

Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis

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Dear Dr. Desdiani,

Congratulations, the manuscript titled "Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis" has been successfully submitted to Evidence-Based Complementary and Alternative Medicine.

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Cover Letter

COVER LETTER

Dear Editor in Chief,

Pharmacology Research & Perspectives Journal

Here, we submit a manuscript entitled “ Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis ” for possible publication in Pharmacology Research & Perspectives Journal. Our study is original, has not already been published, and has not and will not be submitted for publication elsewhere as long as it is under consideration by Pharmacology Research & Perspectives Journal. All authors have read and approved the manuscript and take full responsibility for its content. All authors do not have conflict of interest in regard to this study or its funding.

Thank you very much for your attention,

Best regards,

A handwritten signature in blue ink, appearing to read 'Desdiani Desdiani', with a horizontal line underneath.

Desdiani Desdiani, MD, PhD

Corresponding Author

Submitted Article

Fibropreventive and Antifibrotic Effects of *Uncaria gambir* on Rats with Pulmonary Fibrosis

Desdiani Desdiani ¹, Iris Rengganis ², Samsuridjal Djauzi ², Agus Setiyono ³, Mohamad Sadikin ⁴, Sri Widia A. Jusman ⁴, Nuryati Chairani Siregar ⁵, Suradi Suradi ⁶, and Putri C. Eyaner ⁷

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Abstract: Pulmonary fibrosis causes scar tissue formation that disrupts the functioning of the lungs. *Uncaria gambir* (Hunter) Roxb (hereafter gambir)—a plant native to West Sumatra in Indonesia—contains flavonoid (+)-catechin and has strong antioxidant activity, and it can be used to combat pulmonary fibrosis. This random in vivo experimental study analyzed the antifibrotic effect of gambir on the lungs of rats with bleomycin-induced fibrosis. The subjects were 10 groups of 10-week-old male rats weighing around 200–250 g. All groups were terminated at the end of the seventh week or on day 50. The lungs were cleaned, and tissues were taken to analyze inflammatory cell counts and TGF- β 1 levels using bronchoalveolar lavage (BAL) with ELISA; type I collagen and tissue inhibitor of metalloproteinase 1 (TIMP-1) levels using immunohistochemistry (IHC); and activation of NF- κ B using ELISA and Western blot assays. The most severe histopathological characteristic based on the modified Ashcroft score was in the bleomycin group (BG), whereas the mildest was in the 262 mg/kg of bodyweight antifibrotic gambir-dosed group (AF G262). The results showed a significant difference in the BAL inflammatory cell count ($p = 0.017$; $p < 0.05$). AF G262 differed most from the other antifibrotic groups in terms of the number of inflammatory cells (0.63), TGF- β 1 levels (3.80), and NF- κ B levels (0.48), followed by the 131 mg/kg of bodyweight antifibrotic gambir-dosed group (AF G131), which also differed most from other antifibrotic groups in terms of NF- κ B (0.48), TIMP-1 (11.74), and collagen I (14.50) levels. Western blot analysis showed that the fibropreventive and antifibrotic groups had a specific band size of p65, whereas no specific band binding existed in the control group. This study concluded that the administration of AF G262 could improve fibrosis by lysing the extracellular matrix (ECM) in rat lungs.

Keywords: inflammatory cells; fibropreventive; antifibrotic; gambir

1. Introduction

Pulmonary fibrosis is a condition that causes scar tissue formation and involves an infiltration of inflammatory cells, a proliferation of fibroblasts and reactive oxygen species (ROS), and excessive extracellular matrix accumulation in the pulmonary parenchymal tissue, which can inhibit pulmonary functioning [1]. The pathogenesis of fibrosis also involves the growth factor TGF- β as an important fibrosis mediator because it is a potential target for antifibrotic therapy [2]. Based on data from the Indonesian Pulmonary Doctors Association (Persatuan Dokter Paru Indonesia; PDPI), the prevalence of pulmonary fibrosis in Indonesia in 2017 reached 6.26–7.73 percent per 1,000,000 population and was predicted to increase. Only 700–1500 patients were detected due to a lack of treatment [3]. Green tea, with its catechin (flavonoid) content, proved to have fibrolytic and fibropreventive effects in a study on rats [1]. *Uncaria gambir* (Hunter) Roxb (hereafter gambir) is a member of the Rubiaceae family and is abundant in Southeast Asia, especially in the Sumatra region. This plant's flavonoid (+)-catechin is known for its strong antioxidant activity [4]. Catechin can repair alcohol-induced liver damage through the mechanism of the in vivo NF- κ B anti-inflammatory pathway [5]. Based on the previous study [1], gambir can also be used for pulmonary fibrosis treatment; therefore, an in vivo study was required to examine its mechanism as an antifibrotic and/or fibropreventive substance. Intratracheal bleomycin induction is the best preclinical test to study fibrosis in animal models. Bleomycin can induce oxidative stress in pulmonary tissue, triggering an inflammatory reaction and the formation of fibrotic tissues [2]. As a new therapy for pulmonary fibrosis (which is currently irreversible), the administration of gambir was expected to prevent and treat extensive pulmonary fibrosis in rat models. Green tea was used for fibropreventive and antifibrotic comparison.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted according to the requirements of the Helsinki Declaration, and approval was obtained from the Health Research Ethics Committee, Faculty of Veterinary Medicine IPB University, Bogor, Indonesia, under registration number 114/KEH/SKE/XII/2018, and the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia.

2.2. Rats and Gambir Dosing

This random in vivo analytical study, with an experimental design, was performed on healthy 10-week-old Sprague–Dawley male rats weighing \pm 200–250 g each. Prior to treatment, the rats were acclimatized for 14 d in individual polycarbonate cages covered with wood shavings containing 1 rat per cage. The rats received standard food and drink ad libitum and an albendazole anthelmintic. The room temperature was kept at 25–30 °C, with 80–90% humidity, under 12 h of light and 12 h of darkness. Besides ensuring adaptation to treatment conditions, acclimatization helped in selecting sick animals. In this study, the subjects were divided into 10 groups of 3 rats each, as follows:

1. negative control group (NG)
2. bleomycin group (BG); rats only given 4 mg/kg of bodyweight bleomycin from day 0 to day 21
3. fibropreventive group (FP GT): rats given 4 mg/kg of bodyweight bleomycin on day 8 and day 28 + green tea daily for 49 days
4. antifibrotic group (AF GT): rats given 4 mg/kg of bodyweight of bleomycin on day 0 and day 21 + green tea from day 15 to day 49

5. fibropreventive group (FP G65): rats given 4 mg/kg of bodyweight of bleomycin on day 8 and day 28 + 65 mg/kg of bodyweight of gambir daily until day 49
6. fibropreventive group (FP G131): rats given 4 mg/kg of bodyweight of bleomycin on day 8 and day 28 + 131 mg/kg of bodyweight of gambir daily until day 49
7. fibropreventive group (FP G262): rats given 4 mg/kg of bodyweight of bleomycin on day 8 and day 28 + 262 mg/kg of bodyweight of gambir daily until day 49
8. antifibrotic group (AF G65): rats given 4 mg/kg of bodyweight of bleomycin on day 0 and day 21 + 65 mg/kg of bodyweight of gambir daily from day 15 to day 49
9. antifibrotic group (AF G131): rats given 4 mg/kg of bodyweight of bleomycin on day 0 and day 21 + 131 mg/kg of bodyweight of gambir daily from day 15 to day 49
10. antifibrotic group (AF G262): rats given 4 mg/kg of bodyweight of bleomycin on day 0 and day 21 + 262 mg/kg of bodyweight of gambir daily from day 15 to day 49

The dose of green tea used was 125 mg/kg of bodyweight given orally. All rats were terminated at the end of the seventh week or on day 50.

