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EFFECT OF *MURRAYA PANICULATA* LEAF EXTRACT ON HEPATIC INTERLEUKIN-6 EXPRESSION IN *PLASMODIUM BERGHEI*-INFECTED BALB/c MICE

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ABSTRAK

Malaria menyebabkan peradangan sistemik yang dapat melibatkan jaringan hati, dengan interleukin-6 (IL-6) sebagai salah satu penanda inflamasi penting. Murraya paniculata mengandung senyawa bioaktif yang berpotensi memiliki aktivitas antioksidan dan antiinflamasi. Penelitian ini bertujuan untuk mengevaluasi pengaruh ekstrak daun M. paniculata terhadap ekspresi IL-6 pada jaringan hati mencit BALB/c yang terinfeksi Plasmodium berghei. Penelitian eksperimental dengan desain post-test-only dilakukan menggunakan mencit BALB/c jantan yang dibagi menjadi enam kelompok (n = 5 per kelompok), yaitu kontrol sehat, kontrol infeksi, kontrol hidroksiklorokuin, serta kelompok ekstrak *M. paniculata* dengan dosis 50, 100, dan 200 mg/kg berat badan. Malaria diinduksi menggunakan *P. berghei* galur ANKA. Satu mencit pada setiap kelompok mati selama penelitian sehingga 24 mencit (n = 4 per kelompok) dianalisis. Ekspresi IL-6 pada jaringan hati dievaluasi menggunakan metode imunohistokimia dan dikuantifikasi menggunakan ImageJ dengan plugin IHC Profiler. Analisis pada tingkat hewan tidak menunjukkan perbedaan yang signifikan antarkelompok (ANOVA satu arah, F(5,18) = 1,974; p = 0,132), dan perbandingan berpasangan tidak menunjukkan perbedaan yang signifikan antara kelompok ekstrak dan kontrol infeksi. Dengan demikian, ekstrak daun *Murraya paniculata* belum menunjukkan penurunan ekspresi IL-6 pada jaringan hati yang signifikan secara statistik dibandingkan dengan kelompok kontrol infeksi. Penelitian lebih lanjut dengan jumlah sampel yang lebih besar serta penambahan penanda inflamasi dan cedera hati diperlukan.

Kata Kunci: *Imunohistokimia, Interleukin-6, Malaria, Murraya paniculata, Plasmodium berghei*

ABSTRACT

Malaria induces systemic inflammation that may involve hepatic tissue, with interleukin-6 (IL-6) serving as an important inflammatory marker. *Murraya paniculata* contains bioactive compounds with potential antioxidant and anti-inflammatory activities. This study aimed to evaluate the effect of *M. paniculata* leaf extract on hepatic IL-6 expression in *Plasmodium berghei*-infected BALB/c mice. A post-test-only experimental study was conducted using male BALB/c mice (n = 5 per group) divided into six groups: healthy control, infection control, hydroxychloroquine control, and *M. paniculata* extract groups receiving 50, 100, and 200 mg/kg body weight. Malaria was induced using *P. berghei* ANKA. One mouse in each group died during the experiment, so 24 mice (n = 4 per group) were analyzed. Hepatic IL-6 expression was evaluated by immunohistochemistry and quantified using ImageJ with the IHC Profiler plugin. Animal-level analysis showed no significant difference among groups (one-way ANOVA, F(5,18) = 1.974, p = 0.132), and none of the extract groups differed significantly from the infection control group in pairwise analysis. Therefore, *Murraya paniculata* leaf

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extract did not demonstrate a statistically significant reduction in hepatic IL-6 expression compared with the infection control group. Further studies using larger sample sizes and additional hepatic inflammatory and injury markers are warranted.
Keywords: *Immunohistochemistry, Interleukin-6, Malaria, Murraya paniculata, Plasmodium berghei*

INTRODUCTION

Malaria remains a major global health challenge despite substantial progress in prevention, diagnosis, treatment, and elimination. The World Health Organization (WHO) estimated that malaria caused approximately 282 million cases and 610,000 deaths worldwide in 2024, representing an increase of about 9 million cases compared with the previous year (World Health Organization [WHO], 2025). Although malaria control interventions have prevented an estimated 2.3 billion cases and 14 million deaths since 2000, progress toward reducing malaria mortality remains insufficient to achieve global targets. The continuing emergence and geographical expansion of antimalarial drug resistance further complicate malaria control and highlight the need for complementary therapeutic strategies beyond parasite clearance alone (WHO, 2025; Zhou et al., 2026). In Indonesia, malaria remains highly heterogeneous geographically. The national elimination roadmap targets a malaria-free Indonesia by 2030, and by 2026, 412 of 514 districts/cities and seven provinces had achieved malaria-free status. Nevertheless, the national burden remains concentrated in eastern Indonesia. According to the Indonesian Ministry of Health, 706,297 malaria cases were reported in 2025, approximately 30% higher than the 543,965 cases reported in 2024, with approximately 95% of cases, or 674,046 cases, concentrated in Tanah Papua (Herdiana et al., 2025). The increase has partly been attributed to intensified active case detection and improvements in digital malaria surveillance rather than a simple deterioration in malaria control (Fitri et al., 2023; Herdiana et al., 2025).

