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Impact of Ethanol Extract of Tapak Liman Leaf (*Elephantopus scaber Linn*.) on IL-6 and TNF-*α*. Cytokine Production in Mice with Bleomycin-Induced Pulmonary Fibrosis

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Abstract

Pulmonary fibrosis is a chronic disease marked by excessive fibroblast proliferation and extracellular matrix accumulation. Tapak Liman, a medicinal plant rich in flavonoids and phenolic acids, has shown promise in reducing oxidative stress and inflammation. **Objective;** To evaluate the efficacy of *Elephantopus scaber* ethanol extract (ESEE) treatment in preventing the development of fibrosis in a murine model of pulmonary fibrosis induced by bleomycin. **Method;** A total of fifty-six healthy male BALB/c mice were randomly assigned to seven experimental groups, with eight mice per group. The groups; healthy controls (NC), vehicle controls (VC), negative controls (C-), positive controls (C+), and treatment groups receiving ESEE at different doses: D1 (0.0504 mg/kg), D2 (0.1008 mg/kg), and D3 (0.2016

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mg/kg). Mice were administered dexamethasone or ESEE orally, with bleomycin given intraperitoneally for 14 days. On days 7 and 14, spleens were harvested, Interleukin-6 (IL-6) and Tumor Necrosis Factor Alpha (TNF-α) production were analysed via flow cytometry. **Results:** Increase of Tumor Necrosis Factor Alpha was found in the macrophage of pulmonary fibrosis mice model from day 7 to 14. The production of Interleukin-6 was reduced in the fibrosis group at day 7 and continued to increase at day 14. Interestingly, ESEE treatment for 14 days could effectively reduce Tumor Necrosis Factor Alpha production, and could maintain a stable production of IL-6 at each time point. ESEE at 0.1008 mg/kg BW (D2) was the most effective dose in reducing pro- fibrotic cytokine. **Conclusion;** this study highlights the therapeutic potential of Tapak Liman in the treatment of pulmonary fibrosis, particularly by reducing inflammation and oxidative stress. Its bioactive compounds (flavonoids and terpenoids), provide promising for the development of natural therapies for chronic lung diseases.

Keywords: *Elephantopus scaber*, Bleomycin, Pulmonary Fibrosis, Tumor Necrosis Factor Alpha, Interleukin-6.

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تأثير المستخلص الإيثانولي لأوراق نبات تاباك ليمان على إنتاج السيتوكينات IL-6 و α- TNF في نموذج الفئران المصابة بالتليف الرئوي المُحفَّز بالبليوميسين

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الملخص

التليف الرئوي مرض مزمن يتميز بفرط تكاثر الخلايا الليفية وتراكم المادة خارج الخلوية. يُظهر نبات تاباك ليمان، وهو نبات طبي غني بالفلافونويد والأحماض الفينولية، إمكانيات في تقليل الإجهاد التأكسدي والالتهاب. **الهدف:** تقييم فعالية المستخلص الإيثانولي لنبات التاباك ليمان في منع تطور التليف في نموذج الفئران المصابة بالتليف الرئوي المُحفَّز بالبليوميسين. **المنهجية:** تم توزيع ستة وخمسين فأرًا ذكرًا من سلالة BALB/c بصحة جيدة عشوائيًا على سبع مجموعات تجريبية (ثمانية فئران لكل مجموعة). شملت المجموعات: المجموعة الضابطة السليمة، المجموعة الضابطة للعامل الناقل، المجموعة الضابطة السالبة، المجموعة الضابطة الموجبة، ومجموعات المعالجة التي تلقت المستخلص الإيثانولي بجرعات مختلفة: D1 (0.0504 مغ/كغ)، D2 ؛ (0.1008 مغ/كغ)، D3؛ (0.2016 مغ/كغ). تم إعطاء الديكساميثازون والمستخلص الإيثانولي عن طريق الفم، بينما أُعطي البليوميسين داخل الصفاق لمدة 14 يومًا. في اليومين7 و14، تم فحص الطحال وتقييم نسبة إنتاج إنترلوكين-6 وعامل نخر الورم ألفا بواسطة تقنية قياس التدفق الخلوي. **النتائج:** لوحظ ارتفاع في إنتاج عامل نخر الورم ألفا في

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نموذج التليف الرئوي (المجموعة السالبة –C) من اليوم 7 إلى 14. بينما انخفض إنتاج إنترلوكين–6 في مجموعة التليف –C في اليوم 7 واستمر في الارتفاع بحلول اليوم 14. المثير للاهتمام أن المعالجة بالمستخلص الإيثانولي لمدة 14 يومًا أدت إلى انخفاض فعال في إنتاج عامل نخر الورم ألفا، كما حافظت على إنتاج مستقر لإنترلوكين–6 في كل نقطة زمنية. كانت الجرعةD2 : (0.1008مغ/كغ) من وزن الجسم هي الأكثر فعالية في تقليل السيتوكينات المؤيدة للتليف. **الاستنتاج:** تسلط هذه الدراسة الضوء على الإمكانيات العلاجية لنبات تاباك ليمان في علاج التليف الرئوي، لا سيما من خلال تقليل الالتهاب والإجهاد التأكسدي. تقدم مركباته النشطة بيولوجيًا (الفلافونويد والتيربينويدات) أملًا واعدًا لتطوير علاجات طبيعية لأمراض الرئة المزمنة.

