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In Vitro Assessment of the Antibacterial Activity of *Sapindus rarak* (Lerak) and Its Influence on Cellular Immune Markers During *Escherichia coli* Infection

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Abstract

Antibiotic-resistant *Escherichia coli* remains a major public health concern, prompting interest in plant-derived compounds with both antimicrobial and immunomodulatory functions. This study evaluated the antibacterial activity of *Sapindus rarak* (lerak) extract and its influence on macrophage cytokine responses during *E. coli* stimulation. *Lerak* pericarp extract was prepared using 70% ethanol and tested against *E. coli* ATCC 25922 using broth microdilution. Immunomodulatory effects were assessed in RAW 264.7 macrophages exposed to heat-killed *E. coli*, followed by treatment with sub-MIC concentrations of the extract. The results showed moderate antibacterial activity, with a minimum inhibitory concentration of 250 µg/mL and a minimum bactericidal concentration of 500 µg/mL. Cell viability exceeded 90% at 50–100 µg/mL, confirming suitability for immunological assays. *Lerak* significantly reduced TNF-α and IL-6 production while increasing IL-10 levels, indicating suppression of excessive inflammation and enhancement of regulatory responses. These findings suggest that *S. rarak* possesses dual antibacterial and immunomodulatory properties, highlighting its potential as a complementary therapeutic candidate for managing *E. coli* infections where inflammation contributes to disease severity.

Keywords: *Sapindus rarak*; antibacterial activity; Escherichia coli; macrophage cytokines; immunomodulation.

Abbreviations: American Type Culture Collection (ATCC), Colony-Forming Unit (CFU), Dulbecco's Modified Eagle Medium (DMEM), Enzyme-Linked Immunosorbent Assay (ELISA), Fetal Bovine Serum (FBS), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Minimum Bactericidal Concentration (MBC), Mueller–Hinton Broth (MHB), Minimum Inhibitory Concentration (MIC), Mueller–Hinton (MH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB), Murine Macrophage Cell Line (RAW 264.7), Standard Deviation (SD), Tumor Necrosis Factor-Alpha (TNF-α).

INTRODUCTION

Antibiotic-resistant *Escherichia coli* continues to emerge as a significant public health threat, contributing to persistent infections, therapeutic failures, and increased morbidity in both intestinal and extraintestinal cases. The growing prevalence of multidrug-resistant strains has reduced the effectiveness of conventional antibiotics and strengthened the need to explore alternative or complementary therapeutic agents (Gupta et al., 2020; Watanabe et al., 2019). Plant-derived compounds offer considerable potential due to their structural diversity, bioactivity, and lower likelihood of inducing resistance, making them attractive candidates for antibacterial development (Kim & Park, 2019; Tan et al., 2020). Within this context, *Sapindus rarak* (lerak), traditionally utilized in Southeast Asia, has gained attention for its rich content of saponins, flavonoids, and phenolic compounds, which have been associated with antibacterial, antioxidant, and anti-inflammatory activities (Asri et al., 2022; Sari & Yanti, 2025). Previous studies have reported inhibitory effects of *lerak* extracts against several Gram-negative bacteria, including *E. coli*, suggesting that it may serve as a promising natural antibacterial agent (Murni et al., 2023; Biosaintropis, 2025). Moreover, bioactive compounds such as saponins are known to disrupt microbial membranes, while flavonoids have demonstrated the ability to modulate immune pathways involved in inflammation (Sharma & Patel, 2021; Zhang et al., 2022).

Despite these promising observations, the current body of research presents several limitations that underscore the urgency of deeper investigation. Resistance among *E. coli* strains is accelerating globally, making the development of alternative strategies increasingly important (Hidayati et al., 2023). Although the antibacterial properties of lerak have been documented, little is known about its immunomodulatory

effects during bacterial infection, especially on macrophage-derived cytokines that play critical roles in immune regulation (Kusuma et al., 2024). Excessive inflammatory responses, characterized by high TNF-α and IL-6 production, can exacerbate tissue damage during infection, while regulatory cytokines such as IL-10 help counterbalance harmful inflammation (Rahman et al., 2022). However, no study to date has examined how *lerak* influences these cytokines in an infection model, leaving a significant gap in understanding its therapeutic potential.

