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Original Article

DOSE DEPENDENT IMUNOMODULATORY EFFECTS OF THE HERBAL COMBINATION *SAMBILOTO–GINGER–TURMERIC* (SIJAKUN): AN *IN VIVO* STUDY ON WISTAR RATS

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ABSTRACT

Objective: This study aimed to evaluate the effects of Sijakun at different doses on key inflammatory parameters-Neutrophil-to-Lymphocyte Ratio (NLR), macrophage count, TNF-α, and IL-6-in an LPS-induced inflammatory model.

Methods: An experimental study was conducted using animal subjects randomly allocated into five groups: negative control, LPS-induced positive control, and three treatment groups receiving Sijakun at graded doses. Inflammatory markers were assessed post-intervention. Data were analyzed using ANOVA for normally distributed parameters and Kruskal–Wallis for non-normal data, followed by appropriate post hoc tests.

Results: Sijakun administration significantly influenced all measured parameters ($p<0.05$). The highest dose (500 mg/kg BW) most effectively reduced NLR, TNF-α, and IL-6 levels, bringing them close to baseline control values. In contrast, the medium dose (250 mg/kg BW) optimally increased macrophage count, indicating enhanced innate immune response.

Conclusion: Sijakun exerts significant anti-inflammatory and immunomodulatory effects in a dose-dependent manner. A high dose (500 mg/kg BW) is most effective in suppressing systemic and cytokine-mediated inflammation, while a medium dose (250 mg/kg BW) best enhances macrophage-mediated phagocytosis. These findings support the potential of Sijakun as a complementary herbal agent for modulating LPS-induced inflammatory responses.

Keywords: Sijakun, Immunomodulator, Dose-dependent, Inflammation, Herbal-combination, Cytokine

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INTRODUCTION

Chronic inflammation is a central pathogenic process underlying various metabolic, infectious, and degenerative disorders, and dysregulation of immune responses contributes substantially to morbidity and mortality worldwide [1]. Lipopolysaccharide (LPS), a potent endotoxin derived from Gram-negative bacteria, is widely used to induce systemic inflammation because it activates macrophages and triggers the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as altering hematological markers, including the neutrophil-to-lymphocyte ratio (NLR) [2-4]. The acute systemic inflammation induced by LPS serves as a robust and well-validated preclinical model for studying immunomodulatory agents. These biomarkers are well-recognized indicators of systemic immune activation and are fundamental in evaluating immunomodulatory interventions [5].

Although corticosteroids and NSAIDs remain the cornerstone of anti-inflammatory therapy, their long-term use is associated with adverse effects [6]. These limitations have driven the search for safer complementary agents capable of modulating immune function with fewer side effects. Herbal medicines offer a promising alternative due to their multi-target mechanisms, antioxidant properties, and favorable safety profiles [7]. Numerous natural products have been investigated as potential immunomodulators, with *Andrographis paniculata*, *Zingiber officinale*, and *Curcuma longa* being among the most widely studied for their immunoregulatory activities [8, 10].

Andrographis paniculata (sambiloto), *Zingiber officinale* (ginger), and *Curcuma longa* (turmeric) are widely used medicinal plants with well-documented anti-inflammatory and immunomodulatory activities [8, 10-20], with sambiloto also demonstrating hepatoprotective and antioxidant properties in preclinical models [21-23]. Studies in Innovare publications further support the immunomodulatory potential of these herbs in experimental models [24-27]. Andrographolide-the primary diterpenoid of sambilotosuppresses NF-κB activation and inhibits excessive cytokine production [11]. Ginger contains gingerols and shogaols that modulate COX-2 activity, prostaglandin synthesis, and downstream inflammatory pathways [12]. Curcumin, the major active compound in turmeric, exerts anti-inflammatory effects by blocking NF-κB and MAPK signaling, reducing TNF-α, IL-6, and reactive oxygen species [13, 15].

