Basic Medical Research

Effects of *Physalis angulata* extracts on bleomycin-induced rat: analysis on lung inflammation and fibrosis

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ABSTRACT

BACKGROUND Scleroderma is an immune-mediated connective tissue disease, with interstitial lung disease as one of its manifestations. *Physalis angulata* (*P. angulata*) or ciplukan has shown potential in treating fibrosis, but its role in preventing lung inflammation and fibrosis remains unknown. This study aimed to evaluate the effect of *P. angulata* extract in a bleomycin (BLM)-induced scleroderma rat.

METHODS Sprague-Dawley rats were divided into 6 groups. For lung inflammation prevention, 3 groups received: (1) BLM only, (2) BLM+50 mg/kgBW *P. angulata*, and (3) BLM+100 mg/kgBW *P. angulata*. After 14 days, rats were sacrificed and bronchoalveolar lavage (BAL) leukocyte count, interleukin-6 (IL-6) levels, and lung injury score were assessed. For fibrosis prevention, another 3 groups received the same interventions and were sacrificed after 51 days. Fibrosis score, fibrosis area, hydroxyproline, transforming growth factor-beta (TGF-β), and matrix metalloproteinase-13 (MMP-13) levels were analyzed. BLM was administered subcutaneously, while *P. angulata* was given orally for 14 days. IL-6, TGF-β, and MMP-13 were measured by ELISA and hydroxyproline by colorimetric method. Mean differences and *p*-values were calculated using appropriate statistical tests.

RESULTS *P. angulata* extract did not prevent lung inflammation, as there were no differences in BAL leukocyte count ($p = 0.126$), IL-6 levels ($p = 0.173$), or lung injury scores ($p = 0.397$) between the BLM-only group and those receiving *P. angulata*. The extract also did not prevent lung fibrosis, with no differences in fibrosis scores ($p = 0.173$), fibrosis area ($p = 0.359$), hydroxyproline ($p = 0.295$), TGF-β ($p = 0.374$), or MMP-13 ($p = 0.088$) levels among groups.

CONCLUSIONS *P. angulata* extract did not prevent the development of lung inflammation or fibrosis.

KEYWORDS animal model, bleomycin, lung inflammation, *Physalis*, pulmonary fibrosis, scleroderma

Systemic sclerosis (scleroderma) is an autoimmune disease characterized by inflammation, skin and internal organs fibrosis, and vasculopathy.[1] Fibrosis of internal organs, such as interstitial lung disease (ILD), is a leading cause of death in several scleroderma-related cohorts.[2] The treatment for ILD in scleroderma, including immunosuppressants and antifibrotics, remains inadequate.[3] Immunosuppressive treatment poses potential side effects such as infection.[4] Nintedanib, an approved antifibrotic, slows down ILD progression; however, it is costly and not universally available.[3,5,6] To date, the treatment for preventing ILD development in scleroderma remains lacking.

Physalis angulata (*P. angulata*) or ciplukan is a common medicinal plant in Indonesia.[7] Dewi et al[8] demonstrated its potential in reducing skin fibrosis in

patients with scleroderma. Similarly, several *Physalis* species have prevented fibrosis in animal models, such as pulmonary and liver fibrosis,[9,10] including reduced pulmonary fibrosis during the fibrotic phase in mice models.[11] However, no study has assessed its effects during the inflammatory phase, particularly in preventing pulmonary fibrosis in scleroderma. This study aimed to evaluate the effects of *P. angulata* extract on lung inflammation and fibrosis in an animal model of bleomycin (BLM)-induced scleroderma. The assessment included histopathological analysis and bronchoalveolar lavage (BAL) leukocyte count, as well as interleukin (IL)-6, transforming growth factor-beta (TGF-β), and matrix metalloproteinase (MMP)-13 levels, as markers of inflammatory and fibrotic processes.

METHODS

Experimental design

Male Sprague-Dawley rats (*Rattus norvegicus*) aged 10–12 weeks were obtained from the Laboratory Animal Management Unit, School of Veterinary Medicine and Biomedical Sciences, IPB University. The rats underwent a 7-day acclimatization before treatment.

