

## RESEARCH ARTICLE

## Immunomodulatory and Acute Toxicity Studies of *Peronema canescens* Jack Leaves: *in vivo* Hematological Analysis and *in vitro* IL-6 Gene Expression Inhibition

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### Abstract

**BACKGROUND:** *Peronema canescens* Jack is traditionally employed in the treatment of inflammation, malaria, and immune-related disorders. Despite its traditional use, scientific evidence on its immunomodulatory effects, interleukin (IL)-6 modulation, hematological impact, as well as its related acute toxicity data remains limited. Therefore, this study was conducted to investigate the immunostimulatory potential of *P. canescens* leaf extract through *in vitro* and *in vivo* assessments and evaluate its acute toxicity profile.

**METHODS:** The *in vitro* immunomodulatory activity of *P. canescens* ethanolic extract, n-hexane fraction (NHF), ethyl acetate fraction (EAF), and water fraction (WF) were assessed by measuring IL-6 inhibition in lipopolysaccharide-stimulated RAW 264.7 macrophages. For *in vivo* analysis, Balb/C mice were divided into six groups: a normal control (Na-CMC), a positive control (50 mg/kg BW/day Stimuno *Phyllanthus niruri* extract), a negative control (80 mg/kg BW/day cyclophosphamide as immunosuppressant), and 3 treatment groups receiving *P. canescens* extract at doses of 100, 200, and 400 mg/kg BW/day. Hematological parameters, including white blood cell (WBC) counts, lymphocyte percentages, and neutrophil percentages, were analyzed. Acute toxicity studies were performed by administering *P. canescens* extract at doses of 300, 2000, and 5000 mg/kg BW over observation period.

**RESULTS:** EAF exhibited the most pronounced IL-6 inhibition *in vitro*. *In vivo*, the administration of *P. canescens* extract at 200 and 400 mg/kg BW significantly elevated WBC and lymphocyte levels while concurrently reducing neutrophil counts. No mortality or neurotoxic manifestations were observed, confirming the *P. canescens* extract's safety profile up to 5000 mg/kg BW.

**CONCLUSION:** *P. canescens* leaf extract, particularly EAF, demonstrates robust immunomodulatory activity with a favorable safety margin. These findings underscore its potential therapeutic application in immune modulation.

**KEYWORDS:** *Peronema canescens* Jack, immunomodulatory, IL-6 inhibition, acute toxicity, hematology, cytokines

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## Introduction

Medicinal plants have long been integral to traditional medicine due to their diverse pharmacological properties. *Peronema canescens* Jack, commonly known as Sungkai, is an Indonesian medicinal plant traditionally used to treat fever, infections, and immune-related disorders. Recent phytochemical investigations have identified flavonoids, saponins, and triterpenoids in *P. canescens* leaves, suggesting potential immunomodulatory properties.(1) These plants play an essential role in healthcare systems, particularly in regions where traditional medicine is deeply rooted in cultural practices and local knowledge. Over time, the global scientific community has increasingly recognized the value of medicinal plants (2), emphasizing the need for empirical studies to validate their efficacy and safety. Approximately 20–28% of the global population relies on traditional or alternative medicine, and about 59.12% in Indonesia (3), underscoring the importance of integrating traditional remedies into modern healthcare systems through scientific validation (4).

*P. canescens* is a member of the Verbenaceae family and is native to Indonesia, particularly in Sumatra and Kalimantan.(5) Traditionally, *P. canescens* leaves have been utilized for managing antidiabetic (6), anti-obesity (7), inflammation, malaria, immune-related disorders, and more recently, during the Covid-19 pandemic as an immune booster (8). The leaves of *P. canescens* are rich in bioactive compounds, including flavonoids, alkaloids, saponins, tannins, and phenolics, which exhibit antioxidant, anti-inflammatory, and immunomodulatory properties. (9,10) Among these compounds, flavonoids and tannins are particularly known for their ability to counteract oxidative stress (11) and regulate inflammatory responses (5).

Immunomodulatory are substances that enhance the immune system by triggering the production of immune cells, such as macrophages and lymphocytes, and increasing cytokine production.(12,13) Interleukin (IL)-6 is a key pro-inflammatory cytokine implicated in immune regulation. (14) While physiological levels of IL-6 support the immune response to infections, elevated levels are associated with chronic inflammation and autoimmune diseases.(15) Herbal immunomodulatory have the potential to regulate IL-6 expression, either by stimulating its production during acute infections or by reducing it to manage excessive inflammation in chronic conditions.(16) This dual regulation highlights the importance of studying herbal immunomodulatory in targeting inflammation and immune-related diseases.

(17) In contrast, at high levels, IL-6 can cause excessive inflammation and is associated with chronic inflammatory diseases.(18) Identifying natural immunomodulatory capable of modulating IL-6 production is crucial for the development of alternative immunotherapeutic agents.

Despite its traditional use as an immunomodulatory, scientific evidence regarding the immunomodulatory effects of *P. canescens* remains limited (19), particularly at the molecular and *in vivo* levels. To date, no comprehensive studies have explored its potential to modulate IL-6 expression and its impact on hematological parameters, leaving a significant gap in understanding its immunological mechanisms.(20–22) Additionally, while various bioactive compounds have been identified in *P. canescens* leaves, their specific roles in immune regulation have not been fully elucidated. Another critical gap in the current literature is the absence of acute toxicity data, which is essential for establishing the safety profile and dosage limits of *P. canescens* extract.

