Trop J Nat Prod Res, September 2024; 8(9):8554-8560
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Elephantopus scaber Ethanol Extract Suppresses Inflammation via Regulation of the NF-κB Pathway Expression in Pulmonary Fibrosis

Salma R. Firdausi[1], Riska A. R. Nur'aini[1], Fathiyah N. Izzah[1], Sarah N. Nabilah[1], Yuyun I. Christina[1,2,3], Dinia R. Dwijayanti[1,2,3], Sri Rahayu[1], Muhaimin Rifa'i[1,3], Muhammad S. Djati[1,2,3]*

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang 65145, East Java, Indonesia*
[2]*Research Center of Complementary Medicine and Functional Food, Brawijaya University, Malang 65145, East Java, Indonesia*
[3]*Dewan Jamu East Java Region, Malang 65145, East Java, Indonesia*

ARTICLE INFO

Article history:
Received 27 May 2024
Revised 29 June 2024
Accepted 21 July 2024
Published online 01 October 2024

ABSTRACT

Pulmonary fibrosis is a chronic inflammatory and progressive disease that scars and stiffens the lung, leading to organ function failure and death. There is no effective treatment to prevent this condition. *Elephantopus scaber* is a traditional medicinal plant known for its anti-inflammatory activity, but its prospective role in pulmonary fibrosis needs further investigation. This study aimed to investigate the effect of *Elephantopus scaber* ethanol extract (ESEE) treatment on the expression of nuclear factor kappa of activated B cell (NF-κB) and the production of interleukin (IL)-1β and IL-10 cytokines produced by macrophage and dendritic cells in mice after bleomycin exposure. Mice were randomly grouped and then received doses of ESEE: 0.0504 mg/kg (E1), 0.1008 mg/kg (E2), and 0.2016 mg/kg (E3) or 3 mg/kg dexamethasone (D) as positive control orally, followed by intraperitoneal injection of bleomycin (2 mg/kg) daily for 14 days. Mice were sacrificed on days 7 and 14, and then the splenocytes were isolated to determine the relative number of macrophage and dendritic cells expressing NF-κB, IL-1β, and IL-10 using flow cytometry. The results showed that NF-κB and IL-1β expressing cells in mice-induced bleomycin were significantly declined in all groups receiving ESEE compared to groups that received only bleomycin. However, IL-10-expressing cells, on the contrary, were increased in the ESEE treatment group. Treatment with 0.1008 mg/kg ESEE exhibited the most optimal regulating cytokine activity than the dexamethasone group. Therefore, ESEE treatment effectively suppresses inflammation in pulmonary fibrosis mice models.

Keywords: *Elephantopus scaber*, Inflammation, Pulmonary fibrosis, NF-κB pathway, Flow cytometry.

Introduction

Pulmonary fibrosis (PF) is a common condition in chronic obstructive pulmonary disease.[1] It is a chronic inflammatory lung disease that causes obstructed airflow from the lungs. This condition is typically caused by long-term exposure to irritating or hazardous gasses like cigarette smoke and air pollution. This condition can also be caused by virus infection.[2] The secretion of pro-inflammatory cytokines such as interleukin (IL)-1β, interleukin (IL)-6, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) by activated macrophages and dendritic cells as the body's first defense through the NF-κB pathway is known to increase at the beginning of inflammation.[3] This causes a strong tissue inflammatory response and can disrupt healthy tissue. The average age of affected patients is 60-70 years old, yet this condition can also affect younger people. PF also affects males more, considering the increased risk factors like smoking.[1]

Common pathological features in this condition are fibroblast proliferation and differentiation, abnormal deposition of many extracellular matrices (ECM), inflammatory cell infiltration, and destruction of alveolar structures.[4]

*Corresponding author. E-mail: msdjati@ub.ac.id
Tel: +62 81336668909

Citation: Firdausi SR, Nur'aini RAR, Izzah FN, Nabilah SN, Christina YI, Dwijayanti DR, Rahayu S, Rifa'I M, Djati MS. *Elephantopus scaber* Ethanol Extract Suppresses Inflammation via Regulation of the NF-κB Pathway Expression in Pulmonary Fibrosis. Trop J Nat Prod Res. 2024; 8(9): 8554-8560 https://doi.org/10.26538/tjnpr/v8i9.44

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

High production of ECM can lead to lung stiffness and loss of lung cell function, which can happen progressively.[5] Thus, it can cause difficulty breathing and even lead to death. Pulmonary fibrosis is a progressive disease that can be worsened by inadequate treatment. Most cases also need a longer time to be diagnosed. Until now, there are no efficient treatments for PF, and lung transplantation is the only option in most cases. Patients with a high degree of severity only rely on anti-fibrotic therapies.[1,6] However, anti-fibrotic therapy is costly and also has several adverse effects, including abdominal pain and abnormal liver function[6]. Treatment with new potential low-risk drugs is urgently needed to help more people with PF achieve good symptom control and better quality of life and reduce the risk of other associated conditions. Tapak liman or *Elephantopus scaber* is a herbaceous tropical plant widely used as a traditional medicine. It can be found in many tropical countries such as Africa, West Asia, India, South Asia, and Australia.[7] This plant was believed to help medical conditions like headache, cough, fever, hepatitis, and cancer.[8,9] Its leaves are known for containing secondary metabolites that are beneficial as antioxidants against free radicals. Essential oils like sesquiterpene lactone, triterpenoids, steroids, lupeols, and flavonoids are found in this leaf extract.[7] Deoxyelephantopin and isoscabertopin are active compounds belonging to the sesquiterpene lactone group found in *Elephantopus scaber* with anti-inflammatory and anti-cancer properties.[9,10,11] Previous studies have shown that it could act as NF-κB activation inhibitor *in vitro*.[10,12] However, the therapeutic effects of *Elephantopus scaber* on the pulmonary fibrosis mice model are yet to be investigated. Therefore, this study aimed to investigate the effect of ESEE on the expression of NF-κB, the production of pro-inflammatory cytokines IL-1β, and anti-inflammatory cytokines IL-10 by macrophage and dendritic cells in the early stages of bleomycin-induced pulmonary fibrosis in mice.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Animals

Fifty-six (56) 7-week-old BALB/c male mice weighing 25-30 g were used in this experiment. Animals were purchased from the Pusat Veteriner Farma, Surabaya, Indonesia. All animals were in healthy condition. Animals were acclimatized for 2 weeks in 12 h day and night light cycle, with an average temperature of 25°C before the experiment. Mice were given access to feed and water *ad libitum*. This study was ethically approved by the Animal Care and Use Committee of Universitas Brawijaya, Indonesia (NO:182-KEP-UB-2023).

