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SONGGA WOOD (*STRYCHNOS LIGUSTRINA* BLUME) ASSOCIATED WITH REDUCE TUMOR NECROSIS FACTOR-ALPHA LEVELS IN ARTEMINISIN-BASED-COMBINATION THERAPY-TREATED MALARIA

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ABSTRACT

Background: The emergence of resistance to artemisinin-based combination therapies (ACTs) complicates malaria control strategies, necessitating novel therapeutic approaches and enhanced surveillance in affected regions. Traditional herbal remedies, such as Songga wood (*Strychnos ligustrina* Blume), are gaining attention for their potential antimalarial and immunomodulatory properties, offering promising alternatives to combat drug-resistant malaria. **Objective:** This study aimed to evaluate the effect of ethanolic extracts from the stem of Songga wood (EESWS) on the modulation of TNF-α in malaria treatment with ACTs. **Methods:** A study used a post-test-only-control-group-design with simple-random-sampling involved two treatment-groups and three control-groups, each with five Swiss-Webster-mice. The C1-group was the healthy-control, the C2-group was untreated, and the C3-group received ACT. The P1-group was given EESWS at preventive and therapeutic-doses, while the P2-group received a combination of EESWS-ACT. Blood samples were collected on the day-8-infection to assess parasitemia percentage and plasma TNF-α-levels. **Results:** On day-7-PbA-infection, the untreated-C2-group exhibited the highest parasitemia-levels (13.20 ± 4.18), while the C3, P1, and P2-groups showed significantly lower levels, with no significant differences among these treated-groups ($p > 0.05$). Significant differences in TNF-α-levels were observed, with the untreated-C2-group having higher levels compared to the healthy-C1-group and all treatment-groups. Among the treatments, the P1-group had higher TNF-α levels than both the P2 and C3-groups, while no significant difference was observed between the C3 and P2-groups. **Conclusions**: All treatment regimens effectively promoted recovery from PbA-infection, and the combination of EESWS with ACT appears to facilitate a more balanced modulation of inflammatory responses during the malaria recovery phase.

INTRODUCTION

Malaria remains a significant global health challenge, with the World Health Organization (WHO) highlighting its persistent prevalence, particularly in Southeast Asia. This region reports millions of cases annually, contributing substantially to the global burden of approximately 247 million malaria cases reported worldwide, resulting in an estimated 619,000 deaths in 2022, with children under five years old accounting for a substantial proportion of these fatalities.[1] The emergence of resistance to artemisinin-based combination therapies (ACTs) further complicates malaria control strategies, threatening the efficacy of current treatment protocols.[2, 3] The spread of artemisinin resistance across Southeast Asia, including Indonesia, underscores an urgent need for enhanced surveillance and novel therapeutic approaches.[4-7] This scenario necessitates robust pharmacologic and non-

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pharmacologic interventions to mitigate malaria's impact in these endemic regions.[8]

In response to the escalating challenge of drug-resistant malaria, there has been a resurgence of interest in traditional herbal medicine. These remedies with antiplasmodial activity, rooted in centuries of therapeutic use, present promising alternatives to conventional.[9, 10] The ethnobotanical approach, which has historically led to the development of quinine and artemisinin, continues to underscore the importance of accessible treatments.[2, 11, 12] The integration of herbal remedies with standard antimalarial drugs presents a promising avenue for addressing malaria resistance.[10, 12] The traditional knowledge of medicinal plants, combined with modern pharmacological research, could lead to more effective treatment strategies in malaria-endemic regions. Immunomodulatory strategies have also emerged as vital components in managing severe malaria complications. An immunomodulatory medication as adjuvant therapy of antimalaria artemisinin derivatives have shown promise in modulating immune responses, potentially improving outcomes in severe cases.[13] These therapies highlight the potential benefits of immunomodulation in preventing organ damage and enhancing parasite clearance.[14, 15]

S. ligustrina Blume, a plant traditionally used in Indonesian medicine, has garnered attention for its potential antimalarial and immunomodulatory properties. Empirical evidence suggests that extracts from *S. ligustrina* Blume exhibit significant antiplasmodial activity, supporting its traditional use in treating febrile conditions, including malaria.[16] The plant's secondary metabolites, such as alkaloids and flavonoids, contribute to its therapeutic efficacy by potentially inhibiting *Plasmodium* parasite growth.[17] Furthermore, *S. ligustrina* Blume's immunomodulatory effects, particularly its influence on cytokine production, may enhance immune responses, aiding in malaria parasite clearance.[18] Tumor necrosis factor-alpha (TNF-α), a cytokine which plays a crucial role in the immune response during malaria infection, promoting both protective immunity and potential pathogenesis.[19, 20] While direct research linking TNF-α modulation with *S. ligustrina* Blume in ACT treated malaria is limited, the plant's immunomodulatory properties suggest a potential role in balancing TNF-α responses, thereby optimizing therapeutic outcomes.[21, 22] This theoretical framework supports further investigation into the synergistic effects of *S. ligustrina* Blume on TNF-α modulation in malaria treatment, potentially enhancing the efficacy of current therapies.