Therefore, this study was conducted to evaluate the *in vitro* inhibitory effects of *P. canescens* extract and its fractions on IL-6 gene expression in LPS-induced RAW 264.7 cells, and to investigate the *in vivo* immunomodulatory effects by assessing hematological parameters such as white blood cell (WBC) counts, lymphocyte percentages, and neutrophil percentages, and assess the acute toxicity profile of *P. canescens* extract. This study integrates molecular and *in vivo* analyses with toxicity assessments, which contribute valuable insights into the potential development of *P. canescens* as a safe and effective immunomodulatory agent for managing inflammation-related disorders.

## Methods

### Plant Collection, Processing, Extraction, and Fractionation

*P. canescens* leaves were collected from Kayutanam, West Sumatra, Indonesia. Taxonomic identification was confirmed at the Biosystematics and Molecular Herbarium Jatinangoriensis Laboratory (Reference No.: 05/LBM/IT/11/2021). After washing and air-drying for three days, 100 g of dried leaves were macerated in 1 L of 96% ethanol at room temperature for three days, with occasional stirring. The solvent was evaporated at 45°C using a rotary evaporator, yielding 35 g of crude ethanol extract. The extract was sequentially fractionated into n-hexane (NHF), ethyl acetate (EAF), and water (WF) fractions to separate compounds based on polarity. The *in vitro* studies used *P.*

*canescens* ethanol extract, NHF, EAF, and WF; while *in vivo* immunomodulatory and acute toxicity studies used *P. canescens* ethanol extract only.

### ***in vitro* IL-6 Inhibition Assay**

IL-6 inhibition was evaluated in LPS-induced RAW 264.7 macrophage cells. RAW 264.7 macrophages were seeded in 96-well plates and stimulated with 1 µg/mL LPS to induce IL-6 expression. Each treatment group consist of: untreated cell control (CC group), LPS-induced cell control (CC+LPS group), administered with dimethyl sulfoxide (DMSO) solvent control (DMSO group), administered with *P. canescens* NHF (NHF group), administered with *P. canescens* EAF (EAF group), and administered with *P. canescens* WF (WF group). Other than the CC group, after the LPS stimulation, samples including ethanol extract and its fractions (NHF, EAF, and WF), were tested at a final concentration of 6.125 µg/mL, which was determined based on unpublished preliminary cytotoxicity screening to ensure non-toxic and biologically active levels. *IL-6* mRNA expression was quantified via quantitative polymerase chain reaction (qPCR) using  $\beta$ -actin as the housekeeping gene. The primer sequences used were as follows: *IL-6* forward primer: 5'-CTGCAAGAGACTTCCATCCAG-3'; *IL-6* reverse primer: 5'-AGTGGTATAGACAGGTCTGTTGG-3';  $\beta$ -actin forward primer: 5'-AGAGGGAAATCGTGCGTGAC-3'; and  $\beta$ -actin reverse primer: 5'-CAATAGTGATGACCTGGCCGT-3'.

RNA was isolated using Ribozol reagent (Amresco, Solon, OH, USA), with purity confirmed by A260/A280 ratios, and transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, Taunton, MA, USA). The analysis with quantitative polymerase chain reaction (qPCR) was conducted using the SensiFAST™ SYBR No-ROX Kit, with primers designed for *IL-6* and  $\beta$ -actin. qPCR was performed under the following cycling conditions: an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds. The relative expression levels of *IL-6* were calculated using the  $2^{-\Delta\Delta C_t}$  method, and statistical significance was assessed via one-way ANOVA ( $p < 0.05$ ). Absorbance measurements were conducted using a microplate reader (Infinite M200 Pro, TECAN, Männedorf, Switzerland), and RNA quantification utilized a NanoQuant Plate (TECAN). Relative gene expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method, where  $\beta$ -actin was used as the housekeeping gene and the normal control (CC group) served as the calibrator (baseline) for fold-change determination.

### **Animal Model and Treatment**

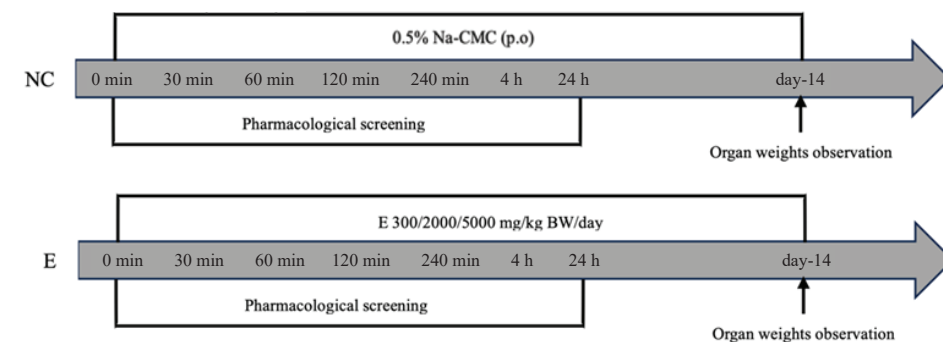
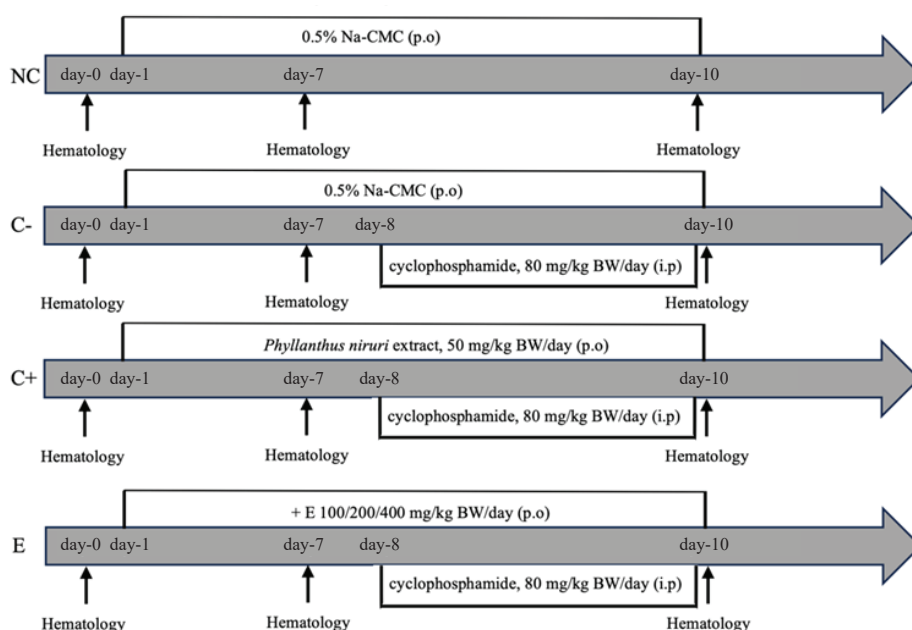
Male Balb/C mice aged 8–10 weeks and weighing 25–30 g were used for both immunomodulatory and acute toxicity studies. The animals were housed under standard laboratory conditions (temperature 22±2°C, humidity 50–60%, 12-hour light/dark cycle), with food and water provided ad libitum. The protocol of the animal study was approved by the Research Ethics Commission of Universitas Padjadjaran (Approval No. 434/UN.6.KEP/EC/2022 for immunomodulatory activity and No. 471/UN.6.KEP/EC/2023 for acute toxicity studies).