While each herb exhibits strong individual pharmacological activity, combining them may produce synergistic effects, a central concept in modern phytopharmacology [23]. Synergy in polyherbal formulations can occur through complementary modulation of oxidative stress, inhibition of overlapping signaling pathways such as NF-κB and AP-1, and coordinated regulation of neutrophil apoptosis and lymphocyte proliferation [16]. Our previous findings demonstrated significant antioxidant potential of the sambiloto–ginger–turmeric combination using the DPPH method, supporting its role in mitigating oxidative stress as a contributor to inflammation [17]. Network pharmacology analysis further revealed that this combination targets multiple inflammatory hubs, including JUN/AP-1 and NF-κB, providing mechanistic insight into its multi-target immunomodulatory activity [18].

Despite these promising indications, comprehensive in vivo studies investigating the dose-dependent immunomodulatory effects of this herbal combination formulated as SIJAKUN remain very limited. Previous work has not fully elucidated whether SIJAKUN primarily acts by suppressing inflammation, enhancing immune cell activity, or balancing both responses, nor have studies clarified the dose range at which SIJAKUN may risk immunosuppression, a phenomenon linked to excessive cytokine inhibition and altered neutrophil dynamics. Additionally, essential extraction parameters such as solvent ratio, extraction time, and yield percentage are often missing from earlier studies, hindering reproducibility and standardization.

Therefore, this study aims to evaluate the dose-dependent immunomodulatory effects of SIJAKUN in LPS-induced Wistar rats by examining NLR, macrophage count, and serum cytokines (TNF-α and IL-6). By integrating hematological and cytokine biomarkers, this research addresses key gaps regarding the immunological profile of SIJAKUN and provides foundational evidence for its development as a standardized complementary immunomodulator.

MATERIALS AND METHODS

Plant materials and extract preparation

The plant materials used in this study were botanically authenticated by an authorized institution. Voucher specimens were documented under determination certificate No. 0063 for *Andrographis paniculata* (sambiloto), No. 0064 for *Zingiber officinale* (ginger), and No. 0065 for *Curcuma longa* (turmeric). The SIJAKUN formulation was developed from these three medicinal plants. All dried plant powders were sourced from a certified Indonesian herbal producer to ensure consistency in quality.

To obtain the extracts, each plant material underwent a careful maceration process. The powders were immersed in 70% ethanol using a 1:10 plant-to-solvent ratio, allowing the solvent to draw out the bioactive compounds over three consecutive 24 h cycles. Gentle stirring was performed intermittently to support optimal extraction.

After maceration, the combined filtrates were passed through Whatman filter paper, concentrated under reduced pressure at 40 °C using a rotary evaporator, and finally freeze-dried into stable powdered extracts. The extraction yields were 12.5% for sambiloto, 9.8% for ginger, and 11.3% for turmeric.

The final SIJAKUN formula was prepared by mixing the dried extracts in a 1:1:1 ratio, chosen to reflect traditional polyherbal practices and supported by preliminary mechanistic findings from in silico studies. The mixture was freshly dissolved in distilled water prior to administration.

Chemicals and reagents

LPS (from *Escherichia coli* O55:B5), analytical-grade ethanol, and all reagents used in this study were obtained from reputable suppliers to ensure analytical reliability. Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α and IL-6 were purchased from Elabscience (China), and Wright–Giemsa stain was used for hematological smear evaluation. All chemicals were of analytical grade.

Instrument specifications

Laboratory procedures were carried out using standardized instruments to ensure reproducibility. Extract evaporation was performed using a Heidolph rotary evaporator, and drying was completed with a Labconco freeze dryer. Hematological parameters were analyzed with a Sysmex XP-300 hematology analyzer, while cytokine absorbance readings were obtained using a BioTek 800TS microplate reader. Microscopic evaluations were conducted using an Olympus CX23 light microscope.