Elephantopus scaber extract preparation

Dried *Elephantopus scaber* leaf powder was purchased and then identified by Balai Materia Medika, Batu, Indonesia, in March 2023 (determination number: 067/656/102.20/2023). A total of 500 g powdered leaves was extracted using 5,000 mL of 96% ethanol (ratio 1:10) in a glass jar and stored in a dark. The mixture was stirred continuously every hour for 24 h. After 24 h, the mixture was filtered using Whatman No. 1 filter paper to separate the filtrate. The filtrate was then processed with a Vacuum Pump Evaporator (IKA® RV10, Germany) to remove the solvent. The extract was then stored at 4°C in a closed pot until further use by the animals.

Experimental design

Mice were randomly divided into seven experimental groups (n = 4). The healthy control group (N); vehicle group (V) were given oral corn oil only; the fibrosis model group (F) received daily bleomycin (MedChemExpress LLC, USA) at a dose of 2 mg/kg BW[13] intraperitoneally for 7 days and 14 days; and the dexamethasone control group (D) that was received daily bleomycin at a dose of 2 mg/kg BW intraperitoneally and dexamethasone at a dose of 3 mg/kg BW orally every day for 7 days and 14 days. *E. scaber* ethanol extract (ESEE) treated group are low dose ESEE group (E1) that was treated with daily oral ESEE at a dose of 0.0504 mg/kg BW and intraperitoneal bleomycin (2 mg/kg BW); moderate dose ESEE group (E2) that was treated with daily oral ESEE at a dose of 0.1008 mg/kg BW and intraperitoneal bleomycin (2 mg/kg BW); and high dose ESEE group (E3) that was treated with daily oral ESEE at a dose of 0.2016 mg/kg BW and intraperitoneal bleomycin (2 mg/kg BW). ESEE was dissolved in corn oil and given orally. Bleomycin was dissolved in PBS following the manufacturer's instruction. Mice were sacrificed on days 7 and 14 by cervical dislocation.[14,15]

Organ isolation and flow cytometry analysis

After the mice were sacrificed, spleens were washed in PBS twice. The spleen was dissected and crushed clockwise in 5 mL PBS to isolate the splenocytes. Cells suspension was centrifuged at 2500 rpm and 10°C for 5 min. Supernatants were discarded, and 1 mL of PBS was added and used to homogenate the pellets. Then, 0.05 mL cells were moved to microtubes to perform antibody staining. For macrophage and dendritic cell markers, extracellular antibody staining was carried out by adding 0.05 mL of FITC-labeled anti-CD11b and PE-labeled anti-CD11c extracellular antibodies to the microtube, then incubated at 4°C in a dark place for 20 min. Then, intracellular antibody staining was performed by adding 0.05 mL of intracellular fixation buffer (IC) and incubating at 4°C in the dark for 20 min. Next, 0.5 mL of permeabilization buffer 10X (PB 10X) was added and centrifuged at 2500 rpm at 10°C for 5 min. Next, the supernatant was discarded, and 0.05 mL of specific intracellular antibodies PerCP-labeled NF-κB, PE-Cy5-labeled anti-IL-1β, and PE-labeled anti-IL-10 were added to the microtube, then incubated at 4°C in the dark for 20 min. Next, 0.4 mL of PBS was added to the samples, homogenized, and transferred into a cuvette for flow cytometry analysis (BD Biosciences FACSCalibur™, San Jose, CA). The data were analyzed using the BD CellQuest™ Pro application to obtain the relative number of $CD11b^+NF\text{-}\kappa B^+$, $CD11b^+IL\text{-}1\beta^+$, $CD11b^+IL\text{-}10^+$, $CD11c^+NF\text{-}\kappa B^+$, $CD11c^+IL\text{-}1\beta^+$, and $CD11c^+IL\text{-}10^+$ cells.

Statistical analysis

Statistical analysis was performed using SPSS 25 for Windows (SPSS Inc., Armonk, NY, USA). Statistical significance was analyzed by two-way ANOVA followed by Duncan multiple comparison tests with $p<0.05$, which was considered significant. Data were presented as mean ± standard deviation.

Results and Discussion

Effect of ESEE treatment on NF-κB expression by macrophage and dendritic cells

Pulmonary fibrosis is a fast-progressing disease with no effective treatment. It is initiated by massive inflammation that later induces irregular tissue restoration characterized by high extracellular matrix secretion. Therefore, research in pulmonary fibrosis has focused on targeting pro-inflammatory cytokines. Traditional plant medicine exploration is preferred nowadays due to its composition of multifunctional compounds. NF-κB is a crucial transcription factor linked closely to inflammation and immunity. Its activation has a significant role in regulating inflammatory response.[16] *Elephantopus scaber* is a traditional plant medicine containing high sesquiterpenes lactones, such as deoxyelephantopin and isodeoxyelephantopin, known as NF-κB activation inhibitor.[17] Sesquiterpenes lactones contained in ESEE inhibit the translocation of NF-κB complex to the nucleus, resulting in lower transcription of inflammatory mediators, such as IL-1β, IL-6, and TNF-α cytokines. Macrophage and dendritic cells have a major role in the innate immune system. Both cells, phagocytes, and present antigens secrete inflammatory mediators to recruit more immune cells to the damaged site. Therefore, this study is needed to investigate the effect of ESEE treatment on NF-κB expression, its downstream cytokine IL-1β secretion, and anti-inflammatory cytokine IL-10 secretion in macrophages and dendritic cells in early bleomycin lung injury.

