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Immunostimulant Properties and Acute Toxicity Studies of Sungkai Leaves (*Peronema canescens* Jack): *In Vivo* Hematological Analysis and *In Vitro* IL-6 Gene Expression Inhibition

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Abstract

Background: *Peronema canescens* Jack (PC), commonly known as Sungkai, is a medicinal plant indigenous to Indonesia, traditionally employed in the treatment of inflammation, malaria, and immune-related disorders. This study aims to investigate the immunostimulatory

potential of PC leaf extract through *in vitro* and *in vivo* assessments while concurrently evaluating its acute toxicity profile

Methods: The *in vitro* immunomodulatory activity was assessed by measuring IL-6 inhibition in LPS-stimulated RAW 264.7 macrophages. *In vivo*, Balb/C mice were divided into six groups: a normal control (Na-CMC), a positive control (Stimuno® *Phyllanthus niruri* extract, 50 mg/kg BW/day), a negative control (cyclophosphamide, 80 mg/kg BW/day, immunosuppressant), and three treatment receiving PC extract at doses of 100, 200, and 400 mg/kg BW/day. Hematological parameters, including WBC counts, lymphocyte percentages, and neutrophil percentages, were analyzed. Acute toxicity studies were performed by administering PC extract at doses of 300, 2000, and 5000 mg/kg BW over a 14-day observation period.

Results: The ethyl acetate fraction (EAF) exhibited the most pronounced IL-6 inhibition *in vitro*. *In vivo*, administration of PC 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 extract's safety profile up to 5000 mg/kg BW.

Conclusion: PC leaf extract, particularly EAF, demonstrates robust immunostimulatory activity with a favorable safety margin. These findings underscore its potential therapeutic application in immune modulation. Future research should explore its bioactive constituents and long-term pharmacological effects.

Keywords: *Peronema canescens* Jack, Immunostimulant, IL-6 inhibition, Acute toxicity, Hematology, Cytokines.

Introduction

Medicinal plants have long been integral to traditional medicine due to their diverse pharmacological properties (1). *Peronema canescens* Jack (PC), 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 PC leaves, suggesting potential immunomodulatory properties (2). 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, 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, with the figure reaching 59.12% in Indonesia (3), underscoring the importance of integrating traditional remedies into modern healthcare systems through scientific validation.

Peronema canescens Jack (PC), commonly known as Sungkai, is a member of the Verbenaceae family (4) and is native to Indonesia, particularly in Sumatra and Kalimantan (5). Traditionally, PC leaves have been utilized for managing inflammation, malaria, immune-related disorders, and more recently, during the Covid-19 pandemic as an immune booster (6). The leaves of PC are rich in bioactive compounds, including flavonoids, alkaloids, saponins, tannins, and phenolics, which exhibit antioxidant, anti-inflammatory, and immunomodulatory properties (7,8). Among these compounds, flavonoids and tannins are particularly known for their ability to counteract oxidative stress and regulate inflammatory responses (5).

Immunostimulants are substances that enhance the immune system by triggering the production of immune cells, such as macrophages and lymphocytes, and increasing cytokine production [8, 11]. Interleukin-6 (IL-6) is a key pro-inflammatory cytokine implicated in immune regulation (10). While physiological levels of IL-6 support the immune response to

infections, elevated levels are associated with chronic inflammation and autoimmune diseases (11). Herbal immunostimulants 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 (12). This dual regulation highlights the importance of studying herbal immunostimulants in targeting inflammation and immune-related diseases (13). In contrast, at high levels, IL-6 can cause excessive inflammation and is associated with chronic inflammatory diseases (14). Identifying natural immunostimulants capable of modulating IL-6 production is crucial for the development of alternative immunotherapeutic agents.

Despite its therapeutic potential, limited research has comprehensively explored the immunomodulatory effects of PC at the molecular level, its *in vivo* efficacy, and its safety profile. Acute toxicity studies in animal models are critical for determining safe dosage ranges, often assessed by calculating the median lethal dose (LD_{50}) (15). These studies provide essential preclinical data to ensure the safety of herbal medicines for human use (16).

The aim of this study is to evaluate the *in vitro* inhibitory effects of PC extract and its fractions on IL-6 gene expression in LPS-induced RAW 264.7 cells, 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 PC extract. This study integrates molecular and *in vivo* analyses with toxicity assessments, contributing valuable insights into the potential development of PC as a safe and effective immunomodulatory agent for managing inflammation-related disorders.