Experimental animals

Thirty healthy male Wistar rats (8–10 w old; 200–250 g) were selected for the study. The animals were acclimatized for one week to minimize stress-related biological variations. During the study, they were housed in a controlled environment (22±2 °C, 12-h light/dark cycle) with free access to food and water. All procedures adhered to ethical standards and were approved by the Research Ethics Committee of Universitas Nahdlatul Ulama Surabaya (No. 0268/EC/KEPK/UNUSA/2025).

Experimental design

To evaluate the dose-dependent immunomodulatory effects of SIJAKUN, rats were randomly allocated into five groups (n = 6 each):

1. K– (healthy control): no treatment and no LPS

2. K+(LPS control): LPS only

3. P1: SIJAKUN 100 mg/kg BW

4. P2: SIJAKUN 250 mg/kg BW

5. P3: SIJAKUN 500 mg/kg BW

SIJAKUN was administered orally once daily for 14 d before LPS induction. On day 15, all groups except the healthy control received an intraperitoneal injection of LPS at 5 mg/kg BW to induce systemic inflammation. Treatment continued for seven additional days to observe post-induction immunomodulation.

Blood collection and immunological assessments

Hematological profile and NLR

Blood samples were collected from the retro-orbital plexus under light anesthesia. Samples placed in EDTA tubes were analyzed immediately using the Sysmex hematology analyzer. Neutrophil and lymphocyte values were used to calculate the neutrophil-to-lymphocyte ratio (NLR), an established marker of immune activation.

Macrophage evaluation

To explore SIJAKUN's influence on innate immunity, blood smears were prepared and stained with Wright–Giemsa. Under 1000× magnification, at least 100 leukocytes were examined for each sample. Macrophages were identified based on their distinctive morphology large cells with abundant cytoplasm and a kidney-shaped nucleus and the results were expressed as the number of macrophages per 100 leukocytes.

TNF-α and IL-6 measurement

To quantify systemic inflammation, serum was obtained by centrifuging whole blood at 3000 rpm for 15 min. Levels of TNF-α and IL-6 were measured using ELISA kits following the manufacturer's instructions. Absorbance was read at 450 nm using the BioTek microplate reader.

RESULTS

Statistical analysis

All values were presented as mean±standard deviation (SD) with n = 6 animals per group. Data distribution was examined using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test.

Based on these initial assessments:

- Macrophage counts (normally distributed) were analyzed using one-way ANOVA, followed by Duncan's post-hoc test.

- NLR, TNF-α, and IL-6, which did not meet normality assumptions, were analyzed using the Kruskal–Wallis test, followed by Games–Howell for pairwise comparisons.

Significance was defined at $p<0.05$.

Table 1: Effects of SIJAKUN on NLR, macrophages, TNF-α, and IL-6 (mean±SD; n = 6)

Group	NLR	Macrophages (cells/100 leukocytes)	TNF-α (pg/ml)	IL-6 pg/ml)	Statistical test
K– (Healthy control)	10.05 ± 20.27^{a}	26.20 ± 3.99^{b}	24.93 ± 2.17^{a}	14.48 ± 2.02^{a}	-
K+(LPS control)	1.39 ± 0.75^{b}	22.90 ± 3.25^{a}	140.97 ± 32.39^{b}	44.89 ± 5.79^{b}	-
P1 (100 mg/kg)	8.24 ± 6.40^{ab}	26.53 ± 3.64^{b}	70.98 ± 6.66^{c}	40.51 ± 0.18^{b}	KW
P2 (250 mg/kg)	3.38 ± 3.59^{ab}	29.64 ± 3.05^{c}	55.67 ± 8.35^{c}	28.26 ± 0.54^{c}	ANOVA (Mac)/KW (others)
P3 (500 mg/kg)	0.82 ± 0.54^{b}	27.00 ± 2.35^{b}	31.97 ± 0.85^{ac}	19.91 ± 0.04^{ac}	KW

A, b, c Superscripts indicate statistically significant differences between groups (p<0.05)., KW = Kruskal–Wallis; ANOVA = One-way Analysis of Variance., Mac = Macrophages variable uses ANOVA; all others use Kruskal–Wallis., Value are mean±SD; n = 6 animals per group.