The result demonstrated that there were significant differences ($p<0.05$) in the relative number of $CD11b^+NF\text{-}\kappa B^+$ in the healthy mice (N) and fibrosis group (F) on day 7 after bleomycin induction. The standard drug control group with dexamethasone (D) could not ($p>0.05$) decrease the relative number of $CD11b^+NF\text{-}\kappa B^+$ cells compared to the fibrosis group (F). All doses of the ESEE treatment group exhibited a significant decrease in the relative number of $CD11b^+NF\text{-}\kappa B^+$ cells compared to the fibrosis model group (F). Interestingly, the highest decrease in the relative number of $CD11b^+NF\text{-}\kappa B^+$ cells was found in the E2 group ($p<0.05$) than in other ESEE groups (Figure 1A, 1C). Furthermore, after 14 days of bleomycin induction, the E2 group significantly reduced the relative number of $CD11b^+NF\text{-}\kappa B^+$ cells than other ESEE groups. A significant reduction in the expression of NF-κB was also found in fibrosis mice after 14 days of dexamethasone treatment. These results indicated that the administration of ESEE in fibrosis mice could reduce NF-κB expression.

The present study indicated that bleomycin injection for 7 days could induce more $CD11b^+NF\text{-}\kappa B^+$ cells than healthy mice. It was indicated that there was an inflammatory response after bleomycin exposure in lung cells. Bleomycin induces DNA oxidative damage in lung cells and provokes programmed cell death or apoptosis.[18] Apoptotic lung cells act as damage-associated molecular patterns (DAMPs) signals to innate immune cells, such as macrophage and dendritic cells, so they can eliminate damaged cells and secrete inflammatory mediators.[19] Increasing NF-κB expression is the marker of this process. Increasing NF-κB expression resulted in increasing production of IL-1β, which later contributes to the pro-fibrosis environment by inducing the Epithelial-Mesenchymal Transition process (EMT) of fibroblast cells to myofibroblast cells that secrete massive extracellular matrices to lung tissue, and later formed a fibrotic mass deposition.[5,18,19] Interestingly, all ESEE doses treatment could suppress NF-κB expression in macrophage cells, with the most significant decrease found in moderate doses (E2) (0.1008 mg/kg BW).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