Methods

Materials

Ethanol (96% purity, Brataco, Indonesia), dimethyl sulfoxide (DMSO, ≥99% purity, Sigma-Aldrich, USA), lipopolysaccharide (LPS, Sigma-Aldrich, USA), phosphate-buffered saline (PBS, Thermo Fisher Scientific, USA), and WST-8 reagent for cell viability assays were sourced as indicated. Total RNA mini kit (Geneaid, Taiwan) was used for RNA extraction, and reverse transcription and PCR amplification reagents, including random primers and PCR master mix, were supplied by Promega (USA). Solvents such as *n*-hexane, ethyl acetate, and distilled water, were analytical grade (Merck, Germany). Stimuno® (PT Dexa Medica, Indonesia) was purchased locally as a positive control. Cyclophosphamide, used as an immunosuppressant, was sourced from a standard pharmaceutical supplier.

Instrumentation

Cell culture was conducted in a biosafety cabinet (ESCO, Singapore), with cell growth facilitated by a CO_2 incubator (Thermo Scientific, USA). Absorbance measurements were conducted using a microplate reader (Infinite M200 Pro, TECAN, Switzerland), and RNA quantification utilized a NanoQuant Plate. PCR amplification was performed with a Bio-Rad T100 thermal cycler (USA), and gel visualization employed the Gel Doc XR+ System (Bio-Rad, USA). Hematological parameters were analyzed with a Sysmex hematology analyzer (Japan).

Collection, Plant Processing, Extraction, and Fractionation

PC leaves were collected from Kayutanam, West Sumatra, Indonesia. 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.

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 LPS (1 μg/mL) to induce IL-6 expression. PC extract fractions (NHF, EAF, WF) were administered at varying concentrations. IL-6 mRNA expression was quantified via qPCR using β-actin as the housekeeping gene. RNA was isolated using Ribozol reagent, with purity confirmed by A260/A280 ratios. RNA was transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, USA). Quantitative Real-Time PCR (qPCR) was conducted using SensiFAST™ SYBR No-ROX Kit, with primers designed for IL-6 and β-actin. Data were analyzed using the ΔΔCt method and statistical significance assessed via one-way ANOVA ($p < 0.05$).

In vivo Immunomodulatory Assay

Thirty Balb/C mice were randomly allocated into six groups (n = 5 per group): normal control (Na-CMC 0.5%), positive control (Stimuno®, 50 mg/kg BW/day), negative control (cyclophosphamide, 80 mg/kg BW/day), and treatment groups receiving PC extract at 100, 200, and 400 mg/kg BW/day. Hematological parameters were assessed on days 0, 7, and 10, including WBC, lymphocyte, and neutrophil counts, were measured using a Sysmex hematology analyzer.

Acute Toxicity Study

Acute toxicity was evaluated in compliance with OECD guidelines. Balb/C mice were administered PC extract at doses of 300, 2000, and 5000 mg/kg BW. Toxicity markers,

mortality, body weight fluctuations, and organ weights were monitored over 14 days. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test ($p < 0.05$).

Results

Plant Collection

PC leaves were collected from Kayutanam, Padang Pariaman, West Sumatra, Indonesia. Taxonomic identification was confirmed at the Biosystematics and Molecular Herbarium Jatinangoriensis Laboratory (reference number: 05/LBM/IT/11/2021).

Ethical Approval

This study received ethical approval from the Universitas Padjadjaran Research Ethics Commission (Approval No: 434/UN.6.KEP/EC/2022 for immunostimulant activity and 471/UN.6.KEP/EC/2023 for acute toxicity studies).

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 untreated control (CC) group showed a baseline IL-6 expression of 0.230 ± 0.005. In contrast, the LPS-induced group (CC+LPS) exhibited a marked increase in IL-6 expression (0.406 ± 0.032). Treatment with DMSO alone (CDMSO) resulted in an IL-6 expression level of 0.174 ± 0.025, indicating minimal interference by the solvent.

Among the tested fractions, the ethyl acetate fraction (EAF) demonstrated the most significant inhibition of IL-6 expression at 6.125 μg/mL (0.221 ± 0.058, $p < 0.05$ vs. CC+LPS). The *n*-hexane fraction (NHF) exhibited a higher expression level (0.382 ± 0.112), showing

limited inhibitory effects, while the water fraction (WF) showed the least inhibition. EAF exhibited the highest potency in inhibiting IL-6 expression, indicative of bioactive flavonoids and phenolic acids modulating inflammatory pathways.