2.3. Lung Tissue Histology

Prior to staining, the organs were fixed with paraffin to maintain the cell texture and structure during the cutting and staining process. The hydration stage aimed to facilitate the entry of dyes because water-soluble materials were used. Staining to observe lung cells/tissue was performed with hematoxylin eosin using Hematoxylin-Mayer reagent and Eosin solution (HE) and with Masson's trichrome (MT) using Carrazi's hematoxylin reagent, orange G 0.75%, Ponceau xylidine fuchsin, 2.5% phosphotungstic acid solution, and aniline blue. Staining was carried out on tissue section thicknesses of 3–5 μ m. Thereafter, dehydration was conducted using a suitable xylene-soluble material (DPX). Tissue staining was followed by observing the collagen structure formation under a microscope and evaluating the fibrosis in each treatment group. The fibrosis images were analyzed using a modified Ascroft score.

Immunohistochemical staining was carried out using the Dako Real Envision (Dako) procedure with a special object glass coated with poly-L-lysine. The percentage of fibrosis area was calculated, and the protein expression level was tested using immunohistochemical examination. The percentage of fibrosis area was the ratio of the total area of brown collagen to the total area of one visual field, and the calculation of the extracellular collagen I and TIMP-1 expression levels was carried out using image analysis techniques with the ImageJ program.

2.4. Bronchoalveolar Lavage Fluid Collection, Cell Counts, and TGF- β 1 ELISA

The lungs of all the groups were cleaned and prepared to analyze the inflammatory cells and TGF- β 1 levels using BAL and the ELISA method. The debris was separated from the BAL fluid at a centrifugation speed of 5000 rpm for 5 min. Absorption was read with an ELISA reader at a wavelength of 450 nm. The results of the absorbance measurements were calculated as averages and then tabulated prior to preparing a concentration diagram of the absorbance. After obtaining the results of the formula, using absorbance data, the sample concentration was calculated.

2.5. NF- κ B ELISA and Western Blot Analysis

Activation of NF- κ B was assessed using ELISA and Western blot assays. The ELISA procedure was carried out after the tissue was homogenized by centrifugation for 15 min at a speed of 10,000 rpm to prepare an antibody cocktail as an ELISA reagent and standard preparation. The results were read with the ELISA reader at 450 nm. The results of the absorbance measurements were calculated as averages and

tabulated, and then a concentration diagram of the absorbance was prepared. After obtaining the results of the formula, using absorbance data, the sample concentration was calculated.

The lung tissue was homogenized in PBS containing a protease inhibitor. After homogenization, it was centrifuged for 15 min at 14,000 rpm at 4 °C. The supernatants of the lung tissue were collected, and the protein concentration of each sample was measured using a bicinchoninic acid (BCA) assay kit according to the BSA standard. Then, 150 µg of protein for each sample was dissolved in 10% Tris-glycine SDS polyacrylamide gel. The protein bands were transferred to the nitrocellulose membranes, and after 1 h of incubation in liquid (5% dry milk and Tris-buffered saline/Tween 20) at room temperature, the membranes were incubated for 24 h with anti TGF-β1. Secondary antibodies were added at a dilution of 1:10,000 and incubated at room temperature for 1 h. The densitometry of nitrocellulose membrane, antibodies, and secondary antibodies was conducted with ImageQuant LAS4000.

The resulting data were analyzed with SPSS v. 20. An ANOVA test was performed to check for any differences between variables, followed by post hoc tests if the results were significant.

3. Results

3.1. Formation of Fibrotic Tissue in the Lung Tissue of Mice Exposed to Bleomycin

The baseline characteristics of the subjects included demographic characteristics (bodyweight and lung weight) and pulmonary macroscopic images, as shown in Table 1.

Table 1. Characteristics and Macroscopic Imaging of Rat Lungs

Group n = 3	Bodyweight, mean (g)	Lung weight, mean (g)	Relative organ weight	Macroscopic Image
NG	218 g	3.7	0.016	No damage to the lungs.
BG	232 g	4.3	0.018	Damage to the lungs, marked by bleeding, edema, and lumps.
FP GT	246 g	4	0.016	Little damage to the lungs.
AF GT	235 g	3.3	0.014	Little damage to the lungs.
FP G65	212 g	3	0.014	Little damage to the lungs, with several parts seeming normal.
FP G131	243 g	3.9	0.016	Extensive damage to the lungs, marked by edema, lumps, bleeding, and a bluish-red tone.
FP G262	210 g	2.8	0.013	Damage to the lungs, bleeding, and lumps, but not as severe as for FP G131.
AF G65	221 g	4	0.018	Little damage to the lungs, and several parts seemed normal.
AF G131	240 g	4.1	0.017	Extensive damage to the lungs, marked by edema, lumps, bleeding, and a bluish-red tone.
AF G262	229 g	3	0.013	Little damage to the lungs, and several parts seemed normal.

The subjects were 10-week-old male rats assigned to 10 groups of 3 rats each with bodyweights of approximately 200–250 g. The lowest bodyweight mean average was in FP G262, at 210 g, whereas the highest was in FP GT, at 246 g. After termination, the lungs were weighed and their macroscopic characteristics assessed. The lung weights of all subjects were approximately 2.8–4.1 g. Relative organ weight was calculated as the lung weight divided by the bodyweight. NG had the highest relative organ weight, at 0.018 g, whereas FP G262 and AF G262 had the lowest relative organ weights, at 0.013. The macroscopic imaging of the subjects is shown in Figure 1.

