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Research

Enhanced Malaria Survival: The Synergistic Effect of Aqueous Extracted *A. muricata Leaves* with ACT

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Abstract

Background: Combination therapy is essential for safeguarding both current and future antimalarial medications. Some argue that managing cerebral malaria necessitates adjunctive therapy. The efficacy of *Annona muricata* as an adjunct to Artemisinin-based-combination-therapy (ACT) remains uncertain, with evaluation based on host-survival in malaria.
Objective: To demonstrate the impact of aqueous extracted *A. muricata* leaf (AEAML) on the host-survival and parasite load in ACT-treated-malaria.
Method: A post-test-only-control-group-design-study was conducted and used 48-Swiss-mice which divided into four-groups. The K-group received water, whereas the P1-group was administered with AEAML. The P2-group received ACT, while the P3-group received an AEAML-ACT-combination. All mice were infected with 10^7 *Plasmodium berghei* ANKA (PbA). Parasitemia and survival were monitored.
Results: The lowest-parasitemia-percentages of the P3 and P2-groups was no significant-different at day-5,7,9,17-PbA-infection ($p>0.05$). Conversely, both P1 and K-groups displayed the highest-parasitemia-percentages at these time-points, with no notable differences between them ($p>0.05$). From day-13-infection onward, the number of surviving-mice in P1-group significantly differed from that in K-group, marked by a notable decrease in K compared to P1-group. The survival-rate in P1-group sharply-declined in comparison to P2-group. By the end of the study, 11 mice in P2 and 10 in P3 survived. Notably, the mean-survival-time was higher in groups-P2 and P3, contrasting with the lower in groups-K and P1. Significantly differing outcomes among the four test groups were confirmed by the log-rank test (Mantel-Cox), with a p-value of 0.0001.
Conclusion: *Annona muricata* demonstrates a more significant impact on both the survival-rate and parasite-load of ACT-treated-malaria compared to those not receiving ACT.

Keywords: *Annona muricata*, ACT, survival, parasitemia, *Plasmodium*.

INTRODUCTION

According to the World Health Organization's 2021 report, nearly half of the global population was at risk of malaria, with about 247 million cases and 619,000 deaths that year.[1] In response, Indonesia has been working with international organizations, including WHO, to combat malaria.[2] A significant global concern is the resistance to antimalarial drugs, especially artemisinin-based combination therapies (ACTs).[3-5] The spread of these resistant strains could undermine malaria control efforts, leading to more severe and prolonged infections. Research continues to seek more effective treatments, such as triple artemisinin-based combination therapies (TACTs), which have shown effectiveness even in areas with multidrug-resistant malaria.[6] Despite recommendations, TACTs face challenges in implementation due to higher costs and logistical issues, particularly in resource-limited and remote areas. Traditional herbal remedies offer potential benefits against ACT resistance, but require rigorous validation and regulation to ensure safety and efficacy. Collaborative efforts with healthcare providers and local communities are essential to maximize benefits and minimize risks. The aqueous leaf extract of *Annona muricata* (AEAML) shows significant antimalarial activity against *Plasmodium berghei* in infected mice.[7] Additionally, the treated mice had prolonged survival times, and the extract was found to be non-toxic up to a dose of 4000 mg/kg, suggesting the potential for developing new antimalarial drugs. Similarly, the ethanolic leaf extract of *Annona muricata* (EEAML) exhibits significant antimalarial activity, demonstrated through both in vivo and in silico approaches.[8] This extract showed a notable reduction in parasitemia levels and improvements in liver biochemical parameters in treated mice. Its effectiveness is attributed to its hypolipidemic effect, which deprives the malaria parasite of essential lipid molecules, and the inhibitory action of compounds like luteolin and apigenin on proteins crucial for the parasite's metabolic pathway. EEAML intervention enhances the production capacity of splenocyte-interleukin (IL)-10 in Swiss mice severely infected with *Plasmodium berghei* ANKA (PbA), an experimental model of malaria.[9, 10] IL-10, an anti-inflammatory cytokine, helps prevent the development of immunopathology and reduces mortality in severe malaria cases.[11, 12] Significantly, increased concentrations of CXCL10 have been observed in both the plasma and saliva of individuals suffering from severe malaria, highlighting its significance in the context of malaria infection.[13, 14] Treatment which reduces CXCL10 levels, in combination with artemether significantly reduced systemic and brain inflammation, increased survival rates in mice with experimental cerebral malaria, and improved outcomes compared to artemether alone.[15] These findings suggest that adjunctively targeting CXCL10 during anti-malarial therapy could offer a promising strategy to enhance survival and mitigate CM-related mortality. Interestingly, AEAML administration modulates CXCL10 expression in the brain, particularly when combined with standard antimalarial ACT, leading to improved recovery and reduced parasitemia.[16] However, it remains unknown whether AEAML enhances the effectiveness of ACT to increase survival time alongside better malaria parasite control.

MATERIAL AND METHOD

Research design and experimental animal

This study was post-test only control group 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, and this period included in the 30 observation-days for survival study. The forty-eight 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 ethical clearance of this study was given by ethical committee of Faculty of medicine Diponegoro University and Dr. Kariadi Hospital (Survival study: No. 642/EC/FK-RSDK/2016).