### **Acute Toxicity Study**

For the acute toxicity test, 15 Balb/C mice (aged 8–10 weeks, 25–30 g) were divided into four groups: Normal control (NC) received 0.5% Na-CMC; E300 received 300 mg/kg BW *P. canescens* ethanol extract; E2000 received 2000 mg/kg BW *P. canescens* ethanol extract; and E5000 received 5000 mg/kg BW *P. canescens* ethanol extract. All treatments were administered orally as a single dose, and animals were observed for 0, 30, 60, 120, 240 min, 4 hour, and 24 hours for signs of toxicity, behavioral changes, weight fluctuation, and mortality. At the end of the observation on day-14, organ weights (liver, kidney, heart, lungs, spleen) were recorded and analyzed statistically using one-way ANOVA followed by Tukey's post hoc test ( $p < 0.05$ ). The schematic diagram and timeline was presented in Figure 1.

### ***in vivo* Immunomodulatory Assay**

A total of 30 mice were randomly divided into six groups (n=5 per group): Normal control (NC) received 0.5% Na-CMC; Negative control (C-) received 80 mg/kg BW cyclophosphamide; Positive control (C+) received 50 mg/kg BW Stimuno® (*Phyllanthus niruri* extract); Treatment groups received *P. canescens* ethanol extract at 100 (E100), 200 (E200), and 400 mg/kg BW/day (E400), respectively. Blood samples were collected on day-0 as baseline data, oral treatments were administered daily according to group designation from day-1 to -7, with a second blood sampling performed at the end of day-7. On day-8 to 10, cyclophosphamide was additionally administered via intraperitoneal injection (i.p.) to all groups, except the NC, while continuing daily oral treatments according to each group's respective regimen. On day-10, the final blood sampling was conducted to assess hematological parameters. Hematological data including WBC, lymphocyte, and neutrophil counts were collected on day -0, -7, and -10 during the treatment period using a Sysmex hematology analyzer (Sysmex, Kobe, Japan) (Figure 1).

**A. Acute Toxicity Study****B. *in vivo* Immunomodulatory Assay**

**Figure 1. Schematic diagram of acute toxicity study and *in vivo* immunomodulatory study design and timeline.** NC: normal control, C-: negative control, C+: positive control, E: extract *P. canescens*/treatment group.

## Results

***in vitro* IL-6 Inhibition**

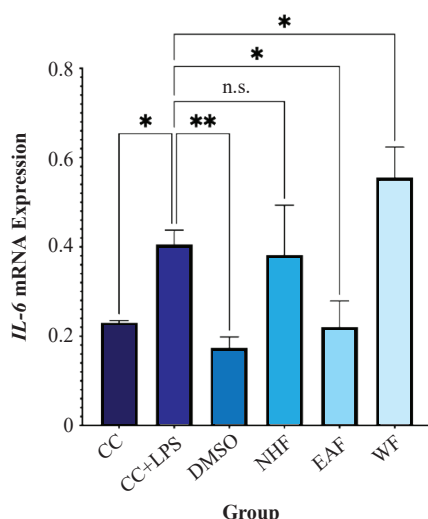
LPS stimulation in RAW 264.7 cells effectively induced inflammation, significantly increasing *IL-6* mRNA expression compared to the untreated control. The CC group showed a baseline *IL-6* expression of  $0.230 \pm 0.005$ . In contrast, the CC+LPS group exhibited a marked increase in *IL-6* expression ( $0.406 \pm 0.032$ ). Treatment with DMSO alone resulted in an *IL-6* expression level of  $0.174 \pm 0.025$ , indicating minimal interference by the solvent.

Among the tested fractions, the EAF demonstrated the most significant inhibition of *IL-6* expression at  $6.125 \mu\text{g/mL}$  ( $0.221 \pm 0.058$ ,  $p < 0.05$  vs. CC+LPS). The NHF exhibited a higher expression level ( $0.382 \pm 0.112$ ), showing limited inhibitory effects, while the WF showed the least inhibition. Among the tested fractions, EAF demonstrated the most

significant inhibition of *IL-6* expression at  $6.125 \mu\text{g/mL}$  ( $0.221 \pm 0.058$ ,  $p < 0.05$  vs. CC+LPS). The NHF exhibited a higher expression level ( $0.382 \pm 0.112$ ), showing limited inhibitory effects, while the WF showed the least inhibition. As shown in Figure 2, the *IL-6* mRNA expression levels in RAW 264.7 cells were significantly reduced after treatment with *P. canescens* fractions.