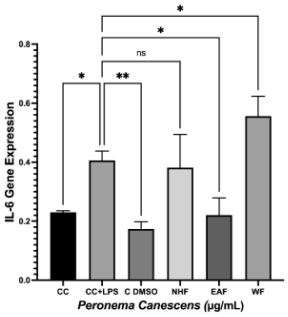


Figure 1. IL-6 mRNA expression levels in RAW 264.7 cells treated with PC fractions.

*CC: Cell Control; CC+LPS: Cell Control+LPS induced; DC: DMSO Control; NHF: PC leaves *n*-Hexane fraction; EAF: PC leaves Ethyl acetate; WF: PC leaves Water fraction.

* The data were presented as mean + standard deviation. The different notations (*, **) signify statistical differences, (ns) not significant among the groups regarding the Dunnett at $p \leq 0.05$.

In vivo Immunomodulatory Activity

The hematological analysis revealed that PC extract at doses of 200 mg/kg BW and 400 mg/kg BW increased white blood cell (WBC) counts and lymphocyte percentages while reducing neutrophil percentages in cyclophosphamide-induced mice. Groups treated with 200 mg/kg BW and 400 mg/kg BW showed WBC counts of $9.15 \pm 0.43 \times 10^3/\mu L$ and $10.22 \pm 0.65 \times 10^3/\mu L$, respectively, compared to the cyclophosphamide group ($5.47 \pm 0.28 \times 10^3/\mu L$, $p < 0.05$).

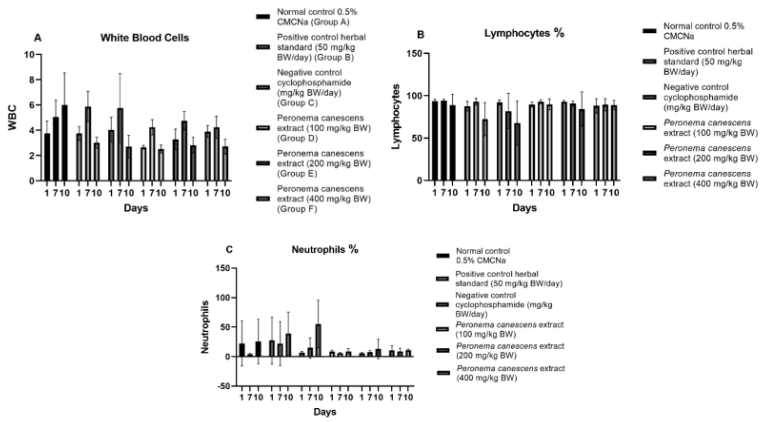


Figure 2. Hematological parameters of cyclophosphamide-induced mice treated with PC extract (A) White blood cells, (B) Percentage of lymphocytes, (C) Percentage of neutrophils.

Lymphocyte percentages in the 200 mg/kg BW and 400 mg/kg BW groups were 65.2 ± 1.8% and 69.8 ± 2.1%, respectively, significantly higher than the cyclophosphamide group (41.3 ± 2.5%, $p < 0.05$). Neutrophil percentages were reduced to 25.6 ± 1.2% and 22.4 ± 1.5% in the 200 mg/kg BW and 400 mg/kg BW groups, respectively, compared to 49.6 ± 2.7% in the cyclophosphamide group.

In vivo Acute Toxicity

Pharmacological Screening

The pharmacological screening was conducted to evaluate the potential neurotoxic effects of PC 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. Observations were categorized into central and autonomic nervous system parameters, with results summarized in Table 1.

Tabel 1. Results of Pharmacological Screening.

Nervous System	Observed Behavioral Values
Central Nervous System	
Posture	100%
Convulsions	0%
Tremors	0%
Writhing	0%
Catalepsy	100%
Flexion	100%
Corneal reflex	100%
Pineal Reflex	100%
Hanging	100%
Autonomous Nervous System	
Defecation	0%
Urine	0%
Salivation	0%
Pylorection	0%
Lacrimation	0%

In Table 1, a "0%" value represents the absence of a specific behavior or symptom, while "100%" indicates its consistent presence across all subjects. Behavioral classifications were determined using standard pharmacological screening protocols. Positive findings, such as intact reflexes and normal posture, confirm the normal function of the central and autonomic nervous systems. Conversely, the absence of adverse behaviors, such as tremors, convulsions, or salivation, suggests the safety of PC extract under the experimental conditions.

