Indonesian Journal of Pharmaceutical Education (e-Journal) 2026; 6 (2): 201– 224
ISSN: 2775- 3670 (electronic)
Journal Homepage: http://ejurnal.ung.ac.id/index.php/ijpe/index
DOI: 10.37311/ijpe.v6i2.37699

Micrometer-Sized *Artocarpus integer* (Thunb.) Merr. Peel Extract Emulsion: Hemoglobin and Lung Histopathology Assessment in Cigarette Smoke-Exposed Wistar Rats

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Article Info:
Received: 7 March 2025
in revised form: 12 April 2026
Accepted: 21 May 2026
Available Online: 30 May 2026

Keywords:
Acute toxicity
Artocarpus integer;
Cigarette smoke;
Emulsion;
Hemoglobin;
Pulmonary histopathology

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ABSTRACT

This study aimed to develop a cempedak fruit peel extract (*Artocarpus integer* (Thunb.) Merr.) emulsion and evaluate its effect on hemoglobin levels and lung histopathology in cigarette smoke-exposed Wistar rats. This experimental study used a pre–post control group design involving 36 male Wistar rats divided into six groups: negative control, cigarette smoke-exposed positive control, non-emulsified extract, and emulsion groups at doses of 25, 50, and 75 mg/kg body weight. Among the 36 rats, 26 survived until the end of the study and were included in the final analysis. Cigarette smoke exposure was performed for 14 days using a smoking chamber. Particle size was characterized by laser diffraction, hemoglobin levels were measured before and after treatment, and lung histopathology was assessed semiquantitatively. Data were analyzed using paired t-tests, Pearson correlation, and two-way mixed-effects models. The emulsion showed a micrometer-sized particle profile with a median diameter (D50) of 16.17 µm. Hemoglobin levels decreased at the threshold of statistical significance in the pooled pre–post analysis ($p=0.05$), but no significant treatment-related effect was found. Lung histopathology showed moderate inflammation in the positive control group, while severe inflammation with alveolar–interstitial hemorrhage was observed in the higher-dose emulsion groups. Overall, the current micrometer-sized emulsion did not demonstrate protective effects on hemoglobin levels or lung histopathology. Further nanoemulsion optimization and safety evaluation are required before continued biological testing.

How to cite (APA 6th Style):
Ayudia,E.I.,Hafizah., Miftahurrahmah., Lipinwati.,Dewi,H., Hardiningsih,D.T.(2026). *Micrometer-Sized Artocarpus integer (Thunb.) Merr. Peel Extract Emulsion: Hemoglobin and Lung Histopathology Assessment in Cigarette Smoke-Exposed Wistar Rats. Indonesian Journal of Pharmaceutical Education (e-Journal),* 6(2), 201-224.

ABSTRAK

Penelitian ini bertujuan mengembangkan emulsi ekstrak kulit buah cempedak (*Artocarpus integer* (Thunb.) Merr.) serta mengevaluasi pengaruhnya terhadap kadar hemoglobin dan histopatologi paru pada tikus Wistar yang dipaparkan asap rokok. Penelitian eksperimental ini menggunakan desain kelompok kontrol pra–pasca perlakuan dengan 36 tikus Wistar jantan yang dibagi menjadi enam kelompok, yaitu kontrol negatif, kontrol positif dengan paparan asap rokok, ekstrak tanpa emulsifikasi, serta emulsi dosis 25, 50, dan 75 mg/kg berat badan. Dari 36 tikus, 26 tikus bertahan hingga akhir penelitian dan dimasukkan dalam analisis akhir. Paparan asap rokok dilakukan selama 14 hari menggunakan smoking chamber. Ukuran partikel dikarakterisasi dengan difraksi laser, kadar hemoglobin diukur sebelum dan sesudah perlakuan, sedangkan histopatologi paru dinilai secara semikuantitatif. Data dianalisis menggunakan *paired t-test*, korelasi Pearson, dan model efek campuran dua arah. Emulsi yang dihasilkan memiliki profil partikel berukuran mikrometer dengan diameter median (D50) sebesar 16,17 µm. Kadar hemoglobin menurun pada ambang batas signifikansi statistik dalam analisis pra–pasca gabungan (p=0,05), tetapi tidak ditemukan efek perlakuan yang bermakna. Histopatologi paru menunjukkan inflamasi sedang pada kelompok kontrol positif, sedangkan inflamasi berat disertai perdarahan alveolar–interstisial ditemukan pada kelompok emulsi dosis tinggi. Secara keseluruhan, emulsi berukuran mikrometer ini belum menunjukkan efek protektif terhadap kadar hemoglobin maupun histopatologi paru. Optimasi menuju nanoemulsi dan evaluasi keamanan lebih lanjut diperlukan sebelum pengujian biologis lanjutan.

Kata Kunci: Toksisitas akut; *Artocarpus integer*; Asap rokok; Emulsi; Hemoglobin; Histopatologi paru-paru.

1. Introduction

Cigarette smoke exposure is a global health risk factor with widespread effects on the hematological and respiratory systems. The prevalence of active smoking and secondhand smoke exposure remains high globally, especially in developing countries, including Indonesia, where the prevalence of adult smokers is 28.8% [1]. This condition significantly affects the hematological status of the smoking population; epidemiological studies report that the prevalence of anemia in active smokers is 17–32% higher than that in nonsmokers, with an average decrease in hemoglobin levels of 0.5–1.5 g/dL during chronic exposure [2],[3].

Exposure to cigarette smoke induces systemic oxidative stress and chronic inflammation, which directly disrupt erythrocyte homeostasis through the formation of carboxyhemoglobin, erythrocyte membrane lipid peroxidation, and the suppression of erythropoiesis, ultimately reducing hemoglobin levels and blood oxygen transport capacity [3],[4],[5],[6],[7],[8]. To date, conventional pharmacological approaches to address anemia caused by cigarette smoke exposure have been limited to iron and erythropoietin supplementation, which does not directly address the underlying oxidative and inflammatory mechanisms [9], [10]. Therefore, approaches based on natural antioxidants from phytochemical sources are relevant alternatives.

Cempedak fruit peel (*Artocarpus integer* (Thunb.) Merr.) is an abundant agricultural waste product in the Sumatra and Kalimantan regions and contains bioactive flavonoids, tannins, and polyphenols with documented antioxidant and anti-inflammatory activities [11],[12],[13],[14]. Specifically, using the DPPH method, Hakim *et al.* [11] reported that an ethanol extract of *cempedak* fruit peel had an antioxidant IC50 value of 650.07 ppm, whereas Ayudia *et al.* [12] confirmed an antioxidant activity of 8,872 ppm. However, these studies were conducted in vitro, and none has evaluated the biological effectiveness of *A. integer* peel extract in cigarette smoke-exposed animal models. Moreover, high antioxidant activity in vitro does not guarantee equivalent biological efficacy *in vivo*, as the oral bioavailability of flavonoids and polyphenol compounds is inherently limited by poor aqueous solubility, rapid intestinal metabolism, and first-pass hepatic elimination factors that reduce the plasma concentration available for systemic antioxidant protection [15],[16].

In vivo testing of the bioactivity of *cempedak* fruit peel extract is limited by low bioavailability because of the limited solubility and stability of the active compounds in biological systems [17]. Emulsion-based delivery systems, particularly at the micro- and nanoscale, have been shown to increase the solubility, stability, and absorption of lipophilic active compounds by reducing droplet size, which expands the contact surface area and facilitates the penetration of biological membranes [18],[19],[20],[21]. Conventional emulsions with droplet sizes in the micrometer range (>1 μm) provide a limited increase in surface area relative to the bulk oil phase and are therefore insufficient to significantly enhance the dissolution rate and membrane permeability of lipophilic bioactive compounds. In contrast, nanoemulsions with droplet sizes below 200 nm substantially increase the contact surface area, enhance the thermodynamic activity of dissolved compounds, and facilitate lymphatic uptake, thereby improving oral bioavailability [20],[22].

