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**Research**

# *Annona muricata* effect on parasitemia and lymphocyte formation of act treated malaria

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## Abstract

Combination therapy is essential to safeguard existing and future antimalarial drugs, prompting exploration into adjuvant therapies for managing cerebral malaria. This study aimed to assess the effectiveness of *Annona muricata* leaves extracted by water (AME) as an adjuvant for Artemisinin-based combination therapy (ACT) in mice infected with *Plasmodium berghei* ANKA (PbA) malaria. The research employed a Post Test Only Control Group Design for the study. A few of 24 Swiss mice were subjected to various treatments, including AME, ACT, and their combination, with a focus on host survival, lymphocyte generation, and migration. Results demonstrated that mice treated with the combination of AME and ACT exhibited significantly reduced parasitemia, and lower percentages of splenic lymphoblasts compared to control groups. Specifically, the mean percentage of splenic lymphoblasts in the AME and ACT combination group was substantially lower than in the control groups and individual treatment groups. Parasitemia levels were significantly reduced in the combination group, emphasizing the synergistic effect of *A. muricata* and ACT. In conclusion, *A. muricata* demonstrated a superior impact on parasitemia, and splenic lymphoblasts in malaria mice treated with ACT, highlighting its potential as a valuable adjuvant therapy in the fight against malaria.

**Keywords:** Plasmodium, Annona muricata, spleen, brain

## INTRODUCTION

According to the World Health Organization's 2021 report, almost 50% of the global population faced the risk of malaria.[1] The report also approximated a total of 247 million malaria cases worldwide in that year, resulting in around 619,000 malaria-related deaths. Indonesia, in collaboration with international organizations and agencies like

the World Health Organization (WHO), has been working to combat malaria effectively.[2] The problem of antimalarial drug resistance, particularly to artemisinin-based combination therapies (ACTs), remains a significant concern globally.[3-5] The concern is that if these resistant strains of malaria spread to other regions, it could severely compromise malaria control and elimination efforts. Malaria parasites capable of withstanding artemisinin treatment could lead to longer-lasting and more severe infections. Research has been conducted to discover more effective treatments for malaria. Triple artemisinin-based combination therapies (TACTs) have proven effective in trials, even in regions with multidrug-resistant malaria.[6] TACTs are currently suggested for use in communities but have not been put into practice yet. In the other hand, traditional medicine offers supplementary methods to conventional antimalarial treatments. Although it is not a complete solution on its own, integrating traditional medicine into malaria control strategies, alongside conventional treatments, research, and prevention initiatives, can substantially aid the global battle against malaria and address the issue of drug resistance.