METHODS

Preparation of Ethanol Extract from the Stem of Songga Wood (*S. ligustrina* Blume).

The ethanolic extracted stem of Songga Wood stem (EESWS) involves several meticulous steps to ensure the purity and efficacy of the extract (ethanolic extracted methode done in Integrated Biomedical Laboratory, Unissula, Semarang). Initially, the wood is separated from the serpent wood stem and its bark is meticulously shaved and ground into a fine powder. This powdered form is then subjected to successive maceration using ethanol as the solvent. The maceration process is conducted over a period of three days, with an extension until the added solvent no longer exhibits a change in color, signifying the exhaustion of extractable components. Daily filtration is performed, and the residual powder is re-macerated with fresh solvent until no further color change is observed. On the fourth day, the filtered extracts are collected and concentrated to dryness using a rotary evaporator, resulting in the acquisition of the ethanol extract. This methodical approach ensures the comprehensive extraction of bioactive compounds present in the Songga wood, contributing to its potential applications in various fields.

Malaria Parasite

Plasmodium berghei ANKA (PbA) was provided by the Integrated Biomedical Laboratory of Unissula, having originally been sourced from Universitas Gajah Mada (UGM). The whole blood containing PbA-infected erythrocytes was injected into donor mice. The blood from these donor mice underwent multiple processing stages to ensure the successful transmission of parasitized erythrocytes to the experimental mice. Each experimental mouse received an infectious dose of 10^7 erythrocytes in 0.2 cc of sterile physiological saline (NaCl) solution. The detail procedure was mentioned elsewhere.

Experimental design and animal handling

This experimental laboratory study utilized a post-test-only control group design. The mice used

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were housed in Experimental Animal Laboratory of the Integrated Biomedical Laboratory of Sultan Agung University (Unissula). The inclusion criteria were 8-week-old healthy female Swiss Webster mice, each weighing between 30 and 35 grams and exhibiting normal anatomical features, activity, and behavior. A simple random sampling method was employed for sample selection. Following a 7-day adaptation period at the Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung Semarang, random grouping was conducted. This process resulted in the formation of two treatment groups and three control groups, each comprising five mice, totaling 25 Swiss Webster mice distributed across five groups. The C1-group served as the healthy control group. The groups of C2, C3, P1 and P2 were PbA-infected in the dose of 10^7 infected red blood cells/200mL physiologic NaCl, and the detail procedure mentioned elsewhere. The C2-group was untreated mice, while the C3-group received ACT that was dihydroartemisinin piperaquine (DHP-Frimal; produced by KBN-Zhejiang Pharmaceutical Co.,Ltd) at a dosage of 0.819 mg/kg body weight per day. Group P1 was administered EESWS at a preventive dose of 0.15 mg/kg body weight per day for 10 days and then a therapeutic dose of 0.30 mg/kg body weight per day starting day 4^{th} infection. Group P2 received a combination of EESWS at the same preventive and therapeutic dosages, along with ACT at 0.819 mg/kg body weight per day starting day 4^{th} infection. On the 8^{th} infection day, blood samples were collected via tail vein sampling to assess parasitemia percentage, with observations conducted under a light microscope at 400x magnification. The mice were then anesthetic using chloroform. The mice were euthanized, and then blood collection was done from plexus orbitalis for plasma TNF-α measurement. The plasma TNF-α levels were measured by using ELISA kit (LegendMAx Cat No 430907, Biolegend Inc, San Diego, USA). The TNF-α measurement was done in the GAKY Laboratory of the Faculty of Medicine, Diponegoro University.

Statistical analysis

Data analysis was performed by using SPSS statistical software provided by Diponegoro University. Each data was tested for normality using Saphiro-Wilk-test. The Kruskal Wallis test was carried out to see a different mean between the five research-groups. The magnitude of the difference in the mean between two-groups was further analyzed using the Mann Whitney U-Test for plasma-TNF-α levels. The significant difference was indicated by $p < 0.05$.