The results indicate that PC 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

Body Weight Changes

Body weight was monitored over a 14-day period to assess the metabolic and systemic effects of PC 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 days 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. The average body weights recorded during the study are summarized in Table 2.

Tabel 2. Result Of Body Weight Measurement by Days and Doses

Days	Control (mg/kg BW)	700 (mg/kg BW)	2000 (mg/kg BW)	5000 (mg/kg BW)
1st day	30.33	24.83	30.50	30.83
7th day	31.38	27.50	31.60	29.83
14th day	35.33	29.67	36.00	31.00

The consistent weight gain observed in the control group and groups treated with 700 mg/kg and 2000 mg/kg BW suggests that PC 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 PC extract at lower and moderate doses, while indicating the need for further investigation into its metabolic effects at higher doses.

Observation of Organ Weights

Relative organ weights were measured to assess the potential dose-dependent effects of PC 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 ($p < 0.05$), as shown in Table 4. 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 PC leaves extract. The kidneys and liver showed significant weight increases, possibly due to their roles in metabolizing and excreting the extract's bioactive compounds.

Tabel 3. Result of relative organ weight measurement

Organ	Control mg/kg BW	700 mg/kg BW	2000 mg/kg BW	5000 mg/kg BW
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)

Mortality Observation

During the 14-day observation period, no mortality was recorded in any experimental group, including those treated with PC 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 are summarized in Table 5.

Tabel 4. Mortality Observation Results

Group (mg/kg BW)	Percentage of mortality (days)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5000	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The absence of mortality in all groups, including those treated with the highest dose of 5000 mg/kg BW, confirms the safety of PC leaves extract under acute exposure conditions. These results suggest that PC 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.

Discussion

In vitro Immunomodulatory Activity

This study provides evidence of the immunomodulatory potential of PC leaves extract, with the ethyl acetate fraction (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 (17). The findings support the hypothesis that PC extract, particularly the EAF, modulates inflammatory pathways, highlighting its therapeutic relevance. These findings align with previous studies indicating that PC leaves extracts possess anti-inflammatory activity (18).

The observed reduction in IL-6 expression by the EAF suggests its ability to interfere with the TLR4-NF-κB signaling pathway. This pathway, activated by LPS binding to TLR4 on antigen-presenting cells (APCs), is a key regulator of pro-inflammatory cytokines, including IL-6, TNF-α, and COX-2. By inhibiting NF-κB activation, (19). the EAF potentially suppresses the cascade of inflammatory responses, restoring immune balance and reducing cytokine-mediated tissue damage. This mechanism aligns with reports on flavonoids and phenolic acids, compounds abundant in PC leaves, known for their ability to modulate NF-κB signaling.

The phytochemical composition of PC leaves—rich in flavonoids, phenolic acids, and triterpenoids—plays a pivotal role in its anti-inflammatory effects. Flavonoids, such as kaempferol, have been shown to inhibit NF-κB activation, while phenolic acids exhibit antioxidant properties that reduce oxidative stress and cytokine production. Additionally, saponins may enhance macrophage activity, contributing to immune regulation. These findings emphasize the potential of PC extract as a multi-targeted therapeutic agent for managing immune dysregulation.

The results align with prior studies on other flavonoid-rich extracts, such as *Alternanthera sessilis*, which similarly inhibit NF-κB activation and reduce IL-6 expression. Furthermore, the anti-inflammatory activity observed in this study corroborates previous MTT assay results, which demonstrated the ability of PC leaves extract to modulate inflammatory

signaling pathways. The relevance of natural compounds, such as phenformin and docosahexaenoic acid (DHA), as IL-6 inhibitors further underscores the therapeutic potential of phytochemicals in addressing hyperinflammatory responses (20). According to (21) the combination of metformin and magnesium citrate demonstrated the strongest anti-inflammatory activity, leading to a significant reduction in TNF-α, NF-κB, and IL-6 levels. Meanwhile, another study reported that consuming beetroot juice or extract, either independently or in combination with simvastatin, effectively lowered LDL, increased HDL, and reduced IL-6 levels. These findings suggest that beetroot may offer potential benefits in both the prevention and treatment of dyslipidemia (22).