Since the treatments were administered after LPS stimulation, the observed inhibition of *IL-6* expression reflects the extract's anti-inflammatory activity rather than a direct immunostimulatory or immunomodulatory effect. Therefore, we acknowledge that the EAF primarily exhibited an inhibitory effect on inflammation, specifically through downregulation of *IL-6* gene expression, a key pro-inflammatory cytokine involved in immune response pathways.

The EAF's strong inhibition of *IL-6* expression suggests the presence of bioactive compounds such as



**Figure 2. Normalized *IL-6* mRNA expression levels in RAW 264.7 cells treated with *P. canescens* fractions.** CC: untreated cell control; CC+LPS: untreated cell control+LPS induction; DMSO: DMSO control; NHF: *P. canescens* leaves NHF; EAF: *P. canescens* leaves EAF; WF: *P. canescens* leaves WF at a concentration of 6.125 µg/mL. Gene expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method, with CC used as the baseline. Data are presented as fold change relative to the normal control (mean±SD), with n=3. \*Significantly different with  $p<0.05$ ; \*\*Significantly different with  $p<0.01$ ; ns: not significant, compared to the CC+LPS group.

flavonoids and phenolic acids that are known to interfere with the NF-κB signaling pathway, a major transcription factor responsible for the expression of various inflammatory cytokines, including *IL-6*. Thus, the findings from this study highlight the anti-inflammatory potential of EAF in LPS-induced macrophage activation.

#### ***in vivo* Acute Toxicity: Pharmacological Screening**

The pharmacological screening was conducted to evaluate the potential neurotoxic effects of *P. canescens* leaves extract on the central and autonomic nervous systems. The absence of toxic symptoms, reflected by consistent data and minimal variability, indicates that the extract does not induce significant neurotoxic effects at the tested doses. Pharmacological screening was conducted using *P. canescens* extract at a dose of 5000 mg/kg BW, the highest dose used in the acute toxicity study, to evaluate potential neurobehavioral effects. Observations were categorized into central and autonomic nervous system parameters. Pharmacological screening of the central and autonomic nervous systems revealed no signs of neurotoxicity. In the central nervous system (CNS), posture, catalepsy, flexion, corneal reflex, pineal reflex, and hanging behavior were observed consistently in 100% of animals, indicating normal neurological responses. Conversely, no signs of

tremors, convulsions, or writhing were detected, suggesting the absence of neuroexcitatory or neurodepressive effects. In the autonomic nervous system, defecation, urination, salivation, piloerection, and lacrimation were not observed in any animal (0%), further confirming the extract's safety at the tested dose. These results collectively suggest that *P. canescens* extract does not impair central or autonomic nervous system function at 5000 mg/kg BW.

*P. canescens* leaves extract does not induce neurotoxic effects at the tested doses. The presence of normal reflexes (e.g., corneal and pineal reflexes) and the absence of adverse behaviors (e.g., convulsions or tremors) confirm the extract's safety for the central and autonomic nervous systems. These findings support its potential for safe therapeutic applications

#### ***in vivo* Acute Toxicity: Body Weight Changes**

Body weight was monitored over a 14 day period to assess the metabolic and systemic effects of *P. canescens* leaves extract. Mice in the control group and those treated with 700 mg/kg and 2000 mg/kg BW of the extract exhibited consistent weight gain by day-7 and -14, indicating normal metabolic activity. In contrast, the group treated with 5000 mg/kg BW showed a slight weight reduction on day-7, which may suggest a potential metabolic impact at higher doses. Table 1 displayed data from the acute toxicity study, which included four groups: control, and extract-treated groups at 300, 2000, and 5000 mg/kg BW. These doses were selected based on the Organisation for Economic Co-operation and Development (OECD) 425 Guidelines and are distinct from the immunomodulatory dosing groups. Data are expressed as mean±SD (n=5). Statistical comparisons among groups were analyzed using one-way ANOVA followed by Tukey's post hoc test, with  $p<0.05$  considered statistically significant (Table 1).

The consistent weight gain observed in the control group and groups treated with 300 mg/kg and 2000 mg/kg BW suggests that *P. canescens* leaves extract does not interfere with normal metabolic processes at moderate doses. The slight weight reduction in the 5000 mg/kg BW group on day-7 could reflect a compensatory metabolic response, potentially linked to increased energy expenditure or reduced food intake at higher doses. By day-14, the body weights in the 5000 mg/kg BW group showed partial recovery, suggesting a transient effect rather than prolonged metabolic disruption. These findings support the safety of *P. canescens* extract at lower and moderate doses, while indicating the need for further investigation into its metabolic effects at higher doses. Statistical analysis using One-way

**Tabel 1. Result of body weight measurement by days and doses.**

| Day    | NC         | E300       | E2000      | E5000      |
|--------|------------|------------|------------|------------|
| Day-1  | 30.33±0.52 | 24.83±0.75 | 30.50±0.44 | 30.83±0.41 |
| Day-7  | 31.83±0.45 | 27.50±0.60 | 31.60±0.68 | 29.83±0.58 |
| Day-14 | 35.33±0.67 | 29.67±0.49 | 36.00±0.77 | 32.00±0.65 |

No statistically significant differences were observed between groups or between days within each group ( $p>0.05$ ), as determined by One-way ANOVA followed by Tukey's post hoc test.