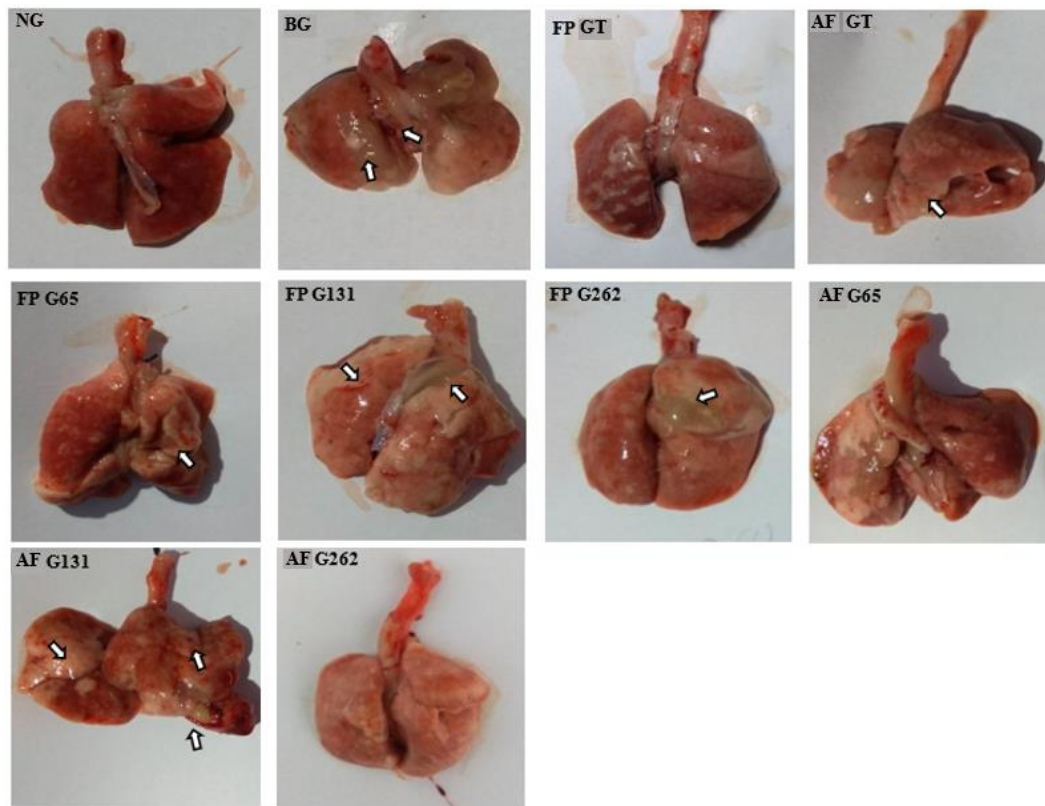
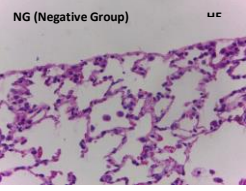
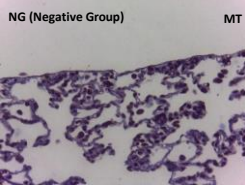
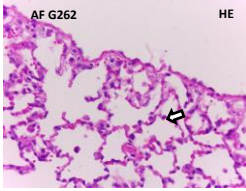
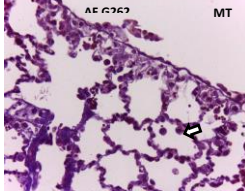
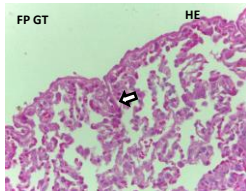
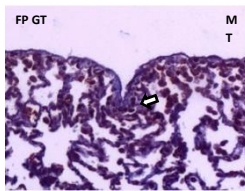
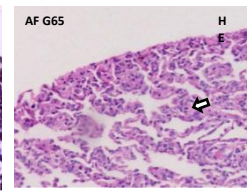
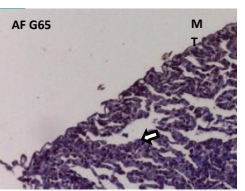
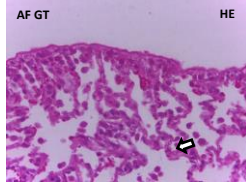
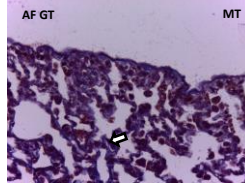
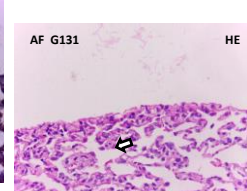
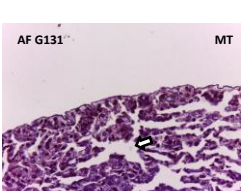
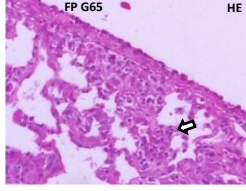
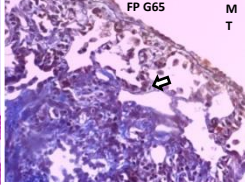
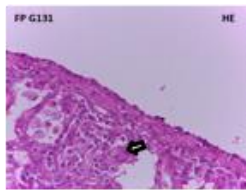
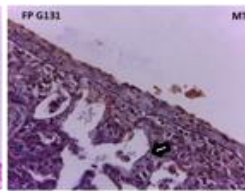
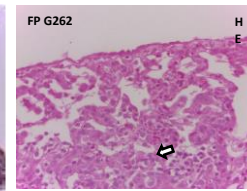
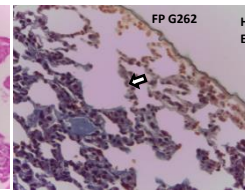
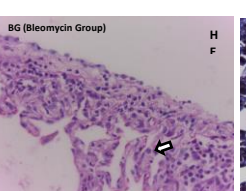
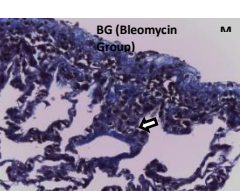


Figure 1. Macroscopic image of pulmonary tissue of the subjects. Arrow indicated bleeding and lumps in the lung tissue. BG group was smaller compared to NG, although had heavier relative weight (0.016 vs 0.018). FP GT, AF GT, FP G65, AF G65 and AF G262 showed less damage compared to other groups.

3.2. Effects of Gambir on Bleomycin-Induced Pulmonary Fibrosis

According to the histopathological characteristics, each group was assessed using a modified Ashcroft score to determine the stages of fibrosis. The most severe histopathological characteristic based on the modified Ashcroft score was observed in BG. In the gambir groups, the most severe was observed in FP G131 and FP G262, as shown in Figure 2.

Group	Stage of Fibrosis	Histopathological image of the lungs with HE & MT			
• NG	Stage 0				
• AF G262	Stage 3				
<ul style="list-style-type: none"> • FP GT • AF GT • FP G65 • AF G65 • AF G131 	Stage 5				
					
					
•	Stage 6				
• BG	Stage 7				

NG = Negative control group; BG = Bleomycin group; FP GT = fibropreventive green tea; AF GT = antifibrotic green tea; FP G65 = 65 mg/kg bodyweight of fibropreventive gambir; FP G131 = 131 mg/kg bodyweight of fibropreventive gambir;

FP G262 = 262 mg/kg bodyweight of fibropreventive gambir; AF G65 = 65 mg/kg bodyweight of antifibrotic gambir; AF G131 = 131 mg/kg bodyweight of antifibrotic gambir; AF G262 = 262 mg/kg bodyweight of antifibrotic gambir.

Figure 2. Histopathological changes in pulmonary tissues of treatment groups using Hematoxylin-Eosin (HE) and Masson Trichome (MT) staining.

3.3. Gambir Alters Inflammatory Cell Profiles in Lungs

The inflammatory cell count results from the bronchoalveolar lavage (BAL) assessment for groups 1–10 were 2.90 (2.3–4.0), 5.70 (5.0–5.8), 2.25 (2.0–2.8), 3.35 (2.7–3.4), 3.0 (2.8–3.2), 4.75 (4.6–5.3), 2.60 (2.0–3.5), 3.70 (1.6–3.8), 4.00 (3.9–4.8), and 2.10 (2.0–3.5), respectively. The statistical test showed a significant difference in the BAL inflammatory cell count ($p = 0.017$; $p < 0.05$). The post hoc analysis of the inflammatory cell count differences between the fibropreventive, antifibrotic green tea, and gambir groups revealed significant differences between several groups, as shown in Figure 3.