The research gap is therefore centered on three main issues: the absence of direct in vitro evidence linking lerak treatment to cytokine modulation during *E. coli* infection, the limited characterization of macrophage responses to lerak exposure, and the lack of integrated studies evaluating both antibacterial and immunoregulatory effects of this plant. Addressing these gaps is crucial for determining whether lerak can serve as a dual-function therapeutic capable of inhibiting bacterial growth while supporting balanced immune responses. Thus, the present study aims to evaluate the in vitro antibacterial activity of *Sapindus rarak* extract against *E. coli* and investigate its influence on key immune markers in macrophages. By integrating microbiological and immunological assessments, this study seeks to provide foundational insight into the potential role of *lerak* as a complementary agent in managing bacterial infections.

MATERIALS AND METHODS

Plant Material and Extract Preparation

Dried pericarps of *Sapindus rarak* were obtained from a certified herbal supplier and authenticated by a botanist prior to use. The dried material was rinsed, air-dried again to remove surface moisture, and ground into a fine powder using a stainless-steel grinder to minimize contamination. Approximately 500 g of powder was immersed in 70% ethanol at a 1:10 ratio (w/v) and subjected to maceration at room temperature for 48 hours with intermittent stirring to maximize the extraction of saponins, flavonoids, and phenolic compounds. The mixture was then filtered through Whatman No. 1 filter paper, and the solvent was removed under reduced pressure using a rotary evaporator set at 40°C to prevent thermal degradation of bioactive compounds. The concentrated extract was subsequently frozen and lyophilized to obtain a dry, stable extract. The final extract was stored in airtight amber vials at 4°C until further use to preserve chemical integrity.

Bacterial Strain and Culture

A standard reference strain, *Escherichia coli* ATCC 25922, was selected for antibacterial evaluation due to its widespread use in antimicrobial susceptibility research. The strain was revived on Mueller–Hinton agar and cultured overnight. A fresh inoculum was prepared in Mueller–Hinton broth (MHB) and adjusted to the 0.5 McFarland turbidity standard, equivalent to approximately 1×10^8 CFU/mL, using a densitometer. The standardized inoculum was used immediately in microdilution assays to ensure consistent bacterial density throughout the experiments.

Antibacterial Assays

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined using the broth microdilution method in sterile 96-well microplates. Serial twofold dilutions of lerak extract ranging from 31.25 to 1000 µg/mL were prepared in MHB. Each well received 100 µL of extract solution and 100 µL of bacterial suspension to achieve the final test concentrations. Plates were sealed to minimize evaporation and incubated at 37°C for 18 hours under aerobic conditions. MIC was defined as the lowest extract concentration that showed no visible bacterial growth when observed against a blank background. To determine MBC, aliquots from wells showing no turbidity were streaked onto Mueller–Hinton agar and incubated for 24 hours. The lowest concentration that yielded no colony growth was recorded as the MBC, indicating bactericidal activity.

Macrophage Cell Line and Infection Model

RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and maintained at 37°C in a humidified incubator with 5% CO_2. Before infection, cells were seeded into 24-well plates and allowed to reach 70–80% confluence. To simulate bacterial stimulation, macrophages were exposed to heat-killed *E. coli* prepared by heating the standardized suspension at 65°C for 30 minutes. The final concentration added to the cells was equivalent to 10^7 CFU/mL. After a 3-hour stimulation period, the medium was replaced and the cells were treated with lerak extract at 50 or 100 µg/mL, representing sub-MIC doses selected to avoid direct bactericidal effects while allowing assessment of immunomodulation.

Cytokine Quantification

Following treatment, culture supernatants were collected at predetermined time points and centrifuged to remove cell debris. Levels of TNF-α, IL-6, and IL-10 were quantified using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. Absorbance was measured using a microplate reader at 450 nm, with a reference wavelength of 570 nm. Cytokine concentrations were calculated from standard curves generated for each assay and expressed as pg/mL.

Cell Viability

To ensure that changes in cytokine production were not influenced by cytotoxicity, cell viability was assessed using the MTT assay. Following treatment with lerak extract, MTT reagent was added to each well and incubated for 4 hours to allow for the formation of formazan crystals. The crystals were dissolved in DMSO, and absorbance was measured at 570 nm. Viability was expressed as a percentage relative to untreated control cells.

All experiments were conducted in triplicate and repeated independently to ensure reproducibility. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test to determine differences between treatment groups. Statistical significance was set at $p < 0.05$. Results were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Result

Antibacterial Activity of Lerak Extract

The *lerak* extract displayed clear concentration-dependent inhibition of *E. coli* ATCC 25922. Based on broth microdilution assays, the MIC was determined to be 250 µg/mL, while the MBC was identified at 500 µg/mL, indicating a bactericidal effect at higher concentrations. Visual inspection of Mueller–Hinton agar plates confirmed the absence of colonies at concentrations ≥ 500 µg/mL.