ANOVA followed by Tukey's post hoc test revealed that the 300 mg/kg BW group consistently had significantly lower body weight compared to all other groups on day-1, -7, and -14 ( $p<0.05$ ). No significant differences were observed among the control, 2000, and 5000 mg/kg BW groups on day-1. However, on day-14, the 2000 mg/kg BW group exhibited significantly higher body weight than the 5000 mg/kg BW group, suggesting a dose-dependent pattern in growth regulation at higher concentrations.

#### ***in vivo* Acute Toxicity: Observation of Organ Weights**

Relative organ weights were measured to assess the potential dose-dependent effects of *P. canescens* leaves extract on major organs. Significant changes were observed in the kidneys, liver, and heart at higher doses (2000 mg/kg and 5000 mg/kg BW) compared to the control group ( $p<0.05$ ). Post hoc analysis confirmed the statistical significance of these differences. No significant changes were noted in the weights of the lungs or lymphatic tissues, suggesting that these organs were less affected by the extract.

The increase in relative organ weights at higher doses (2000 mg/kg and 5000 mg/kg BW) suggests potential metabolic or physiological responses to *P. canescens* leaves extract. The kidneys and liver showed significant weight increases, possibly due to their roles in metabolizing and excreting the extract's bioactive compounds. Table 2 presented the results of relative organ weight measurements, highlighting significant differences in kidney, liver, and heart weights among the treatment groups.

#### ***in vivo* Acute Toxicity: Mortality Observation**

During the 14 days of observation period, no mortality was recorded in any experimental group, including those treated with *P. canescens* leaves extract at doses of 300, 2000, and 5000 mg/kg BW. This consistent 0% mortality rate across all groups, including the control, indicates that the extract does not induce acute toxicity, even at the highest tested dose of 5000 mg/kg BW.

The results of the mortality observation, indicate that no mortality was recorded in any experimental group, including the control and those treated with *P. canescens* extract at doses of 300, 2000, and 5000 mg/kg BW over the 14 days observation period. The absence of fatalities across all treatment groups suggests that the extract exhibits a high safety margin, even at the highest administered dose.

The absence of mortality in all groups, including those treated with the highest dose of 5000 mg/kg BW, confirms the safety of *P. canescens* leaves extract under acute exposure conditions. These results suggest that *P. canescens* extract is non-toxic, even at high doses, supporting its potential for safe therapeutic applications. Further studies could evaluate subchronic and chronic toxicity to reinforce these findings and assess long-term safety.

#### ***in vivo* Immunomodulatory Activity**

The hematological analysis revealed that *P. canescens* extract at doses of 200 mg/kg BW and 400 mg/kg BW increased WBC counts and lymphocyte percentages while reducing neutrophil percentages in cyclophosphamide-

**Tabel 2. Result of relative organ weight measurement.**

| Organ  | NC        | E300      | E2000      | E5000      |
|--------|-----------|-----------|------------|------------|
| Kidney | 0.93±0.08 | 0.95±0.11 | 1.27±0.15* | 1.34±0.14* |
| Lung   | 0.33±0.06 | 0.44±0.18 | 0.47±0.12  | 0.51±0.14  |
| Liver  | 5.44±0.83 | 5.47±1.04 | 6.84±0.84* | 6.62±0.94* |
| Lymph  | 0.51±0.16 | 0.53±0.24 | 0.85±0.24* | 0.63±0.22  |
| Heart  | 0.23±0.03 | 0.27±0.05 | 0.36±0.06* | 0.40±0.07* |

\*Significant at  $p<0.05$  compared to control. The  $p$ -values were obtained using One-way ANOVA followed by Tukey's post hoc test to evaluate significant differences between groups.

induced mice. On day-1, the average WBC counts across all groups remained relatively low, ranging between approximately  $2.6\text{--}4.5 \times 10^9/\text{L}$ . No substantial differences were observed between groups at this stage, indicating a baseline immune status prior to any significant immune response activation. By day-7, a notable increase in WBC was observed in several groups. The most prominent elevation occurred in the negative control group, suggesting an acute inflammatory response due to LPS induction. This aligns with the known mechanism of LPS stimulating pro-inflammatory cytokine release and leukocytosis. The extract-treated groups (200 and 400 mg/kg BW) also showed elevated WBC levels compared to day-1, indicating that *P. canescens* extract may promote immune activation. This moderate WBC elevation suggests potential immunostimulatory effects of the extract. Interestingly, the positive control group showed increased WBC on day-7, followed by a sharp decline by day-10. This may reflect a natural regulatory feedback after peak stimulation, leading to normalization of leukocyte levels. By day-10, WBC counts declined across most groups, including treatment groups. This reduction suggests a homeostatic regulatory mechanism as the immune system returns to baseline levels after the peak response. Overall, these results indicated that *P. canescens* extract, particularly at 200 and 400 mg/kg BW, enhances WBC production around day-7, supporting its role as a mild immunomodulatory. Importantly, the effect appears to be controlled and not excessive, as evidenced by the subsequent decline on day-10.