الكلمات المفتاحية: تاباك ليمان، البليوميسين، التليف الرئوي، عامل نخر الورم ألفا، إنترلوكين–6.

Introduction

medicinal plants;

Globally, plant-based compounds are widely used as natural therapeutics for numerous diseases [Newman, & Cragg. 2020, and Atanasov et al. 2015]. Their application is growing in modern healthcare as a preferred substitute for synthetic drugs [WHO. 2023]. Due to their health-promoting qualities, these botanicals are also heavily marketed in diverse commercial products such as pharmaceuticals, cosmetics, salves, and essential oils [Shukla et al. 2021]. Using medicinal plant extracts for disease control is not only economical but also lowers the risk of side effects, offering a valuable and efficient healthcare strategy. Scientific investigations have further clarified the biological mechanisms of these plants and their constituents on human health [Van and Wink. 2004]. Among the many plants used in traditional medicine worldwide for ailments like lung diseases is Tapak Liman (*Elephantopus scaber* Linn), a herb indigenous to Southeast Asia known for its antioxidant and anti-inflammatory capabilities [Rahman et al. 2020]. Traditionally, its leaves and roots have been applied to treat fevers, respiratory problems, wounds, and skin infections, uses that are corroborated by its documented healing effects [Kota, et al. 2023, Lin et al. 2015 and Huang et al. 2013]. Numerous medicinal plants are the subject

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of extensive scientific investigation due to their broad application in traditional medicine. A prominent example is the use of a single plant as a multi-target therapeutic, demonstrating anti-inflammatory, antipyretic (fever-reducing), antimicrobial (antibiotic), antitussive (cough-suppressing), and diuretic activity. This polypharmacological profile is not coincidental but is underpinned by a complex phytochemical composition. The concurrent presence of diverse bioactive compounds (such as flavonoids, phenolic acids, and saponins) enables a single plant extract to mediate these multiple, often interlinked, therapeutic effects through synergistic or complementary biological pathways [Heinrich et al., 2022]. The whole Tapak liman plant has been extensively researched and is commonly employed as an anti-inflammatory, fever-reducing, antibiotic, cough-suppressing, and diuretic agent [Goyal, P. 2021, Hiradeve& Rangari. 2014 and Mei et al. 2012]. The antioxidant properties of Tapak Liman are driven by its rich profile of bioactive compounds, including flavonoids, terpenoids, and phenolic acids. These compounds help neutralize free radicals and alleviate oxidative stress, which are linked to the development of chronic illnesses, cancer, and age-related conditions [Lin et al. 2015, Zakaria et al. 2012, and Abdelwahab. 2011]. Its well-documented anti-inflammatory effects make it a useful treatment for disorders such as arthritis, bronchitis, and skin inflammation [Tan et al. 2019, and Lin et al. 2015]. Laboratory tests, including DPPH and ABTS free radical scavenging assays, have verified the significant antioxidant potential of Tapak Liman extracts, a trait likely due to its phenolic and flavonoid content [Kumar et al. 2018]. Further in vitro studies by Chandraker et al. (2021) confirmed the potent free radical scavenging ability of its ethanolic extract and showed that it suppresses the production of inflammatory mediators like nitric oxide and prostaglandins in macrophages. This anti-inflammatory efficacy was also demonstrated in vivo, where extracts significantly reduced induced edema in rats [Chandraker et al. 2021].

- Pulmonary diseases;

The respiratory system is directly linked to the external environment through the process of breathing. Environmental factors, including air pollutants (such as smoke, dust, and chemicals), microbes (such