Annona muricata extract and anti-malaria

The leaves of *Annona muricata* (AM) were extracted using water, resulting in an aqueous extract (AEAML), which was processed and analyzed for free by Sido Muncul Company. The treatment involved AEAML combined with an artemisinin-based combination therapy. Preventive dose of AEAML was administered over a 10-day period, starting 7 days before and for 3 days after the *Plasmodium berghei* ANKA (PbA) inoculation. The preventive dose of AEAML was 4.68 mg per day for each mouse weighing 30 grams. This was followed by a therapeutic dose of 9.36 mg per day, starting from the 4th day of confirmed PbA infection. Additionally, ACT (Dihydroartemisinin 40 mg and Piperaquine Phosphate 320 mg; DHP-FRIMAL; PT Mersifarma TM, Sukabumi, Indonesia) at a dose of 0.819 mg per day was administered from the 4th day of PbA infection.

Parasite and infection dose used

The Parasitology Department of Universitas Gajah Mada provided *Plasmodium berghei* ANKA (PbA). Three Swiss mice inoculated with PbA served as donor mice. Blood was collected from these donor mice once their parasitemia levels reached 15-20%. The dose of PbA used for infection was 10^7 parasitized red blood cells (pRBC) in 0.2 ml of sterile physiological NaCl.

Parasitemia measurement

The parasitemia level was monitored using a light microscope to examine thin blood smears. This level was determined by calculating the percentage of infected red blood cells (iRBCs) out of a total of 1000 red blood cells, including both infected and uninfected cells. Parasitemia percentage was measured on specific days: 3, 5, 7, 9, and 17 following PbA infection.

Statistical analyzes used

Survival analysis used the Kaplan-Meier curve. Descriptive analysis displayed the mean and standard deviation values for parasitemia. 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 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

Experimental animal survival analyzes

The Kaplan-Meier curve, illustrated in Figure 1 and detailed in Table 1 (Chi-Square $p = 0.001$), effectively captures the distinction between the K-group and P1-group. Notably, both the K-group and P1-group exhibited a pronounced decline, indicating a noteworthy decrease in survival. Intriguingly, the curve reveals an extended lifespan for the P1-group compared to the control group from day-13-infection (day-20-observation shown at Fig1), with all mice succumbing to their respective conditions during the period 30 and 29 days (Fig1). Furthermore, a comparative analysis of the curve showcases a sharp decline in the P1-group when contrasted with P2-group, signifying a longer duration of survival for the P2-group. All mice of the P1 group were died during the period of 30 observation-days, while only one mouse in the P2 group met this fate on day 18. Notably, the P3 group exhibited two fatalities on days 8 and 11, but thereafter, no additional deaths occurred up to day 30. Most of the mice in P2 and P3-groups were survived until day 30 which was the end period of this study. These findings underscore the distinct survival trajectories among the groups, providing valuable insights into the varied outcomes of the experimental conditions. The survival study, however, indicated that there was no clear evidence of the beneficial effect of AEAML in individuals receiving ACT during malaria treatment.

Parasitemia measurement

The parasitemia percentage on day 3 of the infection survival study showed no significant difference among all animal groups (Kruskal-Wallis-test, $p = 0.291$) (Table 2). However, significant differences were observed among the groups on days 5 and 7 (one-way ANOVA test, $p = 0.0001$ and Kruskal-Wallis-test, $p = 0.0001$, respectively). The parasitemia percentages in the P2 and P3 groups were not significantly different from each other on days 5 and 7 (Bonferroni post hoc test, $p = 1.000$ and Mann-Whitney test, $p = 1.000$, respectively), and these findings were consistent on days 9 and 17 (Table 2). This indicates that the interventions in the P2 and P3 groups were similarly effective in controlling malaria parasites. The K and P1 groups had the highest parasitemia percentages on days 5 and 7, with no significant difference between these groups on those days (Bonferroni post hoc test, $p = 1.000$ and Mann-Whitney test, $p = 1.000$, respectively), a pattern that was continued on day 9. This suggests that the P1 group intervention had no effect on parasitemia percentages on days 5, 7, or 9 of malaria infection. The parasitemia percentages in the P2 and P3 groups were significantly lower than in the K group on days 5, 7, and 9. Similar results were observed when comparing the P2 and P3 groups to the P1 group. These findings suggest that the treatments in the P2 and P3 groups were more effective than the P1 group in controlling the infection.

