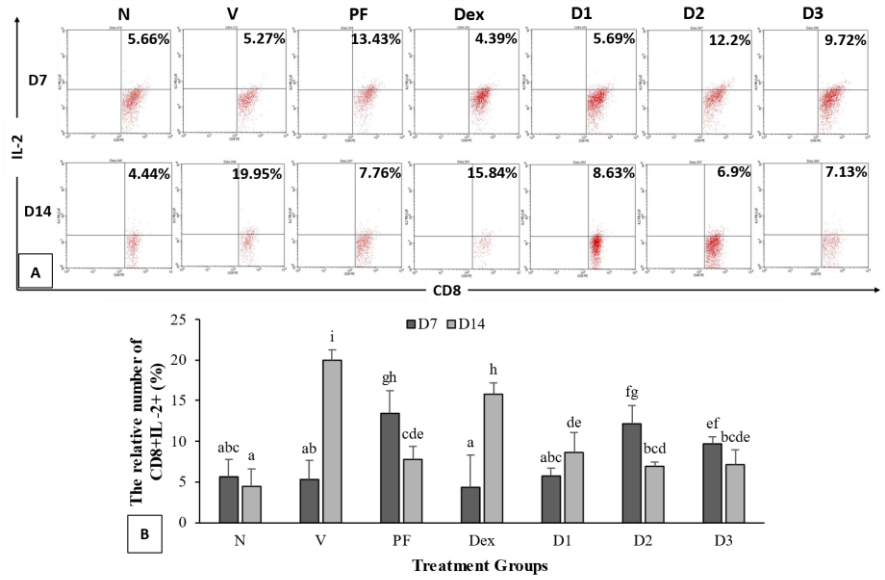

Figure 2. Flow cytometry analysis results of IL-2 produced by $CD8^+$ T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of $CD8^+IL\text{-}2^+$. (B) The bar chart showed the relative 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.

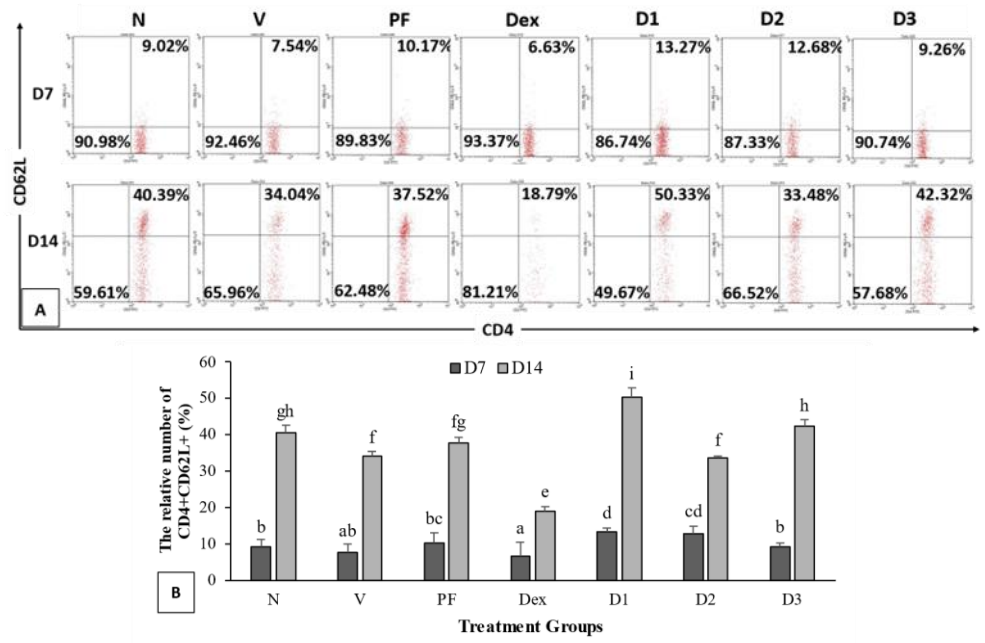

Figure 3. Flow cytometry analysis results of CD4¬ naive T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and *E. scaber* ethanol extract treatment. (A) Dot plot diagrams showed the relative number of CD4+CD62L+. (B) The bar chart showed the relative number of $CD4^{+}CD62L^{+}$ 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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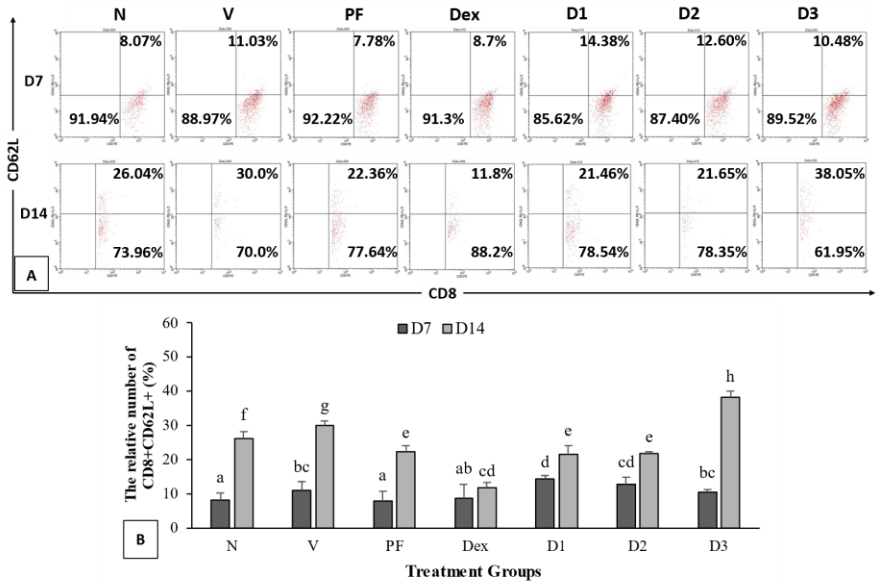

Figure 4. Flow cytometry analysis results of CD48 naive T cells in pulmonary fibrosis mice model after two weeks of bleomycin injection and E. scaber ethanol extract treatment. (A) Dot plot diagrams showed the relative number of CD8+CD62L+. (B) The bar chart showed the relative number of CD8+CD62L+ represented 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.

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