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as bacteria, viruses, and fungi), and climate conditions (such as cold or dry air), have the potential to negatively impact respiratory health, leading to the development of diseases such as asthma, bronchitis, and lung cancer [Schraufnagel, D. E. 2019]. Furthermore, smoking and exposure to second hand smoke represent the most deleterious environmental factors for the respiratory system, as they result in the damage of lung tissue and the development of chronic diseases such as chronic obstructive. So**,** Lung diseases are among the most prevalent medical conditions globally, affecting tens of millions of people worldwide [World Health Organization,WHO, 2022]. Idiopathic Pulmonary Fibrosis (IPF), a progressive and often fatal interstitial lung disease, remains underdiagnosed and poorly recognized within the Indonesian healthcare landscape, and that's why this disease experiences nearly 50 percent misdiagnosis because many of its symptoms are similar to other chronic lung diseases, such as TB (tuberculosis), pneumonia, and asthma [Frieda– detik-Health - Friday, 02 Mar 2018. WIB]. Pulmonary fibrosis is a chronic respiratory disease. It is characterised by excessive fibroblast proliferation and extracellular matrix accumulation, resulting in the destruction of normal tissue architecture and function [Martinez, et al. 2017]. A significant pathological feature of pulmonary fibrosis is the aberrant repair of lung tissue. Chronic inflammation and the progressive fibrotic remodelling of the pulmonary interstitial are central to the disease's pathology [Martinez, F. J., et al. 2017**].**

- Bleomycin;

Bleomycin, a glycopeptide antibiotic derived from *Streptomyces verticillus*, is a common chemotherapeutic agent. Its efficacy stems from causing DNA strand breaks, which trigger programmed cell death (apoptosis) in fast- proliferating cancer cells [Ayoubifar & Arasteh,O, 2023 and Chen & Stubbe. 2005]**.** Bleomycin's unique mechanism of action, which involves the induction of DNA strand breaks through oxidative damage, along with its clinical efficacy in treating malignancies such as testicular cancer, Hodgkin's lymphoma, and squamous cell carcinomas, has established it as an essential component of many combination chemotherapy regimens [Chabner, B. A., & Longo, D. L. (Eds.). 2022]**.** However, its use is often limited by potential toxic side effects, particularly pulmonary

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toxicity. Furthermore, bleomycin is a chemotherapeutic agent that is widely used to induce pulmonary fibrosis in experimental animal models, a response that is particularly robust in mice and rats following intratracheal, intraperitoneal, or intravenous administration [Tashiro, J., et al. 2017]. Bleomycin causes oxidative stress and DNA damage in lung tissues, leading to inflammation, epithelial injury, and eventual fibrosis. It closely mimics the mechanisms of IPF and is one of the most established models for studying the disease [Tashiro, et al. 2017]. Bleomycin-induced lung injury in experimental models features interstitial edema, inflammatory cell infiltration, and immune cell activation, which can lead to pulmonary fibrosis [Mouratis and Aidinis 2011].

Objectives:

To evaluate the efficacy of *Elephantopus scaber* ethanol extract (ESEE) treatment in preventing the development of fibrosis in a murine model of pulmonary fibrosis induced by bleomycin
Duration and place of the study: This study experiment was conducted between August 2023 and November 2023. In the Physiology Laboratory and Animal Cell Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia.

Methodology

Plant Material and Extraction:

The leaves of *Elephantopus scaber Linn were* purchased and identified by Unit Pelaksana Teknis, (UPT). Laboratorium Herbal Materia Medica Batu (specimen number 067/656/102.20/2023). Subsequently, the sample was air-dried at room temperature and ground into a fine powder. One hundred grams of the powdered plant material was macerated in 1,000 milliliters of 96% ethanol at a ratio of 1:10 (w/v) for 24 hours at room temperature with intermittent stirring. Following maceration, the mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated using a vacuum evaporator at 40- 60°C until a paste-like consistency was achieved. The final extract was stored at 3°C in a refrigerator for further analysis.

Animal model of pulmonary fibrosis:

Utilized fifty-six healthy, 6-7-week-old male Balb/C mice

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weighing roughly 30 grams. Sourced from Pusvitma in Surabaya, the mice were housed under standard environmental conditions with free access to food and water. Following a two-week acclimatization period in individual plastic cages, any animals displaying illness were excluded from the research. This study is reported in accordance with the ARRIVE guidelines 2.0 (Percie et al., 2020).

Ethical approval:

This study received ethical clearance from the Research Ethics Commission of Brawijaya University (No. 182-KEP-UB-2023).

Duration and place of the study:

This study was conducted from August 2023 to November 2023 at the Physiology Laboratory and Animal Cell Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia.

Induction of Pulmonary Fibrosis

The IPF was induced with bleomycin (MedChemExpress LLC, USA) in groups C-, C+, D1, D2, and D3 (Table 1). A total of 10 grams of bleomycin was dissolved in 1 ml of phosphate-buffered saline (PBS) and separated into five 200-µl propylene tubes. 7.8 ml of PBS was then added to each propylene tube. Each mouse was injected with bleomycin dissolved at a concentration of 2 mg/kg/body weight daily for 2 weeks [Van et al. 2022]. Bleomycin was injected intraperitoneally [Gul. 2023]. The justification for the three doses investigated (D1: 0.0504, D2: 0.1008, D3: 0.2016 mg.kg-1 BW) stemmed from a preliminary pilot study carried out in our lab. In this initial experiment, a single dose of 0.1008 mg.kg-1 BW was given, revealing a notable biological effect without any signs of toxicity. As a result, D2 was designated as the primary effective dose, while D1 (half of D2) and D3 (double D2) were chosen to assess a dose-dependent response and validate the therapeutic window.