To date, no studies have evaluated the effectiveness of emulsion systems based on *cempedak* fruit peel extract on hemoglobin levels and lung tissue integrity in animal models exposed to cigarette smoke. These conditions raise several questions, such as whether the *cempedak* fruit peel extract emulsion formulation can maintain hemoglobin levels and suppress pulmonary histopathological damage under chronic cigarette smoke exposure conditions. This study aimed to develop a *cempedak* fruit peel extract emulsion formulation and evaluate its effectiveness on hemoglobin levels and lung histopathology in Wistar rats exposed to cigarette smoke. The findings of this study provide a scientific basis for optimizing local phytopharmaceutical delivery systems and developing natural-based supportive intervention strategies for populations vulnerable to cigarette smoke exposure.

2. Methods

Study design and setting

This laboratory experimental study with a pre–post-test control group design incorporated repeated measures conducted *in vivo* using Wistar strain rats (*Rattus norvegicus*) as test animals. This study aimed to evaluate the effects of *cempedak* fruit peel extract (*Artocarpus integer*) emulsion on hemoglobin levels and lung histopathology profiles in a cigarette smoke exposure model. The study was conducted at the Biomedical and Animal Laboratory, Faculty of Medicine and Health Sciences, University of Jambi, for 6 months (July–December 2025). The research subjects used in this study

were obtained from the laboratory collection, where the study was conducted, and underwent species determination.

Ethical considerations and animal welfare

All research procedures involving laboratory animals were conducted in accordance with Indonesian Food and Drug Administration (BPOM) Regulation No. 20 of 2023 concerning Guidelines for Preclinical Pharmacodynamic Testing of Traditional Medicines, applying the 3Rs principle (replacement, reduction, and refinement) as stipulated in the Guide for the Care and Use of Laboratory Animals [23], and the ARRIVE 2.0 reporting guidelines [24]. The research protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, University of Jambi (protocol number 2361/UN21.8/PT.01.04/2025, dated July 15, 2025).

Inclusion and exclusion criteria

The inclusion criteria for the experimental animals were as follows: male Wistar rats aged 8–12 weeks, weighing 180–250 g, in good physical condition, as indicated by clean and nonshedding fur, clear eyes, no skin lesions, active movement, and normal appetite. The exclusion criteria were as follows: rats with body weights outside the specified range, rats showing signs of illness or anatomical abnormalities before the start of treatment, and rats that died due to procedures outside the specified treatment period. Test animals that died during the treatment period were excluded from the final analysis, but their mortality and toxicity data were recorded.

Animals and randomization

The test animals were male Wistar white rats aged 8–12 weeks, with a body weight of 180–250 g. The rats were acclimated for seven days prior to treatment under standard conditions, including a temperature of 22–25°C, a 12-h light/12-h dark cycle, and free access to standard feed and unlimited drinking water [25]. A total of 36 rats that met the inclusion criteria were randomly divided (*simple randomization*) into six groups. Simple randomization was performed by assigning each rat a unique number and using a computer-generated random number sequence (Microsoft Excel® RAND function) to allocate animals to one of the six groups, ensuring an equal number of six rats per group before the start of the experimental period.

The negative control (NC) group was not exposed to cigarette smoke or treated with the extract and emulsion; the positive control (PC) group was not exposed to treatment; the T1 group was exposed to cigarette smoke and *cempedak* fruit peel extract without emulsification; the T2 group was exposed to cigarette smoke and a 25 mg/kg body weight (BW) emulsion; the T3 group was exposed to cigarette smoke and a 50 mg/kg BW emulsion; and the T4 group was exposed to cigarette smoke and a 75 mg/kg BW emulsion. The selection of doses in this study was based on a modified version of the methods of Hardiningsih *et al.* [17]. At baseline, each group comprised six rats (n=6 per group; total n=36). Due to mortality during the 14-day treatment period, the final number of animals available for analysis was as follows: NC (n=6), PC (n=6), T1 (n=3), T2 (n=4), T3 (n=4), and T4 (n=3), totaling 26 surviving animals. Mortality and associated toxicity observations for the 10 animals that died during treatment were recorded separately and are reported as part of the acute toxicity profile

Procedure

Extraction of *cempedak* fruit peel

The *cempedak* fruit peel used in this study was sourced from the Telanaipura area of Jambi City, Indonesia. Fresh *cempedak* fruit peels were washed thoroughly, dried at 40–60°C, and ground into a fine powder. The powder was extracted using the maceration method with 96% ethanol at a ratio of 1:10 (w/v) for 3×24 h at room temperature with periodic stirring every 24 h. The macerate was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at 40°C until a concentrated extract was obtained. The yield of the *cempedak* fruit peel extract was 10% (w/w).

Formulation and characterization of the emulsion

The emulsion was prepared by mixing *cempedak* fruit peel extract as the active phase, Tween 80 (3% v/v) as the surfactant, and distilled water as the continuous phase. The mixture was homogenized using a sonicator probe for 10 min at an amplitude of 80% and temperature of 25–30°C. The particle size distribution of the emulsion was measured using a Fritsch Analysette 22 NeXT laser diffraction instrument (Fritsch GmbH, Germany) with a Fraunhofer calculation model, measurement range of 0.01–3,800 µm, pump speed of 40%, and ultrasonication amplitude of 80%. Measurements were performed in three independent replicates, and the reported parameters included D10, D50, D90, coefficient of variation (CV<5), RMS error, and beam obscuration.

Cigarette smoke exposure and emulsion/extract administration

Before administration, the six groups of rats were weighed to obtain pretreatment data for each group. Rats in the PC, T1, T2, T3, and T4 groups were exposed to cigarette smoke in a closed smoking chamber. The cigarettes used were Aspro Bold® kretek cigarettes (tar content: 32.4 mg; nicotine content: 1.95 mg per cigarette). Three cigarettes were exposed per session per day for ±45 min, with a five-min interval between cigarettes, accompanied by the oral administration of extracts and/or emulsions in a tube with a volume of 2.5 mL per day for 14 days. The NC group received neither cigarette smoke exposure nor vehicle or extract administration throughout the 14-day period. The PC group was exposed to cigarette smoke only, with no extract or emulsion. For the treatment groups (T1–T4), oral administration of the extract or emulsion was performed 30 min prior to daily cigarette smoke exposure, consistent with the rationale that pre-treatment with antioxidant compounds may attenuate the acute oxidative burden induced by smoke inhalation [3]. After the 14th day, all rats were euthanized.

Euthanasia and histopathological preparation

All surviving rats were sacrificed on day 15 using the ketamine-xylazine euthanasia method, in accordance with ethical guidelines. The injection solution consisted of 100 µL of a 10:1 mixture of ketamine (100 mg/mL; Ketaset, Fort Dodge, IA, USA) and xylazine (100 mg/mL; AnaSed, Lloyd Laboratories, Shenandoah, IA, USA), administered via injection into the lateral tail vein [26]. Lungs were then collected from each group. The lung organs were fixed in 10% buffered formalin for 24 h, subjected to graded dehydration, cleared with xylene, infiltrated and paraffin embedded, cut into 4–5 µm thick sections, and then stained with hematoxylin–eosin (HE) [27],[28].

Measurements, data collection, and operational definitions

Body weight

Body weight was measured using digital scales (0.1 g accuracy) on days 0, 7, and 14 of the experiment. Body weight change was calculated as the difference between the final and initial body weights (ΔBW = final BW − initial BW), expressed in grams. A body weight loss of >10% of the initial weight during the treatment period was used as an indicator of acute toxicity, according to the OECD guideline 423 [29].