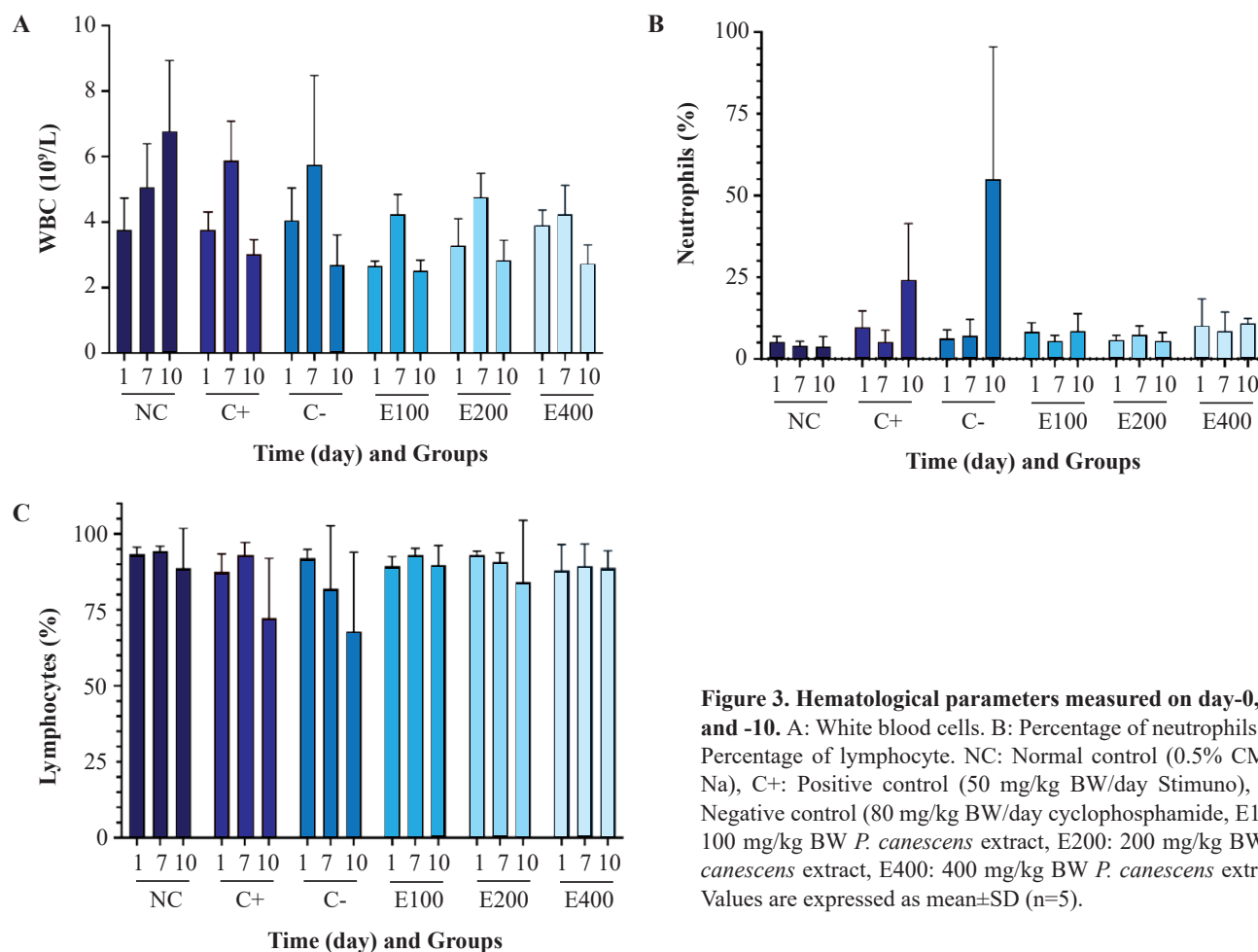
Neutrophil percentage is a critical marker of acute inflammatory response and is highly responsive to LPS stimulation and immunosuppressive agents. On day-1, all groups showed relatively low neutrophil percentages (<15%), indicating a normal immune balance without inflammation. By day-7, an increase in neutrophil levels was observed in several groups, especially the negative control, reaching up to 14.4%. This indicates systemic inflammation caused by LPS induction, which typically shifts immune response toward neutrophil dominance while lymphocyte levels drop. The groups treated with *P. canescens* extract, particularly at 200 and 400 mg/kg BW, were able to maintain neutrophil percentages below 10% through day-7 and -10. This suggests a protective, potentially anti-inflammatory effect of the extract, demonstrating its immunomodulatory capability. By day-10, the negative control group exhibited extreme neutrophil dominance (>90%), confirming the unresolved acute inflammatory state. In contrast, the extract-treated groups maintained much lower neutrophil levels, mostly under 15%, suggesting a stabilized immune response.

These patterns highlight the potential of *P. canescens* extract in modulating neutrophil elevation during inflammation, supporting its role as a beneficial immunomodulator under acute immune stress conditions.

Lymphocyte percentage reflects the relative abundance of adaptive immune cells, which play a crucial role in specific immune responses. On day-1, all groups showed high lymphocyte percentages ranging between 84–96%, indicating a normal immune state without inflammation-induced neutrophil dominance. By day-7, the negative control group showed a significant reduction in lymphocyte percentages, dropping as low as 45.9% in some individuals. This suggests the immunosuppressive effects of cyclophosphamide and the acute inflammatory response triggered by LPS, which typically leads to neutrophilia and a relative drop in lymphocytes. In contrast, the treatment groups receiving *P. canescens* extract at 200 and 400 mg/kg BW maintained high lymphocyte percentages on day-7 and -10, consistently above 85–90% on average. This suggests a protective effect against immunosuppression and supports the extract's potential as an immunomodulator. The positive control group also displayed fluctuations, with some individuals showing reduced lymphocyte levels on day-10 (55.1%), possibly indicating transient immune regulation after peak stimulation. Overall, these findings support the role of *P. canescens* extract, particularly at 200 and 400 mg/kg BW, in maintaining lymphocyte populations during inflammation and immune challenge an indicator of promising immunomodulatory activity. Figure 3 illustrated the hematological parameters of cyclophosphamide-induced mice treated with *P. canescens* extract.