Experimental Study Design

The experimental design followed a completely randomized design (CRD), utilizing a total of 56 mice across two time series. The mice underwent a 14 days acclimatization period prior to being randomly assigned to seven groups, with each group consisting of

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eight mice (n = 8). Details of the group allocation are presented in Table 1.

Table 1. The experimental design
E. scaber: *Elephantopus scaber*

Administration of Dexamethasone and Ethanol Extract of Tapak Liman Leaves;

Different groups	Treatment type	
	Therapy given for 7- and 14- days	**Intraperitoneal dosing of Bleomycin**
Healthy control (NC)	No treatment was administered. The subjects were provided with ad libitum access to water and a standard commercial diet.	Fibrosis was not induced
Vehicle control (VC)	Received Corn oil o.3 ml	Did not experience fibrosis Induction
Negative control (C-)	Did not receive any treatment	Bleomycin injection (2mg/kg BW) daily
Positive control (C+)	Received dexamethasone (Drug control) 0.3 mL from a solution concentration of 333.3 mg/mL corn oil (dose 3 mg/kg BW).	Bleomycin injection (2mg/kg BW) daily
Dose 1 (D1)	Ethanol extract of *E. scaber* leaves (0.0504 $mg.kg^{-1}$BW)	Bleomycin injection (2mg/kg BW) daily
Dose 2 (D2)	Ethanol extract of *E. scaber* leaves (0.1008 $mg.kg^{-1}$ BW)	Bleomycin injection (2mg/kg BW) daily
Dose 3 (D3)	Ethanol extract of *E. scaber* leaves (0.2016 $mg.kg^{-1}$ BW)	Bleomycin injection (2mg/kg BW) daily

In this study, dexamethasone was used as a positive control at a dose of 3 mg/kg body weight, based on previous research [Hu et al. 2015]. Both dexamethasone and the ethanol extract of Tapak Liman were dissolved in corn oil at doses determined by their IC50 values. Mice were administered oral doses of Tapak Liman and dexamethasone for 7 and 14 days in a pulmonary fibrosis model induced by bleomycin [Shi et al. 2014].

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Lymphocyte Isolation;

Mice were euthanized to facilitate spleen isolation. The spleens were rinsed three times with phosphate-buffered saline (PBS) and then crushed in a clockwise motion using the bottom of a syringe in a petri dish containing 1 mL of PBS. An additional 4 mL of PBS was added to the dish, and the contents were transferred to a 15-mL polypropylene tube. The mixture was then centrifuged at 2,500 rpm for 5 minutes at 10°C. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 mL of PBS. A 50 µL aliquot of the cell suspension was then transferred to a 1.5 mL microtube for antibody staining [Roffico and Djati. 2014].

Antibody Staining Procedure

Antibody staining was conducted using both extracellular and intracellular methods. A 50 µL cell suspension was mixed with 50 µL of FITC-conjugated anti-mouse CD4 antibody for extracellular staining (Biolegend, USA) and incubated at 4°C for 20 minutes in the dark. Intracellular staining involved adding 50 µL of intracellular fixation buffer (eBioscienceTM, Thermo Fisher Scientific, USA) to the cell suspension, followed by a 20-minute incubation at 4°C in the dark. The cells were then mixed with 400 µL of permeabilization buffer, centrifuged at 2,500 rpm and 10°C for 5 minutes. The pellet was resuspended in 50 µL of intracellular antibody solutions (PE/Cy5 conjugated anti-mouse TNF-α, and IL-6; Biolegend, USA), the mixture was incubated at 4°C for 20 minutes in the dark, followed by the addition of 400 µL of PBS. The samples were prepared for flow cytometric analysis using BD CellQuest Pro™ software and BD FACS Calibur™. Data analysis followed the staining procedures [Roffico and Djati, 2014].

Histological Analysis

For histological examination, lungs were perfused via the main bronchus with 10% neutral-buffered formalin for 24 hours. After fixation, tissue blocks were excised, dehydrated in graded ethanol, embedded in paraffin and sectioned at a thickness of 4 µm. Subsequently, the sections were subjected to staining with eosin and haematoxylin (E&H) in order to identify inflammatory cells, and with Masson's trichrome in order to assess collagen deposition.