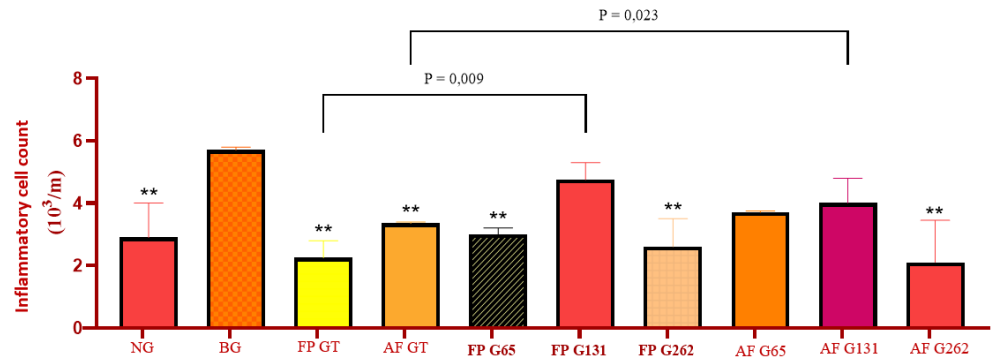


Figure 3. Comparison of inflammatory cell count between groups. Noted significant decrease of BAL inflammatory cell count caused by the administration of green tea and gambir compared to bleomycin group (P < 0,05 vs BG)**

3.4. Gambir Has No Effect on Preventing TGF-β1 Expression and Activation

The results of the TGF-β1-level measurements (pg/mg protein) for groups 1–10 were 0.88 (0.3–2.3), 4.52 (2.6–6.2), 1.14 (0.1–2.6), 2.30 (1.0–4.4), 1.12 (0.4–1.6), 2.58 (1.2–6.7), 2.97 (1.6–3.5), 1.50 (0.3–2.3), 1.02 (0.1–3.1), and 0.64 (0.5–0.8), respectively. The statistical results of all groups showed insignificant differences in TGF-β1 levels ($p = 0.117$; $p > 0.05$), as shown in Figure 4.

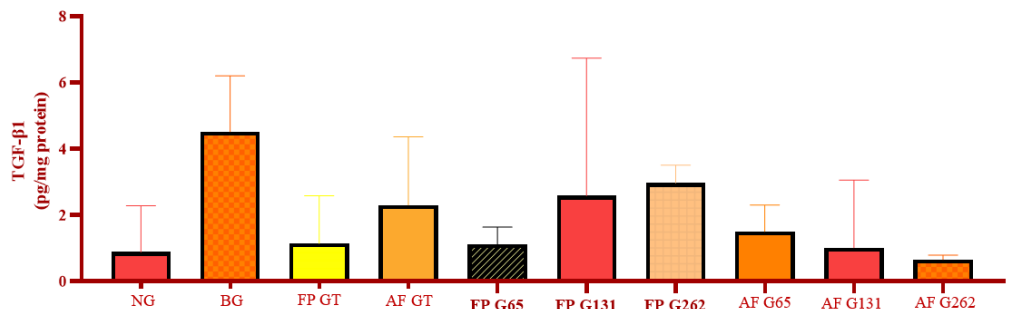
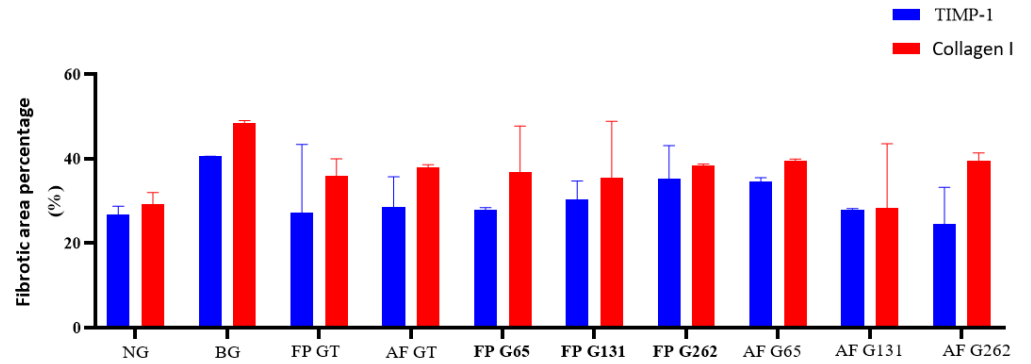


Figure 4. Comparison of TGF- β 1 BAL between groups. There was insignificant difference of TGF- β 1 BAL level between groups ($P > 0.05$).

The results of the TIMP-1 expression assessment using Image J for groups 1–10 were 26.77 (16.76–28.77), 40.5 (32.44–40.6), 27.28 (21.36–43.34), 28.56 (22.62–35.70), 27.87 (23.14–28.41), 30.37 (20.97–34.70), 35.30 (32.46–43.13), 34.58 (26.50–35.51), 27.84 (22.28–28.20), and 24.52 (23.03–33.22), respectively. Additionally, the results of the collagen I expression assessment using Image J for groups 1–10 were 29.2 (26.35–32.03), 48.36 (44.51–49.02), 36.04 (26.17–39.99), 38.02 (29.40–38.64), 36.82 (34.88–47.71), 35.59 (32.53–48.90), 38.4 (29.82–38.77),



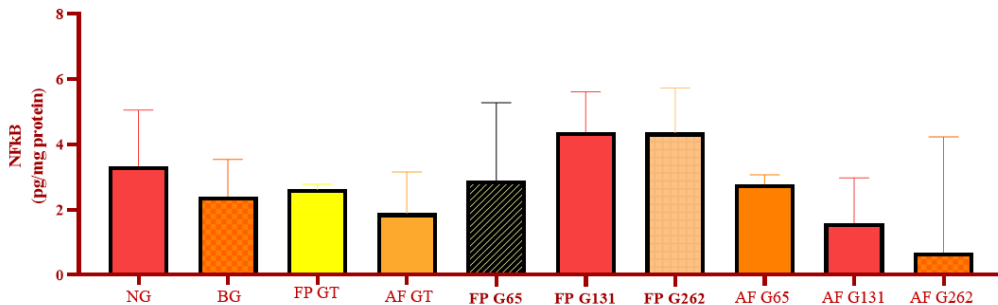
39.46 (33.43–39.93), 28.41 (26.42–43.57), and 39.43 (37.95–41.40), respectively. The statistical results of all groups revealed no significant differences in TIMP-1 and collagen I expression levels ($p = 0.31$ and $p = 0.168$; $p > 0.05$), as shown in Figure 5.

Figure 5. Comparison of TIMP-1 and collagen I expression between groups. There was insignificant difference in TIMP-1 and collagen I expression between groups ($P > 0.05$).

3.5. Gambir Does Not Prevent Bleomycin-Induced Activation of NF- κ B

The results of the NF- κ B assessment (pg/mg protein) for groups 1–10 were 3.34 (2.5–5.1), 2.41 (1.0–3.5), 2.62 (2.2–2.8), 1.89 (1.9–3.2), 2.89 (2.2–5.3), 4.38 (3.4–5.6), 4.36 (3.3–5.7), 2.79 (1.7–3.1), 1.59 (1.0–3.0), and 0.70 (0.6–4.2), respectively. The statistical results of all groups showed no significant differences in NF- κ B levels ($p = 0.134$; $p > 0.05$), as shown in Figure 6.

Figure 6. Comparison of pulmonary tissue NF- κ B level between groups.



There was insignificant difference in pulmonary tissue NF- κ B level between groups ($P > 0.05$).