Hemoglobin level

Hemoglobin levels were defined as the concentration of proteins that bind and carry oxygen and distribute it throughout the body. Point-of-care hemoglobin strips (Easy Touch®) were selected for hemoglobin measurement based on their validated applicability in small laboratory animal research, their minimal blood volume requirement (≤20 µL), which reduces procedural stress during repeated sampling in rats, and their operational feasibility in resource-limited laboratory settings. However, this method has lower precision than that of automated hematology analyzers, which is a limitation of the present study. Measurements were taken at two time points: before treatment (pre-test, day 0) and after 14 days of treatment (post-test, day 15). Blood (0.5 mL) was collected from the retro-orbital sinus using heparinized capillaries under mild anesthesia. Changes in hemoglobin levels were expressed as the difference between the post-and pretest values (ΔHb = Hb posttest − Hb pretest) in g/dL.

Acute toxicity profile and pulmonary histopathology

Acute toxicity was assessed based on two main parameters: (a) changes in body weight during the treatment period and (b) changes in pulmonary histopathology. Histopathological changes were determined by a trained observer (anatomical histology-pathology specialist) who was blinded to group identity using a 0–3 semiquantitative scoring system on five parameters: alveolar-interstitial inflammation, alveolar septal thickening, epithelial damage and loss of architecture, alveolar-interstitial hemorrhage, and protein debris/mild edema, as defined in **Table 1**. The assessment was performed at 400× magnification (10 FOV) for cellular parameters and 100× magnification (five FOV) for area parameters. The total score per rat was calculated as the sum of the means of the five parameters (range, 0–15), with the following interpretation: 0, no lesions; 1–4, mild; 5–9, moderate; and 10–15, severe. Pulmonary histopathology was also used as an acute toxicity assessment parameter along with body weight changes, in accordance with Food and Drug Supervisory Agency Regulation No. 20 of 2023 [30].

Table 1. Semiquantitative histopathological assessment and operational definitions

Observation parameter	Operational definition	Outcome/assessment
Alveolar-interstitial inflammation	Degree of mononuclear inflammatory cell infiltration (lymphocytes, macrophages, few neutrophils) around and within the alveoli; assessed at 400× magnification, 10 FOV	Score 0: None Score 1: Mild/focal Score 2: Moderate/multifocal Score 3: Severe/diffuse
Thickening of alveolar septa	Degree of alveolar wall thickening and increased interstitial *cellularity*; assessed at 100× magnification, 5 FOV	Score 0: Normal Score 1: Mild/focal Score 2: Moderate/multifocal Score 3: Severe/diffuse
Epithelial/ alveolar	Presence of alveolar epithelial necrosis, loss of alveolar structure (collapse	Score 0: None Score 1: Mild/focal

damage and loss of architecture	consolidation/), or destruction of septa; assessed at 100× magnification, 5 FOV	Score 2: Moderate/multifocal Score 3: Heavy/consolidated
Alveolar-interstitial hemorrhage	Degree of hemorrhage in the lung parenchyma; assessed at 100× magnification, 5 FOV	Score 0: None Score 1: Mild/focal Score 2: Moderate/multifocal Score 3: Severe/consolidated
Protein debris/mild edema	Presence of proteinaceous material in the alveolar lumen or interstitial space; assessed at 100× magnification, 5 FOV	Score 0: None Score 1: Mild/focal Score 2: Moderate/multifocal Score 3: Severe/consolidated

Note: *The total score per rat is calculated as the sum of the mean scores of the five parameters: total score = mean P1 + P2 + P3 + P4 + P5, with a total score of 0–15. The degree of damage was interpreted based on the total score as follows: 0 = no lesions; 1–4 = mild; 5–9 = moderate; and 10–15 = severe.*

Statistical analysis

Statistical analyses were performed as follows: (1) a paired t-test was used to evaluate pre–post differences in hemoglobin levels and body weight within the combined dataset, appropriate for normally distributed paired observations; (2) a two-way mixed-effects model (REML) was applied to assess the effects of treatment group (between-subject factor) and measurement time (within-subject factor) on hemoglobin levels, body weight, and histopathological scores, with Geisser–Greenhouse epsilon correction applied to adjust for violations of sphericity; (3) Dunnett's multiple comparisons test was used for post hoc comparisons of each treatment group against the positive control (PC); and (4) Tukey's post hoc test was used for pairwise comparisons between histopathological sub-parameters within groups [31]. This analytical framework was selected to account for the repeated-measures structure of the data while controlling for the family wise error rate across multiple comparisons. All data obtained were tabulated using Microsoft Excel ® for MacOS 2021. All analyses were performed using GraphPad Prism version 10.3.1 for macOS, with significant P values of 0.12 (ns), 0.033 (*), 0.002 (**), and <0.001 (***), and a 95% confidence level ($p<0.05$). The histopathological profile data are presented as images for each treatment group.

3. Results and Discussion

Characterization of emulsion particle size

The emulsion particle size of the *cempedak* fruit peel extract was characterized using three measurements, as shown in **Figure 1**. The measurement results revealed that the median particle diameter (D50) in the three consecutive measurements was 15.96, 16.17, and 16.40 µm, with an average D50 of 16.17 µm and a coefficient of variation (CV) of 1.11%. The D10 and D90 values were 3.35 µm (0.82% CV) and 46.20 µm (0.65% CV), respectively. The root mean square (RMS) error of all measurements was 0.025, and the beam obscuration ranged from 14% to 16%, which was within the optimal range. A CV value of <1.12% indicated good measurement reproducibility. Our findings confirmed that the particle size profile distribution was unimodal, with a D10–D90 range of approximately 3.35–46.20 µm (**Table 2**).

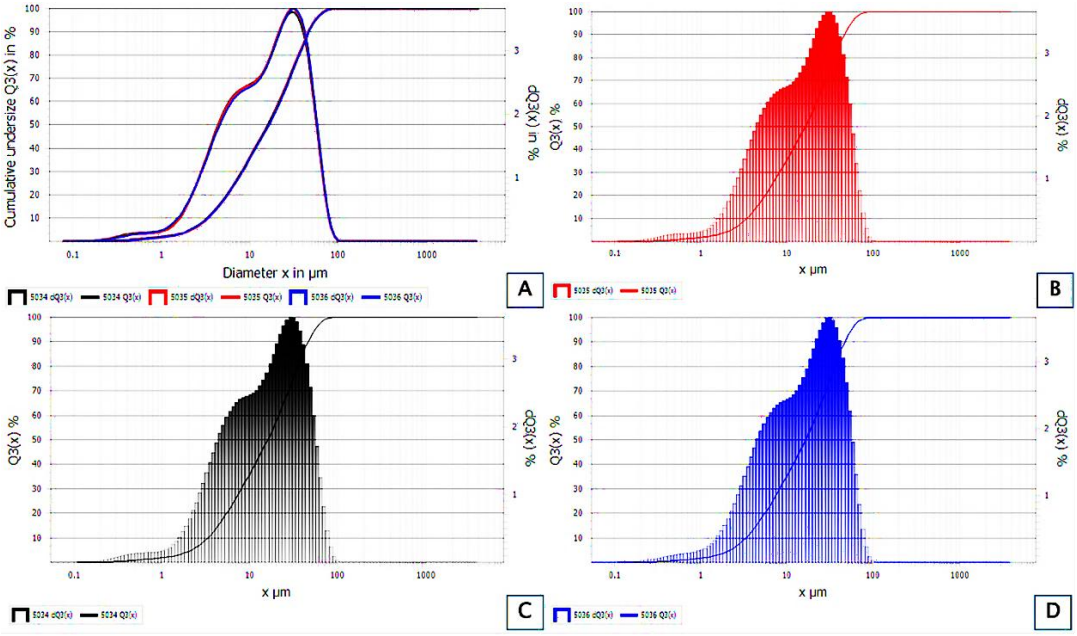

Figure 1. Particle size distribution profile of the *cempedak* fruit peel extract emulsion based on laser diffraction (Fritsch Analysette 22 NeXT). (**A**) Overlay of three repeated measurements (M5034, M5035, M5036) showing the cumulative distribution curve Q3(x) and the density curve dQ3(x); (**B**) Measurement M5035; (**C**) Measurement M5034; (**D**) Measurement M5036.