This inflammatory response is essential for controlling parasite replication, but excessive or dysregulated inflammation can contribute to tissue injury and severe disease. IL-6 is of particular interest because it functions as a pleiotropic cytokine linking innate immune activation with systemic inflammatory and acute-phase responses. However, the biological role of IL-6 in malaria is complex and context dependent, IL-6 can participate in host defense and parasite control while excessive inflammatory signaling may contribute to pathological tissue responses (Gowda & Wu, 2018; Popa & Popa, 2021). Thus, assessment of IL-6 provides an informative approach for investigating the inflammatory component of malaria pathogenesis, particularly when evaluated at the tissue level rather than solely through systemic circulating concentrations.

Among the organs affected during malaria infection, the liver represents an important immunological and pathological site. The liver is involved in the Plasmodium life cycle and contains specialized immune cells, including Kupffer cells, that continuously interact with circulating microbial and parasitic products (Glennon et al., 2022; Hirako et al., 2022). Experimental malaria studies have demonstrated that *Plasmodium berghei* infection can induce hepatic inflammation and tissue injury, characterized by inflammatory-cell recruitment, hepatocellular degeneration, and accumulation of malarial pigment within the liver (Albohiri & Alzanbagi, 2021). In particular, *P. berghei* ANKA infection has been associated with hepatic inflammation, leukocyte infiltration, hepatocellular injury, and increased inflammatory signaling in murine models (Albohiri & Alzanbagi, 2021; Hildebrandt et al., 2024). Hemozoin

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accumulation and parasite-associated inflammatory responses can alter the hepatic microenvironment and contribute to oxidative and inflammatory stress. Importantly, experimental studies have also demonstrated a close relationship between *P. berghei* infection and cytokine-mediated hepatic responses, while IL-6 has been shown to participate in host responses to Plasmodium during hepatic infection (Hildebrandt et al., 2024; Scaccabarozzi et al., 2018).

The identification of interventions capable of modulating malaria-associated inflammation has consequently increased interest in medicinal plants and their bioactive constituents. *Murraya paniculata* (L.) Jack, commonly known as orange jasmine, is a medicinal plant belonging to the Rutaceae family and contains a diverse array of secondary metabolites, including phenolic compounds, flavonoids, coumarins, carbazole alkaloids, polymethoxyflavonoids, and terpenoid constituents (Fatima et al., 2024; Yohanes et al., 2023). Phytochemical investigations of its leaves have demonstrated substantial variation in metabolite composition depending on extraction solvent and geographical origin, supporting the importance of extract-specific characterization (Fatima et al., 2024). Experimental studies have reported antioxidant and membrane-stabilizing activities of *M. paniculata* leaf extracts, while isolated flavonoids from the plant have demonstrated anti-inflammatory effects in experimental cellular models (Sonter et al., 2021). Notably, several flavonoids isolated from *M. paniculata* inhibited inflammatory mediator production, including nitric oxide and IL-6, in activated murine macrophages, providing biological evidence that constituents of the plant can influence inflammatory signaling. Other studies have identified a broad phytochemical profile containing alkaloids, flavonoids, phenolics, terpenes, and related metabolites, with antioxidant activity varying according to the extraction solvent (Kumar et al., 2023). Together, these findings provide a biological rationale for investigating *M. paniculata* as a potential modulator of inflammation. However, evidence from general anti-inflammatory or antioxidant models cannot by itself establish an effect in malaria or in malaria-associated hepatic inflammation.

Although *M. paniculata* has demonstrated diverse pharmacological activities and some of its constituents have shown antiplasmodial or anti-inflammatory properties, an important knowledge gap remains regarding its effects on malaria-associated hepatic inflammation. Previous evidence related to *M. paniculata* and malaria has largely focused on phytochemical activity, isolated compounds, or parasite-related outcomes. For example, flavonoids derived from *M. paniculata* have demonstrated in vitro activity against *Plasmodium falciparum*, while experimental work involving *M. paniculata* in murine malaria has investigated parasite growth and hemozoin deposition rather than tissue-specific inflammatory bioms (Shanta et al., 2025; Wiratama et al., 2026). These observations indicate that the antimalarial potential of the plant cannot be assumed to reflect its effects on host inflammatory responses. To our knowledge, evidence remains limited regarding the effect of *M. paniculata* leaf extract on hepatic IL-6 expression in *P. berghei*-infected BALB/c mice, particularly using immunohistochemical assessment of IL-6 within liver tissue. This distinction is important because modulation of hepatic inflammation represents a different biological endpoint from direct parasite suppression. Consequently, investigating tissue-specific IL-6 expression may provide new insight into whether the biological activity of *M. paniculata* extends beyond potential antiparasitic effects toward modulation of the host inflammatory response during malaria.

Therefore, this study aimed to evaluate the effect of *Murraya paniculata* leaf extract on hepatic IL-6 expression in BALB/c mice experimentally infected with *Plasmodium berghei*. Hepatic IL-6 expression was assessed using immunohistochemical staining followed by digital

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quantitative analysis to characterize differences in tissue-level inflammatory responses among experimental groups. By integrating an established experimental malaria model with tissue-specific immunohistochemical assessment, this study sought to provide experimental evidence regarding the potential role of *M. paniculata* leaf extract in modulating hepatic inflammation associated with malaria.