Compared to the herbal standard (Stimuno), *P. canescens* extract at 200 and 400 mg/kg BW demonstrated comparable or better immune-modulatory effects. While Stimuno showed moderate enhancement of WBC and lymphocyte levels on day-7, its effects declined more noticeably by day-10. In contrast, *P. canescens* extract maintained a more consistent lymphocyte percentage and better suppressed neutrophil elevation, indicating sustained immune protection and a potential anti-inflammatory role. These findings suggest that *P. canescens* extract could offer immunomodulatory benefits on par with, or potentially superior to, the reference herbal standard.

In the normal control group, the slight increase in WBC over the 10 days observation period could be due to physiological fluctuations or environmental factors such as handling stress and circadian rhythm. The peak of WBC count in the negative control group on day-7 is likely due to acute inflammatory response following LPS induction. A



**Figure 3. Hematological parameters measured on day-0, -7, and -10.** A: White blood cells. B: Percentage of neutrophils. C: Percentage of lymphocyte. NC: Normal control (0.5% CMC-Na), C+: Positive control (50 mg/kg BW/day Stimuno), C-: Negative control (80 mg/kg BW/day cyclophosphamide, E100: 100 mg/kg BW *P. canescens* extract, E200: 200 mg/kg BW *P. canescens* extract, E400: 400 mg/kg BW *P. canescens* extract. Values are expressed as mean±SD (n=5).

decrease observed on day-10 in the positive control group might indicate a natural regulatory response of the immune system after early immune activation. Although the WBC levels increased on day-7 and declined on day-10 across all groups, the extent of change varied significantly. This pattern likely reflects a general immune activation phase followed by natural immune resolution. Notably, the extract-treated groups maintained higher WBC and lymphocyte levels than the negative control group, indicating their protective role against cyclophosphamide-induced immunosuppression. Baseline WBC differences may have arisen due to biological variability, individual adaptation to laboratory conditions, and pre-treatment stress, all of which can influence leukocyte levels despite standardized procedures.

## Discussion

This study provides evidence of the immunomodulatory potential of *P. canescens* leaves extract, with the EAF demonstrating significant *IL-6* inhibition in LPS-induced

RAW 264.7 macrophage cells. *IL-6* is a critical pro-inflammatory cytokine implicated in chronic inflammatory diseases and acute conditions such as cytokine storms during Covid-19.(23) Although the inhibition of *IL-6* gene expression by the EAF is primarily indicative of anti-inflammatory activity, it also reflects immunomodulatory potential. Immunomodulation involves both the stimulation and suppression of immune responses to restore immune homeostasis. In this study, EAF downregulated the excessive *IL-6* expression induced by LPS, suggesting its role in modulating overactive inflammatory pathways rather than broadly suppressing immune function. Therefore, the reduction of *IL-6* can be interpreted as part of a regulatory mechanism that contributes to immune balance, consistent with the definition of immunomodulation.

Determining the best fraction activity *in vitro* is an important initial step to identify the most potent and bioactive fraction. This information provides a foundation for understanding which fraction contributes most significantly to the overall biological activity of the extract. Although the *in vivo* tests in this study still used the crude

extract, the *in vitro* findings offer valuable guidance for future studies, such as active compound isolation or further *in vivo* assays using selected fractions. Therefore, the *in vitro* data serves as a strategic guide to streamline and focus subsequent research efforts.

These findings may indicate a lack of anti-inflammatory constituents in NHF and WF or the presence of pro-inflammatory compounds that might potentiate *IL-6* expression. The NHF, being rich in nonpolar components such as terpenoids and fatty acids (24), and the WF, potentially containing hydrophilic sugars and polysaccharides (1), could interact differently with macrophage signaling pathways. Therefore, rather than exerting inhibitory effects, these fractions may not contain sufficient bioactive compounds to counteract LPS-induced inflammation or might even contribute to it. In contrast, the EAF enriched in phenolic and flavonoid compounds, effectively inhibited *IL-6* expression and demonstrated anti-inflammatory potential.(25)

The findings support the hypothesis that *P. canescens* extract, particularly the EAF, modulates inflammatory pathways, highlighting its therapeutic relevance.(26) Future studies should focus on addressing these gaps to further validate its therapeutic potential. Further *in vivo* studies and compound characterization are needed to confirm its therapeutic potential, particularly for conditions involving *IL-6* overexpression such as rheumatoid arthritis and cytokine storms.

WBCs, lymphocytes, and neutrophils serve as key indicators of immune function. In this study, *P. canescens* extract exerts dose-dependent immunomodulatory effects, as reflected by significant changes in WBC counts, lymphocyte percentages, and neutrophil percentages.(27) Treatment with 200 mg/kg BW and 400 mg/kg BW of *P. canescens* extract significantly increased WBC counts compared to the negative control, suggesting enhanced immune activity. This indicates that the extract may counteract cyclophosphamide-induced immunosuppression, which typically reduces leukocyte levels.(28) The increased WBC counts at moderate to high doses further support the extract's potential in stimulating immune cell proliferation, aiding in immune recovery.(29)

Increased lymphocyte percentages at these doses also indicate enhancement of adaptive immunity, likely through stimulation of T and B lymphocyte proliferation.(30) This effect is beneficial in conditions of immune suppression or chronic inflammation. Neutrophil responses, representing innate immunity, were similarly modulated. The 400 mg/kg BW dose improved neutrophil activity without inducing

excessive inflammatory responses, suggesting balanced immune regulation. Additionally, the extract helped regulate neutrophil-driven inflammation, maintaining stable neutrophil levels at moderate doses.(30)

Pharmacological screening confirmed that *P. canescens* leaf extract does not cause neurotoxic effects at any tested dose. The absence of tremors, convulsions, and impaired reflexes indicates that the extract maintains central nervous system stability without affecting sensory-motor function. Similarly, normal defecation and urination patterns suggest no adverse effects on the autonomic nervous system, supporting its overall safety profile in acute exposure. The absence of neurotoxicity in *P. canescens* leaf extract aligns with previous studies on flavonoid-rich herbal extracts, which are known for their neuroprotective properties.(31) Phenolic acids, including kaempferol, help reduce oxidative stress and modulate inflammatory pathways in the central nervous system (CNS) (32), similar effects have been observed in *Albuca amoena* extract, which aids in CNS regulation and metabolic stability (33). The results further support the extract's tolerability and its potential role as a safe natural immunomodulator. Nonetheless, further studies, including chronic toxicity and mechanistic evaluations, are necessary to confirm its long-term safety.