The rats were divided into three groups to evaluate the preventive effect of *P. angulata* on inflammation. All groups received a daily subcutaneous injection of BLM (200 mcg) on the shaved back for 14 days (according to the method of Lam et al[12]). Two groups received *P. angulata* extract at 50 (BLM2-14+CIP50) and 100 (BLM2-14+CIP100) mg/kg body weight (BW), whereas the other group was the positive control group (BLM2-14). On Day 15, all rats were sacrificed.

For the fibrosis prevention study, the rats were divided into three groups. All groups received BLM for 14 days, with two groups receiving *P. angulata* extract at 50 (BLM2-51+CIP50) and 100 (BLM2-51+CIP100) mg/kgBW, and one group not receiving *P. angulata* (BLM2-51). The rats were euthanized after 51 days.

The *P. angulata* dosage was adopted from a study by Dewi et al,[8] where 750 mg/day (or 50 mg/kgBW) of *P. angulata* was administered to patients with scleroderma, corresponding to 92.5 mg/kgBW in rats, and subsequently rounded to 100 mg/kgBW. The sample size was calculated using Federer's formula, with six rats per group. The rats were placed in standard cages with food and clean water *ad libitum* under a 12-hour light-dark daily cycle. The conditions of the animals were evaluated daily by an attending veterinarian and two laboratory technicians. After the experiment, all rats were euthanized with an overdose of ketamine HCl and xylazine.

All animal experiments and histopathological examinations were conducted at the School of Veterinary Medicine and Biomedical Sciences, IPB University, while TGF-β and MMP-13 levels were examined at the Integrated Laboratory of the Faculty of Medicine, Universitas Indonesia. This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia – Cipto Mangunkusumo Hospital (No: KET-136/UN2.F1/ETIK/PPM.00.02/2022) and the Animal Ethics Committee, Faculty of Veterinary Medicine, IPB University (No: 008/KEH/SKE/VI/2022).

Histopathological examination

Following euthanasia, lung tissues were extracted, fixed in 10% neutral buffered formalin solution for at least 6 hours, and embedded in paraffin blocks. Tissue sections (3–5 µm) were prepared from paraffin blocks and stained with hematoxylin and eosin, and Masson's trichrome. For inflammation assessment, 20 lung fields per lobe were evaluated at 400× magnification using the acute lung injury scoring system of the American Thoracic Society.[13] Fibrosis severity was assessed using the Ashcroft score (0–8 scale) across 10 fields per lobe at 100× magnification.[14] The extent of lung fibrosis was calculated using the ImageJ software (Wayne Rasband, USA) and presented as percentages.[15]

BAL fluid examination

Following the euthanasia, we performed tracheal cannulation and bronchoalveolar washing with a total volume of 3 ml. The leukocyte count in the BAL fluid was measured using a Mindray BC-2800 Vet Hematology Analyzer (Shenzhen Mindray Animal Medical Technology Co., China), as described by Qiao et al.[16]

IL-6, hydroxyproline, TGF-β, and MMP-13 examinations

IL-6, TGF-β, and MMP-13 levels in lung homogenates were quantified using an enzyme-linked immunosorbent assay kit (MyBiosource, Inc., USA for IL-6; FineTest®, China for TGF-β; LSBio®, USA for MMP-13). Hydroxyproline levels in the lung homogenates were quantified using a hydroxyproline assay kit (colorimetric) (Abcam, UK). A microplate reader

Multiskan GO (Thermo Fisher Scientific, USA) was used to measure optical density at 450 nm.

Scleroderma-inducing agent

Scleroderma was induced using 15 mg dissolved BLM hydrochloride (Bleocin®; PT Kalbe Farma Tbk, Indonesia) in 7.5 ml of sterile distilled water. A 100 µl daily subcutaneous injection was administered for 14 days.

***P. angulata* extract**

The ethanolic extract of *P. angulata* was obtained from the Center for Herbal Medicine, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia. The aerated parts of *P. angulata* were powdered and extracted using maceration. Active compounds, angulatin A and quercetin, were measured using thin-layer chromatography, and their general composition was analyzed via spectrophotometry. *P. angulata* was orally administered for 14 days at 50 and 100 mg/kgBW daily.