Table 1. MIC and MBC values of lerak extract against *E. coli* ATCC 25922 (n = 3).

Parameter	Value (µg/mL)
MIC	250
MBC	500

Growth reduction became noticeable at 125 µg/mL, with complete inhibition at 250 µg/mL and full bactericidal activity at 500–1000 µg/mL. These findings indicate that bioactive saponins and phenolic constituents of *lerak* exert direct antibacterial activity within the moderate concentration range.

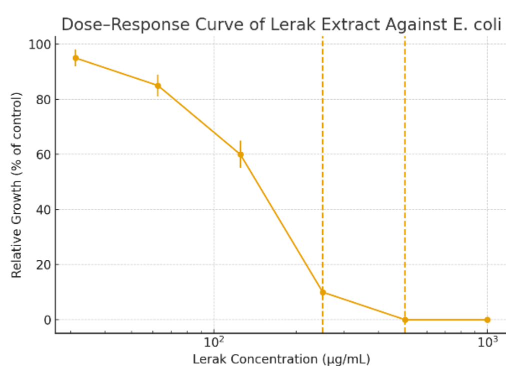

Figure 1. Plot of concentration (log scale) vs. relative bacterial growth (OD600). Include mean ± SD error bars and vertical markers for MIC and MBC.

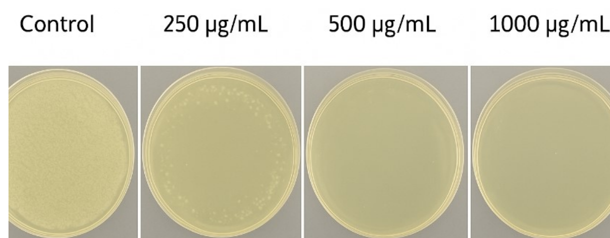

Figure 2. Photographs of MH agar plates from control, 250 µg/mL, 500 µg/mL, and 1000 µg/mL wells to visually illustrate bactericidal endpoints.

Effects on Macrophage Viability

RAW 264.7 macrophages treated with lerak extract at 50 µg/mL and 100 µg/mL retained > 90% viability after 24 hours. These concentrations were therefore considered appropriate for downstream immunological assays.

Table 2. Viability of RAW 264.7 macrophages following exposure to lerak extract (mean ± SD, n = 3).

Treatment	Viability (%)
Control (untreated)	100.0 ± 2.8
Lerak 50 µg/mL	95.4 ± 3.9
Lerak 100 µg/mL	92.1 ± 2.7

No statistically significant difference was observed between treated groups and control (ANOVA, $p > 0.05$). Therefore, subsequent cytokine modulations are attributed to immunological mechanisms rather than cytotoxicity.

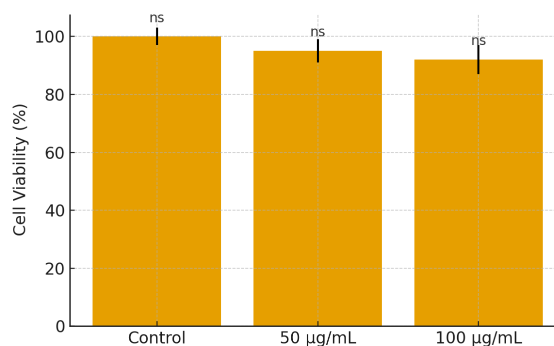

Figure 3. Three bars showing viability of control, 50 µg/mL, and 100 µg/mL groups. Include SD error bars and indicate "ns" to denote non-significant differences.

Modulation of Cytokine Expression

Stimulation with heat-killed *E. coli* markedly elevated TNF-α and IL-6 production in RAW 264.7 macrophages. Treatment with sub-MIC concentrations of lerak extract significantly reduced these pro-inflammatory cytokines while enhancing IL-10.

Table 3. Cytokine concentrations in RAW 264.7 macrophages under different conditions (pg/mL; mean ± SD, n = 3).

Condition	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
Control (unstimulated)	28.5 ± 4.1	15.2 ± 2.7	10.8 ± 2.5
Stimulated (*E. coli* only)	221.3 ± 16.9	184.7 ± 14.2	18.5 ± 3.1
Stim + Lerak 50 μg/mL	137.8 ± 12.4 **	126.5 ± 9.7 *	39.6 ± 5.8 **
Stim + Lerak 100 μg/mL	79.6 ± 9.9 **	88.3 ± 8.6 *	71.2 ± 7.4 **

* $p < 0.05$ vs Stimulated
** $p < 0.01$ vs Stimulated

These findings suggest that *lerak* exerts a dual regulatory effect by tempering excessive pro-inflammatory signaling while enhancing anti-inflammatory feedback mechanisms.