RESEARCH METHODS

Study Design, Location, Duration and Statistical Analysis

This study employed a true experimental *in vivo* design using a post-test-only control group approach. The experimental procedures were conducted over a period of ten months. Preparation of the *Murraya paniculata* leaf extract and all animal experiments were conducted at the Animal Laboratory, Faculty of Medicine, Universitas Negeri Malang, Indonesia. Histological tissue processing and immunohistochemical analyses were subsequently performed at the Pathology and Biomedical Laboratory, Faculty of Medicine, Universitas Negeri Malang, Indonesia. The study involved six experimental groups to evaluate hepatic interleukin-6 (IL-6) expression under different experimental conditions. The collected liver tissue samples were examined using immunohistochemical staining, and IL-6 expression was quantified using the H-score method. All statistical analyses were performed using IBM SPSS Statistics version 25**.**

Ethical Approval

All experimental procedures involving laboratory animals were reviewed and approved by the Health Research Ethics Committee (KEPK), Faculty of Medicine, Universitas Negeri Malang (Ethical Clearance No. 13.05.06/UN32.14.2.8/LT/2026). All procedures were conducted in accordance with internationally accepted principles for the care and use of laboratory animals. Animal welfare was maintained throughout the experiment by providing standardized housing conditions, including a controlled ambient temperature of 22 ± 2°C, a 12-h light/12-h dark cycle, and unrestricted access to commercial pellet feed and sterile drinking water (Çelik et al., 2025). Procedures were performed with efforts to minimize pain, distress, and unnecessary handling of the animals.

Experimental Animals and Group Allocation

The experimental animals were healthy male BALB/c mice (*Mus musculus*) aged 8–12 weeks and weighing 25–30 g. Animals exhibiting abnormal physical characteristics, signs of illness, or reduced baseline activity were excluded before the initiation of the experiment. Following acclimatization, 30 mice were randomly allocated into six experimental groups, with five mice initially assigned to each group (n = 5/group), using a computerized randomization procedure. All animals were maintained under standardized laboratory conditions throughout the experimental period to minimize environmental variation, and the mice were monitored regularly to assess their general condition. During the experimental period, one mouse in each group died before the study endpoint and was excluded from the analysis, so the sample size was reduced from 30 mice (n = 5/group) to 24 mice (n = 4/group), as reported in the Results.

Preparation of *Murraya paniculata* Leaf Extract

Fresh leaves of *Murraya paniculata* were collected and taxonomically authenticated before extraction. The leaves were subsequently dried and processed into dried plant material.

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A total of 500 g of dried *M. paniculata* leaf material was extracted by maceration using 5 L of 96% ethanol (Akomeng & Adusei, 2021; Nurhaslina et al., 2022). The maceration process was performed for 72 h, corresponding to three consecutive 24-h extraction periods. During the extraction process, the plant material was maintained in contact with the ethanol solvent with periodic stirring to facilitate extraction of the soluble phytochemical constituents. Following maceration, the extract was separated from the plant residue by filtration (Akomeng & Adusei, 2021). The collected ethanolic filtrate was subsequently concentrated to obtain the crude *M. paniculata* leaf extract. The resulting crude extract was stored under appropriate conditions until administration to the experimental animals (Zubaydah et al., 2023).

Malaria Induction and Treatment Protocol

Experimental malaria was induced by intraperitoneal injection of 1×10^7 *Plasmodium berghei* ANKA-infected erythrocytes suspended in 0.2 mL phosphate-buffered saline (PBS) (Basir et al., 2012; Sinha et al., 2022). Following inoculation, the progression of parasitemia was monitored through microscopic examination of Giemsa-stained thin blood smears obtained from the tail vein (Wirawan & Haryatmi, 2025). Treatment administration was initiated on Day 4 after *P. berghei* infection following confirmation of established parasitemia and was continued once daily until Day 7. The assigned *M. paniculata* leaf extract doses and hydroxychloroquine were administered orally once daily using an intragastric gavage, and all doses were adjusted to the individual body weight of each animal. Mice in the infection-control group received distilled water by the same route and schedule, whereas the negative-control group consisted of non-infected mice receiving distilled water.

Digital Quantification of Hepatic IL-6 Expression

Hepatic IL-6 expression was quantitatively assessed using a semi-automated digital image analysis procedure to minimize observer-dependent variability. Following immunohistochemical staining, microscopic images were captured using a trinocular light microscope at 400× magnification (Ariesaka et al., 2024). Five randomly selected, non-overlapping microscopic fields were captured from each animal, ensuring that the analyzed fields were representative of the liver tissue and did not overlap. The acquired images were saved in TIFF format and analyzed using the IHC Profiler plugin integrated into ImageJ software (Varghese et al., 2014; Wen et al., 2024). The IHC Profiler was used to analyze the intensity and distribution of cytoplasmic DAB staining. The staining was classified into four intensity categories: high positive, positive, low positive, and negative. The proportions of cells within the respective staining categories were subsequently used to calculate the H-score. The H-score was calculated using the following equation:

$$\textbf{H-score} = (0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)$$

RESULT AND DISCUSSIONS

Result

Experimental Animals and Study Completion

Thirty male BALB/c mice were initially allocated to six experimental groups, with five mice assigned to each group. During the experimental period, one mouse from each experimental group died, resulting in four surviving animals in each group. Thus, a total of 24 mice completed the experimental protocol and were included in the final analysis, consisting of four mice in the negative-control group, four in the infection-control group, four in the drug-

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control group, and four in each of the three *Murraya paniculata* leaf extract treatment groups (50, 100, and 200 mg/kg body weight). All immunohistochemical and quantitative analyses were performed using these 24 animals, and for inferential statistics the five field-level values of each mouse were averaged so that the unit of analysis was the animal (n = 4 per group).