Table 2. Particle size distribution of *cempedak* fruit peel extract emulsions

Parameter	Repetition (n=3)			Mean (µm)	CV (%)
	M5034 (µm)	M5035 (µm)	M5036 (µm)		
D10 (µm)	3.33	3.39	3.34	3.35	0.82
D50 (µm)	15.96	16.17	16.40	16.17	1.11
D90 (µm)	45.99	45.99	46.62	46.20	0.65
RMS Error	0.025	0.025	0.025		
Beam Obscuration (%)	16	15	14		

Note: *D10, D50, and D90 are the particle diameters at the 10th, 50th, and 90th percentiles of the cumulative volume distribution, respectively. CV is the coefficient of variation. The RMS error is the root mean square error of diffraction fitting.*

Hemoglobin levels

Hemoglobin levels were measured before and after treatment in all experimental animals that survived until the end of the study period. Of the 36 rats initially included, 26 completed the full treatment period and were available for paired t-tests, Pearson's correlation analysis, and mixed-effects model analysis. Ten rats died during the treatment period and were excluded from the final analysis; their mortality data and associated observations were recorded as part of their acute toxicity profile. The pre- and post-test hemoglobin level data met the assumptions of a normal distribution, as determined by the Shapiro–Wilk test (pretest: W=0.9522, p=0.154; posttest: W=0.9888, p=0.987); therefore, a paired t-test was performed.

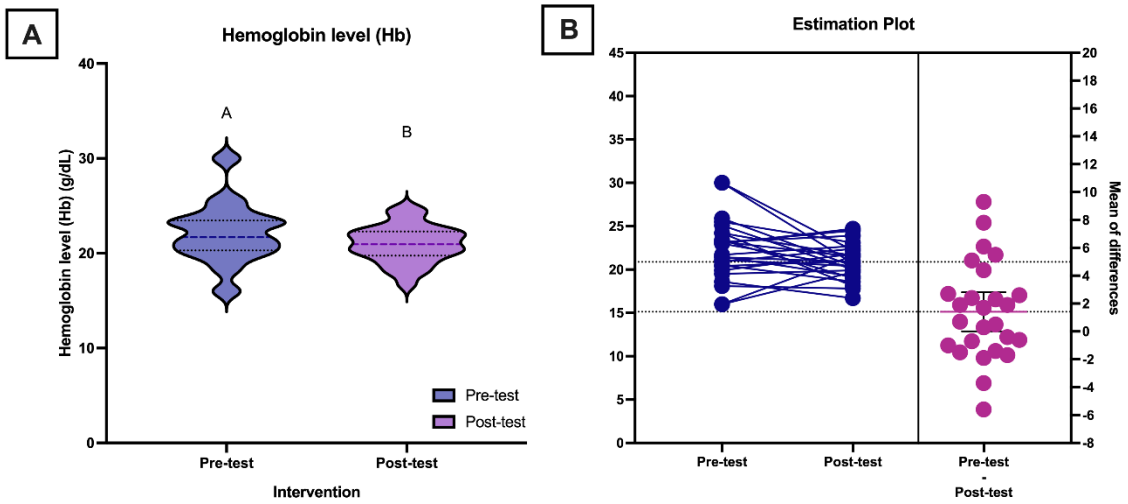

Figure 2. Distribution and changes in hemoglobin levels before (pre-test) and after treatment (post-test). Remarks: (**A**) Violin plot of pretest (blue) and post-test (light purple) hemoglobin level distributions. The vertical dotted lines indicate the median and quartiles of each group. Letters A and B indicate statistically significant differences between the pre- and post-tests (p=0.05). (**B**) Estimation plot showing changes in individual hemoglobin levels of each rat from pretest to posttest (left panel) and mean differences with 95% confidence intervals (right panel). The horizontal dashed lines in the right panel mark the 95% confidence interval limits.

Based on the results of the paired t-test (**Figure 2**), there was a significant difference in pretest and posttest hemoglobin levels (t=2.070; df=25; p=0.05; 95% CI difference: 0.007–2.816 g/dL). The mean hemoglobin concentration decreased by 1.412 g/dL (SD=3.477) from 22.28 g/dL during the pretest period to 20.87 g/dL during the post-test period. The effect size was calculated using an R^2 (partial eta squared) of 0.1463, indicating that 14.63% of the variation in hemoglobin levels could be explained by the model. Furthermore, the Pearson correlation test results between the pre- and post-test hemoglobin levels revealed a weak positive correlation that was not statistically significant (r=0.2791; 95% CI: −0.1213 to 0.6015; p=0.17). These results indicate that the administration of *cempedak* fruit skin emulsion did not increase hemoglobin levels in rats exposed to cigarette smoke.

Table 3. Two-way ANOVA (mixed-effects analysis) on hemoglobin levels

Group	Mean± SD (pretest)	Mean± SD (posttest)	Pre-Post difference	Fixed effects (type III)		
				Row Factor (Pre-Post)	Time (between groups)	Row Factor x Time
NC	21.41± 2.51	19.8± 1.23	1.61± 1.28			
PC	24.05± 5.78	20.67± 2.27	3.38± 3.51	F (1, 10)	F (2.434,	F (5, 39)
T1	20.44± 2.94	22.7± 1.55	-2.26± 1.39	= 1.633	18.98) =	= 2.154
T2	22.65± 2.21	19.02± 0.98	3.63± 1.23	(p =	0.8373	(p =
T3	21.76± 1.51	22.4± 1.52	-0.64± -0.01	0.23) ^{ns}	(p=0.47)^{ns}	0.08)^{ns}
T4	22.06± 1.22	21.7± 1.28	0.36± -0.06			

Note: *All groups were normally distributed and met the assumption of homogeneity (p>0.05). The measurement model used two-way ANOVA (or mixed models) in GraphPad Prism 10 for macOS (version 10.3.1). Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (negative control, no treatment), PC (positive control, cigarette*

smoke), T1 (smoke + extract without emulsification), T2 (smoke + emulsion, 25 mg/kg BW), T3 (smoke + emulsion, 50 mg/kg BW), and T4 (smoke + emulsion, 75 mg/kg BW).

The results of the two-way ANOVA (mixed-effects analysis) revealed no significant effect of the measurement time factor (pretest vs. posttest; F(1,10)=1.633; p=0.23), treatment group factor (F(2,434; 18.98)=0.837; p=0.47), or interaction between the two (F(5,39)=2.154; p=0.08). The overall mean predicted hemoglobin concentration decreased from 22.07 g/dL in the pretest to 21.05 g/dL in the posttest, with a difference of 1.012 g/dL (95% CI: −0.753–2.776; SE=0.792), which was not significantly different from zero, and the Geisser–Greenhouse epsilon was 0.4867. Dunnett's multiple comparisons test on the pre- and post-treatment groups confirmed that no group was significantly different from the positive control (PC) either before or after treatment (all p>0.50). The interaction p-value of 0.08 indicates a trend toward differences in the pattern of hemoglobin level changes between groups that approached but did not reach statistical significance (**Table 3** and **Figure 3**).