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Statistical analysis;

A statistical analysis was conducted on the data set, with the percentage of each parameter analysed using the IBM SPSS Statistics version 21 for Windows. Data were first assessed for normality and homogeneity. Subsequently, parametric analysis was performed through two-way ANOVA (analysis of variance) with a significance level set at $P < 0.05$

Results

Changes in production of IL-6 by $CD4^+$ ($CD11^+IL\text{-}6^+$)

The capacity of bleomycin to elicit a fibrotic response in mice has been previously demonstrated in scientific studies in several studies. The administration of bleomycin to mice has been demonstrated to elicit an inflammatory response, which represents a form of the body's intrinsic defense mechanism against injury [Necas et al. 2013]. During the inflammatory process, there is an accumulation of leukocytes and an increase in the synthesis and secretion of pro-inflammatory cytokines, one of which is IL-6 [Ayoub. 2017]. In the present study, and based on Figure 1, in the measurements on day 7, we can see a clear increase in the production of CD4+IL-6+ (interleukin-6) in the groups exposed to bleomycin compared to the normal control group (NC) and vehicle control group (VC), which explains the body's response (immune system) to this infection. As for the measurements on the 14th day, we note a decrease in the production of CD4+IL-6+ in the group treated with dexamethasone (positive group; C+) and also in the groups treated with the ethanolic extract of the leaves of Tapak liman, which confirms that this medicinal plant contains anti- inflammatory and also antioxidant compounds. The ethanolic extract of Tapak liman (*Elephantopus scaber L*) leaves exhibits anti-inflammatory properties by inhibiting the production of interleukin-6 (IL-6), a cytokine involved in the immune response during inflammation. Inhibiting IL-6 can mitigate excessive inflammation and help prevent related conditions, such as pulmonary fibrosis. The ethanol extract of *Elephantopus scaber* L. (tapak liman) significantly altered the population of naïve helper T cells (CD4+IL-6+) in mice with bleomycin-induced pulmonary fibrosis ($F = 8.977$; $P = 0.001$). As shown in Figure 1, a significant temporal shift occurred between day 7 and day 14. On day 7, all bleomycin-injected groups (C-: 26.77%, C+: 40.97%, D1: 35.47%, D2: 16.62%, D3: 27.28%) showed elevated levels compared to the

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normal control (NC: 19.66%), indicating a pronounced inflammatory effect. By day 14, while most groups returned to levels similar to the control. Notably, the D2 treatment group (0.1008 mg.kg^{-1} BW), which started at 16.62%, showed a marked increase, contrasting with the general trend of decline in other groups. This suggests that the medium dose of the extract has a specific immunomodulatory effect that alters the typical resolution of the acute inflammatory response.

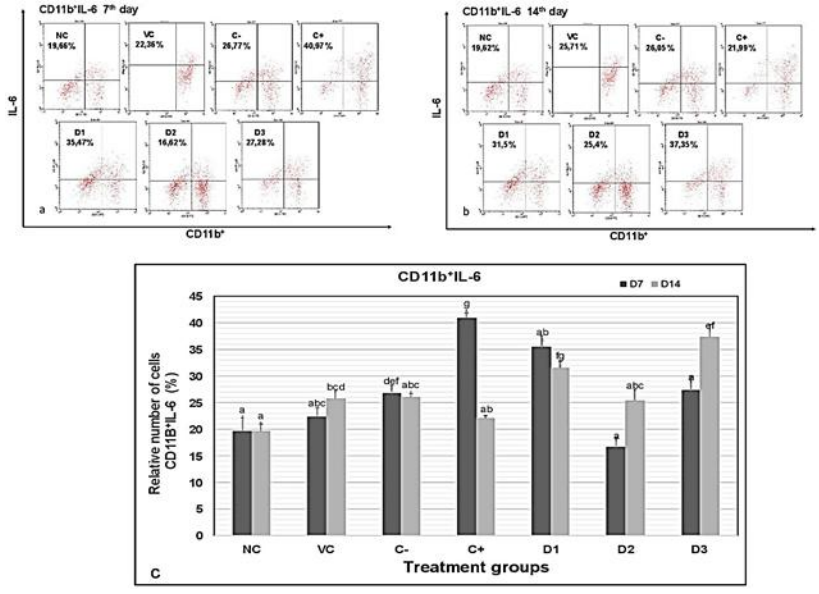

Figure 1. Relative number of CD4+IL-6+ cells

As seen in figure 1: (a) is a dot plot representing the percentage of CD4+IL-6+ cells for the 7-day treatment groups; (b) is a dot plot displaying the percentage of CD4+IL-6+ cells for the 14-day treatment groups, and (c) is a bar graph illustrating the relative number of CD4+IL-6+ cells for each treatment group.
For groups: Healthy control (NC); Vehicle control (VC); Pulmonary fibrosis negative control (C-); Drug control (positive control, C+); D1: ESEE administered at 0.0504 mg/kg; D2: ESEE administered at 0.1008 mg/kg; D3: ESEE administered at 0.2016 mg/kg

Changes in production of CD11b^{+}TNF-α^{+};