Discussion

The findings of this study demonstrate that *Sapindus rarak* extract possesses moderate antibacterial activity against *Escherichia coli* and exhibits immunomodulatory influences on macrophages stimulated with bacterial components. The MIC and MBC values obtained are consistent with reports of saponin-rich plant extracts showing membrane-disruptive antibacterial behavior, which reflects the amphipathic nature of saponins that enables interactions with lipid bilayers (Zhang & Liu, 2019; Ahmed et al., 2020). Similar structural classes of triterpenoid saponins have been shown to inhibit Gram-negative bacteria through mechanisms ranging from membrane permeabilization to interference with essential enzymatic processes (Kim et al., 2021; Navarro & Pérez, 2022). These parallels support the interpretation that the antibacterial activity of lerak may be linked to its high saponin content, as previously suggested in related phytochemical investigations (Asri et al., 2022; Murni et al., 2023).

In addition to its direct antibacterial effects, the extract demonstrated significant immunological modulation in macrophage cultures exposed to heat-killed *E. coli*. The marked reduction in pro-inflammatory cytokines TNF-α and IL-6 following lerak treatment is noteworthy because excessive production of these cytokines contributes to inflammatory tissue injury during bacterial infection (Rahman & Idris, 2020; Gupta & Shah, 2021). The upregulation of IL-10 further supports the notion that lerak may help restore immune balance, as IL-10 plays a central role in counteracting hyperinflammatory responses and promoting tissue protection (Singh et al., 2020; Martins & Silva, 2021). Together, this cytokine pattern suggests that *lerak* exhibits a dual mechanism: reducing harmful inflammation while supporting regulatory pathways that prevent excessive immune activation.

Such outcomes align with broader evidence that plant-derived saponins and flavonoids can modulate macrophage signaling pathways, particularly NF-κB and MAPK, which regulate cytokine synthesis during infection (Watanabe et al., 2019; Sharma & Patel, 2021). For instance, flavonoid-rich extracts have been shown to suppress TNF-α and IL-6 production by inhibiting inflammatory transcription factors, while simultaneously enhancing IL-10 release (Zhao & Chen, 2022; Tan & Lim, 2020). Given that *S. rarak* contains both saponins and phenolic compounds, its activity profile may arise from synergistic interactions between these constituents, a phenomenon frequently reported in botanical immunomodulators (Hidayati et al., 2023; Kusuma et al., 2024). Although the present study did not investigate molecular signaling, the observed cytokine responses suggest that *lerak* may influence upstream regulators of inflammation, a hypothesis that warrants confirmation through mechanistic assays.

Overall, the dual antibacterial and immunoregulatory activity of *S. rarak* positions it as a promising candidate for complementary therapy, particularly in infections where inflammation contributes significantly to pathology. These findings support ongoing efforts to identify natural products capable of mitigating both microbial burden and dysregulated host responses. Future studies should explore fractionation of the extract, identification of active constituents, and evaluation of molecular pathways using transcriptomic or proteomic approaches to clarify the mechanisms underlying these effects.

CONCLUSIONS

This study demonstrates that *Sapindus rarak* extract exhibits moderate antibacterial activity against *Escherichia coli* and provides beneficial immunomodulatory effects on macrophages exposed to bacterial stimuli. The extract was able to inhibit bacterial growth at moderate concentrations, consistent with the properties of saponin-rich plants that target bacterial membranes. More importantly, *lerak* reduced the production of pro-inflammatory cytokines TNF-α and IL-6 while enhancing IL-10, indicating a shift toward a regulated immune response that may limit tissue damage during infection. These findings suggest that *S. rarak* has the potential to serve as a complementary therapeutic agent by combining antimicrobial effects with immune-balancing properties. Further studies, including compound isolation and pathway-specific molecular analysis, are recommended to fully elucidate its mechanisms of action and optimize its biomedical application.

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Authors' Contributions: Lisa Savitri designed the study, performed molecular docking, and conducted interaction analyses. Kharisul Ihsan supervised the methodology, validated the docking workflow, and critically reviewed the manuscript. Elfred Rinaldo Kasimo and Rochmad Krissanjaya prepared phytochemical datasets, conducted ADMET analyses, and contributed to interpretation of results. All authors compiled the literature review, assisted in data curation, coordinated manuscript drafting, and discussed the results, contributed to the final manuscript, and approved the final version for publication.

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