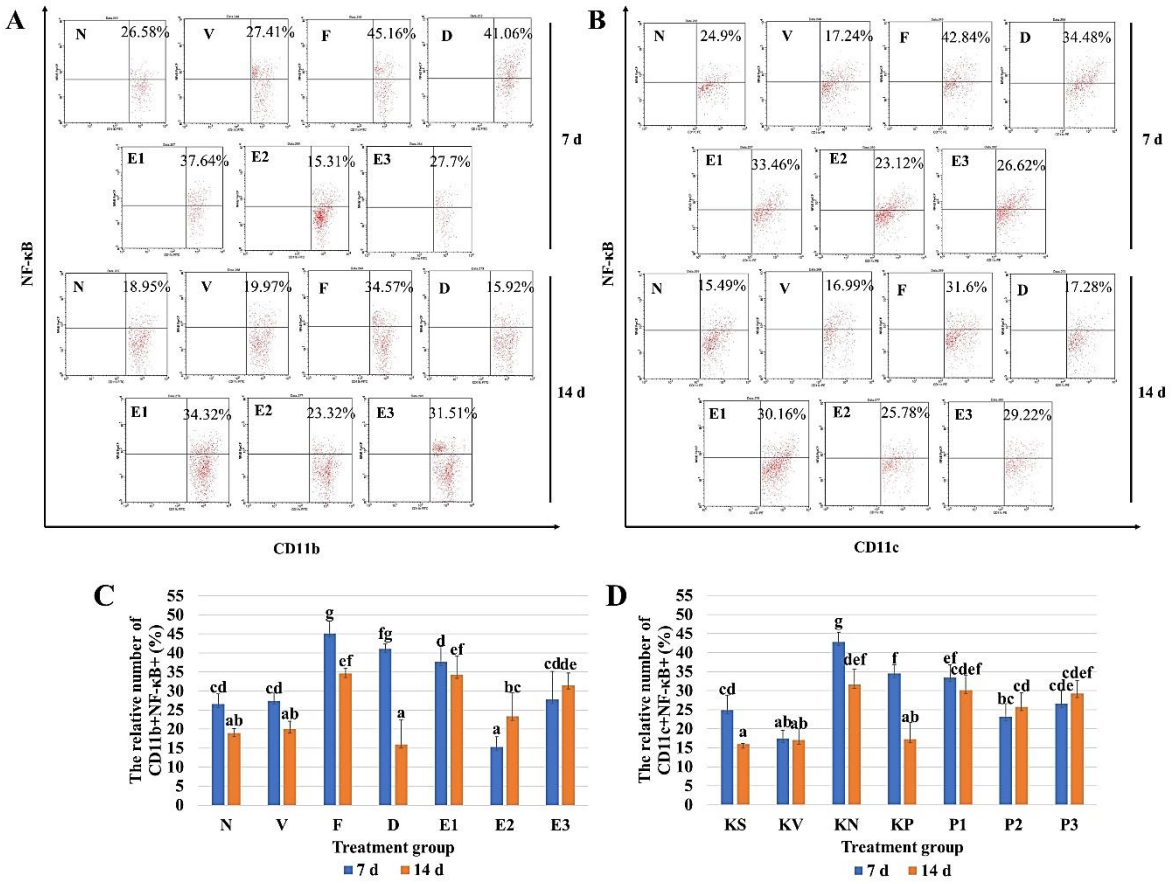

Figure 1: Flow cytometry analysis results of CD11b$^+$NF-κB$^+$ and CD11c$^+$NF-κB$^+$ cell populations on days 7 and 14, evaluated by flow cytometry analysis. **(A, B)** Dot plot diagrams showed the percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. **(C, D)** Chart data showed the mean percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. The data were presented as mean ± standard deviation (SD), and different notations showed a significant difference based on the Duncan HSD test ($p<0.05$). N: healthy control; V: vehicle control; F: fibrosis model with bleomycin (2 mg/kg); D: dexamethasone treatment (3 mg/kg); E1: ESEE low dose treatment (0.0504 mg/kg); E2: ESEE moderate dose treatment (0.1008 mg/kg); E3: ESEE high dose treatment (0.2016 mg/kg)

In contrast, dexamethasone treatment showed no significant effect in suppressing NF-κB on day 7. However, on day 14, dexamethasone treatment possessed a lower NF-κB expression. This delayed effect of dexamethasone may be due to the repetitive injury in alveolar epithelial cells caused by bleomycin. This result was also found in a previous study by Vega *et al*.[20], which states that dexamethasone could not ameliorate acute alveolar injury after bleomycin exposure in terms of immune cell infiltrations and lung edema due to its remodeling activity in membrane lamellipodia and filopodia that cause irregular cell repair process. Nonetheless, moderate dose ESEE treatment (E2) showed the best result in suppressing NF-κB expression. Tashiro *et al*. (2017) stated that inflammation response occurred from day 3 to 9 after bleomycin's first injury, followed by fibroblast activation and matrix deposition at day 10 to 21, indicating fibrosis initiation.[21] Therefore, ESEE could early suppress NF-κB expression in macrophage cells.

This study also investigates the expression of NF-κB in mature dendritic cells. The result showed significant differences in CD11c$^+$NF-κB$^+$ cell populations between the fibrosis model and healthy control groups, indicating the increase of NF-κB expression at day 7 in response to bleomycin exposure. Dexamethasone (D) and the E1 group showed a significant decrease in cell populations compared to the fibrosis group but still higher than other ESEE treatment groups, indicating weak suppression activity. Besides, the E2 group showed optimum suppression activity, resulting in a significant decrease of CD11c$^+$NF-κB$^+$ cell populations compared to the fibrosis model group (F). At day 14, all ESEE treatment groups showed an insignificant change in cell population compared to day 7, indicating continuous inflammation.

In early lung tissue damage, inactivated resident dendritic cells attract DAMP signals released by injured lung cells. Inactivated dendritic cells then phagocytes cell debris, then migrate to the lymph nodes to present the antigen to T cells and become activated.[22] A recent study found that immature dendritic cells tend to infiltrate lung fibrosis sites massively, leading to continuous chronic tissue inflammation and in situ activation.[23] These explain the decrease in cell population in the fibrosis group on day 14. The result also showed no significant change in the relative number of CD11c$^+$NF-κB$^+$ cells at all doses of ESEE on day 14 compared to day 7. Although longer ESEE treatment did not show a significant decrease in the CD11c$^+$NF-κB$^+$ cell population, ESEE treatment nonetheless showed its benefit in preventing worse inflammation.

Effect of ESEE treatment on IL-1β production by macrophage and dendritic cells

IL-1β cytokine is one of the key markers of inflammation.[24] The existence of unknown substances or damaged cells triggers its secretion through TLRs/ NF-κB pathway activation. Activation of the NF-κB pathway results in translocation of its dimer structure to the nucleus, then induces proIL-1β cytokine and NLRP3 inflammasome release to the cytoplasm. NLRP3 inflammasome then combined with procaspase-1 and formed an inflammasome complex. Inflammasome complex also induces autocleavage of caspase-1, and later, it will induce maturation of proIL-1β and its release to the extracellular environment as IL-1β.[25] As the result of the process mentioned, higher NF-κB activation results in higher IL-1β cytokine maturation and secretion. IL-1β cytokine secretion by macrophage cells also indicates its polarization to the M1

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

type, which is known to act pro-inflammatory by secreting more inflammatory cytokines.[26] Increasing numbers of IL-1β leads to massive inflammation in the lung tissue and creates a pro-fibrotic environment as IL-1β induces tumor growth factor (TGF)-β secretion and promotes the EMT process, which results in myofibroblast activation.[27] Activated myofibroblast, known as the main secretor of the extracellular matrix, led to fibrosis development in the tissue.

This study showed that the relative number of $CD11b^+IL\text{-}1\beta^+$ increased significantly ($p<0.05$) in the fibrosis model group (F) at day 7 compared to the healthy control group (N) and vehicle control group (V). This result was in line with the high expression of NF-κB because of IL-1β as downstream from NF-κB pathway. Treatment with different doses of ESEE for 7 days indicates significant ($p<0.05$) alleviation of IL-1β production in the fibrosis mice model, with no significant difference between the E1, E3, and dexamethasone group (D). Interestingly, E2 showed the best and most significant ($p<0.05$) alleviation of $CD11b^+IL\text{-}1\beta^+$ cell number (Figure 2A, 2C). Moreover, on day 14, all groups showed no significant average changes with day 7, but slight decreases were found in the F, D, and E1 groups, and in contrast with that, the E2 and E3 groups showed a slight average increase in cell percentage. Thus, these results suggested that peak inflammation caused by bleomycin happened on day 7, and ESEE treatment in proper time showed more benefit in regulating initial inflammation.