Immunohistochemical Assessment of Hepatic IL-6 Expression

Immunohistochemical staining demonstrated distinct patterns of IL-6 immunoreactivity in the hepatic tissue across the experimental groups. IL-6 immunoreactivity was identified by the presence of brown 3,3′-diaminobenzidine (DAB) staining predominantly within the cytoplasm of hepatic cells. Based on staining intensity and distribution, the immunoreactivity was classified into three categories: negative, low positive, and positive. Representative immunohistochemical staining patterns for each category are presented in Figure 1.

The negative category was characterized by the absence or minimal presence of brown DAB staining, with predominantly hematoxylin-counterstained tissue. The low-positive category showed detectable brown DAB staining with relatively low intensity and limited distribution. In contrast, the positive category demonstrated more pronounced brown DAB staining with greater intensity and distribution within the hepatic tissue. These differences in staining patterns provided a qualitative basis for assessing variations in hepatic IL-6 immunoreactivity among the experimental groups. Digital image analysis using ImageJ with the IHC Profiler plugin was subsequently used to characterize the intensity distribution of DAB staining. The analysis generated pixel-based classification of the stained tissue according to the corresponding immunoreactivity categories. The resulting pixel distribution was used to determine the proportion of tissue pixels assigned to each staining-intensity category and to support the subsequent quantitative assessment of hepatic IL-6 expression.

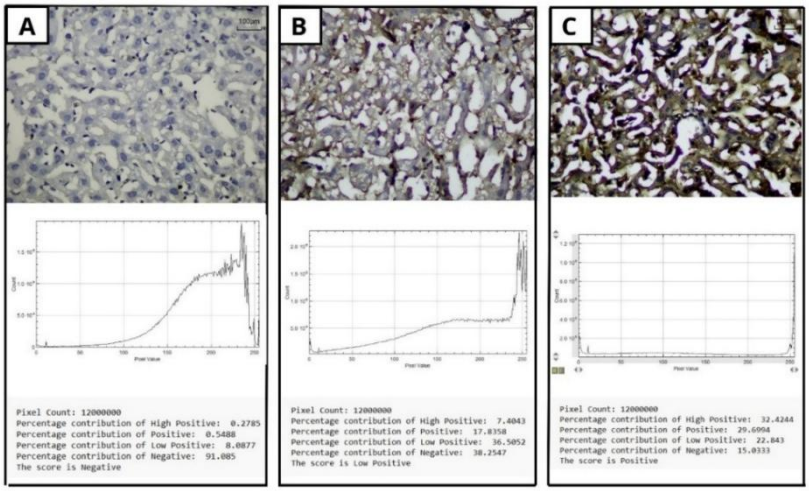


Figure 1. illustrates representative examples of the digital interpretation of hepatic IL-6 immunohistochemical staining, consisting of (A) negative, (B) low positive, and (C) positive immunoreactivity. For each category, the figure presents the original tissue image together with the corresponding intensity histogram, pixel count, and percentage contribution generated by the IHC Profiler analysis.

Distribution of Immunohistochemical Staining Categories

The distribution of hepatic IL-6 immunohistochemical staining categories varied across the experimental groups (Table 1). A total of 120 microscopic fields were analyzed, consisting

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of five fields from each of the 24 mice (20 fields from each experimental group). The analyzed fields were classified into three staining categories: negative, low positive, and positive. These field-level counts are presented descriptively and were not used for statistical inference, because fields from the same mouse are not independent observations.

The negative-control group showed predominantly negative staining, with 12 of 20 analyzed fields (60.0%) classified as negative and the remaining 8 fields (40.0%) classified as low positive. No fields in this group were classified as positive. In contrast, the infection-control group demonstrated a shift toward greater IL-6 immunoreactivity, with only 1 field (5.0%) classified as negative, 12 fields (60.0%) as low positive, and 7 fields (35.0%) as positive.

Table 1. Distribution of Immunohistochemical Staining Categories Across Study Groups

Group	(N), n (%)	(LP), n (%)	(P), n (%)	Total, n (%)	Mean ± SD H-Score (per mouse, n = 4)
Group 1	12 (60.0)	8 (40.0)	0 (0.0)	20 (100.0)	41.79 ± 19.40
Group 2	1 (5.0)	12 (60.0)	7 (35.0)	20 (100.0)	71.77 ± 27.83
Group 3	8 (40.0)	12 (60.0)	0 (0.0)	20 (100.0)	47.21 ± 18.72
Group 4	0 (0.0)	15 (75.0)	5 (25.0)	20 (100.0)	76.23 ± 14.69
Group 5	2 (10.0)	14 (70.0)	4 (20.0)	20 (100.0)	56.79 ± 29.51
Group 6	0 (0.0)	12 (60.0)	8 (40.0)	20 (100.0)	74.28 ± 9.37
Overall	23 (19.2)	73 (60.8)	24 (20.0)	120 (100.0)	

Abbreviations: N, negative; LP, low positive; P, positive. Staining-category counts are based on microscopic fields (20 per group); H-scores are presented as the mean ± SD of the per-mouse mean H-score (n = 4 mice per group).

In the drug-control group, 8 of 20 fields (40.0%) were classified as negative and 12 fields (60.0%) as low positive, with no fields classified as positive. Among the *Murraya paniculata* leaf extract-treated groups, the 50 mg/kg body weight group showed 15 fields (75.0%) classified as low positive and 5 fields (25.0%) as positive, with no negative fields. The 100 mg/kg body weight group showed 2 fields (10.0%) classified as negative, 14 fields (70.0%) as low positive, and 4 fields (20.0%) as positive. Meanwhile, the 200 mg/kg body weight group showed 12 fields (60.0%) classified as low positive and 8 fields (40.0%) as positive, with no fields classified as negative.