Data processing and analysis

Data were analyzed using the SPSS software version 29.0 (IBM Corp., USA). Numerical data were presented as mean and standard deviation for normally distributed data or as median and range (minimum–maximum) for non-normally distributed data. The mean differences in BAL leukocyte count, lung inflammation score, Ashcroft lung fibrosis score, the extent of lung fibrosis, as well as IL-6, hydroxyproline, TGF-β, and MMP-13 levels, between groups, were analyzed using the analysis of variance (ANOVA) test with post-hoc analysis (Bonferroni or Games–Howell) for normally distributed data. For non-normally distributed data, the Kruskal–Wallis test and post-hoc analysis (Mann–Whitney) were applied. Statistical significance was defined as $p<0.05$.

RESULTS

All experimental animals survived until the end of the study, with no observable changes in their physical appearance or behavior. All rats showed an increased BW during the 14- and 51-day observations. However, the increase was less pronounced in the groups that did not receive *P. angulata* than in those that did. BW and lung weight are presented in Table 1.

Lung inflammation was represented by the IL-6 levels in the lung tissue, leukocyte count from BAL, and lung injury score from lung histopathology (Table 2). The highest IL-6 levels were observed in the BLM-only group, whereas the 50 mg/kgBW *P. angulata* extract group exhibited the lowest IL-6 levels. Similarly, leukocyte counts from the BAL fluid were the highest in the BLM-only group and lowest in the *P. angulata* extract (100 mg/kgBW) group. Lung injury scores, based on histopathological analysis, were highest in the BLM-only group and lowest in the *P. angulata* extract (100 mg/kgBW) group (Figure 1). There were no significant differences in IL-6 levels, BAL fluid, or lung injury scores among the groups.

Lung fibrosis was assessed by evaluating the degree of lung fibrosis quantified as the Ashcroft fibrosis score and extent of lung fibrosis, as well as hydroxyproline, TGF-β, and MMP-13 levels, in the lung tissue. The lowest fibrosis score was observed in the BLM2-51+CIP50 group, whereas the BLM2-51 group

Table 1. BW and lung weight of the experimental animals

Animal group (N = 6)	Initial BW (g), mean (SD)	Final BW (g), mean (SD)	Δ BW (g), median (min–max)	Total lung weight (g), mean (SD)	Relative lung weight
BLM2-14+CIP50	210 (25.1)	218 (25.78)	5.5 (2–22)	1.70 (0.3)	0.00781
BLM2-14+CIP100	176.5 (17.43)	184.67 (18.3)	7.5 (2–14)	1.68 (0.23)	0.00913
BLM2-14	200.17 (28.41)	199.83 (40.24)	3 (–43–33)	1.9 (0.29)	0.0987
BLM2-51+CIP50	249.3 (31)	289 (31)	30.5 (6–118)	1.62 (0.1)	0.0057
BLM2-51+CIP100	248.5 (38.6)	263 (47.2)	10.5 (6–27)	1.57 (0.4)	0.0063
BLM2-51	213.5 (31.6)	222 (29.5)	8.5 (–4–17)	1.54 (0.2)	0.0071

BW=body weight; SD=standard deviation

BLM2-14+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (14 days observation); BLM2-14+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (14 days observation); BLM2-14: bleomycin (200 mcg) (14 days observation); BLM2-51+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (51 days observation); BLM2-51+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (51 days observation); BLM2-51: bleomycin (200 mcg) (51 days observation)

Table 2. Lung inflammation

Lung inflammation variables	BLM2-14+ CIP50	BLM2-14+ CIP100	BLM2-14	*p*
IL-6 levels on lung tissue (pg/mg protein), GM in 95% CI	9,956 (8,804–11,259)	10,760 (7,362–15,725)	13,035 (10,226–16,615)	0.173*
Leukocyte count from BAL fluid (number of cells cell/µl), median (min–max)	550 (0–900)	200 (0–500)	750 (200–900)	0.126†
Lung injury score, mean (SD)	0.0485 (0.0173)	0.0362 (0.0114)	0.0499 (0.025)	0.397*

BAL=bronchoalveolar lavage; CI=confidence interval; GM=geometric mean; IL-6=interleukin-6; SD=standard deviation
*Analysis of variance (ANOVA) test; †Kruskal–Wallis test
BLM2-14+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (14 days observation); BLM2-14+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (14 days observation); BLM2-14: bleomycin (200 mcg) (14 days observation)