While the study demonstrates the efficacy of PC leaves extract in inhibiting IL-6 expression, several limitations should be acknowledged. The research relies on *in vitro* models, which do not fully replicate the complexity of inflammatory responses in living organisms. Additionally, the study does not isolate specific bioactive compounds within the EAF, leaving gaps in understanding the precise molecular interactions responsible for the observed effects. Furthermore, long-term safety and efficacy remain untested, as the study did not include *in vivo* models of chronic inflammation or subchronic toxicity assessments.

The significant IL-6 inhibition by the EAF fraction underscores its potential as a therapeutic agent for managing chronic and acute inflammatory conditions, including rheumatoid arthritis, sepsis, and COVID-19-related cytokine storms. By targeting NF-κB signaling, PC extract offers a promising avenue for restoring immune homeostasis and preventing cytokine-mediated damage (23). Future studies addressing the identified limitations will further validate its therapeutic value and elucidate its mechanisms of action.

***In vivo* Immunomodulatory Activity**

White blood cells (WBCs), lymphocytes, and neutrophils are critical indicators of immune system dynamics, reflecting the balance between adaptive and innate immunity. This study demonstrates that PC leaves extract exhibits dose-dependent immunomodulatory effects, as evidenced by significant changes in WBC counts, lymphocyte percentages, and neutrophil percentages (24).

The WBC counts in groups treated with PC extract showed significant dose-dependent increases at 200 mg/kg BW and 400 mg/kg BW compared to the negative control group, indicating enhanced immune activity. This suggests that the extract counteracts cyclophosphamide-induced immunosuppression (25), which is often characterized by reduced leukocyte counts. The higher WBC counts at moderate to high doses support the extract's role in stimulating immune cell proliferation or activation, which is crucial for restoring immune homeostasis in immunosuppressed conditions (26).

Lymphocyte percentages were significantly increased in groups treated with 200 mg/kg BW and 400 mg/kg BW of PC extract, demonstrating its ability to enhance adaptive immunity. Lymphocytes, particularly T and B cells, are central to immune memory and pathogen-specific responses (27). The dose-dependent increase in lymphocyte percentages suggests that PC extract enhances the proliferation or activation of these immune cells. This property is particularly relevant for managing conditions where adaptive immunity is compromised, such as chronic inflammation or immunosuppressive therapies.

Neutrophil percentages, a marker of innate immune activity, were also modulated by PC extract. While cyclophosphamide-induced groups showed initial suppression and rebound of neutrophil counts, the 400 mg/kg BW dose of PC extract demonstrated enhanced neutrophil activity, indicating its ability to support innate immune functions. Interestingly, the extract also mitigated excessive neutrophil-driven inflammation, as evidenced by more stable neutrophil percentages at moderate doses (27). This dual regulation suggests that PC extract balances

neutrophil activity, reducing the risk of hyperinflammatory responses often associated with chronic diseases and cytokine storms.

The combined increase in lymphocyte percentages and regulation of neutrophil activity highlights the dual immunomodulatory effects of PC extract. By enhancing adaptive immunity and attenuating excessive neutrophil-driven inflammation, PC extract addresses both immune activation and regulation. This dual mechanism makes it a promising candidate for managing immune dysregulation, particularly in conditions involving chronic inflammation, autoimmune disorders, or cytokine storms, such as those observed in severe COVID-19 cases. The observed immunomodulatory effects are consistent with prior studies on herbal extracts rich in flavonoids and phenolic acids, which modulate immune responses by targeting key inflammatory pathways, including NF-κB signaling. The ability of PC extract to restore immune homeostasis aligns with the therapeutic potential of other natural immunomodulators.

The observed immunomodulatory effects are consistent with prior studies on herbal extracts rich in flavonoids and phenolic acids, which modulate immune responses by targeting key inflammatory pathways, including NF-κB signaling. The ability of PC extract to restore immune homeostasis aligns with the therapeutic potential of other natural immunomodulators.

Pharmacological Screening

The pharmacological screening results indicate that PC leaves extract does not induce neurotoxic effects on the central or autonomic nervous systems. The absence of tremors, convulsions, or impaired reflexes demonstrates the extract's ability to maintain neuronal stability without interfering with key sensory-motor pathways. The consistent results across all dose levels suggest that the extract does not disrupt motor coordination, pain signaling, or behavioral responses, reinforcing its safety for acute use.