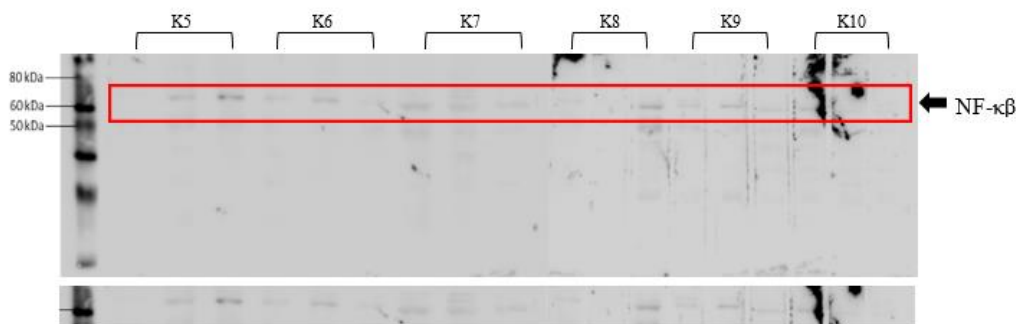
Mean discrepancy values were obtained by deducting the mean value for the bleomycin group from the mean value for the treatment group. The largest

discrepancy in the inflammatory cell count was observed in FP GT. The largest discrepancies in the TGF- β 1 and NF- κ B levels were seen in AF G262. The largest discrepancies in TIMP-1 and collagen expression levels were seen in AF G131. Among the fibropreventive groups, FP GT had the largest discrepancy in terms of the inflammatory cell count (3.15) and NF- κ B (-0.22) and collagen I expression (13.23) levels, followed by FP G65, which had the largest discrepancy among the fibropreventive groups in terms of the TGF- β 1 (3.38) and TIMP-1 expression (11.37) levels. In regard to the inflammatory cell count, increasing or decreasing the gambir dose had no effect on reducing the inflammatory cell count. By contrast, in terms of the TGF- β 1 and collagen I expression levels, the lower the gambir dose, the better its influence in reducing the TGF- β 1 and collagen I expression levels (i.e., decreasing the mean discrepancies for the TGF- β 1 and collagen I expression levels in line with increasing gambir doses). The mean discrepancy for the NF- κ B levels was negative, meaning that the fibropreventive gambir and green tea groups had a higher mean NF- κ B level compared to BG, and the higher the gambir dose, the higher the NF- κ B level.

AF G262 had the largest discrepancy among the antifibrotic groups in terms of the number of inflammatory cells (0.63) and the TGF- β 1 (3.80) and NF- κ B (0.48) levels, followed by AF G131, which also had the largest discrepancy among the antifibrotic groups in terms of the NF- κ B (0.48), TIMP-1 (11.74), and collagen I (14.50) levels. The Western blot examination showed that the fibropreventive and antifibrotic groups had a specific band size of p65, whereas no specific band binding existed in the green tea group.

Western blot assay revealed that the fibropreventive and antifibrotic groups of gambir was seen in a specific band of p65, as shown in Figure 7.

Figure 7. Western blot result on gambir group.



Meanwhile, the negative control group, bleomycin group, and fibropreventive and antifibrotic groups of green tea extract did not show a specific band.

3.6. Gambir is an Effective Antifibrotic Treatment

The results of our study demonstrated that gambir decreased the number of inflammatory cells, as well as the levels of TGF- β 1, NF- κ B, TIMP-1, and collagen I expression. However, fibropreventively, the increase in the dose of gambir was not in line with the decrease in the TGF- β 1 and TIMP-1 expression levels. On the contrary, a lower dose of gambir produced a decrease in TGF- β 1 levels and better TIMP-1 expression. The antifibrotic groups showed an increase in the gambir dose in line with a decrease in the TGF- β 1 and NF- κ B levels, and lower TIMP-1 expression levels. This was supported by macroscopic and microscopic images that revealed less lung tissue damage and a lesser degree of fibrosis based on the modified Ashcroft score, which was better than that for BG and other gambir groups.

4. Discussion

4.1. Characteristics of Green Tea and Gambir Subjects

No study on gambir has examined its ability to decrease or prevent pulmonary fibrosis in humans and animals. Gambir is known to have a strong antioxidant effect

because it contains abundant catechin [6–8]. Melia et al. revealed that, besides its high antioxidant activity, gambir also exhibits antimicrobial activity [9]. Idiopathic pulmonary fibrosis (IPF), which is a fatal lung disease marked by chronic, progressive fibrosis, inhibits the functioning of the lungs, and oxidative stress is the main pathway in IPF [10]. According to Tsai et al. [11], the antifibrosis, anti-inflammatory, and antioxidative effects of catechin have been reported. Several in vitro and in vivo studies have shown that catechin can weaken fibroblast proliferation, increase antioxidant protection, and inhibit inflammation.

The administration of 262 mg/kg of bodyweight and 65 mg/kg of bodyweight of gambir to the antifibrotic groups and 65 mg/kg of bodyweight of gambir to the fibropreventive groups reduced macroscopic damage to pulmonary tissues compared to BG. This study showed that the administration of 131 mg/kg of bodyweight of gambir to the fibropreventive and antifibrotic groups resulted in extensive pulmonary tissue damage, characterized by pulmonary edema, lumps, bleeding, and a bluish-red tone. This may have been caused by the inhibition of the drug side-effect elimination system and toxic effect of gambir at a certain dose on the lungs of animals.

4.2. The Administration of Green Tea Reduced the Inflammatory Cell Count

Furthermore, this study showed that the administration of green tea could reduce the number of inflammatory cells caused by intratracheal bleomycin. Fibrotic pulmonary tissue first undergoes cellular inflammation and histologically exhibits an accumulation of inflammatory cells, such as lymphocytes, macrophages, plasma cells, eosinophils, and neutrophils. Inflammatory response is one of the paths to pulmonary fibrosis [12].

In this study, the administration of green tea to the antifibrotic and fibropreventive groups also decreased the levels of TGF- β . TGF- β induces myofibroblast proliferation and extracellular matrix protein synthesis, and suppresses ECM degradation by increasing antiproteinase or reducing metalloprotease, causing fibrosis. TGF- β 1 also stimulates ROS production, leading to oxidative stress by reducing the antioxidant enzymes in the lungs and hepatocytes, such as catalase, GSH, and SOD, which can inhibit fibrogenic responses due to oxidative stress [13]. The administration of bleomycin to rats increased cytokine and chemokine levels on days 7 and 14 compared to a control [14]. This study concluded that bleomycin administered intratracheally and fibropreventive green tea could reduce TGF- β 1 levels, inducing fibrosis. Tabuchi et al. [15] proposed that green tea could suppress TGF- β 1 by interacting with type 2 TGF- β receptors.

NF- κ B is a type of regulator protein for the immune system and inflammatory responses [16]. Damaged pulmonary tissues activate NF- κ B through phosphorylation, mediated by I κ B kinase (IKK); the degradation of inhibitory molecules, such as I κ B α ; and phosphorylation of the NF- κ B p65 subunit. The activated NF- κ B then binds in a specific DNA sequence to regulate several inflammatory gene expressions. Additionally, NF- κ B regulates three main functions: activation of inflammatory T cells, survivability, and inflammatory responses. Activation of NF- κ B is responsible for profibrogenic signaling pathways. Based on the results of this study, bleomycin administration leads to a significant increase in NF- κ B levels, triggering inflammatory cytokines, such as TNF- α and IL-1 β , and chemokines, such as MCP-1 (monocytes chemoattractant protein-1). S. Beigh et al. [17] found that TNF- α and transcription factor NF- κ B levels increased in fibrotic pulmonary tissue induced by intratracheal 5 mg/kg bleomycin. Moreover, they stated that suppressing the NF- κ B signaling pathway could prevent the myofibroblast differentiation and fibrosis caused by bleomycin, followed by a reduction in β -catechin expression. Green tea has an antioxidant effect, inhibiting the activation of NF- κ B by suppressing collagen formation and TIMP-1 through in vivo antifibrotic and fibropreventive mechanisms in the liver [4,18].