The alkaloid of *Annona sp* has anti-infectivity and anti-cancer.[7] The combination of ethanolic extracted *Annona muricata* and *Khaya grandifoliola* reduce oxidative stress *in vitro*, and reduce parasitemia, pro-inflammatory cytokines in experimental malaria mouse model.[8] *A. muricata* extract (AME) intervention increases splenocyte-interleukin (IL)-10 production capacity during severe *Plasmodium berghei* ANKA (PbA)-infected Swiss mice, an experimental malaria. IL-10, an anti-inflammatory cytokine, protects the development of immununopathology and mortality during severe malaria.[9, 10] CXCL10, a pivotal chemokine, plays a significant role in regulating inflammation and orchestrating cellular immune responses amid infectious diseases. Notably, elevated levels of CXCL10 have been detected in the plasma and saliva of severe malaria patients, underscoring its importance in the context of malaria infection.[11, 12] *Plasmodium* possesses the capability to inhibit the production of CXCL10 by both monocytes and neutrophils. This finding contradicts the previous observation of heightened CXCL10 levels in severe malaria cases. To resolve this inconsistency, researchers have discovered that elevated CXCL10 levels surprisingly promote increased *Plasmodium* growth. This discovery provides a rationale for the observed phenomenon, shedding light on the complex relationship between CXCL10 and *Plasmodium* infection in severe malaria cases.[13] AME administration modulates the CXCL10 expression in the brain.[14] Remarkably, when combined with standard antimalarial ACT, AME administration leads to a notable reduction in brain-CXCL10 expression and parasitemia percentage during the recovery phase in malaria-infected Swiss-mice. This synergistic AME–ACT therapy emerges as a superior intervention strategy compared to AME alone.[15] Moreover, the absence of CXCL10 is linked to the accumulation of CXCR3+ CD4+ T-follicular helper cells in the spleen, which are crucial for antibody production, thereby aiding in the control of PbA infection.[16] CXCL10 neutralization or the use of CXCL10 knockout mice has been shown to mitigate brain microvascular inflammation, enhance parasite control, and confer protection against experimental cerebral malaria (ECM) during PbA infection.[17-20] Notably, an upregulation of the innate immune sensor STING1 by brain vascular endothelial cells is observed in PbA-infected mice, subsequently leading to increased CXCL10 expression. This heightened CXCL10 level is pivotal in driving leukocyte recruitment to the brain, thereby inducing inflammation and causing tissue damage.[21] In the context of severe PbA-infection, it has been established that AME augments spleen-CXCL10 expression,[22] a significant finding that had yet to be explored in the context of AME-ACT combination therapy. Furthermore, the potential of AME as an adjuvant therapy in conjunction with ACT treatment, particularly its influence on the splenic-lymphoblast severe malaria patient, remained unexplored. This study, therefore, aimed to fill these critical gaps by investigating the impact of the combined AME-ACT approach the lymphoblast count in the spleen, providing a more thorough understanding of the intricate mechanisms at play.

## MATERIAL AND METHOD

**Research design and experimental animal**

This study was posttest control only design. Swiss mice were purchased commercially from private mice breeding, and the mice strain was confirmed by Indonesian government institution. The mice were acclimated for 7 days upon arrival. The twenty-four mice included in this study, were healthy and they were positive malaria after PbA inoculation. The mice were kept in clean and aseptic room in Parasitology Department of Faculty of medicine Diponegoro University. The mice receive adequate pellet food and healthy drinking water. The mice were terminated for spleen lymphoblast measurement. The mouse groups were named as K, P1, P2, P3 group. The K-group did not receive any treatment, while P1, P2 and P3-group treated with AME, ACT and combination AME-ACT, respectively. The ethical clearance of this study was given by ethical committee of Faculty of medicine Diponegoro University and Dr. Kariadi Hospital (No 174/EC/FK-RSDK/ 2016).

**Annona muricata extract and anti-malaria**

The leaves of AM were extracted by using water. The AM extract (AME) processed and analyzed for free by Sido Muncul Company. The treatment used was AME, artemisinin-based combination dose for period of 10 days (7 days before PbA-inoculation and 3 days after PbA infection) followed by 9.36 mg/day as therapy dose (since day 4 of PbA-infection when parasitaemia had been confirmed). ACT (0.819 mg/day) was given since day 4 of PbA-infection. Spleens were isolated, processed and cultured on day 7 of PbA-infection.

**Parasite and infection dose used**

*P. berghei* ANKA (PbA) was provide by Parasitology Department of Universitas Gajah Mada. Three Swiss mice inoculated with PbA was used as donor mice. The blood was collected from donor mice when they had 15 – 20% parasitemia. The PbA-infection dose used was $10^7$ in 0.2 ml sterile-physiologic NaCl.

**Parasitemia measurement**

The parasitemia level was observed in the thin blood smear by the used of light microscope. The level was based on the percentage of the ratio infected red blood cells (iRBCs) to the total of 1000 RBCs included iRBC and uninfected RBCs. These were observed in the mice in survival study and the mice used in the study of the spleen-lymphoblast and the leukocytes in brain microvascular. Parasitemia percentage was measured at the time point days which were day 3 and 7 of PbA-infection.