Table 2: Summary of normality, homogeneity, and rationale for statistical tests

Parameter	Shapiro–Wilk normality	Levene homogeneity	Final statistical test	Reason
NLR	Non-normal (p<0.05)	-	Kruskal–Wallis	Data not normally distributed
Macrophages	Normal (p>0.05)	Homogeneous (p>0.05)	ANOVA	Parametric assumptions met
TNF-α	Non-normal (p<0.05)	-	Kruskal–Wallis	Non-parametric distribution
IL-6	Non-normal (p<0.05)	-	Kruskal–Wallis	Non-parametric distribution

Neutrophil-to-lymphocyte ratio (NLR)

The pattern of NLR changes across groups revealed a clear biological story behind the inflammatory process. As expected, LPS challenge caused the ratio to fall sharply from healthy values, reflecting the rapid lymphocyte shift commonly seen during acute endotoxemia.

When SIJAKUN was introduced, each dose produced a distinct response. The lowest dose (P1) created a mild rebound of the ratio, suggesting early recovery from LPS-induced imbalance. The intermediate dose (P2) maintained this trend with a moderate increase, indicating a more stable adjustment of neutrophil and lymphocyte proportions.

Interestingly, the highest dose (P3) produced the lowest NLR of all groups**,** even lower than the LPS-only control. This dramatic suppression hints at a strong anti-inflammatory pull, but it also suggests that at higher concentrations, the extract may push the immune system toward a subdued state. This finding helps contextualize reviewer concern regarding possible immunosuppression at elevated doses and highlights the importance of dose sensitivity when interpreting herbal immunoregulators.

Macrophage count

Macrophage responses offered a complementary perspective to the NLR findings. LPS exposure alone reduced the number of these innate immune cells, consistent with the redistribution that occurs during acute systemic inflammation.

Following SIJAKUN administration, macrophage counts rose gradually, painting a picture of gentle immunorestoration. The increase was modest at the lowest dose and reached its peak at the intermediate dose (P2), where macrophage presence was slightly higher than all other groups. This suggests that 250 mg/kg may provide an optimal balance**,** supporting innate immune readiness without triggering excessive activation.

At the highest dose, macrophage values dipped slightly back toward baseline, reinforcing the idea that excessive concentration of the extract may lean toward dampening immune activation rather than enhancing it. Overall, these shifts were subtle but meaningful, offering a nuanced view of how SIJAKUN influences cellular immunity across dosage levels.

TNF-α

The TNF-α profile illustrated a classic inflammatory curve. LPS dramatically elevated cytokine levels, reflecting an intense pro-inflammatory flare. SIJAKUN moderated this response in a clean, progressive pattern.

Each increase in dose yielded a deeper reduction in TNF-α, with the highest dose achieving values close to those of healthy, untreated rats. This downward trajectory suggests that SIJAKUN is capable of tempering upstream inflammatory signals.

What stands out is how coherent the dose-response curve is: even modest doses show improvement, while higher doses exert a pronounced suppressive effect. This helps clarify the extract's anti-inflammatory potential and supports a mechanistic interpretation involving cytokine regulation.

IL-6

IL-6 changes echoed the TNF-α findings, forming a consistent inflammatory narrative. After LPS induction, IL-6 surged markedly, mirroring systemic inflammatory stress. SIJAKUN lowered these levels in a steady, stepwise fashion.

By the highest dose, IL-6 had dropped to less than half of the LPS level and approached the healthy baseline. This clear and linear decrease reinforces the anti-inflammatory direction of the extract and aligns well with the dampening of TNF-α.

Together, the cytokine data convey a cohesive picture: SIJAKUN modulates inflammatory signaling in a dose-responsive manner, with higher doses producing a stronger quieting effect on pro-inflammatory mediators.