4.3. Administration of 262 mg/kg of Bodyweight of Gambir Reduced the Inflammatory Cell Count

The inflammatory cell count results of the BAL assessment for 65 mg/kg of bodyweight, 131 mg/kg of bodyweight, and 262 mg/kg of bodyweight of gambir for the fibropreventive/antifibrotic and control groups showed that AF G262 had the lowest count, whereas BG had the highest. Tsai et al. [11] reported that several in vitro and in vivo studies indicated various effects of catechin on fibroblasts, such as suppressing cell proliferation, increasing antioxidant protection, and inhibiting inflammation.

The lowest TGF- β 1 level was in AF G262, followed by NG, and the highest was in BG. You et al. [19] stated that epigallocatechin gallate (EGCG) could inhibit the proliferation of myofibroblasts by suppressing the secretion of TGF- β 1. This study demonstrated that the administration of gambir reduced TGF- β 1 levels, which could induce fibrosis.

The lowest NF- κ B level was in AF G262, whereas the highest was in FP G131, followed by FP G262. Paola [20] revealed that catechin reduced the exudate level of TNF-alpha, active cytokines, and several signaling pathways, leading to the activation of the NF- κ B transcription factor. Polyphenol inhibited the induction of tumor necrosis factor-alpha in macrophages by suppressing the activation of NF- κ B.

4.4. The Administration of 262 mg/kg of Bodyweight of Gambir to the Antifibrotic Groups Reduced the Inflammatory Cell Counts Compared to Fibropreventive Green Tea

The lowest inflammatory cell count was in AF G262, whereas the highest was in FP G131. Dona et al. [21] stated that green tea strongly inhibits in vitro and in vivo neutrophil activities and, as an orally available medicine, may be effective in treating and preventing fibrosis.

The lowest TGF- β 1 level was in AF G262, whereas the highest was in FP G262. Lago et al. [22] stated that flavonoid-type antifibrotic therapy could reduce TGF- β 1 levels in the lungs of rats with fibrosis.

The lowest NF- κ B level was in AF G262, whereas the highest was in FP G131. The statistical test results did not reveal significant differences in the NF- κ B levels ($p = 0.089$; $p > 0.05$). Gambir has proven to have an antioxidant effect via various mechanisms, including the inhibition of NF- κ B activation. It acts as an antifibrotic agent, not only by inhibiting NF- κ B activation but also through its role as an anti-inflammatory and antioxidant agent. Gambir may suppress the pro-inflammatory pathways related to most chronic diseases and inhibit the production of TNF and cell signaling mediated by TNF in various types of cells. Gambir has an antioxidant effect, inhibiting NF- κ B activation by suppressing the formation of collagen and TIMP-1 through in vivo antifibrotic and fibropreventive mechanisms in the liver [4,5].

4.5. Histopathological Imaging of Pulmonary Tissues Using Hematoxylin-Eosin and Masson's Trichome Staining Based on a Modified Ashcroft Score

From histopathological results based on the modified Ashcroft score, the green tea and gambir groups showed improvement, especially in AF G262, which exhibited a stage 3 modified Ashcroft score. The administration of preventive gambir in 131 mg/kg of bodyweight and 262 mg/kg of bodyweight doses produced histopathological images based on a stage 6 modified Ashcroft score, which tended to be more severe than other groups, albeit better than in BG. This indicated that the administration of preventive gambir and an increased dosage did not improve fibrosis in a dose-dependent manner. Other studies stated that the administration of bleomycin could increase the expression of inflammatory cytokines, followed by an increase in profibrotic mediators, such as TGF- β 1, lysophosphatidic acid (LPA), and matrix deposition. The matrix metalloprotease (MMP) gene includes MMP12, MMP19, and lysyl oxidase-like (LOXL) [23]. Gambir and green tea are believed to suppress the expression of inflammatory cytokines and profibrotic mediators, one of which is TIMP-1 and collagen I expression. This was proved by Sri Ningsih in her study on the role of gambir extract in suppressing the development of liver fibrosis [18]. Seger et al. [24], who examined histopathological images of fibrotic lungs, showed an

accumulation of fibrotic tissue and collagen deposition in the alveolar septum using specific Masson's trichome staining.

4.6. Administration of Bleomycin Increased TIMP-1 and Collagen I Expression Percentages

The lowest TIMP-1 expression was in NG, whereas the highest was in BG. TIMP-1 is believed to have an antiapoptotic effect on activated pulmonary epithelial cells. Hemmann [25] believes that MMP and its physiological inhibitor, TIMP-1, play an important role in the development of fibrotic tissue. Zhen et al. [26] revealed that during fibrosis progression, fibroblasts showed increased proliferation and migration, increased matrix protein expression, and production of MMP and TIMP, causing interstitial collagen or extracellular matrix substitution.

The lowest collagen I expression was in NG, whereas the highest was in BG. Green tea is believed to inhibit the formation of collagen I by increasing the production of MMP and triggering several types of cells, such as macrophages, to increase collagen I degradation. Salem et al. [27] claimed that a green tea group showed thin interalveolar septa, decreased cellular infiltration, red blood cells, and accumulation of collagen fibers. Immunohistochemistry staining was represented by a brown color.

4.7. Administration of 262 mg/kg of Bodyweight Gambir to the Antifibrotic Groups Decreased the TIMP-1 Expression Percentage, and Administration of 131 mg/kg of Bodyweight of Gambir to the Antifibrotic Groups Decreased the Collagen I Expression Percentage

The lowest TIMP-1 expression was in AF G262, whereas the highest was in BG. The PCR results in the study conducted by Dona et al. [21] showed that catechin increased collagen I and TIMP-1 transcription. Catechin inhibited the production of collagen I and increased MMP; therefore, catechin is believed to be a potential therapy for liver fibrosis.

The lowest collagen I expression was in AF G131, and the highest was in BG. FP G131 had lower collagen I expression than the other fibropreventive groups and BG, although it was higher than in NG. AF G131 had lower collagen I expression than the other antifibrotic groups and the control groups (NG and BG). This was caused by the dose-dependent antioxidant activity of gambir, with a lower dose producing a suboptimal response and a higher dose potentially being toxic, which is in accordance with the work of Tsai et al. [11]. Nakamuta et al. [28] concluded that the dose of catechin affected the production of collagen and MMP activity. Catechin could inhibit the production of collagen and increase MMP in liver fibrosis.

4.8. Administration of 262 mg/kg of Bodyweight of Gambir to the Antifibrotic Groups Decreased the Percentage of TIMP-1 Expression, and Administration of 131 mg/kg of Bodyweight of Gambir to the Antifibrotic Groups Reduced the Percentage of Collagen I Compared to Green Tea

The lowest TIMP-1 expression was in AF G262, whereas the highest was in FP G262. Based on microscopic images, TIMP-1 and collagen I expressions were marked by well-defined brown fibers along the alveolar septa. There were various TIMP-1 and collagen I expressions in all groups, but the differences were not significant. Tissue inhibitors of metalloproteinase (TIMPs) are specific, endogenous inhibitor proteins of matrix metalloproteinases (MMPs). Increased TIMP levels result in ECM accumulation, which causes pulmonary fibrosis [29]. In this study, the mean percentages for TIMP-1 and collagen I expressions between groups indicated that AF G262 had a lower percentage than that of BG, although it was higher than that of NG. This was supported by Sri Ningsih, who revealed that the administration of gambir extract decreased TIMP-1 and collagen I expression, which in turn suppressed the development of liver fibrosis [18].