**Lymphoblast isolation and preparation**

The spleen cells were isolated from the mice at the day 7 of PbA-infection. The spleen was pinched in the normal saline solution. The splenocyte solution were then centrifuged at 4°C. The pellet of splenocytes were kept and discard the supernatant. The splenocytes were diluted in the cold ammonium chloride (NH4Cl) and shake in the ice buckets. The splenocyte solution was then and passed through sterile gauze once. The pellet of splenocytes were then washed 2 times using NaCl physiology at 4°C. The count the splenocytes using the Improved Neubauer Hemocytometer, and adjust to the concentration of $10^7$ cells/mL. The thin slides of the splenocytes were prepared on the object glass, fixed by using methanol and stained by using Giemsa. The light microscope was used to observed the lymphoblast number among total of 200 splenocytes in the homogenous area of the slide.

**Statistical analyzes used**

Descriptive analysis displays the mean and standard deviation values for parasitemia and lymphoblast percentage. The percentage of parasitemia in each group was tested for normality of distribution and homogeneity of the data. Difference tests on data with normal and homogeneous distributions were carried out using parametric tests. The one-way ANOVA test shows $p < 0.05$ then proceed with the post hoc test. The spleen lymphoblast data was analyzed by the one-way ANOVA test followed by then the post hoc test. The data distribution is normal but not homogeneous then a nonparametric test is used. The nonparametric tests are also used in the not normally distributed data. The Kruskal Wallis test shows $p < 0.05$ then proceed with the Mann-Whitney test.

## RESULTS

**Parasitemia measurement**

The parasitemia percentage at day 3 infection of the, showed no difference among all animal groups was not different (Kruskal Wallis test, $p = 0.291$; data was not shown). The parasitemia percentage showed significant different among all groups at day 7 infection (Kruskal-Wallis $p = 0.002$). The parasitemia percentage of P2 and P3-groups was not significantly different at day 7 infection ($p = 0.21$; Table1). This indicated that the effect of the interventions of P2 and P3 groups were not different in controlling the malaria parasites. The groups of K and P1 showed the highest parasitemia percentage at 7 infection, and the differences was not found between these groups at those observed days ($p = 0.47$). This suggested that the intervention of P1-group has no effect on parasitemia percentage at 7 of malaria. The parasitemia percentage of P2 and P3-groups were significantly remarkable lower than K-group at day 7 infection. The similar findings were also observed when the P2 and P3-groups were compared to P1-group. These suggested that the treatments of P2 and P3-groups were better than that of P1-group in controlling the infection.

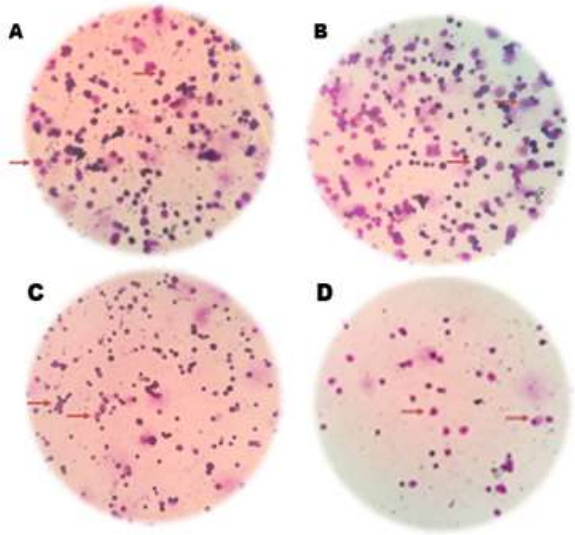
**Spleen Lymphoblast percentage**

The figure of splenic lymphoblasts from groups K, P1, P2, P3 (sorted from the left) in one field of view. Splenic lymphoblasts are indicated by arrows (**Error! Reference source not found.** 2). Lavene Statistical Test p value > 0.01 means the data is homogeneous. One-Way ANOVA test p value <0.01 means there is a significant

difference between the groups. The percentage of lymphoblasts experienced a significant decrease between groups K – P1, K – P2, K – P3, P1 – P3. The results were not different between P1 – P2 and P2 – P3 (Table 2). The lymphoblast percentage all treatment-groups (P1, P2, and P3) showed significantly lower lymphoblast percentage of the spleen. This showed that the treatments used in this study associated with the reduce of the spleen lymphoblast percentage at day 7 PbA-infection. Those received ACT in P2 or P3-group showed significantly lower lymphoblast percentage than P1-group. This demonstrated that ACT-treatment associated with the decrease spleen-lymphoblast percentage at day 7 PbA-infection. Because of no different spleen lymphoblast percentage between P2 and P3-groups, this indicate that ACT alone or its combination with AME had no different effect toward the spleen-lymphoblast percentage at day 7 PbA-infection.