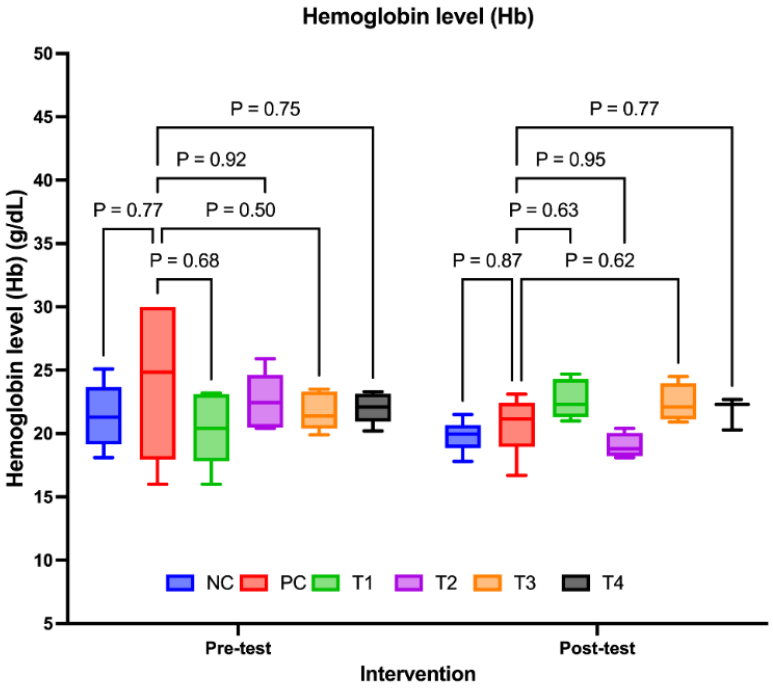

Figure 3. Comparison of hemoglobin levels in rats before and after treatment in each group. Remarks: Two-way ANOVA (or mixed model) was used in GraphPad Prism 10 for macOS version 10.3.1. Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (Negative Control – No treatment), PC (Positive Control – Cigarette smoke), T1 (Smoke + Extract without emulsification), T2 (Smoke + Emulsion 25 mg/kg BW), T3 (Smoke + Emulsion 50 mg/kg BW), and T4 (Smoke + Emulsion 75 mg/kg BW).

The significant pre–post difference detected by the paired t-test (t=2.070; df=25; p=0.05; R^2=0.1463) reflects a borderline overall shift in hemoglobin levels when data from all groups were pooled, thereby increasing the effective sample size and statistical power for detecting a small mean difference of 1.412 g/dL. This finding should be interpreted cautiously, given that p=0.05 is at the conventional alpha boundary and the effect size (partial η^2=0.15) is modest. The absence of significant effects in the two-way mixed-effects model—for measurement time (F(1,10)=1.633; p=0.23), treatment group

(F(2.434,18.98)=0.837; p=0.47), and their interaction (F(5,39)=2.154; p=0.08)—indicates that neither the emulsion nor the cigarette smoke exposure produced treatment group-specific changes in hemoglobin levels beyond the non-specific reduction observed across all groups. Taken together, these results do not support the protective effect of the *cempedak* fruit peel emulsion on Hb levels under the tested conditions.

Pulmonary histopathological changes

Lung histopathology was assessed using five parameters: inflammation, cellular debris, loss of lung architecture, septal thickening, and hemorrhage. Each parameter was semiquantitatively assessed on a scale of 0–3 in five to ten fields of view, per rat. Statistical analysis was performed using a mixed-effects model (REML) with Geisser–Greenhouse correction and Dunnett's post hoc test for the positive control (PC) group and Tukey's post hoc test for comparisons between subparameters within groups. The mean score per parameter per group is presented in **Table 4** and **Figure 4**, and the results of the combined analysis of all parameters are presented in **Table 5**.

Table 4. Mean ± SD histopathology scores of the lungs per parameter/group

Analysis Unit	Group	Mean ± SD	GGE	F	P value	*Dunnett's multiple comparisons test* (PC vs. all doses)		
						Mean Diff.	q	Adjusted P value
Inflammation	NC	1.800±0.5099	0.4926	2.483	0.12^{ns}	-0.6600	2.081	0.29 ^{ns}
	PC	2.450±0.2950						
	T1	2.400±0.3688				-0.05000	0.2319	>0.99 ^{ns}
	T2	2.240±0.1817				-0.2200	1.901	0.34 ^{ns}
	T3	2,383±0.3656				-0.06667	0.3701	>0.99 ^{ns}
	T4	2.433±0.3777				-0.01667	0.1152	>0.99 ^{ns}
Debris	NC	1.440±0.5177	0.5514	0.3240	0.79^{ns}	0.08000	0.4924	0.98
	PC	1.433±0.4633						
	T1	1.167±0.3204				-0.2667	1.512	0.49 ^{ns}
	T2	1.360±0.8050				0.000	0.000	>0.99 ^{ns}
	T3	1.633±0.9158				0.2000	0.4060	0.99 ^{ns}
	T4	1.533±0.8454				0.1000	0.2997	>0.99 ^{ns}
Architectural loss	NC	0.880±0.1789	0.4438	2.885	0.09^{ns}	-0.2400	2.449	0.20^{ns}
	PC	1.100±0.1673						
	T1	0.967±0.1966				-0.1333	1.348	0.57 ^{ns}
	T2	1.640±0.5177				0.5200	1.857	0.36 ^{ns}
	T3	1.700±0.7874				0.6000	1.837	0.34 ^{ns}
	T4	1.667±0.8165				0.5667	1.844	0.34 ^{ns}
Septum Thickening	NC	1.760±0.8295	0.5568	0.3857	0.75 ^{ns}	-0.2400	0.5098	0.97^{ns}
	PC	2.000±0.2530						
	T1	1.567±0.6377				-0.4333	1.857	0.34^{ns}
	T2	1.760±0.2608				-0.2400	1.395	0.56 ^{ns}
	T3	1.800±0.6573				-0.2000	0.7071	0.91 ^{ns}
	T4	1.933±0.7005				-0.06667	0.1932	>0.99 ^{ns}
Hemorrhage	NC	1.640±0.5177	0.4568	0.9578	0.42 ^{ns}	0.2000	0.6202	0.94
	PC	1.500±0.5899						
	T1	1.133±0.3933				-0.3667	1.177	0.67 ^{ns}
	T2	1.400±0.6782				-0.04000	0.1728	>0.99 ^{ns}
	T3	1.933±0.8641				0.4333	0.8924	0.83 ^{ns}
	T4	1.767±0.9668				0.2667	0.6100	0.95 ^{ns}

Note: *All groups were normally distributed and met the assumption of homogeneity (p>0.05); mixed model measurements were conducted using GraphPad Prism 10 for macOS version 10.3.1. Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (negative control, no treatment), PC (positive control, cigarette smoke), T1 (smoke + extract without emulsification), T2 (smoke + emulsion, 25 mg/kg BW), T3 (smoke + emulsion, 50 mg/kg BW), and T4 (smoke + emulsion, 75 mg/kg BW). GGE: Geisser–Greenhouse epsilon. Adj. p: adjusted p-value post-hoc Dunnett test vs. PC.*

Analysis of the combined mixed-effects model (REML) of all histopathological parameters revealed a statistically significant treatment group effect ($F(1{,}705; 8{,}526) = 39.11$; $p<0.001$) with effective subject matching ($\chi^2=85.48$; $p<0.001$), indicating differences in histopathological profiles between treatment groups. Conversely, the effects between histopathological parameters ($F=0.756$; $p=0.51$) and the interaction between groups and parameters ($F=2.063$; $p=0.13$) were not significant, indicating a relatively uniform pattern of scores between the parameters in all groups. Analysis of each parameter revealed no significant differences between the treatment groups, and the results of the post hoc test for PCs confirmed that no treatment group was significantly different from the positive control group for any parameter.