The lowest collagen I expression was in AF G131, whereas the highest was in AF G65. Antifibrotic doses of 131 mg/kg of bodyweight gambir are believed to inhibit the formation of collagen I by increasing the production of MMP and

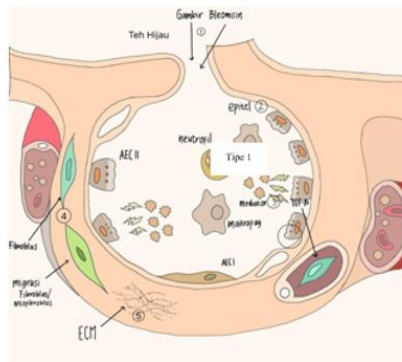
triggering several types of cells, such as macrophages, thus increasing collagen I degradation. Chen et al. [30] stated that the calculation and identification of extracellular matrix and collagen I expression could be accomplished using immunohistochemistry staining and image analysis techniques.

4.9. Interaction between Gambir and NF- κ B Using the Western Blot Method

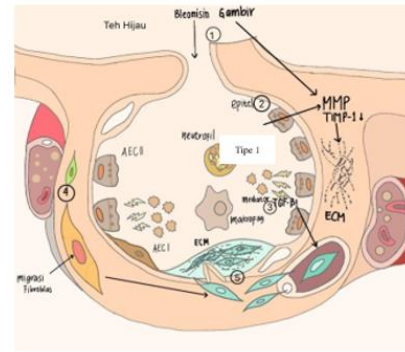
Western blot assays revealed that FP G65, FP G131, FP G262, AF G65, AF G131, and AF G262 had a specific p65 NF- κ B dimer-size band. This indicated that the bonding interaction of gambir with NF- κ B could prevent it from translocating to the nucleus, thus initiating no inflammatory/fibrogenesis process. Bitzer et al. [31] confirmed that the protective effect of an antifibrosis agent was correlated to a high level of Smad7 and inactivation of the NF- κ B and TGF- β 1 signaling pathways by performing Western blot analysis on I κ B α and NF- κ Bp65 antibodies. The fibropreventive and antifibrotic mechanisms of green tea and gambir identified in this study are shown in Figure 8.

Figure 8. Mechanism of fibropreventive and antifibrotic of gambir

A. Fibropreventive



B. Antifibrotic



- A. Gambir and green tea were given initially to suppress the production of ECM by interrupting the activation of type 1 pulmonary epithelial cells, suppressing the activities of ROS, mediators, and inflammatory cells, followed by the administration of bleomycin at the beginning of weeks 2 and 4. (2) The damage to type 1 pulmonary epithelial tissue is decreased. (3) The activities of ROS, inflammatory cells (including neutrophils, macrophages, and lymphocytes), and the activities of the inflammatory mediators TGF- β 1, NF- κ B, and TIMP-1/collagen decreased. (4) The activation of fibroblasts and myofibroblast proliferation decreased. (5) The deposition of the extracellular matrix (especially in FP GT) decreased.
- B. Bleomycin was given first to initiate fibrosis, followed by the administration of gambir and green tea at the beginning of week 3 to lyse ECM and suppression of the activation of type 1 epithelial cells. (2) Type 2 pulmonary epithelial damage improved. (3) The activities of ROS, inflammatory cells (including neutrophils, macrophages, and lymphocytes), and inflammatory mediator TGF- β 1, NF- κ B, and TIMP-1/collagen expression levels were suppressed. (4) Activation of fibroblasts and myofibroblasts decreased. (5) Lysis of the extracellular matrix occurred (especially in AF G262).

The fibropreventive effect of green tea proved to be better than that of the gambir, a result supported by El Safti et al. [32]. Due to different methods of administration, pulmonary fibrosis improvement might occur through natural recovery after arresting bleomycin induction in the moderate stage of fibrosis. Increasing the dose of gambir did not have the expected fibropreventive effect related to the dose-dependent antioxidant activity of gambir as stated by Tsai et al. [11]. Many studies have demonstrated the effectiveness of green tea in inhibiting pulmonary fibrosis. The administration of preventive gambir in 131 mg/kg of

bodyweight and 262 mg/kg of bodyweight doses did not result in low-stage histopathological images based on a stage 6 modified Ashcroft score, which tended to be worse than in the other groups, albeit better than in BG. This showed that the administration of preventive gambir and an increase in the dose did not improve fibrosis in a dose-dependent manner. AF G262 showed a reduced (stage 3) modified Ashcroft score/histopathological image and decreased inflammatory cell counts and levels of TGF- β 1, TIMP-1 and NF- κ B, which prompted the formation of fibrosis; therefore, the administration of 262 mg/kg of bodyweight of gambir could improve fibrosis/lyse ECM in rat lungs.

In summary, AF G262 had the largest discrepancy among the antifibrotic groups in terms of the number of inflammatory cells, the TGF- β 1 and NF- κ B levels. The Western blot examination showed that the fibropreventive and antifibrotic groups had a specific band size of p65, whereas no specific band binding existed in the green tea group. The results of our study demonstrated that gambir decreased the number of inflammatory cells, as well as the levels of TGF- β 1, NF- κ B, TIMP-1, and collagen I expression. However, fibropreventively, the increase in the dose of gambir was not in line with the decrease in the TGF- β 1 and TIMP-1 expression levels. On the contrary, a lower dose of gambir produced a decrease in TGF- β 1 levels and better TIMP-1 expression. The antifibrotic groups showed an increase in the gambir dose in line with a decrease in the TGF- β 1 and NF- κ B levels, and lower TIMP-1 expression levels. This was supported by macroscopic and microscopic images that revealed less lung tissue damage and a lesser degree of fibrosis based on the modified Ashcroft score, which was better than that for BG and other gambir groups. Further studies are needed to consider MMP, ROS, and fibroblast counts after the administration of 262 mg/kg of bodyweight of gambir for 7 weeks in animal models. Further studies are also needed to determine the safety of gambir for humans at a dose equivalent to 262 mg/kg of bodyweight for seven weeks.

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Supervision: Samsuridjal Djauzi, Agus Setiyono, Mohamad Sadikin, Sri Widia A. Jusman, Suradi
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Writing – review & editing: Desdiani Desdiani, Iris Rengganis, Samsuridjal Djauzi, Agus Setiyono

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Data Availability Statement: Data is contained within the article.

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Reviewer Reports 20 April 2022



C

— Editorial Comments

Decision

Valeria Sülsen 28.04.2022

Major Revision Requested

Message for Author

Dear authors,
Revise the manuscript according to the reviewer comments and highlight the changes

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1 submitted

Report

Reviewer 1 20.04.2022

- Unfortunately, this manuscript is written in a very complicated way that makes it very difficult to be understood. The authors have to rewrite it in a simple way.
- In the Ethics Statement, the authors have to mention the number of the Ethical Committee approval.
- The authors mentioned that the rats were acclimatized for 14 d in individual polycarbonate cages containing 1 rat per cage. This means that they used 3 cages/ group. Why they did not use one cage/group?
- The authors have to declare why they used an albendazole anthelmintic.
- The route of administration of Gambir should be mentioned.
- The authors did not mention the method used to euthanize the animals. They have to mention the method used to euthanize the animals.
- The authors did not mention how they count the cells. They have to mention if they used an automated cell counter or manual hemocytometer counting
- Figures for AF G262 = 262 mg/kg bodyweight of antifibrotic gambir (H& Mt) are missed.
- Why the authors used the discrepancy values. The better to use the percentage values.