The findings indicate that *P. canescens* extract influences metabolic parameters in a dose-dependent manner. Weight gain in the control and moderate-dose groups (300 and 2000 mg/kg BW) suggests preserved metabolic function, while a slight weight reduction in the 5000 mg/kg BW group on day-7 may reflect increased energy expenditure or altered appetite regulation at higher doses. Flavonoids in the extract may also influence appetite by modulating gamma-aminobutyric acid type B receptor subunit 1 (GABAB1R) and neuropeptide Y (NPY) expression, leading to reduced food intake and increased energy expenditure, which could explain the observed dose-dependent effects.(34)

Increased liver and kidney weights observed at 2000 and 5000 mg/kg BW indicate heightened metabolic and detoxification activity, likely due to the processing of bioactive compounds. The increase in heart weight at these doses may represent an adaptive response to elevated metabolic demands. These results are consistent with the known effects of flavonoid-rich extracts on energy balance and organ function, supporting the metabolic safety of *P. canescens* extract at low to moderate doses and highlighting the need for further evaluation at higher concentrations. Additionally, plant extracts with immunomodulatory and detoxifying properties often affect liver and kidney weights,

as these organs are key in metabolizing and excreting phytochemicals.(34) The transient increase in lymph node weight at 2000 mg/kg BW, followed by a decrease at 5000 mg/kg BW, suggests a dose-dependent modulation of immune activity. The initial enlargement may reflect immune activation, whereas the subsequent reduction could indicate metabolic compensation or potential immunosuppression at higher doses. Additionally, alterations in liver, kidney, and heart weights point to physiological responses associated with detoxification and metabolic processing of bioactive compounds. These results imply that *P. canescens* extract modulates both immune and metabolic homeostasis in a dose-dependent manner. While moderate doses support immune function and metabolic stability, higher doses may trigger adaptive responses requiring further investigation. Histopathological and biochemical analyses are recommended to confirm the safety and long-term effects of the extract. These findings support the pharmacological potential of *P. canescens* extract as an immunomodulatory and metabolic-regulating agent.(20)

The absence of mortality in all experimental groups, even at the highest dose of 5000 mg/kg BW, confirms the excellent safety profile of *P. canescens* leaf extract during acute exposure. In the present study, no mortality or observable signs of toxicity were detected in mice administered with *P. canescens* extract at doses up to 5000 mg/kg BW. This is consistent with findings an acute toxicity study on *Cassia fistula* extract using distilled water as a negative control.(35) For instance, another acute toxicity study conducted on *Citrullus colocynthis* leaf extract using 0.9% NaCl as a negative control. Their findings indicated no toxic effects at doses up to 2000 mg/kg BW.(36)

As per OECD guidelines, substances with no mortality up to this dose are classified as “practically non-toxic,” reinforcing the extract’s tolerability and therapeutic potential.(37) This finding suggests that *P. canescens* leaves extract is well-tolerated, even at high doses, and provides a strong foundation for further exploration of its therapeutic potential. Acute toxicity studies in animal models are critical for determining safe dosage ranges, often assessed by calculating the median lethal dose ( $LD_{50}$ ). (38) These studies provide essential preclinical data to ensure the safety of herbal medicines for human use.(39)

The inability to determine an  $LD_{50}$  due to 0% mortality further highlights its low acute toxicity. Since no adverse effects were observed, higher-dose testing is unnecessary at this stage, supporting its progression to subchronic and chronic toxicity studies. The safety profile aligns with other flavonoid-rich extracts, known for their antioxidant and

anti-inflammatory properties. Compounds like kaempferol and phenolic acids in *P. canescens* leaves may contribute to their protective effects. These findings confirm *P. canescens* extract’s non-toxic nature and its potential for further therapeutic applications.(40) However, subchronic and chronic toxicity studies, along with comprehensive biochemical and histopathological analyses, are essential to confirm its long-term safety and suitability for prolonged use. Additionally, future acute toxicity tests using intermediate doses between 300 and 2000 mg/kg BW are recommended to more accurately identify the threshold at which toxicity may begin to manifest.

## Conclusion

This study demonstrates the immunomodulatory potential of *P. canescens* leaf extract, particularly its EAF, which significantly inhibits *IL-6* expression in LPS-induced macrophages. *In vivo* findings further support its role in enhancing adaptive immunity by increasing lymphocyte counts while regulating neutrophil activity. The extract also exhibits a favorable safety profile, with no observed neurotoxic or autonomic disturbances, and is classified as “practically non-toxic” based on OECD guidelines. Dose-dependent metabolic effects suggest enhanced detoxification activity at higher doses, warranting further histopathological and biochemical evaluations. These findings support the therapeutic potential of *P. canescens* extract, highlighting the need for future studies on its bioactive compounds and long-term safety.

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## Authors Contribution

MRRR, YS, SAS, and MM. were responsible for conducting the *in vitro* and *in vivo* immunomodulatory studies. FA, GW, SAS, and JL carried out the acute toxicity experiments.

MRRR, YS, and SAS. contributed to the drafting, editing, and revision of the manuscript. All authors reviewed and approved the final version of the manuscript for submission.

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