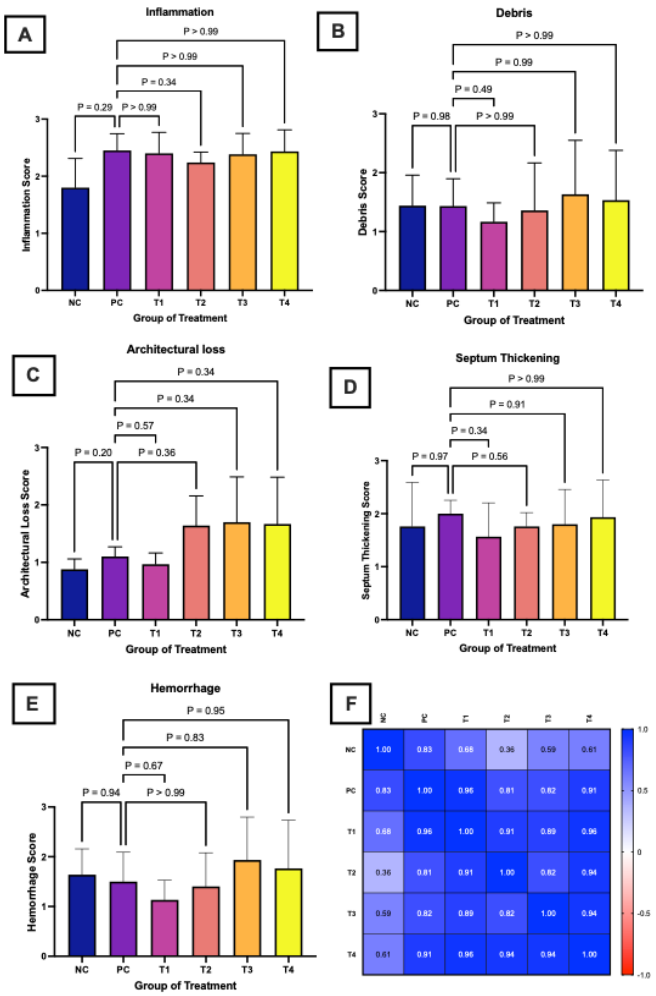

Figure 4. Comparison of lung histopathological profiles for each parameter and their correlations. **Remarks:** Dunnett's post hoc test was performed against the positive control (PC) group. Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (negative control – no treatment), PC (positive control – cigarette smoke), T1 (smoke + extract without emulsification), T2 (smoke + emulsion, 25 mg/kg BW), T3 (smoke + emulsion, 50 mg/kg BW), and T4 (smoke + emulsion, 75 mg/kg BW).

A comparison of the subparameters within the groups using Tukey's post hoc test revealed a consistent pattern. In the NC and PC groups, architectural loss scores were significantly lower than inflammation scores (NC: p=0.03; PC: p<0.001), indicating that inflammation was the dominant parameter under cigarette smoke exposure conditions. In the T1 group, three parameters, namely, debris (p<0.001), architectural loss (p<0.001), and hemorrhage (p=0.004), were significantly lower than inflammation, indicating a different pattern of damage distribution from that in the other groups.

In the PC group, septal thickening was significantly greater than architectural loss (p=0.01), whereas in T3, septal thickening was lower than inflammation (p=0.03), and in T4, debris was lower than inflammation (p=0.03). Pearson's correlation analysis between the total PC histopathology score and treatment groups revealed a significant positive correlation in T1 (r=0.962; p=0.009) and T4 (r=0.911; p=0.03), whereas NC (r=0.832; p=0.08), T2 (r=0.811; p=0.10), and T3 (r=0.818; p=0.09) showed strong but non-significant correlations. The correlation matrix between all groups (**Figure 4F**) revealed r values ranging from 0.36 to 0.96, with the highest correlations in T1 and PC (r=0.96) and T1 and T4 (r=0.96), and the lowest correlation in NC and T2 (r=0.36).

Table 5. The mixed-effects analysis combining all pulmonary histopathology parameters

Fixed effects (type III)	F	df	P Value	GGE
Treatment groups (between groups)	39.11	(1.705; 8.526)	<0.001***	0.4263
Histopathologic changes (between parameters)	0.756	(2.355; 11.77)	0.51 ns	0.4710
Interaction (treatment × parameter)	2.063	(3.847; 17.31)	0.13 ns	0.1924

Note: *All groups were normally distributed and met the assumption of homogeneity (p>0.05); the measurement model used two-way ANOVA (or mixed models) in GraphPad Prism 10 for macOS version 10.3.1. GGE: Geisser–Greenhouse epsilon. Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***).*

Pulmonary histopathology profile

The histopathological features of the lungs in all treatment groups were observed in hematoxylin–eosin (HE)-stained preparations at two magnification levels: 100× for overall tissue architecture assessment and 400× for cellular assessment. Representative microphotographs of all groups are presented in **Figure 5**. The negative control group (NC) showed normal lung findings, with open and uniform alveolar spaces, thin alveolar septa, and no inflammatory cell infiltration, hemorrhage, or intraluminal debris. Compared with the NC group, the positive control group (PC), which was exposed to cigarette smoke, presented histopathological changes characterized by diffuse mononuclear inflammatory cell infiltration (lymphocytes and macrophages) in the interstitial and perialveolar spaces, thickening of the alveolar septa, and uneven narrowing of alveolar spaces. These changes were consistent with chronic inflammatory responses due to exposure to cigarette smoke for 14 days.

Compared with the PC group, the T1 group showed a picture characterized by multifocal inflammatory cell infiltration, focal consolidation areas, and vascular congestion, which were more dominant in several fields of view. Compared with the positive control treatment, the non-emulsified extract treatment did not result in measurable improvements in lung morphology. The alveolar architecture of the T2, T3, and T4 groups appeared better than that of the PC group at low magnification. However, a semiquantitative assessment per rat revealed a different result. The mean inflammation scores at T3 and T4 were higher than those at PC and T1. In the T3 group, two of the six rats achieved a total score in the severe category with histological evidence of

hemorrhage, and similarly, in the T4 group, two rats had a severe total score accompanied by alveolar–interstitial hemorrhage in the observed lung tissue. All rats in the PC group were in the moderate category based on the total score of the five observed parameters.

These findings indicate that the administration of the emulsion at doses of 50 and 75 mg/kg BW did not directly suppress the degree of pulmonary inflammation; rather, the inflammatory response in both groups was more severe than that in the positive control group. The visual improvement in architecture at low magnification seen in T3 and T4 is likely due to a redistribution of the damage pattern, in which the alveolar space appears wider but is accompanied by interstitial hemorrhage and severe inflammatory infiltration in certain focal areas compared with the diffuse and uniform inflammatory pattern seen in the PC group.

Body weight changes

Body weight was measured before and after 14 days of treatment to assess general toxicity. Consistent with the hemoglobin analysis, body weight analysis was performed only in the 26 rats that survived until the end of the study period. Ten rats died during treatment and were not included in the paired body weight analysis; however, their mortality was recorded as part of the acute toxicity profile. The pre- and post-test body weight data met the assumption of normality based on the Shapiro–Wilk and Kolmogorov–Smirnov tests ($p>0.05$). Descriptive statistics for each group and the inferential analysis are presented in **Table 6** and **Figure 6**.

A paired t-test of the combined data for all groups revealed a significant decrease in body weight before and after treatment ($t=10.30$; $df=34$; $p<0.001$; 95% CI: −36.94 to −24.77 g). The mean body weight decreased by 30.86 g (SD=17.72) from 228.3 g at pretest to 197.3 g at posttest, with an effect size $R^2 = 0.757$, indicating that 75.7% of the variation in body weight change could be explained by the treatment. The correlation between pre- and post-test body weights was positive and significant ($r=0.577$; 95% CI: 0.302–0.764; $p<0.001$), indicating that the initial body weight significantly changed because of the administration of *cempedak* fruit peel extract and emulsion.

The mixed-effects model analysis revealed a significant measurement time effect (row factor: $F(1,10) = 30.33$; $p<0.001$), providing evidence that weight loss occurred consistently during the treatment period. Conversely, the treatment group effect ($F(2,941; 28.82) = 0.851$; $p=0.48$) and the interaction between time and group ($F(5,49) = 0.199$; $p=0.96$) were not significant, indicating that the pattern of weight loss did not differ between the groups. Post hoc Dunnett's test confirmed that the no-treatment group was significantly different from the positive control (PC) group in both the pre- and post-tests (all adjusted $p>0.78$). The mean weight loss across all groups ranged from 25.7 g (T2) to 38.3 g (T1), corresponding to a mean percentage body weight loss of 11.6%–16.8%. This range exceeded the 10% threshold used as an early warning indicator for toxicity monitoring. However, because the weight loss pattern did not differ significantly between groups, this finding may reflect the combined effects of cigarette smoke exposure, handling stress, oral administration, and treatment procedures rather than a dose-specific toxic effect of the emulsion alone.