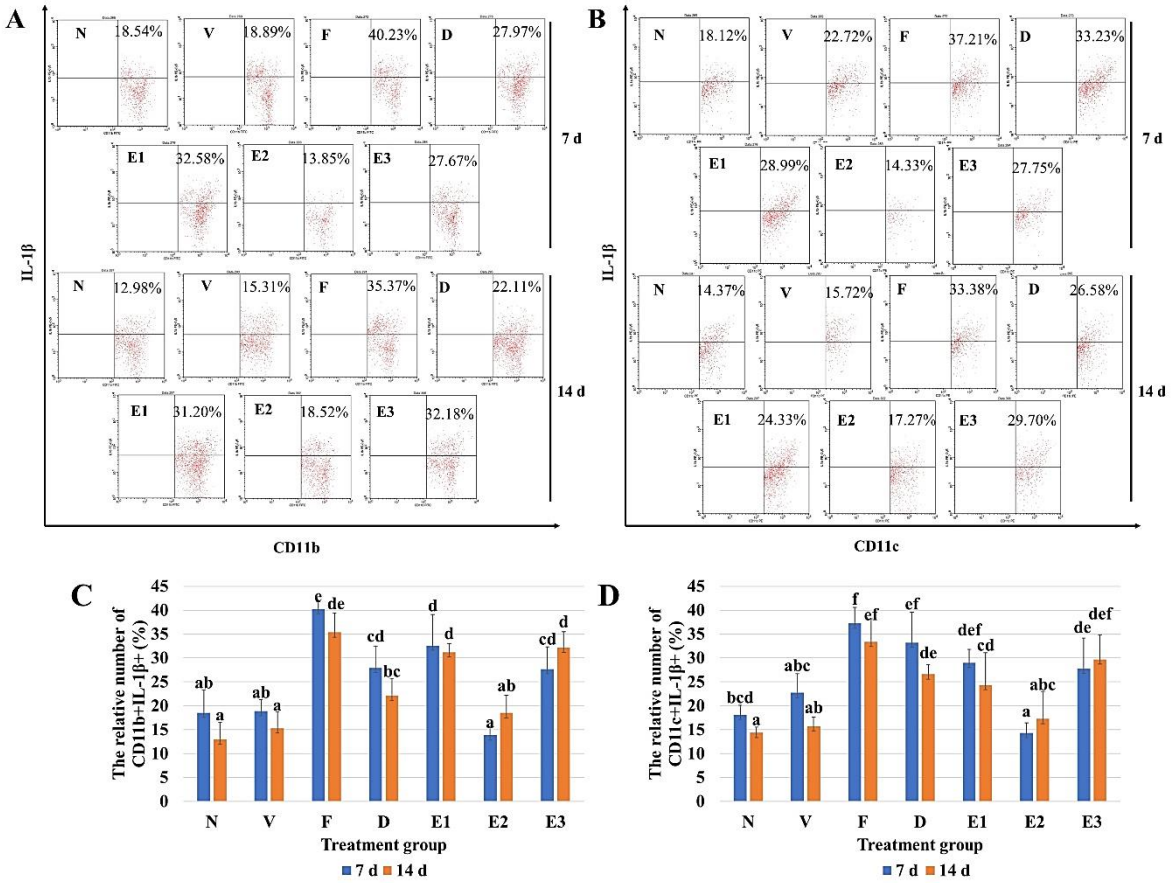

Figure 2: Flow cytometry analysis results of $CD11b^+IL\text{-}1\beta^+$ and $CD11c^+IL\text{-}1\beta^+$ cell populations on days 7 and 14, evaluated by flow cytometry analysis. **(A, B)** Dot plot diagrams showed the percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. **(C, D)** Chart data showed the mean percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. The data were presented as Mean ± standard deviation (SD), and different notations showed a significant difference based on the Duncan HSD test ($p<0.05$). N: healthy control; V: vehicle control; F: fibrosis model with bleomycin (2 mg/kg); D: dexamethasone treatment (3 mg/kg); E1: ESEE low dose treatment (0.0504 mg/kg); E2: ESEE moderate dose treatment (0.1008 mg/kg); E3: ESEE high dose treatment (0.2016 mg/kg)

A significant increase ($p<0.5$) in the production of IL-1β by the dendritic cells was observed in the fibrosis model group (F), indicating activation of the dendritic cells due to bleomycin induction for 7 days (Figure 2B, 2D). On the other hand, there was no significant difference between dexamethasone and a low dose of ESEE treatment with the model group, indicating its weak action in alleviating IL-1β production by the dendritic cells. Interestingly, the E2 group showed the best IL-1β suppression. The inhibitory activity of ESEE to activate NF-κB might be caused by inhibiting IKK complex phosphorylation, which leads to unsuccessful NF-κB translocation to the nucleus and proIL-1β gene transcription.[28] A lower percentage of activated dendritic cells in the E2 group also indicates advantageous $CD4^+$ T cell activation inhibition, as the $CD4^+$ T cell will further produce pro-inflammatory cytokine and pro-fibrotic cytokine-like IL-17A.[29] On the contrary, the overall group result on day 14 showed no significant difference from day 7, which might be caused by the same daily repetitive small exposure to the bleomycin, resulting in precarious resolution of the injury.