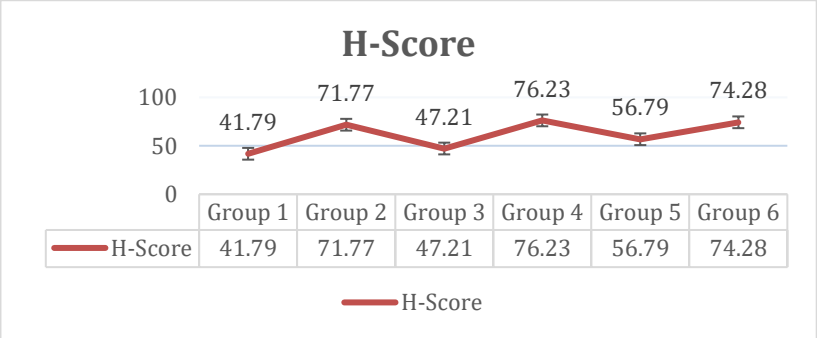


Figure 2. Average H-score Graph

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Overall, low-positive staining was the most frequently observed category, accounting for 73 of 120 analyzed fields (60.8%), followed by positive staining in 24 fields (20.0%) and negative staining in 23 fields (19.2%). The distribution of staining categories demonstrated descriptive differences in hepatic IL-6 immunoreactivity among the experimental groups. Quantitative differences in IL-6 immunoreactivity based on the mean H-score are presented in Figure 2.

Quantitative H-score Analysis

Quantitative assessment of hepatic IL-6 immunoreactivity demonstrated variation in mean H-scores across the experimental groups when the per-mouse mean H-score was used as the unit of analysis (Table 1; Figure 2). The mean H-score was 41.79 ± 19.40 in the negative-control group, 71.77 ± 27.83 in the infection-control group, and 47.21 ± 18.72 in the drug-control group. Among the *Murraya paniculata* leaf extract-treated groups, the mean H-scores were 76.23 ± 14.69, 56.79 ± 29.51, and 74.28 ± 9.37 for the 50, 100, and 200 mg/kg body weight groups, respectively. Individual mouse values were widely dispersed within several groups, for example from 34.73 to 94.89 in the infection-control group and from 33.50 to 99.39 in the 100 mg/kg group, indicating considerable inter-animal variability.

The infection-control group exhibited a higher mean H-score than the negative-control group (71.77 vs. 41.79), suggesting a numerically greater hepatic IL-6 immunoreactivity following *Plasmodium berghei* infection. The drug-control group showed a mean H-score of 47.21, which was close to that observed in the negative-control group. Among the extract-treated groups, the 100 mg/kg body weight group had the lowest mean H-score (56.79), whereas the 50 and 200 mg/kg body weight groups showed higher mean H-scores of 76.23 and 74.28, respectively, which were similar to the infection-control mean. Whether these differences were statistically distinguishable at the animal level is addressed in the following section.

Thus, based on the descriptive H-score values, the 100 mg/kg body weight dose showed the lowest hepatic IL-6 immunoreactivity among the three *Murraya paniculata* leaf extract doses evaluated. However, the standard deviation in this group was the largest among all groups, so the lower mean should be interpreted cautiously. Statistical comparisons were therefore required to determine whether the observed differences between groups were statistically significant. These analyses were performed using the mean H-score of each mouse as the experimental unit.

Statistical Comparison of Hepatic IL-6 Expression

The distribution of the animal-level hepatic IL-6 H-score data was assessed using the Shapiro–Wilk test. No statistically significant deviation from normality was observed in any experimental group, with *p*-values ranging from 0.073 to 0.695 (all $p > 0.05$). The *p*-values for Groups 1–6 were 0.073, 0.398, 0.199, 0.695, 0.240, 0.377, respectively (Table 2). Because the Shapiro–Wilk test has limited power with four observations per group, normality was additionally checked on the ANOVA residuals, which also showed no deviation from normality ($W = 0.949$, $p = 0.264$). These findings indicated that the H-score data were acceptable for parametric analysis

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Table 2. Assessment of Normality of Hepatic IL-6 H-Score Data

Group Treatment	Shapiro-Wilk			*p*
	Statistic	df	Sig.	
Group 1	0.782	4	0.073	$p > 0.05$
Group 2	0.893	4	0.398	$p > 0.05$
Group 3	0.841	4	0.199	$p > 0.05$
Group 4	0.947	4	0.695	$p > 0.05$
Group 5	0.854	4	0.240	$p > 0.05$
Group 6	0.889	4	0.377	$p > 0.05$

Homogeneity of variance was subsequently evaluated using Levene's test. The test based on the mean yielded a Levene statistic of 1.106 ($df1 = 5$, $df2 = 18$; $p = 0.392$), and the median-based test similarly was not significant (statistic = 0.612; $p = 0.692$). These findings indicated that the assumption of equal variances was satisfied (Table 3). Therefore, Tukey's HSD test, which is appropriate for equal variances and equal group sizes, was used for pairwise comparisons, and the Games–Howell procedure was not required. Nevertheless, the group standard deviations ranged from 9.37 to 29.51, and the small group size limits the power of Levene's test to detect unequal variances.