**Table 1: Parasitemia percentage of the *P. berghei* ANKA infected mice**

| Group | Mean ± SD Day 7 | *p* value | | | |
|---|---|---|---|---|---|
| | | K | P1 | P2 | P3 |
| K | 19.88 ± 5.96 | | 0.47 | 0.01 | 0.01 |
| P1 | 18.40 ± 3.17 | | | 0.01 | 0.01 |
| P2 | 0.55 ± 0.13 | | | | 0.21 |
| P3 | 0.41 ± 0.15 | | | | |




*A.Represent the K-group; B. Represent the P1-group; C. Represent the P2-group, and D. Represent the P3-group.*

**Fig 1: Lymphoblast in the spleen of each mouse-group. Lymphoblast pointed with red arrow.**

**Table 2: Lymphoblast percentage statistical analyzes**

| Group | Mean ±SD % | *p* value | | | |
|---|---|---|---|---|---|
| | | Control | P1 | P2 | P3 |
| K | 26.70 ± 4.43 | | 0.0073 | 0.0001 | 0.00001 |
| P1 | 17.38 ± 7.60 | | | 0.0564 | 0.0051 |
| P2 | 11.14 ± 3.69 | | | | 0.2532 |
| P3 | 7.54 ± 0.98 | | | | |

## DISCUSSION

The AME-treatment alone, associated with a significantly lower spleen-lymphoblast percentage (Table 2). This treatment also showed no association with ability to control malaria infection since the parasitemia percentage of those receive AME was not different than control without any treatment (Table 1). Notably, spleen-produced IL-

10 in AME-treated mice surpassed that in the untreated control group.[23, 24] IL-10 is produced by various cell types, including T-reg cells known for suppressing immune responses. Hence, further investigation is required to determine the predominant IL-10-producing cells in this study and ascertain whether they contribute to the survival and spleen immune responses. The cell-type of lymphoblast affected by severe malaria is the TNF receptor II-positive regulatory T (Treg) cells. It was found that a subset of TNFRII+ Treg cells with high expression of Foxp3 was increased in severe relative to uncomplicated malaria.[25] This indicates that TNFRII+ Treg cells are specifically affected by severe malaria. Additionally, implicated lymphocytes in the immunity and pathogenesis of severe malaria, further supporting the involvement of lymphocytes in severe malaria.[26] The spleen plays a crucial role in the immune response during malaria infection, particularly in the erythrocytic stages. It is involved in the development of the immune response and the elimination of parasitized red blood cells (pRBCs).[27] However, during malaria infection, the anatomy of the spleen becomes disorganized, leading to the dissolution of the marginal zone (MZ) and apoptosis of MZ B cells.[28] This disorganization may contribute to the suppression of lymphoblast formation in the spleen during malaria infection. Furthermore, blood stage infection induces dendritic cells (DCs) to suppress CD8+ T cell responses, which may also impact lymphoblast formation in the spleen.[29] The spleen's role in the clearance of damaged RBCs is compromised during malaria infection, potentially reducing the ability of macrophages to control bacterial replication.[30] Additionally, the closed state of splenic circulation protects the spleen from malaria parasites, resulting in lower numbers of activated macrophages and affecting erythropoiesis and lymphopoiesis.[31] The spleen's pathology in human malaria is not well understood, but it is known to contribute to innate resistance and limit the magnitude of parasitemia.[32] Moreover, the spleen senses infected RBCs and is an important organ in the immune response development during malaria infection.[33] Malaria parasites are also known to suppress host immune responses to facilitate their survival, although the underlying mechanisms remain elusive.[34] All of these indicate that the spleen's disorganization, compromised clearance function, and suppression of immune responses during malaria infection likely contribute to the suppression of lymphoblast formation in the spleen. These factors collectively impact the spleen's ability to mount an effective immune response and may lead to the dysregulation of lymphoblast formation.