Effect of ESEE treatment on IL-10 production by macrophage and dendritic cells

As the bleomycin exposure to mice induces an inflammatory response marked by more secretion of IL-1β, recent research on pulmonary fibrosis targets the downregulation of pro-inflammatory cytokine. Many previous studies stated that suppressing inflammation by enhancing anti-inflammatory cytokines in early lung injury helped develop fibrosis. One of the anti-inflammatory cytokines broadly explored in pulmonary fibrosis development is IL-10, as it inhibits the downregulation of IFN-γ and upregulation of TGF-β.[30] Inhibition of TGF-β upregulation is essential in decreasing fibrosis development as TGF-β is known to maintain inflammation and acts as a pro-fibrotic

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

cytokine by activating myofibroblast as the result of the EMT process. Myofibroblast is a cell with few epithelial and endothelial features and contains α-smooth muscle actin (α-SMA). This activated cell will secrete massive amounts of extracellular matrix components such as collagen and fibrin, thereby initiating fibrosis development.[31] IL-10 secretion by macrophage cells also indicates its alternative polarization to M2 type, whose role is still poorly understood in pulmonary fibrosis. However, some studies also revealed its anti-fibrotic role by secreting matrix metalloproteinase-10 (MMP-10) to degrade excessive ECM in lung tissue during repair.[32]

On the first week of bleomycin treatment, the fibrosis model group showed significant ($p<0.05$) higher IL-10 expression by macrophage cells than the healthy group (Figure 3A). Treatment with dexamethasone showed a slight increase in the production of IL-10 secreted by macrophage, which indicated weak anti-inflammatory activity. Different doses of ESEE at 7 days also did not show a significant increase of IL-10 in the model group. In contrast, ESEE treatment for 14 days showed a significant ($p<0.05$) effect in inducing IL-10 production secreted by macrophages. Higher IL-10 level indicates an inflammation suppression phase after bleomycin induction by inhibiting further release of IL-1β and TGF-β. Interestingly, the E2 group showed the highest relative number of $CD11b^+IL\text{-}10^+$ cells compared to other groups after 14 days of treatment (Figure 3C).

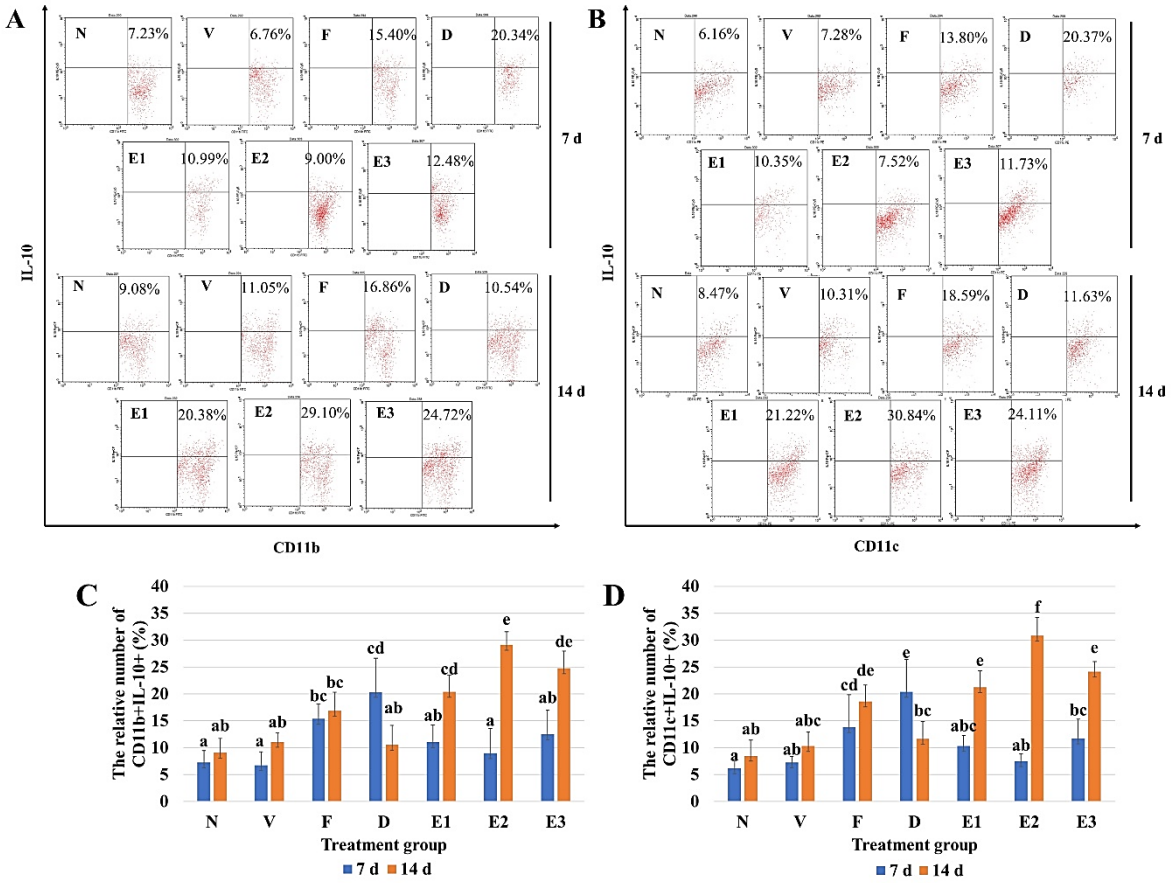

Figure 3: Flow cytometry analysis results of $CD11b^+IL\text{-}10^+$ and $CD11c^+IL\text{-}10^+$ cell populations on days 7 and 14, evaluated by flow cytometry analysis. **(A, B)** Dot plot diagrams showed the percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. **(C, D)** Chart data showed the mean percentage of cell populations after being treated with bleomycin and various doses of *E. scaber* ethanol extract for 7 and 14 days. The data were presented as mean ± standard deviation (SD), and different notations showed a significant difference based on the Duncan HSD test ($p<0.05$). N: healthy control; V: vehicle control; F: fibrosis model with bleomycin (2 mg/kg); D: dexamethasone treatment (3 mg/kg); E1: ESEE low dose treatment (0.0504 mg/kg); E2: ESEE moderate dose treatment (0.1008 mg/kg); E3: ESEE high dose treatment (0.2016 mg/kg)

This study demonstrated fewer $CD11c^+IL\text{-}10^+$ cell populations in the fibrosis model group than in the healthy and vehicle group on day 7 (Figure 3B). However, all ESEE treatment groups for 7 days could not significantly increase the IL-10 production. Moreover, treatment with dexamethasone (D) for 7 days exhibited a significant ($p<0.05$) increase in IL-10 production but then decreased after 14 days of treatment (Figure 3D). Interestingly, all ESEE treatment groups increased ($p<0.05$) $CD11c^+IL\text{-}10^+$ cell population after 14 days of treatment, with the highest number in the E2 group. That indicates dendritic cells dominate tolerogenic function during injury. In previous studies, Lupeol and flavonoid substances contained in ESEE might also play a role in that process, as both showed anti-inflammatory and anti-fibrotic activity.[33,34] Dexamethasone treatment showed a fluctuating effect in stimulating IL-10 production, as shown by the decrease in $CD11c^+IL\text{-}10^+$ cell population after 14 days of treatment.