Table 3. Assessment of Homogeneity of Variance of Hepatic IL-6 H-Score Data

Method	Levene Statistic	df1	df2	Sig. (p)	Interpretation
Based on Mean	1.106	5	18	0.392	Not significant
Based on Median	0.612	5	18	0.692	Not significant

One-way ANOVA of the animal-level data showed no statistically significant overall difference in hepatic IL-6 H-score among the six experimental groups ($F(5, 18) = 1.974$, $p = 0.132$; $\eta^2 = 0.354$; Table 4). This finding indicates that hepatic IL-6 immunoreactivity did not differ significantly among the experimental groups when the mouse was used as the experimental unit. The moderate-to-large effect size alongside a non-significant *p*-value most likely reflects the small number of animals and the high inter-animal variability rather than the absence of any difference. An analysis that treated the 120 microscopic fields as independent observations would have produced a spuriously significant result ($p = 0.002$), illustrating how pseudoreplication inflates the effective sample size and the risk of false-positive findings (Lazic et al., 2018). Pairwise comparisons are nevertheless reported below for completeness.

Table 4. One-Way Analysis of Variance of Hepatic IL-6 H-Score Across Experimental Groups

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4402.10	5	880.42	1.974	0.132
Within Groups	8026.16	18	445.90		
Total	**12428.26**	**23**			

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Tukey's HSD analysis showed that none of the pairwise comparisons reached statistical significance (all $p \geq 0.242$). The difference between the negative-control group (Group 1) and the infection-control group (Group 2) was 29.98 H-score units ($p = 0.376$), and that between the infection-control group and the drug-control group (Group 3) was 24.56 units ($p = 0.582$). Similarly, comparisons between the infection-control group and the *Murraya paniculata* leaf extract-treated groups were not statistically significant, including Group 4 (50 mg/kg body weight; $p = 1.000$), Group 5 (100 mg/kg body weight; $p = 0.911$), and Group 6 (200 mg/kg body weight; $p = 1.000$). The smallest *p*-value was observed for the comparison between Group 1 and Group 4 ($p = 0.242$). The complete pairwise comparisons are presented in Table 5.

Table 5. Tukey HSD Post-Hoc Comparisons of Hepatic IL-6 H-Score Between Experimental Groups

Group Comparison	Mean Difference	Std. Error	*p*-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Group 1 vs. Group 2	-29.98	14.93	0.376	-77.44	17.47
Group 1 vs. Group 3	-5.42	14.93	0.999	-52.87	42.03
Group 1 vs. Group 4	-34.44	14.93	0.242	-81.89	13.01
Group 1 vs. Group 5	-15.00	14.93	0.910	-62.45	32.46
Group 1 vs. Group 6	-32.49	14.93	0.296	-79.94	14.97
Group 2 vs. Group 3	24.56	14.93	0.582	-22.89	72.02
Group 2 vs. Group 4	-4.46	14.93	1.000	-51.91	43.00
Group 2 vs. Group 5	14.99	14.93	0.911	-32.47	62.44
Group 2 vs. Group 6	-2.50	14.93	1.000	-49.96	44.95
Group 3 vs. Group 4	-29.02	14.93	0.410	-76.47	18.43
Group 3 vs. Group 5	-9.58	14.93	0.986	-57.03	37.88
Group 3 vs. Group 6	-27.07	14.93	0.483	-74.52	20.39
Group 4 vs. Group 5	19.44	14.93	0.780	-28.01	66.90
Group 4 vs. Group 6	1.95	14.93	1.000	-45.50	49.41
Group 5 vs. Group 6	-17.49	14.93	0.844	-64.94	29.96

Among the three *Murraya paniculata* leaf extract-treated groups, the 100 mg/kg body weight group (Group 5) showed the lowest mean H-score descriptively (56.79), compared with 76.23 in the 50 mg/kg group and 74.28 in the 200 mg/kg group. However, the comparison between Group 5 and the infection-control group was not statistically significant ($p = 0.911$). Therefore, the lower H-score observed in the 100 mg/kg body weight group should be interpreted as a descriptive finding rather than as evidence of a statistically significant reduction in hepatic IL-6 expression relative to the infection-control group. The differences among the three extract doses also did not demonstrate a clear linear dose-dependent pattern, as the mean H-score decreased from 76.23 at 50 mg/kg to 56.79 at 100 mg/kg but subsequently increased to 74.28 at 200 mg/kg. The relatively large variability within the 100 mg/kg group (SD = 29.51; individual mouse means from 33.50 to 99.39) further limits interpretation of this pattern.

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Discussion

Principal Finding

The present study investigated the effect of *Murraya paniculata* leaf extract on hepatic interleukin-6 (IL-6) expression in BALB/c mice experimentally infected with *Plasmodium berghei*. The principal finding was that, when the individual mouse was used as the experimental unit, no statistically significant differences in hepatic IL-6 immunoreactivity were detected among the six experimental groups (one-way ANOVA, $p = 0.132$). Descriptively, the infection-control group showed a higher mean H-score than the negative-control group (71.77 vs. 41.79), and positive staining was observed in 35.0% of analyzed microscopic fields in the infection-control group but in none of the fields of the negative-control group. However, the difference in mean H-score between these two groups was not statistically significant ($p = 0.376$). These findings therefore indicate a trend toward increased hepatic IL-6 immunoreactivity accompanying *P. berghei* infection that requires confirmation in a larger sample.