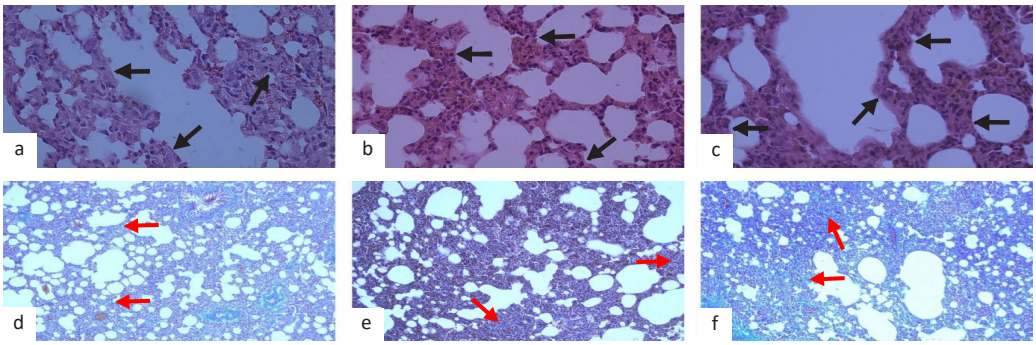

Figure 1. Lung inflammation on histopathology between BLM2-14+CIP50 (a), BLM2-14+CIP100 (b), and BLM2-14 (c) groups (H&E staining with 400× magnification); and lung fibrosis on histopathology between BLM2-51+CIP50 (d), BLM2-51+CIP100 (e), and BLM2-51 (f) groups (MT staining with 100x magnification). Black arrows indicates an increase in alveolar septal thickening in inflammation and red arrows showed distortion of lung architecture with fibrosis. H&E=hematoxylin and eosin; MT=Masson's trichrome; BLM2-14+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (14 days observation); BLM2-14+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (14 days observation); BLM2-14: bleomycin (200 mcg) (14 days observation); BLM2-51+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (51 days observation); BLM2-51+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (51 days observation); BLM2-51: bleomycin (200 mcg) (51 days observation)

Table 3. Lung fibrosis

Variables	BLM2-51+CIP50	BLM2-51+CIP100	BLM2-51	*p*
Ashcroft score, median (min–max)	6.25 (5.88–6.5)	6.26 (5.08–6.74)	6.51 (6.06–6.8)	0.173†
Lung fibrosis area (%), mean (SD)	48.1 (5.7)	50.2 (6.3)	53.8 (7.9)	0.359‡
Hydroxyproline levels (pg/mg protein), mean (SD)*	0.69 (0.46)	0.76 (16)	1.06 (0.55)	0.295‡
TGF-β levels (pg/mg protein), mean (SD)*	19.86 (1.99)	16.08 (4.72)	17.29 (6.12)	0.374‡
MMP-13 levels (pg/mg protein), mean (SD)*	1,511.78 (442.99)	908.64 (359.46)	1,312.67 (517.18)	0.088‡

MMP-13=matrix metalloproteinase-13; SD=standard deviation; TGF-β=transforming growth factor-beta
*Levels of hydroxyproline, TGF-β, and MMP-13 obtained from lung fibrosis tissue samples; †Kruskal–Wallis test; ‡analysis of variance (ANOVA) test
BLM2-51+CIP50: bleomycin (200 mcg)+*P. angulata* extract at 50 mg/kgBW (51 days observation); BLM2-51+CIP100: bleomycin (200 mcg)+*P. angulata* extract at 100 mg/kgBW (51 days observation); BLM2-51: bleomycin (200 mcg) (51 days observation)

exhibited the highest level (Figure 1). However, no statistically significant differences were observed in the fibrosis scores among the groups based on the Kruskal–Wallis test (Table 3).

The fibrotic area was assessed based on the percentage of the affected area relative to the total visual field. Most fibrosis was observed in the BLM2-51 group, while the BLM2+CIP50-51 group had the lowest degree. There were no statistically significant differences in the areas of fibrosis among the groups. Similarly, hydroxyproline levels in the lung tissue were the highest in the BLM2-51 group and lowest in the BLM2+CIP50-51 group; however, the differences were not significant among the groups. The analysis of TGF-β and MMP-13 levels in the lung tissue using ANOVA revealed no significant differences between groups (Table 3).