Tumor necrosis factor alpha (TNF-α) is a pivotal pro-inflammatory cytokine involved in the regulation of immune

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responses, inflammation, and cellular homeostasis. It is primarily produced by activated macrophages, but can also be secreted by other immune cells such as T- cells, B-cells, and natural killer (NK) cells. TNF-α is involved in several physiological and pathological processes, making it a key target in inflammatory diseases. On day 7, mice with bleomycin-induced fibrosis showed a marked increase in CD11b+TNF-α+ cells, a finding statistically validated by ANOVA (F = 13,275; P = 0.001). The fibrosis model group (C-) had the most pronounced effect, with these cells constituting 11.74% of the population, contrast to the healthy control group (Figures 2a, 2b). After 7 days, both dexamethasone (C+) and all ESEE doses significantly increased CD11b+TNF-α+ cells compared to the fibrosis model (C-), with the D2 group showing the lowest level overall (D2= 8.09%). By day 14, the untreated model (C-) and the D1/D3 groups showed worsening inflammation. In contrast, dexamethasone and the mid-dose ESEE (D2, 0.1008 mg/kg) effectively reduced the cell population, demonstrating that the D2 dose was the most effective at controlling inflammation and inhibiting fibrosis. Figure (2).

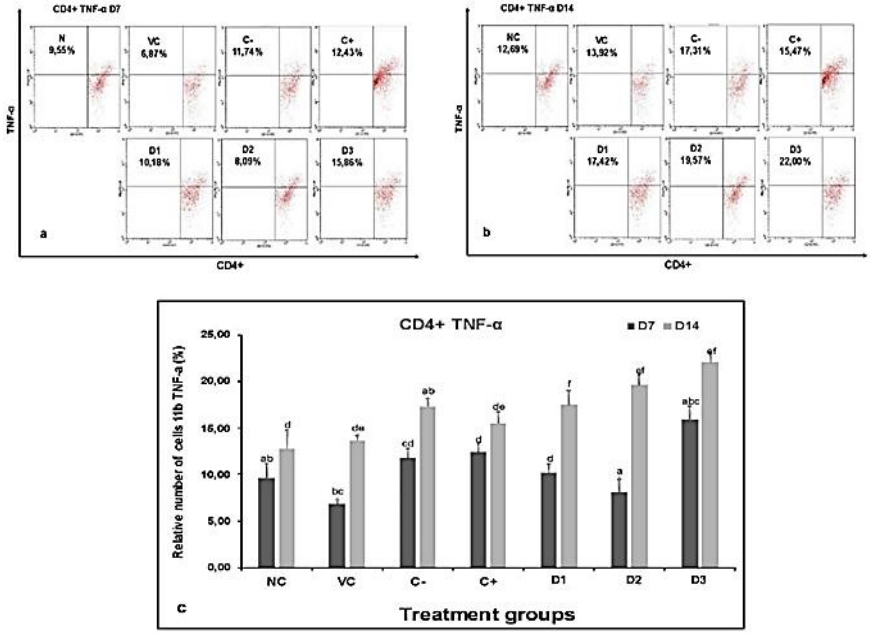

Figure 2. Flow cytometry result of CD4+TNF-α+ cell population at days 7 and 14 for each treatment group

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As seen in figure 2: (a) is a dot plot demonstrating the CD4+TNF-α+ cell population. (b) The representative bar showed the mean relative percentage of the CD4+TNF-α+ cell population. Data were shown as mean ± standard deviation (SD). Lowercase marks showed significant differences based on Duncan's HSD post hoc test (p 0.05).
Groups: NC: healthy mice group; VC: vehicle control group; C-: fibrosis model group with bleomycin; C+: positive control group with dexamethasone; D1: ESEE treatment at a dose of 0.0504 mg/kg; D2: ESEE treatment at a dose of 0.1008 mg/kg; D3: ESEE treatment at a dose of 0.2016 mg/kg

Histological changes in the lung tissues;

Pulmonary fibrosis is a progressive lung disease characterized by the accumulation of scar tissue in the lungs. This fibrotic process leads to thickening and stiffening of the affected tissue, impairing the proper functioning of the lungs.

Consequently, the lungs' ability to absorb oxygen and expel carbon dioxide is reduced, leading to a range of symptoms, including shortness of breath, persistent dry cough, fatigue, and weight loss [Zaghloul et al. 2017 and King et al. 2017]. The main histological feature of idiopathic pulmonary fibrosis is a progressive fibrotic process involving the lung tissue. This process begins with alveolitis, which is followed by a range of degrees of inflammation and subsequent fibrosis. The key histopathological characteristics of pulmonary fibrosis are marked by the excessive deposition of extracellular matrix, proliferation of fibroblasts, collapse of alveoli, and loss of normal lung architecture. Figure (3).