RESULTS

Parasitemia percentage

On day 7 post-PbA infection, parasitemia levels differed significantly among the groups. The C2 group (PbA-infected without treatment) showed the highest parasitemia percentage (Mean ± SD;13.20 ± 4.18), indicating severe infection in the absence of therapeutic intervention. In contrast, parasitemia levels were significantly lower in the treatment groups, including C3 (ACT-treated, 6.40 ± 0.96), P1 (EESWS-treated, 4.68 ± 1.94), and P2 (EESWS + ACT-treated, 4.06 ± 1.13). These reduced parasitemia levels suggest that all treatments, whether with antimalarial-ACT alone, EESWS alone, or the combination of EESWS and ACT, were effective in suppressing parasite load. Moreover, there were no significant differences in parasitemia levels among the treated groups ($p > 0.05$), indicating that all treatment regimens were equally effective in driving the recovery phase of PbA infection.

Plasma TNF-α Levels

The boxplot (Figure 1) demonstrates plasma TNF-α levels across the groups, providing insights into the inflammatory response. The C1 group (healthy mice) had the lowest TNF-α levels, representing a normal baseline. In stark contrast, the C2 group (PbA-infected without treatment) exhibited the highest TNF-α levels with significant variability, indicating a strong systemic inflammatory response due to PbA infection. Following treatment, TNF-α levels were reduced in all treated groups. Specifically, the C3 group (ACT-treated) showed lower levels compared to the C2 group, highlighting the effectiveness of ACT in mitigating inflammation. The P1-group (EESWS-treated) demonstrated a higher median of TNF-α levels than C2-group. The P2 group (EESWS + ACT-treated) showed the most substantial reduction, with TNF-α levels nearing those of the healthy C1 group, suggesting a synergistic effect of the combined therapy in controlling inflammation.

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Statistical comparisons of TNF-α levels among the groups reveal significant differences (Table 1). The C2 group (PbA-infected without treatment) showed significantly higher levels compared to the healthy C1 group (p = 0.004), as well as all treatment groups (p ≤ 0.013). This demonstrated that malaria without conventional antimalaria treatment increase pro-inflammatory response. Among the treatment groups, the P1 group (EESWS-treated) showed higher TNF-α levels compared to the combined P2 group (p = 0.006) and the ACT-only C3 group (p = 0.004), indicating that EESWS alone had a higher pro-inflammatory effect. However, there was no significant difference between the C3 (ACT-treated) and P2 (EESWS + ACT-treated) groups (p = 0.687), suggesting that the combination therapy (P2) achieved a pro-inflammatory effect comparable to ACT alone.

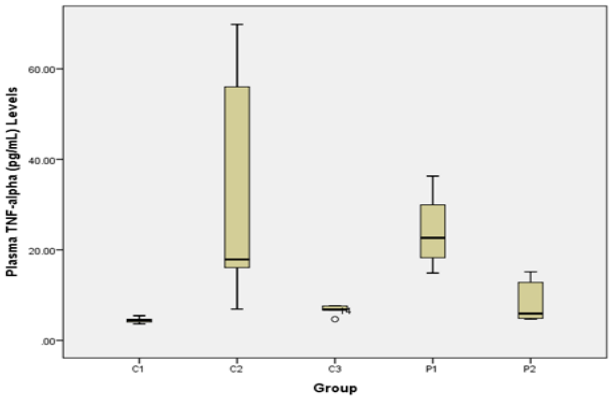

Figure 1. Plasma TNF-α levels (C1=healthy control, C2=PbA-infected untreated, C3=PbA-infected-ACT-treated, P1=PbA-infected-EESWS-treated, P2= PbA-infected-EESWS-ACT-treated)

Table 1. Plasma TNF-α levels

Group	C2	C3	P1	P2
C1	0.004	0.008	0.004	0.013
C2		0.013	0.631	0.010
C3			0.004	0.687
P1				0.006

* Significant ($p \leq 0.05$) (C1=healthy-controls, C2=PbA-infected-untreated-group, C3=PbA-infected-ACT-treated-group, P1=PbA-infected-EESWS-treated-group, P2=PbA-infected-EESWS-ACT-treated-group)