Administration of *M. paniculata* leaf extract produced heterogeneous changes in hepatic IL-6 expression across the three doses investigated. The mean H-scores were 76.23, 56.79, and 74.28 for the 50, 100, and 200 mg/kg body weight groups, respectively. Thus, the observed response did not follow a simple monotonic dose-dependent pattern. Although the 100 mg/kg group exhibited the lowest descriptive H-score among the extract-treated groups, the comparison with the infection-control group was not statistically significant in the animal-level analysis. Therefore, the present findings should not be interpreted as evidence of a dose-dependent anti-inflammatory effect of the extract.

Importantly, the findings provide exploratory tissue-specific data regarding hepatic IL-6 immunoreactivity in a malaria model. The use of immunohistochemistry combined with digital image analysis allowed the distribution and intensity of IL-6 staining to be characterized within hepatic tissue rather than relying solely on circulating cytokine concentrations. Nevertheless, changes in hepatic IL-6 expression alone should not be interpreted as direct evidence of hepatoprotection, because biochemical markers of hepatic injury, histopathological injury scores, and broader inflammatory or oxidative-stress parameters were not assessed in the present study.

Effect of *Plasmodium berghei* Infection on Hepatic IL-6 Expression

The numerically higher hepatic IL-6 immunoreactivity observed following *P. berghei* infection is biologically consistent with the inflammatory response associated with malaria. Following infection, parasite-derived components and host responses to infected erythrocytes can activate innate immune pathways, resulting in the production of inflammatory mediators. The liver represents an important site of host–parasite interaction and immune activity during malaria because it is involved in both the hepatic stage of parasite development and the systemic inflammatory response that accompanies blood-stage infection. Consequently, hepatic tissue may reflect local inflammatory signaling occurring during experimental malaria. However, this difference was not statistically significant in the animal-level analysis and should therefore be regarded as a trend rather than a confirmed effect.

The observed numerical increase in IL-6 immunoreactivity may therefore be interpreted within the sequence of parasite-associated immune activation, inflammatory signaling, and cytokine production. Recognition of parasite-associated molecular signals can activate innate immune cells, including macrophage-lineage cells, and promote intracellular inflammatory

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signaling. These responses can involve transcriptional pathways such as nuclear factor-κB (NF-κB), which regulate the expression of multiple inflammatory mediators. IL-6 is one of the cytokines produced within this inflammatory network and can participate in the amplification and regulation of the acute-phase response. Accordingly, increased hepatic IL-6 staining in the infection-control group may reflect activation of the local inflammatory environment associated with malaria (Mizobuchi et al., 2018).

The categorical field-level distribution points in the same direction. In the negative-control group, 60.0% of analyzed microscopic fields were categorized as negative and none were categorized as positive. In contrast, only 5.0% of fields in the infection-control group were classified as negative, whereas 35.0% were classified as positive. This shift from predominantly negative or low-positive staining toward stronger immunoreactivity is consistent with enhanced hepatic IL-6 expression after *P. berghei* infection. However, because these fields originate from only four mice per group and are not independent observations, the proportions are descriptive and cannot replace the animal-level comparison, in which the difference between the two groups was not statistically significant ($p = 0.376$).

However, IL-6 should not be regarded solely as a pathological mediator. IL-6 is a pleiotropic cytokine involved in both inflammatory and physiological immune responses (Belhimeur et al., 2023). Therefore, an increase in hepatic IL-6 does not necessarily indicate irreversible hepatic damage. Rather, it indicates activation of an inflammatory signaling environment. Demonstrating whether this inflammatory response is accompanied by actual hepatocellular injury would require integration with serum ALT/AST measurements, histopathological scoring, oxidative-stress markers, or other indicators of hepatic dysfunction.

Effect of *Murraya paniculata* Leaf Extract

The biological rationale for investigating *M. paniculata* leaf extract in this context is supported by the diverse phytochemical composition of the plant. Reported constituents of *M. paniculata* include flavonoids, phenolic compounds, coumarins, terpenoids, and alkaloids, several of which have been associated with antioxidant and anti-inflammatory activities. Reviews of *M. paniculata* and the broader *Murraya* genus have documented biological activities involving oxidative stress and inflammatory responses, although the activity of an unfractionated leaf extract cannot be attributed to a single compound without direct chemical characterization.

Particularly relevant to the present findings, experimental evidence has demonstrated that flavonoids isolated from *M. paniculata* can modulate inflammatory responses in murine macrophage models. Three flavonoids—5,7,3′,4′,5′-pentamethoxyflavone, 5,7,3′,4′-tetramethoxyflavone, and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone—were reported to inhibit inflammatory responses in LPS-activated macrophages, including suppression of IL-6 production. Among these compounds, 5,7,3′,4′-tetramethoxyflavone demonstrated particularly strong inhibition of IL-6 production in the reported experimental system (Wu et al., 2016).

This previous evidence provides a plausible biological basis for the possibility that constituents of *M. paniculata* extract may influence IL-6-associated inflammatory signaling in malaria. In addition, reported antioxidant activity of *M. paniculata* leaf extracts suggests that modulation of oxidative stress could contribute indirectly to changes in inflammatory signaling. Nevertheless, the present study did not directly measure oxidative-stress parameters, NF-κB activation, macrophage activation, or individual phytochemical concentrations (Al-Khayri et

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al., 2022). Therefore, these pathways should be regarded as mechanistic hypotheses supported by previous literature rather than mechanisms demonstrated by the present experiment.