The role of immature dendritic cells in contributing to lymphoblast formation during severe malaria is a complex process that involves the interaction between *P. falciparum* and the host immune system. Several studies have highlighted the impact of malaria infection on dendritic cell function. For instance, it has been observed that immature dendritic cells may suffer a maturation defect following interaction with erythrocytes infected with malaria parasites, leading to an inability to induce protective malaria liver-stage immunity.[35] Additionally, it has been suggested that intact malaria-infected erythrocytes can adhere to dendritic cells, inhibiting their maturation and subsequently reducing their capacity to stimulate T cells.[36] Furthermore, the frequency of certain dendritic cell subsets, such as BDCA3-positive dendritic cells, has been found to be increased in the peripheral circulation of children with severe malaria, indicating a potential role in the pathogenesis of the disease.[37] Moreover, the interaction between dendritic cells and malaria parasites has been shown to modulate the immune response, with blood stage infection inducing dendritic cells to suppress CD8+ T cell responses.[29] This suppression of T cell responses has been proposed to be mediated by the induction of regulatory T cells, contributing to immune evasion by the malaria parasites.[38] Additionally, malaria infection has been found to impair T cell clustering and immune priming despite normal signal 1 from dendritic cells, affecting immune responsiveness during the course of the infection.[39] Furthermore, the impact of malaria infection on dendritic cell function extends to the innate immune response, with hemozoin, a byproduct of malaria parasites, inhibiting the differentiation and maturation of human monocyte-derived dendritic cells, possibly contributing to severe immunodepression during acute and chronic *P. falciparum* malaria.[40] Additionally, toll-like receptor 9 has been implicated in mediating innate immune activation by hemozoin, linking the activation of dendritic cells to the immune response to malaria parasites and their metabolites.[41] In summary, the interaction between immature dendritic cells and *P. falciparum* infection is a multifaceted process that involves modulation of dendritic cell function, suppression of T cell responses, and potential immune evasion mechanisms. Understanding the intricate interplay between dendritic cells and malaria parasites is crucial for elucidating the pathogenesis of severe malaria and developing targeted interventions.

The parasitemia and splenic lymphoblast percentage of the mice study was not significantly different between those receive combination AME-ACT and ACT alone. The mice in those groups were enter recovery phase of PbA-infection. This suggested that the reduce lymphoblast formation associated with the recovery of those receive the combination AME-ACT or ACT alone. Although, the additional effect of AME as adjuvant therapy upon those receive ACT was not evident based on malaria parasite control. The inhibition effect of AME toward splenic lymphocyte formation was not interfere malaria control in those receive ACT. Other beneficial effect of AME remained to be explored in malaria treated ACT. AME intervention increases spleen production of IL-10 known as a protective cytokine against immunopathology developed in malaria.[23, 24] ACT increases IL-10 production of splenocytes isolated from PbA-infected mice during recovery phase.[42] The combination ACT and certain herbs such as *Strychnos ligustrina*

restricts the increase spleen IL-10 production.[42] IL-10 has two contradictory effect, the first is that inhibits the present of immunopathology during malaria, and the second is that interferes the parasite clearance.[43, 44] Recent study shows that IL-10 supports the generation of protective antibody against the blood stage of malaria.[45] This process is rapid and transient. Although the better effect AME-ACT than ACT was not evident in recent study. The beneficial combination of AME-ACT effect in detail at molecular protective effect contributed in the multiorgan dysfunction warrants to be studied. The use of active compound of AME remains an interest to be further study.

## CONCLUSION

*A. muricata* has better effect on the percentage of parasitemia and splenic lymphoblasts of malaria mice treated with ACT than those without ACT.

## ACKNOWLEDGMENTS

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