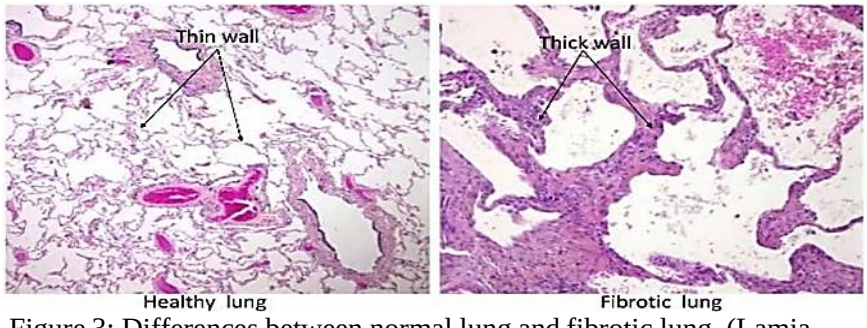

Figure 3; Differences between normal lung and fibrotic lung. (Lamia Yacoubi, et al. 2011)

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Figure 4 displays representative lung tissue images from the histological (H&E) analysis. Lungs from healthy control mice (NC) appeared normal with minimal collagen deposition after 14 days. In stark contrast, the bleomycin-only negative control group (C-) showed severe pathological changes, characterized by inflammatory cell infiltration, alveolar collagen accumulation, extensive edema, and structural disruption. These indicators of fibrosis and inflammation were significantly less severe in all protective effects.

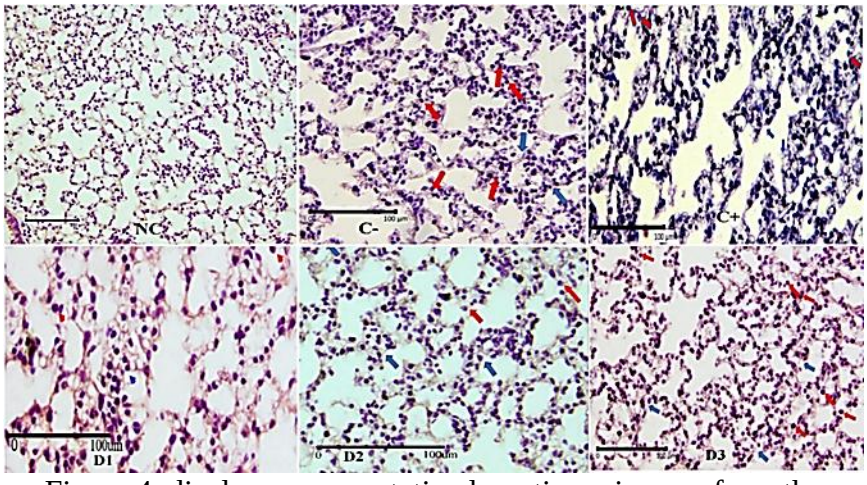

Figure 4. displays representative lung tissue images from the histological (H&E) analysis.

A histological analysis (H&E) was conducted on lung tissues from healthy mice (NC) and the negative control group (C-) that received BLM and no treatment, the positive control group (C+) that received BLM and dexamethasone, and the groups that received BLM and also Tapak Liman leaves ethanolic extract at different doses. The sections demonstrate the infiltration of inflammatory cells (red arrow), disruption of the alveolar architecture with lymphoid follicles and a notable increase in interstitial thickness in the untreated group (C-) (blue arrow). A notable reduction in inflammatory activity was observed in the treated groups (D1, D2, and D3). Lung sections were obtained two weeks following the conclusion of the treatment regimen in all mice. One illustrative example is provided for each group. The image was captured at a magnification of ×100. (Red arrow: Inflammatory cell and blue arrow: Thickened alveolar wall).