DISCUSSION

Our study's findings indicate that mice receiving treatments including ACT, EESWS and combination ACT-EESWS, exhibit significantly lower parasitemia and TNF-α levels compared to untreated control mice. This observation supports existing literature suggesting that TNF-α is crucial for the immune response against malaria, but excessively high levels can be detrimental. Elevated TNF-α levels are associated with severe malaria outcomes, including cerebral malaria and systemic inflammation.[23, 24] In animal models, such as those infected with *Plasmodium berghei*, high TNF-α levels have been correlated with disrupted erythropoiesis, highlighting the cytokine's role in malaria-associated anemia.[25] In contrast, lower TNF-α levels following effective treatment may reflect a resolution of inflammation and a return to homeostasis, which is beneficial for recovery.[22] The relationship between TNF-α levels and parasitemia suggests that effective treatment not only reduces parasite load but also mitigates the inflammatory response, potentially decreasing the risk of complications associated with high TNF-α levels.[26, 27] Thus, the combination of low parasitemia and low TNF-α levels post-treatment may indicate a successful therapeutic outcome, emphasizing the importance of monitoring both parasitic and inflammatory markers in malaria management. Overall, these findings highlight the importance of effective malaria treatment in reducing both parasitemia and TNF-α levels, thereby mitigating severe disease outcomes. Monitoring these parameters can provide valuable insights into the therapeutic efficacy and guide clinical decisions in malaria management.

Artemisinin-based combination therapy (ACT) has been shown to modulate TNF-α levels during malaria treatment. Our findings indicate that TNF-α levels in groups treated with ACT or its combination with EESWS remained significantly higher than those in healthy control mice (Figure 1 and Table 1). This aligns with studies suggesting that ACT can influence the immune response, leading to changes in cytokine profiles, including TNF-α. Patients undergoing ACT for *Plasmodium falciparum* malaria have exhibited elevated TNF-α levels, associated with disease severity.[23, 28, 29] This elevation may result from the immune system's response to dying parasites, triggering pro-inflammatory cytokine production.[26, 30]

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The elevation of TNF-α levels during malaria treatment with ACT is significantly influenced by the presence of dying parasites, which release various malaria toxins, including hemozoin, malaria DNA, and glycosylphosphatidylinositol (GPI). Hemozoin, a byproduct of hemoglobin digestion by the malaria parasite, acts as a pathogen-associated molecular pattern (PAMP) that can stimulate the immune system. It has been shown to bind to Toll-like receptor 9 (TLR9) on dendritic cells and macrophages, leading to the production of pro-inflammatory cytokines, including TNF-α. Similarly, GPI, another component released during parasite lysis, interacts with TLR1/TLR2 and TLR2/TLR6 dimers, further promoting the secretion of pro-inflammatory cytokines. Moreover, the release of malaria DNA from dying parasites can activate multiple innate immune receptors, including TLR7 and TLR9, which also contribute to the inflammatory response.[31] This cascade of immune activation is crucial, as it not only enhances TNF-α production but also leads to increased surface leukocyte adhesion molecules which vacillate leukocyte recruitment and subsequent inflammation in the host.[32] The elevated TNF-α levels, while part of the immune response to control the infection, can also result in detrimental effects, such as endothelial dysfunction and increased permeability of the blood-brain barrier, exacerbating conditions like cerebral malaria.[33] Thus, the interplay between the release of malaria toxins and the host's immune response underscores the complexity of TNF-α modulation during malaria treatment. TNF-α -308G>A polymorphism, linked to increased susceptibility to severe malaria, suggests that genetic factors may also influence how ACT affects TNF-α levels.[24, 26] The interaction between ACT and the host's immune response can create a complex cytokine environment, where elevated TNF-α levels may contribute to both therapeutic effects and potential adverse outcomes, such as inflammation-related complications.[34, 35] While ACT effectively reduces parasitemia, its impact on TNF-α levels highlights the need for careful monitoring of inflammatory responses during treatment.

Our recent study demonstrated that the administration of Songga wood (*S. ligustrina* Blume) in combination with ACT, or ACT alone, did not result in significant differences in TNF-alpha levels in a malaria mouse model. The active compounds in *S.ligustrina*, such as brucine and strychnine, have shown various pharmacological effects that may influence parasitemia and cytokine production during malaria.[36-38] These compounds possess notable antiplasmodial activity, contributing to the reduction of parasitemia by directly targeting malaria parasites. *S. ligustrina* exhibits anti-inflammatory effects in malaria treatment by modulating key cytokines like IL-10 and chemokines like CXCL12.[18, 39] These findings suggest that *S. ligustrina* Blume may help mitigate the immunopathological responses associated with malaria, providing a complementary approach to conventional antimalarial therapies. The studies referenced here highlight the plant's potential in enhancing the efficacy of existing treatments while reducing inflammation-related complications in malaria. The extract alone and in combination with ACT led to an increase in IL-10 production compared to untreated controls, but lower than the levels observed with ACT alone.[18] The regulation of TNF-α by IL-10 is crucial in malaria treatment, as ACT increases IL-10 levels, mitigating excessive inflammation.[18] The balance between IL-10 and TNF-α ensures an effective immune response without exacerbating inflammation. Interestingly, when ACT is combined with *S. ligustrina*, IL-10 levels are lower compared to ACT alone, suggesting that *S. ligustrina* may modulate immune pathways differently, potentially affecting the IL-10/TNF-α ratio. Despite these variations, both treatments maintain stable TNF-α levels, highlighting their ability to control parasitemia while preventing excessive inflammation.[33] Furthermore, the effect of *S. ligustrina*'s active compounds on chemokines may influence leukocyte recruitment and activation, impacting the overall immune response during malaria infections. For example, high levels of CXCL12, a chemokine involved in immune cell trafficking, have been associated with the protective effects of ACT, as it is expected to inhibit Th1 cell migration toward the blood-brain barrier.[39] By potentially reducing harmful inflammatory mediators while promoting protective cytokines, *S. ligustrina* may play a beneficial role in managing malaria, although further investigation is needed to fully elucidate its therapeutic potential.