The stable autonomic nervous system (ANS) parameters, such as normal defecation and urination patterns, further highlight the extract's lack of adverse effects on gastrointestinal and

renal functions. Observations of regular fecal and urinary output, combined with normal body weight across groups, suggest that the extract may support balanced parasympathetic activity. This parasympathetic modulation, potentially mediated by enhanced vagal tone and acetylcholine signaling, could promote homeostatic balance during inflammatory or physiological stress, contributing to metabolic and gastrointestinal health.

The absence of neurotoxicity aligns with previous findings on flavonoid-rich herbal extracts, which are often associated with neuroprotective properties. The role of phenolic acids in protecting the central nervous system by reducing oxidative stress and modulating inflammatory pathways (28). Compounds such as kaempferol and phenolic acids, known to be present in PC leaves, have been shown to mitigate oxidative stress and inflammatory pathways in the central nervous system (CNS) (29), further supporting the extract's safety and potential therapeutic benefits. Similar to PC, extracts from plants like *Albuca amoena* extract have demonstrated the ability to regulate central nervous system activity (30), promoting digestive efficiency and metabolic stability. These findings suggest that PC leaves extract may not only be safe but could also confer additional benefits for managing stress-induced physiological imbalances.

The pharmacological screening results confirm that PC leaves extract is well tolerated and does not induce neurotoxic or behavioral disturbances at the tested doses. Its safety profile, combined with potential parasympathetic modulation, highlights its suitability for therapeutic applications, particularly as an immunomodulatory agent. Future studies should focus on chronic toxicity assessments and mechanistic investigations to fully establish its safety and explore its broader therapeutic potential.

Observation of Body Weight and Organ Weights

The results of this study suggest that PC leaves extract influences metabolic and physiological parameters in a dose-dependent manner. The observed weight gain in the control

and lower-dose groups (700 mg/kg and 2000 mg/kg BW) indicates that the extract does not disrupt normal metabolic processes at moderate doses. However, the slight weight reduction in the 5000 mg/kg BW group on day 7 suggests a potential metabolic impact at higher doses, possibly related to energy expenditure or appetite regulation.

The decrease in body weight at higher doses aligns with previous studies reporting that PC extract exhibits anti-diabetic effects by promoting glucose excretion through urine, leading to reduced glucose availability as an energy source. This glucose deficit could trigger compensatory mechanisms such as increased fat and muscle protein catabolism, which, if prolonged, may contribute to weight loss. The role of flavonoids in appetite modulation is also relevant, as studies have shown that these compounds regulate food intake by altering the expression of gamma-aminobutyric acid B1 receptor (GABAB1R) and neuropeptide Y (NPY) in the hypothalamus. The downregulation of NPY has been associated with reduced appetite and increased energy expenditure, which could explain the dose-dependent effects observed in the present study(31).

The observed changes in relative organ weights further suggest systemic effects of PC extract at higher doses. Significant increases in kidney and liver weights at 2000 mg/kg and 5000 mg/kg BW suggest enhanced metabolic and detoxification activity, possibly due to the increased processing of bioactive compounds present in the extract. Similarly, the increased heart weight at these doses may reflect an adaptive response to cardiovascular metabolic demands, though the precise mechanisms remain unclear.

The findings are consistent with previous research on flavonoid-rich herbal extracts that influence metabolic regulation. Flavonoids are known for their ability to modulate glucose metabolism, lipid oxidation, and appetite control, potentially contributing to the observed body weight changes (31). Additionally, plant extracts with immunomodulatory and detoxification

properties often induce changes in liver and kidney weights, as these organs play central roles in metabolizing and excreting phytochemicals.

The transient increase in lymph node weight at 2000 mg/kg BW followed by a decrease at 5000 mg/kg BW suggests a complex immune response. The initial increase may indicate immune activation or lymphoid tissue proliferation, whereas the subsequent decline may be due to metabolic trade-offs or potential immunosuppressive effects at very high doses. This pattern has been observed in studies evaluating the effects of high-dose flavonoids on immune homeostasis, where excessive exposure can sometimes lead to downregulation of immune function.