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Discussion

Medicinal plants represent a valuable reservoir of bioactive compounds for treating complex diseases. Pulmonary fibrosis, characterized by progressive inflammation, oxidative damage, and scar tissue deposition in the lungs, is one condition where plant-based therapeutics hold significant promise. *Elephantopus scaber Linn*. (Tapak Liman), traditionally used for inflammatory ailments, is rich in flavonoids and terpenoids compounds known for their anti-inflammatory and antioxidant activities [Atanasov, A. G., et al. 2021]. This study investigated the therapeutic potential of an ethanol extract of *E. scaber* leaves in a murine model of bleomycin-induced pulmonary fibrosis, focusing on its modulation of key inflammatory pathways. The bleomycin model was selected for its well-established ability to recapitulate the key pathological features of human idiopathic pulmonary fibrosis (IPF), including alveolar epithelial injury, sustained inflammation, and aberrant collagen deposition [Moeller et al., 2008]. Consistent with this model's pathophysiology, our study observed significant lung inflammation and tissue remodeling in untreated bleomycin-challenged mice. Histological analysis revealed pronounced alveolar thickening, inflammatory cell infiltration, and excessive collagen accumulation, aligning with classic fibrotic progression described in prior studies [Green, 2002; Mouratis and Aidinis, 2011]. Treatment with *E. scaber* extract markedly attenuated these pathological changes. The extract demonstrated a dose-dependent suppression of pulmonary inflammation, as quantified by significant reductions in the pro-inflammatory cytokines IL-6 and TNF-α. Notably, the higher tested dose (0.1008 $mg \cdot kg^{-1}$ BW) achieved a reduction in IL-6 levels by day 14 that was comparable to the effect of the positive control, dexamethasone. This reduction is critically important, as IL-6 is a pivotal mediator that drives fibroblast activation and persistence of the inflammatory milieu in fibrotic lungs. Furthermore, flow cytometric analysis revealed that the extract significantly modulated immune cell populations, reducing the frequencies of $CD4^{+}IL\text{-}6^{+}$ and $CD11b^{+}TNF\text{-}\alpha^{+}$ cells. The decrease in $CD11b^{+}TNF\text{-}\alpha^{+}$ cells, which represent activated myeloid-lineage cells such as macrophages and neutrophils, indicates that *E. scaber* directly tempers the innate immune response central to fibrosis initiation. These findings align with and extend previous reports on the anti-

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inflammatory efficacy of *E. scaber* in other models [Yap et al., 2010]. The coordinated downregulation of these cytokines and their cellular sources strongly suggests that the extract interferes with upstream pro-inflammatory signaling cascades, such as the NF-κB and MAPK pathways, which are known regulators of IL-6 and TNF-α transcription [Necas et al., 2013; Ayoub, 2017]. In addition to its anti-inflammatory effects, the antioxidant capacity of *E. scaber's* phytoconstituents likely contributes to its anti-fibrotic activity. Bleomycin-induced injury is mediated in part by oxidative stress and DNA damage. By scavenging free radicals and bolstering cellular antioxidant defenses a property documented for *E. scaber* flavonoids [Gunasekaran et al., 2014], the extract may protect lung tissue from secondary oxidative damage, thereby breaking the cycle of injury and repair that leads to fibrosis. When compared to dexamethasone, a standard corticosteroid, *E. scaber* extract showed similar efficacy in reducing key inflammatory and histopathological markers at the studied time points. This is a promising finding, as long-term dexamethasone use is associated with considerable adverse effects. *E. scaber*, therefore, presents itself as a potentially safer, natural alternative for managing chronic fibrotic disease [Raghu and Rochwerg, 2020]. However, it is crucial to note that while dexamethasone is a single, potent synthetic molecule, *E. scaber* extract is a complex mixture whose activity may arise from multi-target synergism.

Conclusion;

This study confirms that the ethanol extract of *Elephantopus scaber* leaves possesses significant anti-inflammatory and anti-fibrotic properties in a bleomycin-induced pulmonary fibrosis model. The extract effectively reduced the levels of pivotal cytokines (IL-6 and TNF-α), modulated pathogenic immune cell populations, and ameliorated histological lung damage. These results validate the traditional use of *E. scaber* and underscore its potential as a promising candidate for developing phytotherapeutic interventions for IPF. Nevertheless, several critical steps remain for translational development. Future research must focus on identifying the specific bioactive compound**(s)** responsible for the observed effects, elucidating the precise molecular mechanisms (e.g., direct inhibition of NF-κB translocation or

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MAPK phosphorylation), and conducting long-term toxicity and efficacy studies in comparison to current anti-fibrotic drugs. Such work will be essential to optimize dosing, ensure safety, and fully realize the clinical potential of Tapak Liman in treating pulmonary fibrosis.

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Abbreviations:

IL-6; Interleukin-6, IPF; Idiopathic Pulmonary Fibrosis, BLM; Bleomycin, PBS; Phosphate- Buffered Saline, ESEE; Elephantopus Scaber L. Ethanol Extract, E. scaber L; Elephantopus Scaber Linn, TNF-α; Tumor Necrosis Factor Alpha, MAPK; Mitogen-Activated Protein Kinase, NK; Natural Killer cells, NF-κB; Nuclear Factor Kappa.

Conflicts of interest:

The authors confirm that there are no conflicts of interest associated with this publication.

Contributions of authorship;

The following entities played a pivotal role in the study's design and conceptualization: AEA, SRF, YIC, and MSD. SRF and YIC contributed to laboratory work and data collection.
AEA was responsible for analyzing the data and drafting the initial manuscript. YIC assumed responsibility for the administration of the project and the validation of the data. The subsequent analysis and visualization of the results were conducted by SW. MR's contribution included a thorough revision of the article, which was deemed essential for its advancement in the field. The responsibility for acquiring funding fell under the purview of MSD. All authors have meticulously reviewed the published version of the manuscript and have consented to it.

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