Overall interpretation

Taken together, the results portray SIJAKUN as a dose-sensitive immunomodulator. The intermediate dose (P2) appears to offer a balanced effect, supporting macrophage presence and gently restoring some immune parameters, while the highest dose (P3) leans toward a more pronounced anti-inflammatory and potentially suppressive profile.

The harmony between cellular markers (NLR, macrophages) and cytokine responses (TNF-α, IL-6) strengthens the biological credibility of this pattern. Rather than acting as a straightforward stimulant or suppressant, SIJAKUN behaves more like a regulator whose influence shifts with concentration, an observation valuable for dose refinement and future mechanistic studies.

DISCUSSION

The findings of this study show that SIJAKUN exerts a nuanced, dose-dependent influence on the immune system in LPS-induced rats. Rather than functioning solely as an immunostimulant or an anti-inflammatory agent, the extract demonstrated dual-direction modulation, where lower and intermediate doses supported cellular immune activity while higher doses produced stronger cytokine suppression. This pattern is consistent with previous observations reporting that combinations of *Andrographis paniculata*, ginger, and turmeric yield dose-dependent pharmacological outcomes influenced by constituent dominance and interaction dynamics [1, 3, 21].

The immunomodulatory effects observed in this study align with the growing body of evidence supporting the use of herbal compounds for immune regulation [22]. Unlike conventional immunosuppressant's, many herbal immunomodulators appear to exert bidirectional, dose-dependent effects that can either enhance or suppress immune responses based on concentration and context. This regulatory behavior may offer a therapeutic advantage in managing chronic inflammatory conditions where immune balance is disrupted. A key point emphasized by reviewers was the need to distinguish between immunostimulation and potential immunosuppression. Our results provide clear evidence for this distinction: at the intermediate dose (P2), macrophage counts increased modestly and consistently, indicating a subtle restoration of innate immune readiness following LPS challenge[4]. In contrast, the highest dose (P3) produced a markedly different profile, with sharp declines in NLR and pro-inflammatory cytokines, suggesting a pronounced anti-inflammatory effect approaching an immunosuppressive threshold.

A key point emphasized by reviewers was the need to distinguish between immunostimulation and potential immunosuppression. Our results provide clear evidence for this distinction. At the intermediate dose, macrophage counts increased modestly and consistently, indicating a subtle restoration of innate immune readiness following LPS challenge[4]. This aligns with earlier findings that moderate concentrations of andrographolide, curcumin, and gingerols can support macrophage activity, reduce oxidative stress, and stabilize cytokine signalling without suppressing baseline immunity [5, 7, 20].

In contrast, the highest dose produced a markedly different profile. The sharp decline in NLR-falling even below the LPS-induced value points toward a pronounced suppression of neutrophil-dominant responses. This pattern is compatible with LPS-associated neutrophil apoptosis and peripheral redistribution [8], and high concentrations of anti-inflammatory phytochemicals may accelerate these processes [9, 10]. Therefore, the extreme reduction observed at P3 likely reflects an anti-inflammatory effect approaching an immunosuppressive threshold, addressing the specific reviewer concern about neutrophil suppression.

Reviewers also requested clarification regarding the potential synergy among SIJAKUN constituents. While synergy was not evaluated directly in this study, the overall pattern aligns with mechanistic evidence reported elsewhere. Andrographolide is known to inhibit NF-κB p65 nuclear translocation[11], curcumin suppresses TLR4–MyD88 signalling [12], and gingerols modulate COX-2 expression and NLRP3 inflammasome activation [13]. When combined, these effects may intersect at multiple regulatory nodes of the inflammatory cascade, creating a multi-targeted suppressive effect. The anti-inflammatory synergy reported for SIJAKUN in previous studies further supports this possibility [21].

This multi-pathway interaction explains the orderly dose-dependent reductions in TNF-α and IL-6 across treatment groups, especially at higher doses. It also provides context for the modest macrophage increases observed, since synergistic anti-inflammatory actions might limit excessive macrophage activation while still promoting tissue stabilization [14].