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- The authors did not mention how they count the cells. They have to mention if they used an automated cell counter or manual hemocytometer counting
- Figures for AF G262 = 262 mg/kg bodyweight of antifibrotic gambir (H& Mt) are missed.
- Why the authors used the discrepancy values. The better to use the percentage values.
- Western blot assay revealed that the fibropreventive and antifibrotic groups of gambir was seen in a specific band of p65, as shown in Figure 7. The authors did not clarify the groups in Figure 7.
- Unfortunately, the results and the discussion were written in a very complicated way that makes them difficult to be understood. I have to read them several times to understand. It will be understood if the authors wrote in a more simple way.

Editor Decision 28 April 2022



Dear Dr. Desdiani,

In order for your submission "Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis" to Evidence-Based Complementary and Alternative Medicine to proceed to the review process, there needs to be a revision.

Reason & Details:

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Conflict of interest: Not declared

Data availability statement: Data is contained within the article.

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Thank you for allowing us the opportunity to submit a revised draft of the manuscript “Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis”

We appreciate the time and effort that you dedicated to providing feedback on our manuscript and are grateful for the insightful comments and valuable improvements to our paper. We have incorporated most of the suggestions made by the reviewers. Please see below, for a point-by-point response to the reviewers’ comments and concerns:

1. Unfortunately, this manuscript is written in a very complicated way that makes it very difficult to be understood. The authors have to rewrite it in a simple way.

→ We agree and have updated the manuscript.

2. In the Ethics Statement, the authors have to mention the number of the Ethical Committee approval.

→ The ethics statement already mentioned in Material and Methods section

“This study was conducted according to the requirements of the Helsinki Declaration, and approval was obtained from the Health Research Ethics Committee, Faculty of Veterinary Medicine IPB University, Bogor, Indonesia, under registration number 114/KEH/SKE/XII/2018, and the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia.”

3. The authors mentioned that the rats were acclimatized for 14 d in individual polycarbonate cages containing 1 rat per cage. This means that they used 3 cages/ group. Why they did not use one cage/group?

→ If we use 3 rats/cage, it would affect the growth of the rat (Hughes and Nowak, 1973). Based on Kane (2002), the rats were consistent in their preference for the larger cages and rats should be given larger space allowance. Animals should be able to express normal postures and have some freedom of movement; both conditions were difficult to achieve in the small cages when 2 adult rats and a litter were present (Gaskill and Corning, 2015).

4. The authors have to declare why they used an albendazole anthelmintic.

→ Albendazole anthelmintic used because the mode of action of albendazole anthelmintic is by binding strongly with the tubulin of nematodes leading to nematode death. It has low acute toxicity

when given by the oral route to mice, rats. Furthermore, based on carcinogenicity bioassays, albendazole giving no evidence of neoplasia in rats/mice (European Medicines Agency, 2004).

5. The route of administration of Gambir should be mentioned.

→ The Gambir was given orally and the bleomycin was given intratracheally. We have updated the route of administration into the Material and Methods section:

“..The dose of bleomycin used was 4 mg/kg body weight, given intratracheally. The doses of gambir given were 65 mg, 131 mg, and 262 mg/kg body weight orally..”

6. The authors did not mention the method used to euthanize the animals. They have to mention the method used to euthanize the animals.

→ The rats were euthanizing by injectable anesthetics methods using ketamine lethal dose. After confirming the death by examining the vital signs, the rats were undergoing surgery from lower abdomen to thorax. We also have updated the methods at the “2.2. Rats and Gambir Dosing”

“... All rats were terminated at the end of the seventh week or on day 50 with injectable anesthetics methods using ketamine lethal dose.”

7. The authors did not mention how they count the cells. They have to mention if they used an automated cell counter or manual hemocytometer counting

→The cell counting was conducted using manual hemocytometer. We already add the methods into “2.4. Bronchoalveolar Lavage Fluid Collection, Cell Counts, and TGF-β1 ELISA”

“...The smear placed under microscope and cells was count manually using manual hemocytometer..”

8. Figures for AF G262 = 262 mg/kg bodyweight of antifibrotic gambir (H& Mt) are missed.

→ The figures already add into the “Figure 2” in “3.2. Effects of Gambir on Bleomycin-Induced Pulmonary Fibrosis” section.

9. Why the authors used the discrepancy values. The better to use the percentage values.

→ The discrepancy values was used because the sample size was small and has non-uniform sampling patterns. The drawback using percentage value in statistical analysis based on normal-theory methods is that its distribution may be highly skewed (Berry and Ayers, 2006)

10. Western blot assay revealed that the fibropreventive and antifibrotic groups of gambir was seen in a specific band of p65, as shown in Figure 7. The authors did not clarify the groups in Figure 7.

→ We already add the information regarding the group of treatment at the Western Blot result and “4.7. Interaction between Gambir and NF- κ B Using the Western Blot Method” section in Discussion.

“...This indicated that the bonding interaction of gambir with NF- κ B could prevent the translocation to the nucleus, thus no initiation of inflammatory/fibrogenesis process...”

11. Unfortunately, the results and the discussion were written in a very complicated way that makes them difficult to be understood. I have to read them several times to understand. It will be understood if the authors wrote in a more simple way.

→ We already changed the results and discussion section.

References:

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Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis

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Desdiani Desdiani ¹, Iris Rengganis², Samsuridjal Djauzi², Agus Setiyono³, Mohamad Sadikin⁴, Sri Widia A. Jusman⁴, Nuryati Chairani Siregar⁵, Suradi Suradi⁶, Putri C. Eyoer⁷ + [Show Affiliations](#)

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Tanggal: Senin, 27 Juni 2022 pukul 19.18 GMT+7

Dear Dr. Desdiani,

I am pleased to let you know that your article has been published in its final form in "Evidence-Based Complementary and Alternative Medicine."

Desdiani Desdiani, "Fibropreventive and Antifibrotic Effects of Uncaria gambir on Rats with Pulmonary Fibrosis," Evidence-Based Complementary and Alternative Medicine, vol. 2022, Article ID 6721958, 11 pages, 2022. <https://doi.org/10.1155/2022/6721958>.

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Best regards,

Karthikeyan Kalahasthi
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Fibropreventive and Antifibrotic Effects of *Uncaria gambir* on Rats with Pulmonary Fibrosis

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Abstract

Pulmonary fibrosis causes scar tissue formation that disrupts the functioning of the lungs. *Uncaria gambir* (Hunter) Roxb (hereafter gambir)—a plant native to West Sumatra in Indonesia—contains flavonoid (+)-catechin, which has strong antioxidant activity and can be used to combat pulmonary fibrosis. This random in vivo experimental study analyzed the antifibrotic effect of gambir on the lungs of rats with bleomycin-induced fibrosis. The subjects were 10 groups of 10-week-old male rats weighing around 200–250 g. All groups were terminated at the end of the seventh week or on day 50. The lungs were cleaned, and tissues were taken to analyze inflammatory cell counts and TGF- β 1 levels using bronchoalveolar lavage (BAL) with ELISA; type I collagen and tissue inhibitor of metalloproteinase 1 (TIMP-1) levels using immunohistochemistry (IHC); and activation of NF- κ B using ELISA and Western blot assays. The most severe histopathological characteristic based on the modified Ashcroft score was in the bleomycin group (BG), whereas the mildest was in the 262 mg/kg of the bodyweight

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