DISCUSSION

This study demonstrated that *P. angulata* extract did not prevent the development of lung inflammation or fibrosis in a scleroderma rat model. This finding suggests that the extract lacks preventive effects when administered during the early phase of fibrosis. During the inflammation phase, no significant differences were observed between the BLM-only group and the groups receiving 50 or 100 mg/kgBW *P. angulata* extract. BAL leukocyte counts, IL-6 levels, and lung injury scores were not significantly reduced by the extract. Similarly, in the fibrotic phase, *P. angulata* showed no efficacy in reducing fibrotic markers. Fibrosis scores, fibrosis area, hydroxyproline content, TGF-β levels, and MMP-13 levels showed no significant differences compared with those in the BLM-only group. These findings indicate that the oral administration of *P. angulata* for 14 days, starting concurrently with subcutaneous BLM, was not sufficient to prevent the onset or progression of inflammation and fibrosis in this model.

Scleroderma is an autoimmune disease characterized by skin and internal organ fibrosis. Animal models have been used to explore various aspects of scleroderma, particularly the BLM-induced fibrosis model. This model replicates scleroderma-associated fibrosis in the skin and internal organs.[17] Similarly, BLM is a common agent for inducing pulmonary fibrosis in animal models. However, compared with the effect of intratracheal instillation, subcutaneous injection of BLM resulted in lung fibrosis that resembled ILD in scleroderma.[12] The timing of intervention relative to BLM administration is critical in determining whether a substance is related to lung fibrosis development. When administered within 7–10 days after BLM administration, the substance is considered preventive. Conversely, if administered at least 7 days after the last BLM dose, it is considered curative.[18,19]

Inflammation and immune activation occur early in the pathogenesis of ILD in scleroderma.[20] Repeated endothelial and epithelial injuries trigger inflammatory responses, causing the recruitment and activation of fibroblasts. These fibroblasts differentiate into myofibroblasts that accumulate in the extracellular matrix (ECM),[21] preventing inflammation and fibrosis. Previous studies on various substances have shown that preventing inflammation precludes pulmonary fibrosis.[22,23]

Studies have demonstrated that *P. angulata* exhibits anti-inflammatory properties in various animal models. Choi and Hwang[24] reported that an extract containing *Piper cubeba*, *P. angulata*, and *Rosa hybrida* at 200 mg/kgBW reduced carrageenan-induced paw inflammation. In our study, *P. angulata* extract did not prevent inflammation, as evidenced by the slight differences in the histopathological analysis, BAL, and lung inflammatory marker findings. This discrepancy may due to the higher dosage used in their study compared to our study and included a combined extract of active substances.[24] Similarly, do Espírito Santo et al[25] showed that a concentrated ethanolic extract from *P. angulata* (CEEPA) reduced paw edema in an arthritis animal model. Despite the similar dosages to that of our study, the differences in inflammatory inducers and induced organs may explain the inconsistent results. While CEEPA reduced tumor necrosis factor-alpfa, IL-1 beta, and cyclooxygenase-2 expression, it did not affect IL-6 expression, similar to our results.

Other studies, including those by Almeida Junior et al,[26] Vieceli et al,[27] Rivera et al,[28] and Wiraswati et al,[29] showed that *P. angulata* reduces IL-6 levels or expression *in vitro* and *in vivo* in various inflammation models. However, IL-6 increases and peaks during the lung inflammation stage.[30] Tan et al[31] revealed an IL-6 expression higher on Day 14 than on Day 30 after BLM administration. Dewi et al[11] found that the administration of *P. angulata* during the fibrotic stage reduced IL-6 and lung fibrosis, suggesting that IL-6 plays a role in inducing pulmonary fibrosis beyond the inflammatory stage. However, the effects of *P. angulata* on IL-6 remain unclear. Further studies are required to clarify the effects and time course of *P. angulata* on IL-6.