A dendritic cell is known for its leading role as an antigen-presenting cell and an initiator of T cell response. Activated dendritic cells by stimuli like inflammatory cytokines or damage-associated molecular patterns (DAMPs) will process and present antigens on their surface to be later recognized by naïve T cells and activated. In early injury, dendritic cells can also secrete pro-inflammatory cytokines like IL-1β and Il-6 to spread the signal faster. Despite that, the dendritic cell is also known for its flexibility in shifting between immunogenic and tolerogenic functions.[35] Dendritic cells could increase IL-10 production and promote regulatory T-cell differentiation to help maintain an immune homeostasis environment.

In pulmonary fibrosis, cytokines, especially those produced by macrophage and dendritic cells, are mainly involved in initiating and regulating the inflammation process through their distinct function and ability to stimulate immune cells.[36] Besides inflammation suppression,

cytokine regulation in tissue remodeling after injury is also important to prevent fibrosis development. Beneficial secondary metabolites in ESEE, like sesquiterpene lactones, flavonoids, and lupeol substances in traditional plants, must be explored. This study revealed that ESEE could prove its benefits in counteracting the activation of NF-κB, thereby further regulating the secretion and maturation of pro-inflammatory cytokine IL-1β and maintaining sufficient secretion of anti-inflammatory cytokine IL-10. These results indicated that ESEE treatment potentially protects the lung in the fibrosis mice model by suppressing NF-κB expression and its further downstream in macrophage and dendritic cells.

Conclusion

ESEE could suppress early inflammatory response after bleomycin injury in fibrosis mice models via regulation of the NF-κB pathway by decreasing NF-κB expression and IL-1β production and increasing IL-10 production by macrophage and dendritic cells with the optimum ESEE dose at 0.1008 mg/kg. Further research on the fibrous mass protein expression is required to clearly understand the ESEE's inhibitory mechanism against fibrosis development.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

All authors thank the members of the Animal Physiology, Structure, and Development Laboratory for technical support and discussion. This work was supported by the Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia, through a professor research grant (Grant number. 4158.4/UN10.F09/PN/2023).

References

1. Maher TM, Bendstrup E, Dron L, Langley J, Smith GE, Khalid JM, Patel H, Kreuter M. Global incidence and prevalence of idiopathic pulmonary fibrosis. Respir Res. 2021; 22(197):1–10.
2. Hasan M, Paul NC, Paul SK, Saikat ASM, Akter H, Mandal M, Lee SS. Natural Product-Based Potential therapeutic interventions of pulmonary fibrosis. Molecules. 2022; 27(5):1481.
3. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017; 9(6):7204–7218.
4. Yuan S, Tse WM, Tse KWG, Xu F, Xie H, Aga EB, Xiong H, Ye B. Ethanol extracts of bulbus of *Fritillaria cirrhosa* protects against pulmonary fibrosis in rats induced by bleomycin. J Funct Foods. 2022; 97(105239):1–10.
5. White ES. Lung extracellular matrix and fibroblast function. Ann Am Thorac Soc. 2015; 12(suppl 1):S30–S33.
6. Fernández-Colino A, Iop L, Ferreira MSV, Mela P. Fibrosis in tissue engineering and regenerative medicine: treat or trigger?. Adv Drug Deliv Rev. 2019; 146:17–36.
7. Hiradeve SM and Rangari VD. A review on pharmacology and toxicology of *Elephantopus scaber* Linn. Nat Prod Res. 2014; 28(11):819–830.
8. Christina YI, Rifa'i M, Widodo N, Djati MS. The combination of *Elephantopus scaber* and *Phaleria macrocarpa* leaves extract promotes anticancer activity via downregulation of ER-α, Nrf2 and PI3K/AKT/mTOR pathway. J Ayurv Integr Med. 2022; 13(4):1–14
9. Wang J, Li P, Li B, Guo Z, Kennelly EJ, Long C. Bioactivities of Compounds from *Elephantopus scaber*, an Ethnomedicinal Plant from Southwest China. Evid-Based Complement Alternat Med. 2014; 2014(1):1-7.
10. Pandey V, Tripathi A, Rani A, Dubey PK. Deoxyelephantopin, a novel naturally occurring phytochemical impairs growth, induces G2/M arrest, ROS-mediated apoptosis and modulates lncRNA expression against uterine leiomyoma. Biomed Pharmacother. 2020; 131(110751):1-13.
11. Pan L, Liangyu H, Zhang L, Xu H, Chen Y, Bian Q, Zhu A, Wu H. Deoxyelephantopin decreases the release of inflammatory cytokines in macrophage associated with attenuation of aerobic glycolysis via modulation of PKM2. Int Immunopharmacol. 2020; 79(106048):1–7.
12. Djati MS, Christina YI, Dwijayanti DR, Rahayu S. Synergistic modulation of proinflammatory mediators and cytokines in lipopolysaccharide-activated RAW 264.7 macrophages: The therapeutic potential of *Elephantopus scaber* and *Sauropus androgynus* ethanol extract. Vet World. 2024; 17(3):728–734.
13. Kim SN, Lee JS, Yang HS, Cho JW, Kwon S, Kim Y, Her JD, Cho KH, Song CW, Lee K. Dose-response effects of bleomycin on inflammation and pulmonary fibrosis in mice. Toxicol Res. 2010; 26(3):217–222.
14. Christina YI, Diana MR, Fuzianingsih EN, Nurhayati N, Ridwan FN, Widodo N, Rifa'i M, Djati MS. Hormone-balancing and protective effect of combined extract of *Sauropus androgynus* and *Elephantopus scaber* against Escherichia coli-induced renal and hepatic necrosis in pregnant mice. J Ayurv Integr Med. 2021; 12(2):245–253.
15. Rahayu S, Annisa R, Anzila I, Christina YI, Soewondo A, Marhendra APW, Djati MS. *Marsilea crenata* ethanol extract prevents monosodium glutamate adverse effects on the serum levels of reproductive hormones, sperm quality, and testis histology in male rats. Vet World. 2021; 14(6):1529–1536.
16. Ernst O, Vayttaden SJ, Fraser IDC. Measurement of NF-*K*B activation in TLR-Activated macrophages. In: Methods Mol Biol. 2018; 1714:67–78 p.
17. Amalia R, Widyarti S, Djati MS. The Effect of *E. scaber* Extract to TNF-α and TGF-β on Mice under Carcinogen Treatment. J Exp Life Sci. 2018; 8(2):75–82.
18. Mungunsukh O, Griffin AJ, Lee YH, Day RM. Bleomycin induces the extrinsic apoptotic pathway in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2010; 298(5):L696–L703.
19. Liu X, Cao H, Li J, Wang B, Zhang P, Zhang XD, Liu Z, Yuan H, Zhan Z. Autophagy induced by DAMPs facilitates the inflammation response in lungs undergoing ischemia-reperfusion injury through promoting TRAF6 ubiquitination. Cell Death Differ. 2017; 24(4):683–693.
20. Vega MA, Chupin C, Pascariu M, Privé A, Dagenais A, Berthiaume Y, Brochiero E. Dexamethasone fails to improve bleomycin-induced acute lung injury in mice. Physiol Rep. 2019; 7(21):1–12.
21. Tashiro J, Rubio GA, Limper AH, Williams KJ, Elliot SJ, Ninou I, Aidinis V, Tzouvelekis A, Glassberg MK. Exploring