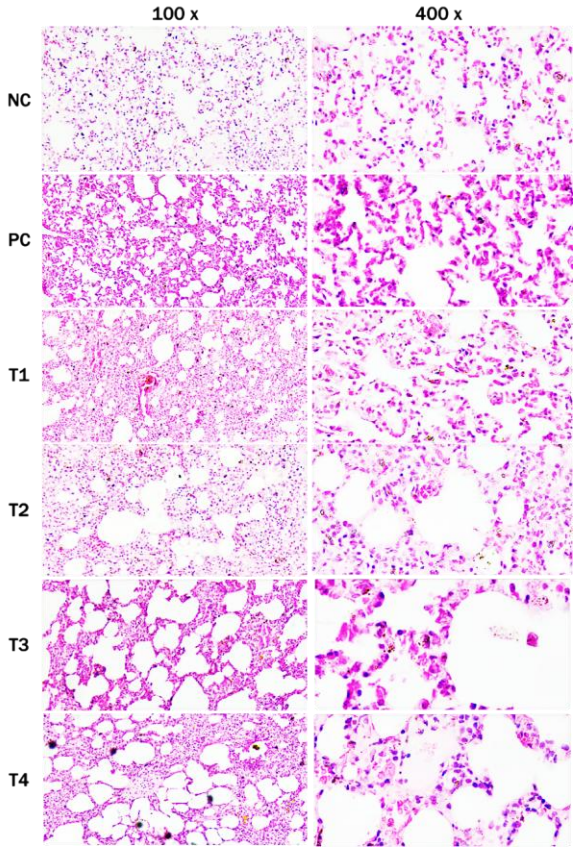

Figure 5. Histopathological microphotographs of rat lungs after treatment. **Remarks**: H&E staining. The left and right columns indicate magnifications of 100× and 400×, respectively. **NC** (normal control), normal alveolar architecture, thin septa, and no inflammatory cell infiltration; **PC** (positive control, cigarette smoke exposure), diffuse mononuclear inflammatory cell infiltration, thickening of septa, and narrowing of alveolar spaces; **T1** (smoke + extract without emulsification), multifocal infiltration, vascular congestion, and focal areas of consolidation; **T2** (smoke + emulsion 25 mg/kg BW), reduced inflammatory cell infiltration, more open alveolar spaces, and partially preserved architecture; **T3** (smoke + emulsion 50 mg/kg BW), mild-to-moderate infiltration, and carbon particle deposits in alveolar macrophages; **T4** (smoke + emulsion 75 mg/kg BW), mild-to-moderate infiltration, relatively preserved architecture, and carbon-laden macrophages visible at high magnification.

Table 6. Two-way ANOVA (mixed-effects analysis) on body weight

Group	Mean± SD (pretest)	Mean± SD (posttest)	Pre-Post difference	Mean± SD (Pre-Post)	Fixed effects (type III)		
					Row Factor (Pre-Post)	Time (between groups)	Row Factor x Time
NC	223.3± 0.000	195.0± 0.000	28.3± 0.00	209.2± 20.03	<0.001***	0.48^{ns}	0.96^{ns}
PC	235.0± 0.000	200.0± 0.000	35.0± 0.00	217.5± 24.75			
T1	228.3± 0.000	190.0± 0.000	38.3± 0.00	209.2± 27.11			
T2	221.7± 0.000	196.0± 0.000	25.7± 0.00	208.8± 18.15			
T3	236.7± 0.000	205.0± 0.000	31.7± 0.00	220.8± 22.39			
T4	225.0± 0.000	196.7± 0.000	28.3± 0.00	210.8± 20.03			

Note: *All groups were normally distributed and met the assumption of homogeneity (p>0.05). The measurement model used two-way ANOVA (or a mixed model) in GraphPad Prism 10 for macOS (version 10.3.1). Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (Negative Control – No treatment), PC (Positive Control – Cigarette smoke), T1 (Smoke + Extract without emulsification), T2 (Smoke + Emulsion 25 mg/kg BW), T3 (Smoke + Emulsion 50 mg/kg BW), and T4 (Smoke + Emulsion 75 mg/kg BW).*

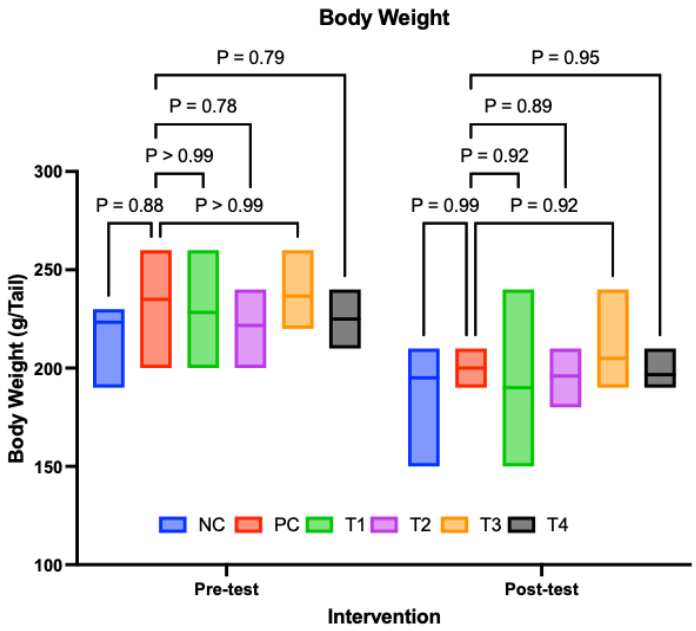

Figure 6. Comparison of body weights before and after each treatment. Remarks: Two-way ANOVA (or mixed model) was used in GraphPad Prism 10 for macOS version 10.3.1. Significant P values: 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***). NC (negative control, no treatment), PC (positive control, cigarette smoke), T1 (smoke + extract without emulsification), T2 (smoke + emulsion, 25 mg/kg BW), T3 (smoke + emulsion, 50 mg/kg BW), and T4 (smoke + emulsion, 75 mg/kg BW).

Discussion

This study aimed to develop an emulsion formulation based on *cempedak* fruit peel extract and evaluate its effectiveness on hemoglobin levels and pulmonary histopathology in Wistar rats exposed to cigarette smoke for 14 days. Overall, the findings revealed that compared with the positive control, the resulting formulation was a conventional micrometer-sized emulsion and not a nanoemulsion and did not significantly protect against hemoglobin levels or pulmonary histopathological damage.

Characterization of the particle size distribution of *cempedak* fruit peel confirmed that the resulting emulsion is a conventional emulsion with a D50 of 16.17 µm, which is still well above the established size for nanoemulsions [32], [33]. The failure to achieve nanoscale droplet size may constrain the biological effectiveness of the preparation, because droplet size strongly determines the contact surface area, dissolution rate, and capacity of lipophilic bioactive compounds to permeate biological membranes [22], [34]. The use of ultrasonication at an amplitude of 80% for 10 min with Tween 80 as the sole surfactant does not appear to generate sufficient energy to break down the droplets to the nanometer scale [35]. Previous nanoemulsion formulation studies have revealed that achieving particle sizes <200 nm requires a well-optimized surfactant–cosurfactant combination, a precise oil–water phase ratio, and a higher homogenization energy intensity, including the use of a high-pressure homogenizer or stepwise ultrasonication at amplitudes >90% [36], [37], [38]. High measurement reproducibility (CV <1.12%) indicates that the emulsion preparation process is stable and consistent [39], [40], [41].