In this study, *P. angulata* extract did not prevent fibrosis, as evidenced by the lack of differences in Ashcroft scores, hydroxyproline levels, and extent of fibrosis among groups. Histopathological examination and hydroxyproline levels are the primary indicators of pulmonary fibrosis in animal models.[19] Inflammation is a significant initial event in the development of pulmonary fibrosis. Inflammation, if not suppressed, results in the initiation of fibrosis.[32] This study showed that *P. angulata* extract did not reduce pulmonary fibrosis, which aligns with its lack of effect in reducing pulmonary inflammation. However, these findings differ from those of previous studies of *P. angulata* in models of patients with scleroderma. Dewi et al[11] reported that *P. angulata* reduced pulmonary fibrosis in BLM-induced animals. Similarly, Imaduddin et al[33]

found that *P. angulata* extract reduced the mRNA expression of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), MMP-8, and Krüppel-like factor 4. NOX4 is crucial in the production of reactive oxygen species and contributes to pulmonary fibrosis. Its expression was increased after BLM instillation, which was abrogated by *P. angulata*.[33,34]

These differences can be explained by differences in the timing of *P. angulata* administration and the duration of BLM exposure.[11,33] Difference in timing relative to BLM administration could influence different pathological events and gene expression.[31] *P. angulata*'s mechanisms of action possibly do not involve targeting the proteins expressed during inflammation; they rather involve the proteins expressed during the fibrotic phase of the disease. Furthermore, Dewi et al[8] demonstrated that patients with scleroderma showed improvement in skin fibrosis after administering *P. angulata* extract, validating the fact that *P. angulata* affects an already-established fibrosis state.

The function of *P. angulata* as an antifibrotic agent is to reduce fibrosis when administered during the fibrotic phase.[18] Fibrosis is a complex process involving various cells, signaling pathways, and cell-tissue interactions. It usually results from chronic inflammatory stimulus, which, if unresolved, triggers fibrotic mediators such as TGF-β. This further stimulates fibroblasts to transform into myofibroblasts and produce ECM. Fibrosis resolution occurs in three steps: removal of fibrotic triggers (such as TGF-β), degradation of ECM, and removal of myofibroblast.[35] TGF-β plays a significant role as a primary stimulator of fibroblasts in collagen production, while MMP-13 is crucial for collagen degradation.[36] In this study, the early administration of *P. angulata* in lung inflammation did not influence TGF-β and MMP-13.

Dewi et al[11] showed that *P. angulata* reduced TGF-β levels, mitigating lung fibrosis, inconsistent with our study. Liu et al[37] and Chaudary et al[30] reported that TGF-β peaked in the third week after BLM instillation, while MMP-13 peaked on Day 35.[38] Thus, administering *P. angulata* during the inflammatory phase is possibly overly early to effectively reduce TGF-β or stimulate MMP-13. This study and that of Dewi et al[11] showed that *P. angulata* does not reduce early-stage inflammation and may contribute to fibrosis if administered unduly early. However, when administered in the later fibrosis stage, it may reduce TGF-β expression, mitigating lung fibrosis. Several components of *P. angulata,* such as quercetin, angulatin A, and physalin A, influence fibrosis resolution by increasing fibroblast apoptosis and inducing myofibroblast dedifferentiation and apoptosis.[39–42]

This study is the first to investigate the role of *P. angulata* in a scleroderma animal model, specifically, its effect in preventing inflammation and fibrosis during the inflammatory phase. In contrast, previous studies by Dewi et al,[8] Imaduddin et al,[33] and Dewi et al[11] evaluated *P. angulata*'s effect after fibrosis had already manifested. Similarly, this study evaluated lung inflammation and fibrosis using methods recommended by the American Thoracic Society.[13,19] However, this study has some limitations. The duration of *P. angulata* administration was relatively short. This study also did not include a healthy control group, compare *P. angulata* with standard treatments, or evaluate its efficacy as a standard treatment. *P. angulata* administration at 50 and 100 mg/kgBW during the inflammatory phase of lung fibrosis did not prevent inflammation or fibrosis in scleroderma animal models. Further studies with a longer duration and involving combination with standard treatments during the inflammatory phase may validate *P. angulata*'s potential role in preventing lung inflammation and fibrosis.

Conflict of Interest

Rianto Setiabudy is the editorial board member of this journal, but was not involved in the decision-making process of this article.

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