TNF-α also plays a role in the development of trained immunity, which can enhance the host's ability to respond to subsequent infections. Trained

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immunity refers to the long-lasting functional reprogramming of innate immune cells, leading to an improved response to pathogens. The relationship between TNF-α and trained immunity, particularly in the context of malaria, reveals significant insights into the mechanisms of immune response modulation. In the studies analyzed, it was observed that after an initial infection or exposure to *Plasmodium falciparum*, monocytes exhibited a trained immunity phenotype characterized by increased TNF-α production upon subsequent stimulations. This enhanced production is associated with epigenetic reprogramming, including changes in histone modifications that prime genes for more robust expression upon re-exposure to stimuli.[40] Moreover, the studies highlight that this trained immunity effect involves a complex interplay between innate and adaptive immune cells. For example, the presence of lymphocytes, particularly T cells, was found to be necessary for the full expression of trained immunity in monocytes, suggesting that adaptive immune signals, such as IFN-γ, can augment the trained response mediated by TNF-α.[20] This intercellular communication emphasizes the importance of TNF-α not only as a marker of trained immunity but also as a mediator that bridges innate and adaptive immune responses. These findings suggest that the modulation of TNF-α and its associated pathways could potentially be leveraged to enhance immune responses against malaria and possibly other infections, offering a pathway for novel therapeutic strategies. The studies highlights the potential of targeting trained immunity mechanisms to improve disease outcomes.[41] In the perspective of malaria, the modulation of TNF-α levels through interventions like *S. ligustrina* may help to establish a protective immune environment without triggering the detrimental effects associated with high TNF-α levels. This balance is crucial, as trained immunity can improve the host's ability to control parasitemia and reduce the severity of malaria clinical manifestation. Moreover, the combination of *S. ligustrina* with ACT may synergistically enhance the immune response by promoting the differentiation of monocytes into macrophages and dendritic cells, which are essential for effective antigen presentation and T-cell activation.[39] This process can further contribute to the development of trained immunity, allowing the immune system to respond more effectively to future malaria infections. Therefore, while lower TNF-α levels may indicate a reduced inflammatory response, they may also facilitate the establishment of trained immunity, ultimately enhancing the host's defense against malaria and reducing the risk of exacerbation during subsequent infections. This therefore the study would be strengthened by observation whether EESWS or EESWS-ACT in combination affected the trained immunity during PbA-reinfection.

The study elucidates the therapeutic potential of combining *S. ligustrina* Blume with ACT in a murine model of malaria. This combination maintains TNF-α levels comparable to those observed with ACT alone, indicating effective management of parasitemia while preventing excessive inflammatory responses. The active constituents of *S. ligustrina*, particularly brucine and strychnine, are known for their antiplasmodial properties and their ability to modulate cytokine production, thus achieving a crucial balance between TNF-α and IL-10. This cytokine balance is pivotal in alleviating severe malaria complications. Furthermore, the synergistic combination may augment immune responses by fostering trained immunity, thereby enhancing the host's capacity to control parasitemia and mount effective responses to subsequent infections. Further research is warranted to fully understand the mechanisms and potential benefits of this combination therapy.

CONCLUSION

In conclusion, our findings highlight the therapeutic promise of combining *S. ligustrina* Blume with ACT, emphasizing the importance of monitoring parasitic and inflammatory markers in malaria treatment.

ETHICAL APPROVAL

This study was approved by Health Research Ethical Committee Faculty of Medicine Universitas Diponegoro (Ethical Clearance No. 145/EC/H/KEPK /FK-UNDIP/XI/2019).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization, Kis; methodology, Kis; data analysis, Kis; data collection, Kis; source of funds, Kis; wrote the original draft, Kis; review and edit, Kis.

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