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).

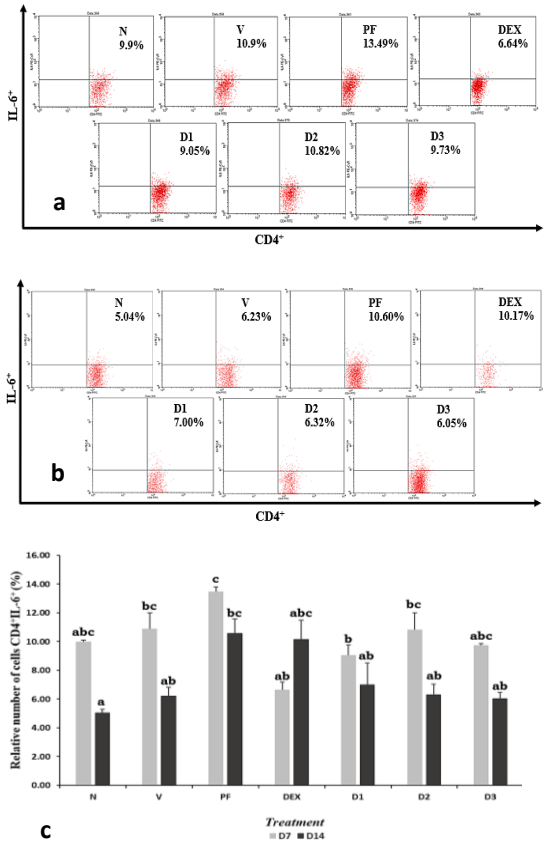

Figure 1. Relative number of $CD4^+IL\text{-}6^+$: (a) Dot plot diagrams showed the percentage of $CD4^+IL\text{-}6^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of CD4+IL-6+ cells for each 14-day treatment group; and (c) The representative bar showed the relative 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).

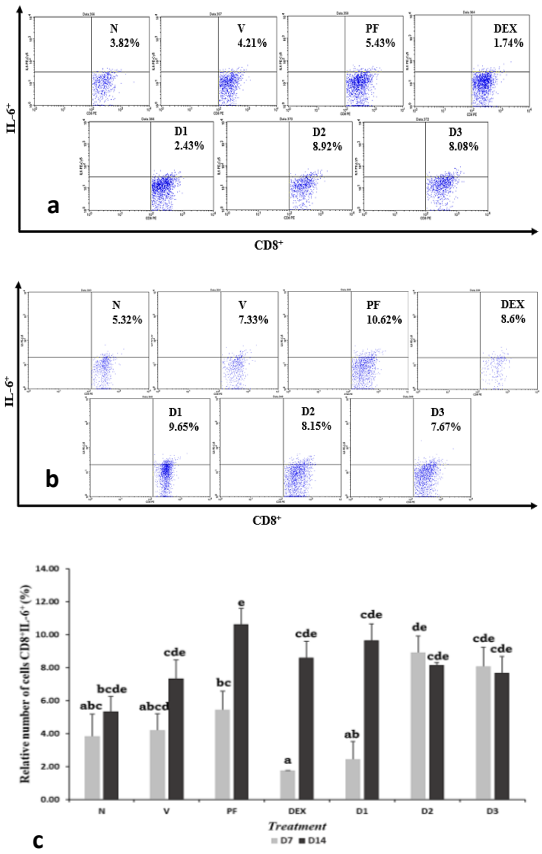

Figure 2. Relative number of $CD8^+IL\text{-}6^+$: (a) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}6^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}6^+$ cells for each 14-day treatment group; and (c) The representative bar showed 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).

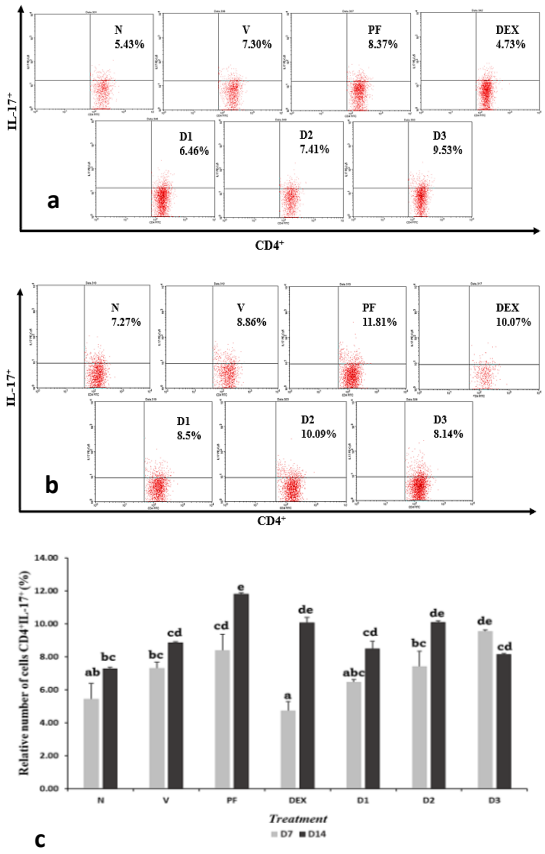

Figure 3. Relative number of CD4⁺IL-17⁺: (a) Dot plot diagrams showed the percentage of CD4⁺IL-17⁺ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of CD4⁺IL-17⁺ cells for each 14-day treatment group; and (c) The representative bar showed the relative number of CD4⁺IL-17⁺ 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].

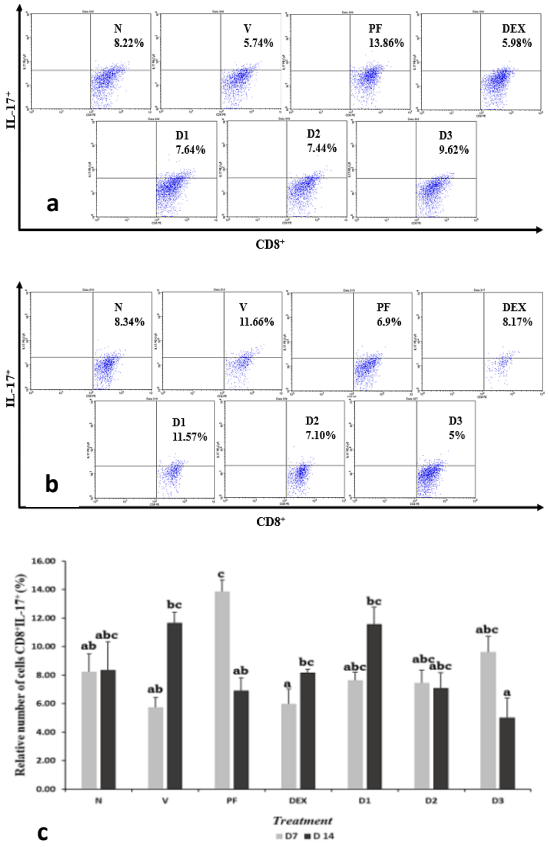

Figure 4. Relative number of $CD8^+IL\text{-}17^+$: (a) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}17^+$ cells for each 7-day treatment group; (b) Dot plot diagrams showed the percentage of $CD8^+IL\text{-}17^+$ cells for each 14-day treatment group; and (c) The representative bar showed 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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