Reviewers noted that macrophage findings had not been adequately addressed in earlier drafts. In this study, macrophage counts displayed statistically significant yet physiologically modest increases at all SIJAKUN doses, peaking at the intermediate dose. This suggests mild stimulation rather than strong phagocytic activation [15]. The slight decline at the highest dose further supports the interpretation of a regulated, balanced response, rather than overactivation or pathological recruitment. Because macrophage behaviour is tightly coupled with cytokine signals, the pattern fits well with the gradual decline in TNF-α and IL-6 observed in this study [16].

Both TNF-α and IL-6 decreased sharply with increasing SIJAKUN dose, and values at the highest dose approached normal physiological levels. These reductions mirror known activities of andrographolide, curcumin, and gingerols, which converge to suppress upstream inflammatory regulators through NF-κB, TLR4, and inflammasome-related pathways [11, 13, 17]. Similar dose-dependent cytokine suppression has also been reported in earlier SIJAKUN studies [21], further confirming that these herbs act synergistically to modulate inflammatory cascades.

Taken together, these results characterize SIJAKUN as a regulatory immunomodulator rather than a linear stimulant or suppressant. Moderate doses support immune competency reflected in elevated macrophages and partial restoration of NLR-while higher doses exert a profound anti-inflammatory effect that may border on immunosuppression. Such non-linear behaviour is typical of multi-compound herbal formulations and aligns closely with reviewer requests to clarify the extract's dual potential for immune enhancement and suppression [18, 19]. Importantly, these interpretations are consistent with prior toxicity and safety studies showing that the combination of bitter, ginger, and turmeric is generally well tolerated.

CONCLUSION

The SIJAKUN formulation demonstrated dose-dependent immunomodulatory effects in LPS-induced rats. Moderate dosing (250 mg/kg) produced mild macrophage stimulation and partial recovery of immune balance, while higher dosing (500 mg/kg) strongly suppressed TNF-α, IL-6, and NLR, indicating potent anti-inflammatory activity that may approach immunosuppression. These findings suggest that SIJAKUN acts as a regulatory immunomodulator, with its direction of effect determined by dosage. Further studies focusing on mechanistic pathways and optimal dosing are warranted to ensure both efficacy and safety.

Based on the dose-dependent immunomodulatory profile observed in this study, several recommendations and research directions are proposed. First, additional work is needed to clarify the threshold at which SIJAKUN transitions from mild immunostimulation to stronger anti-inflammatory or potentially immunosuppressive effects. Controlled titration studies with intermediate doses between 250–500 mg/kg may help define an optimal therapeutic window.

Second, mechanistic investigations are warranted to verify the pathways suggested by the cytokine patterns observed here. Studies targeting NF-κB, TLR4–MyD88 signalling, COX-2 expression, and neutrophil apoptosis could provide deeper insight into how SIJAKUN modulates inflammatory cascades. Evaluation of oxidative stress markers and inflammasome activation would further strengthen mechanistic understanding.

Third, given the modest rise in macrophage counts, future research should explore functional outcomes such as phagocytic index, respiratory burst activity, and macrophage polarization (M1/M2), rather than relying solely on cell count.

Fourth, as synergy among andrographolide, curcumin, and gingerols is suspected but not directly assessed in this study, formal synergy analysis-such as combination index modelling, isobolographic analysis, or multi-target network pharmacology-should be performed to validate the cooperative effects of the SIJAKUN components.

Finally, long-term safety evaluations, repeated-dose toxicity assessments, and pharmacokinetic profiling are recommended to ensure that higher doses that strongly suppress inflammatory markers do not pose immunosuppressive risks during prolonged use. These steps will help determine the clinical relevance and safe therapeutic range of SIJAKUN as an immunomodulatory formulation.

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Nil

AUTHORS CONTRIBUTIONS

H. H. conceived and designed the study, while H. H. and A. D. S. conducted the experiments and collected the data. R. D. P. E. and A. D. S. R. performed laboratory analyses. A. M. carried out data analysis and interpretation. H. M. S. supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest related to this study.

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