The absence of a statistically significant difference between the infection-control group and the extract-treated groups is also important. Although the extract groups showed different descriptive H-scores, the current data do not establish that *M. paniculata* significantly reduced hepatic IL-6 expression relative to malaria infection. This distinction is important because evidence of pharmacological plausibility does not automatically establish efficacy in a specific disease model. The present findings therefore extend previous anti-inflammatory observations by examining hepatic IL-6 immunoreactivity in experimental malaria, but they do not yet demonstrate a definitive therapeutic effect. With only four animals per group and considerable inter-animal variability, the study also had limited power to detect moderate differences between groups, so a true effect of the extract cannot be excluded.

Dose–Response Relationship

An important feature of the present study was the absence of a straightforward dose-dependent reduction in hepatic IL-6 expression. The mean H-score was 76.23 at 50 mg/kg, decreased to 56.79 at 100 mg/kg, and increased again to 74.28 at 200 mg/kg. Thus, the relationship between extract dose and IL-6 immunoreactivity appeared non-linear. The 100 mg/kg group showed the lowest descriptive H-score among the extract groups, but this observation should not be interpreted as evidence that 100 mg/kg represents an optimal therapeutic dose because the between-group differences were not statistically significant in the current analysis. A non-monotonic response is biologically plausible for a crude botanical extract because its pharmacological activity reflects the combined effects of multiple constituents rather than a single purified compound (Rajčević et al., 2022). Different constituents may have different dose–response relationships, absorption characteristics, metabolic pathways, and biological targets. At increasing concentrations, interactions among phytochemicals may also alter the net biological response (Rajčević et al., 2022; Vaou et al., 2022). Therefore, increasing the dose of a crude extract does not necessarily produce a proportional increase in a particular pharmacological effect.

Another possible explanation is that IL-6 expression reflects a dynamic inflammatory process rather than a static linear dose-response relationship. The magnitude of tissue cytokine expression may depend on the timing of treatment relative to infection, parasite burden, cellular composition of the tissue, and the balance between pro-inflammatory and regulatory pathways (Rajčević et al., 2022; Chaachouay, 2025). Because the present study evaluated IL-6 at a single experimental endpoint, temporal changes in IL-6 expression could not be determined. Consequently, the present dose-response findings should be considered hypothesis-generating. Future studies using a larger number of animals, multiple sampling time points, standardized phytochemical profiling, and pharmacodynamic measurements will be necessary to determine whether the apparent non-linear pattern represents a reproducible biological phenomenon or variation within the experimental model.

Relationship Between IHC Distribution and H-Score

The combination of categorical immunohistochemical interpretation and quantitative image analysis provides complementary information regarding hepatic IL-6 expression. The categorical assessment demonstrated a shift in the distribution of staining from negative toward low-positive and positive categories, particularly in the infection-control group. The

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quantitative H-score subsequently incorporated staining intensity into a continuous measure, providing a more detailed representation of the overall immunoreactivity (Varghese et al., 2014). This relationship was particularly evident when comparing the negative-control and infection-control groups. The negative-control group had predominantly negative staining, with 60.0% of analyzed fields classified as negative and no positive fields, whereas the infection-control group had only 5.0% negative fields and 35.0% positive fields. The corresponding mean H-score also increased from 41.79 to 71.77, although this difference was not statistically significant at the animal level. Thus, the categorical and quantitative approaches showed a consistent directional pattern.

Digital image analysis may reduce some of the subjectivity inherent in visual IHC interpretation by applying standardized pixel-based classification to DAB staining. In the present study, ImageJ with the IHC Profiler plugin was used to characterize the intensity distribution of immunohistochemical staining (Ram et al., 2021). The resulting H-score therefore provides a quantitative complement to representative microscopic images and categorical staining assessment.

Nevertheless, digital quantification does not eliminate all sources of variability. Image acquisition, illumination, tissue thickness, antigen retrieval, staining intensity, background staining, and selection of microscopic fields can influence pixel-based measurements. Standardized acquisition and analysis procedures are therefore essential when comparing IHC intensity across experimental groups.

CONCLUSION

This study found that hepatic IL-6 immunoreactivity was numerically higher in *Plasmodium berghei*-infected BALB/c mice than in uninfected controls, suggesting a possible local inflammatory response within the liver, although this difference was not statistically significant in the animal-level analysis. Administration of *Murraya paniculata* leaf extract produced variable changes in hepatic IL-6 expression across the tested doses, without demonstrating a statistically significant reduction in IL-6 expression compared with the infected control group under the conditions of the present experiment. The observed non-linear dose-response pattern should be interpreted cautiously, as it may reflect the complex composition of the crude extract, the inflammatory environment of malaria infection, or variability resulting from the small number of animals per group.

The findings provide preliminary in vivo data regarding the interaction between *M. paniculata* leaf extract and hepatic inflammatory responses during experimental malaria. Although previous studies have demonstrated anti-inflammatory activity of selected *M. paniculata* constituents in cellular models, the present findings indicate that such activity cannot be directly extrapolated to suppression of hepatic IL-6 expression in an established malaria model. Therefore, *M. paniculata* leaf extract should not yet be considered demonstrably hepatoprotective based solely on hepatic IL-6 immunoreactivity. Further studies incorporating larger sample sizes, standardized phytochemical characterization, longitudinal assessment, and complementary endpoints such as serum liver enzymes, histopathological injury scores, oxidative-stress markers, and broader cytokine profiles are warranted to clarify its potential role in modulating malaria-associated hepatic inflammation.

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