In this study, a significant decrease in hemoglobin levels was detected between the pre- and post-test data, revealing that the 14-day cigarette smoke exposure model successfully induced hematological changes in the test animals. Exposure to cigarette

smoke containing carbon monoxide, free radicals, and other toxic compounds is known to disrupt hemoglobin homeostasis through several mechanisms, including the formation of carboxyhemoglobin, which reduces oxygen-binding capacity; increased oxidative stress that damages erythrocyte membranes; and the activation of inflammatory pathways that suppress erythropoiesis [42], [43], [44]. However, group-by-group analysis using the REML model did not reveal significant differences between the treatment groups, indicating that the administration of the *cempedak* fruit peel extract emulsion at the three tested doses did not provide a protective effect on hemoglobin levels. These findings differ from those of several studies reporting the protective effects of flavonoid-rich plant extracts on hemoglobin levels in oxidative models [45], [46], [47], [48]. This difference is most likely due to the micrometer-scale emulsion particle size, which results in suboptimal bioavailability of the active compounds, compounded by high individual variability and an unbalanced number of subjects owing to mortality during treatment [16], [49], [50].

Histopathological examination of the lungs revealed that exposure to cigarette smoke for 14 days induced structural changes consistent with a chronic inflammatory response, characterized by mononuclear inflammatory cell infiltration, thickening of the alveolar septa, and narrowing of the alveolar spaces in the PC group. These findings are consistent with those of previous studies documenting histopathological changes in the lungs of rats exposed to cigarette smoke in the form of macrophage infiltration, thickening of the alveolar walls, and focal emphysema in response to short- to medium-term exposure to cigarette smoke [51], [52], [53], [54], [55].

These findings indicate that statistical analysis revealed no significant differences between groups across all parameters; however, a mixed-effects model analysis confirmed significant differences in histopathological profiles between groups ($p<0.001$). The rats in groups T3 and T4, which received the highest dose of emulsion accompanied by cigarette smoke exposure, had total histopathological scores in the severe category, accompanied by alveolar–interstitial bleeding, while all rats in the PC group were in the moderate category. This pattern indicates a possible proinflammatory effect at high doses, which is a condition in pharmacology known as hormesis or high-dose toxicity in phytochemical compounds [56]. In addition, alveolar hemorrhage was observed in groups T3 and T4, indicating acute toxicity of the established formulation.

The micrometer-scale droplet size (D50=16.17 μm) of the current formulation represents a fundamental constraint on the bioavailability of flavonoids. Droplets in this size range have a substantially smaller surface-area-to-volume ratio compared to nanoemulsion droplets (<200 nm), resulting in a slower dissolution rate, limited permeation through the intestinal epithelium, and reduced lymphatic absorption of lipophilic polyphenols [22], [34]. To achieve nanoscale droplets, future formulation work should explore: (1) optimized surfactant–cosurfactant combinations, such as Tween 80 paired with Span 80 or polyethylene glycol-based co-surfactants at a hydrophilic–lipophilic balance matched to the active phase; (2) higher homogenization energy through high-pressure homogenization (≥500 bar) or stepwise ultrasonication at amplitudes >90%; and (3) phase ratio optimization to reduce the dispersed-phase fraction and minimize re-coalescence [35], [37]. Regarding the proinflammatory pattern observed in the T3 and T4 groups, future studies should incorporate quantitative biomarkers of pulmonary oxidative stress (8-isoprostane, malondialdehyde) and inflammation (interleukin-6, tumor necrosis factor-α, nuclear factor kappa B activation) to determine whether the observed alveolar–interstitial hemorrhage represents dose-

dependent phytochemical toxicity or a vehicle-mediated effect specific to Tween 80 at the administered volume [57].

The presence of carbon-laden macrophages in the T3 and T4 groups served as a histological marker, confirming the success of the cigarette smoke exposure model and the phagocytic activity of alveolar macrophages as a lung defense mechanism against inhaled particles [58], [59], [60], [61]. These findings are consistent with previous reports documenting the accumulation of carbon-laden macrophages as a typical pulmonary response to chronic cigarette smoke exposure [57], [62], [63]. Carbon deposits were more prominent in the high-dose group than in the PC and T1 groups, possibly because of differences in the phagocytic capacity of alveolar macrophages influenced by the interaction of bioactive compounds in *cempedak* fruit skin emulsion with the innate immune system of the lungs [64], [65], [66], [67].

Significant and consistent weight loss across all groups was not different between groups, indicating a physiological response to the exposure conditions and treatment procedures rather than specific toxic effects of the test preparation. No group met the criteria for acute toxicity based on the percentage of weight loss according to OECD 423, although the recorded percentage of weight loss (11.6–16.8%) was at the threshold requiring further monitoring if the treatment duration was extended. This study confirmed that the administration of a *cempedak* fruit peel emulsion to rats exposed to cigarette smoke directly affected their weight loss. Thus, characterization, comparative model studies, and accurate dose determination are necessary to mechanistically confirm the effects of *cempedak* fruit peel.

Clinical and public health implications

The results of this study have implications for the development of phytopharmaceuticals based on local waste as a strategy to prevent the effects of exposure to cigarette smoke. Although its effectiveness has not been proven at this stage, the particle characterization findings confirm that optimization of the drug delivery system is a prerequisite before biological testing can continue. From a public health perspective, the use of *cempedak* fruit peel as a raw material for phytopharmaceuticals has the potential to support the development of affordable, standardized herbal medicines in areas endemic to *cempedak* cultivation, particularly in Jambi Province. The successful development of standardized nanoemulsions from this source in the future could lead to the development of alternative supportive therapies for populations vulnerable to cigarette smoke exposure, particularly for the treatment of mild-to-moderate anemia, especially in developing countries with a high smoking prevalence.

Limitations of the Study

This study has several limitations, including the fact that the preparation produced was a conventional micrometer-sized emulsion rather than a nanoemulsion; therefore, the claim of increased bioavailability cannot be substantiated by the results. The high mortality rate of the test animals resulted in an unbalanced number of subjects per group and significantly reduced the statistical power of intergroup analysis. The 14-day exposure period may not be sufficient to induce measurable hematological changes across all parameters, particularly for hemoglobin parameters. The molecular mechanisms underlying the severe inflammatory response in the high-dose group were not explored in this study and should be investigated in future studies. Hemoglobin measurements using point-of-care strips have lower precision than those using automated hematology analyzers. Furthermore, the total flavonoid and total polyphenol

content of the formulated emulsion were not quantified in the present study, which limits the ability to establish a dose–exposure relationship between the administered emulsion and its bioactive load, and precludes assessment of formulation consistency across batches—a deficiency that should be addressed in future studies through standardized phytochemical quantification prior to biological testing.

4. Conclusion

The cempedak fruit peel extract emulsion produced in this study was a conventional micrometer-sized emulsion (D50=16.17 µm) and did not demonstrate protective efficacy on hemoglobin levels or lung histopathology in Wistar rats exposed to cigarette smoke under the tested conditions. The proinflammatory histopathological pattern and alveolar–interstitial hemorrhage observed at higher doses (50 and 75 mg/kg BW) indicate safety signals that require systematic evaluation through oxidative stress and inflammatory biomarker profiling. Therefore, formulation optimization toward a nanoemulsion system is required before further biological efficacy testing. Future studies should optimize the surfactant–cosurfactant system, increase the number of experimental animals, extend the treatment duration, quantify bioactive markers, and explore inflammatory and oxidative stress pathways using integrated in vitro, in silico, and in vivo approaches.

Acknowledgements

The authors thank all those who assisted with this research. The research team thanks the Institute for Research and Community Service (LPPM) of Universitas Jambi for funding this research through the Internal Grant Program with Research Implementation Agreement Letter Number 2891/UN21.11/PT.O1.05 /SPK/2025 dated July 2, 2025, and in accordance with the Decree of the Rector of Universitas Jambi Number: 1715/UN21/PT/2025 dated June 25, 2025.

Conflict of Interest:

The authors declare that there is no conflict of interest regarding the publication of this article.

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