animal models that resemble idiopathic pulmonary fibrosis. Frontiers Med. 2017; 4(118):1–11.
22. Martín-Fontecha A, Lanzavecchia A, Sallusto F. Dendritic cell migration to peripheral lymph nodes. In: Handb Exp Pharmacol. 2008; 188:31–49 p.
23. Bocchino M, Zanotta S, Capitelli L, Galati D. Dendritic cells are the intriguing players in the puzzle of idiopathic pulmonary fibrosis pathogenesis. Frontiers Immunol. 2021; 12(664109):1–8.
24. Borthwick LA. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. Semin Immunopathol. 2016; 38(4):517–534.
25. Liu D, Zhong Z, Karin M. NF-κB: A Double-Edged Sword Controlling Inflammation. Biomedicines. 2022; 10(6):1250.
26. Kishore A, Petrek M. Roles of Macrophage Polarization and Macrophage-Derived miRNAs in Pulmonary Fibrosis. Front Immunol. 2021; 12(678457):1–8.
27. Zhang S, Fan Y, Qin L, Fang X, Zhang C, Yue J, Bai W, Wang G, Chen Z, Renz H, Skevaki C, Liu X, Xie M. IL-1β augments TGF-β inducing epithelial-mesenchymal transition of epithelial cells and associates with poor pulmonary function improvement in neutrophilic asthmatics. Respir Res. 2021; 22(216):1–15.
28. Mehmood T and Muanprasat C. Deoxyelephantopin and Its Isomer Isodeoxyelephantopin: Anti-Cancer Natural Products with Multiple Modes of Action. Molecules. 2022; 27(7):2086.
29. Hashimoto M, Yanagisawa H, Minagawa S, Sen D, Goodsell A, Ma R, Moermans C, McKnelly KJ, Baron JL, Krummel MF, Nishimura SL. A critical role for dendritic cells in the evolution of IL-1β –Mediated Murine Airway Disease. J Immunol. 2015; 194(8):3962-3969.
30. She YX, Yu QY, Tang XX. Role of interleukins in the pathogenesis of pulmonary fibrosis. Cell Death Discov. 2021; 7(52):1–10.
31. Sziksz E, Pap D, Lippai R, Béres NJ, Fekete A, Szabó AJ, Vannay Á. Fibrosis related inflammatory mediators: Role of the IL-10 cytokine family. Mediators Inflamm. 2015; 2015(1):1–15.
32. Kolahian S, Fernandez IE, Eickelberg O, Hartl D. Immune mechanisms in pulmonary fibrosis. Am J Respir Cell Mol Biol. 2016; 55(3):309–322.
33. Li D, Guo YY, Cen XF, Qiu HL, Chen S, Zeng XF, Zeng Q, Xu M, Tang QZ. Lupeol protects against cardiac hypertrophy via TLR4-PI3K-Akt-NF-κB pathways. Acta Pharmacol Sin. 2021; 43(8):1989–2002.
34. Pan X, Ma X, Jiang Y, Wen J, Yang L, Chen D, Cao X, Peng C. A Comprehensive Review of Natural Products against Liver Fibrosis: Flavonoids, Quinones, Lignans, Phenols, and Acids. Evid-Based Complement Alternat Med. 2020; 2020(1):1–19.
35. Schülke S. Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. Front Immunol. 2018; 9(455):1–18.
36. Ekapratama H, Rahmadi M, Hariyadi DM. Microsphere-Based Drug Delivery to Alveolar Macrophages -a Review. Trop J Nat Prod Res. 2020; 4(10):661–671.