The findings of this study suggest that PC leaves extract influences metabolic homeostasis and organ physiology in a dose-dependent manner. While moderate doses (700 mg/kg and 2000 mg/kg BW) appear to support normal metabolic function, higher doses (5000 mg/kg BW) may induce metabolic adaptations that warrant further investigation. The observed changes in organ weights, particularly in the liver, kidneys, and heart, indicate potential physiological responses to increased detoxification and metabolic demands. However, additional studies, including histopathological and biochemical analyses, are necessary to confirm the safety and long-term implications of PC extract use. These results contribute to the growing body of evidence supporting the pharmacological potential of PC leaves as an immunomodulatory and metabolic-regulating agent. According to (32) levels of TNF-α, IL-6 and IL-10 are associated with the degree of liver fibrosis.

Mortality Observation

The absence of mortality across all experimental groups treated with PC leaves extract, including at the highest dose of 5000 mg/kg BW, underscores its excellent safety profile during acute exposure. According to the OECD guidelines, substances with no mortality at doses up to 5000 mg/kg BW can be classified as “practically non-toxic” (33).This finding suggests that

PC leaves extract is well-tolerated, even at high doses, and provides a strong foundation for further exploration of its therapeutic potential.

The inability to determine the LD_{50} value due to a consistent 0% mortality rate further highlights the low acute toxicity of the extract. As the upper dose limit for acute toxicity testing has been reached without adverse effects, it is unnecessary to test higher doses during this phase. These results validate the short-term safety of PC extract and support its suitability for progression to subchronic and chronic toxicity studies.

The safety profile of PC leaves extract aligns with prior findings on other flavonoid-rich herbal extracts, which often demonstrate minimal acute toxicity due to their natural antioxidant and anti-inflammatory properties. Compounds such as kaempferol and phenolic acids, present in PC leaves, have been reported to exert protective effects on key organs by reducing oxidative stress and inflammation, potentially contributing to the extract's non-toxic nature (34).

The mortality observation results confirm that PC leaves extract is non-toxic at doses up to 5000 mg/kg BW, providing a robust safety profile during acute exposure. These findings support the classification of the extract as "practically non-toxic" and validate its potential for further development as a therapeutic agent. 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.

Conclusion

This study provides compelling evidence of the immunomodulatory and metabolic effects of PC leaves extract, highlighting its potential as a therapeutic agent for managing immune dysregulation and inflammatory conditions. The *in vitro* findings demonstrate that the ethyl acetate fraction (EAF) of PC extract significantly inhibits IL-6 expression in LPS-induced

macrophages, suggesting its role in modulating the TLR4-NF-κB signaling pathway. This mechanism is consistent with the anti-inflammatory properties of flavonoids and phenolic acids, reinforcing the potential of PC extract in mitigating cytokine-mediated immune disorders.

The *in vivo* immunomodulatory study further supports these findings, showing that PC extract enhances adaptive immunity by increasing lymphocyte counts while regulating neutrophil activity. The observed dose-dependent effects, particularly at 200 mg/kg and 400 mg/kg BW, indicate that PC extract may help counteract immunosuppressive conditions, such as those induced by cyclophosphamide. These results align with existing literature on flavonoid-rich herbal extracts that influence immune homeostasis through key inflammatory pathways.

The pharmacological screening results confirm the safety of PC extract, as no neurotoxic or autonomic disturbances were observed. The extract exhibited no adverse effects on motor coordination, sensory-motor reflexes, or autonomic functions, supporting its well-tolerated profile. The absence of toxicity-related behavioral changes further strengthens the case for PC extract as a safe immunomodulatory agent.

Metabolic evaluations indicate that body weight and organ weight changes are dose-dependent. While moderate doses (700 mg/kg and 2000 mg/kg BW) support normal metabolic function, higher doses (5000 mg/kg BW) may induce metabolic adaptations, potentially affecting energy balance and organ function. The observed increases in liver and kidney weights suggest enhanced metabolic and detoxification activity, necessitating further histopathological and biochemical assessments to clarify the extract's long-term safety profile. The mortality assessment demonstrated no fatalities at doses up to 5000 mg/kg BW, classifying PC extract as "practically non-toxic" based on OECD guidelines. This robust safety profile paves the way for further investigations into its chronic toxicity and therapeutic applications.

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Authors' Contributions

M.R.R.R., Y.S., S.A.S., and M.M. were responsible for conducting the *in vitro* and *in vivo* immunomodulatory studies. F.A., G.W., S.A.S., and J.L. carried out the acute toxicity experiments. M.R.R.R., Y.S., and S.A.S. 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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