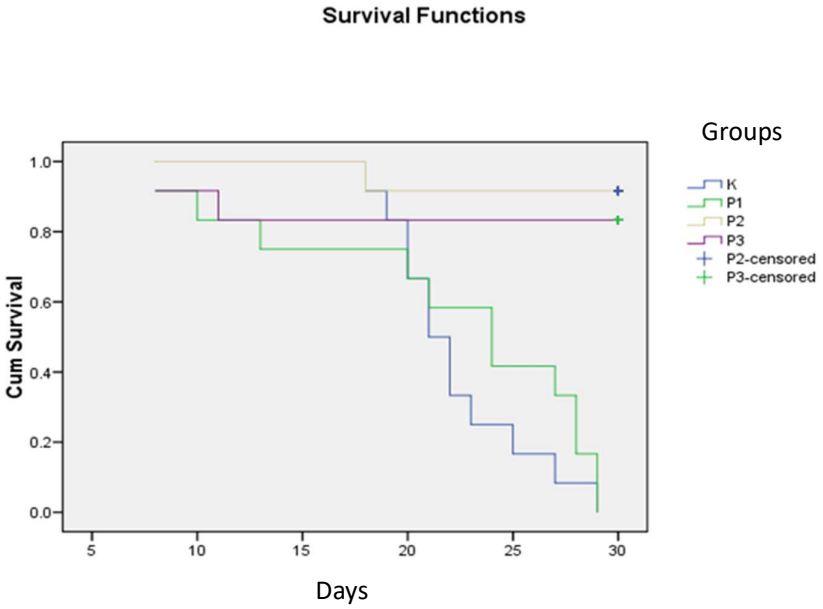

Fig 1: Kapplan Meier Curve

Table 1: Survival Time of *Plasmodium berghei* ANKA infected Swiss mice

Group	Treatment	Survival Time (Mean ± SD days)
K	No treatment	22.25 ± 3.28
P1	AEAML	21.75 ± 7.56
P2	ACT	29.00 ± 3.46
P3	AEAML + ACT	26 ± 8.01

Table 1: Parasitemia percentage and Statistical Analyzes

	Mean (%)					*p* Value				
Group	Day 3	Day 5	Day 7	Day 9	Day17	Day 3	Day 5	Day 7	Day 9	Day17
K	4.04	19.21	24.5	26.7	died	0.291	0.04	0.01	0.00	died
P1	2.93	15.20	19.3	19.1	died		0.04	0.01	0.00	died
P2	2.88	3.38	0.26	0.31	0.136		1.00	0.62	1.00	0.76
P3	1.56	3.17	0.25	0.35	0.130					

The *p* values were for comparing P3-group with other group in each time point.

DISCUSSION

The Kaplan-Meier curve illustrated longer lifespan of the mice treated with AEAML-treatment alone (P1-group) than those controls without any treatment (K-group) after day 20 (Fig.1). This might be explained by the increase splenocyte-IL-10 production of malaria mice model treated EEAML.[9, 10] Interleukin-10's capacity to counteract haemozoin-induced pro-inflammatory effects could potentially prevent tissue damage due to immunopathology.[17] These therefore, studies are needed to determine whether IL-10 produced by those treated with AEAML is sufficient to protect the mice toward the malaria immunopathology. Fatal malaria cases occur in those with hyperparasitemia patients.[18] IL-10 contributes in the reduce ability to control malaria parasite load. The transcription factor basic helix-loop-helix family member e40 (Bhlhe40) controls *Plasmodium yoelii* infection by regulating IL-10 and IFN-γ levels in CD4+ T cells, and its loss leads to higher IL-10, increased parasitemia, delayed clearance, and compromised immune response.[19] The mean of parasitemia percentage of those receive AEAML were lower than controls, and these were observed day 5, 7, 9 PbA-infection (Table 2). This in somehow explained by the finding is that the levels of IL-10 correlate with parasite load of malaria patients. Those malaria patients have a high IL-10 and specific-MSP3-IgG levels.[20] This is noted in children with asymptomatic malaria (AM) and mild malaria (MM). The malaria mouse model demonstrated a decrease in parasitemia in mice treated with Human Immune IgG and anti-MSP3-antibody.[21] In addition, there is a relationship between antibody

responses and parasitemia, which depends on IgG anti-MSP3 in children and IgG anti-MSP1p19 in adults living in urban areas of endemic regions.[22] Recent study shows that the absence of antibodies to locally expressed PfEMP1 types is associated with severe malaria and may partially explain the severity of malaria in Papuan adults.[23] Whether AEAML intervention is able to increase level of protective antibody contribute to lower parasitemia level in PbA-infected mice, warrant to be further study. Immunity against malaria relies on germinal center (GC)-derived antibody responses orchestrated by T follicular helper (TFH) cells. IL-10 produce by T follicular helper (TFH) cells and acts in B cells within the first 96 hours of malaria infection to support humoral immunity.[24] This rapid, transient IL-10 provision promotes B cell survival and interaction with CD4 T cells. IL-10 also restores the compromised function of monocytes and macrophages linked to malaria infection.[17] Whether AEAML involve in the increase B cells and monocyte-macrophage function need to be elucidated.

The Kaplan-Meier curve showed that mice treated only with AEAML (P1 group) had a shorter lifespan compared to the untreated control group (K group) before day 20 (Fig. 1). This might be related to the present of malaria-associated acute respiratory distress syndrome (MA-ARDS) due to the increase IL-10 level in AEAML-treated PbA-infected mice. The host microbiota influences the development of MA-ARDS and mortality, with IL-10 playing a crucial role.[25] Parasite sequestration in the lungs leads to prolonged immune activation and IL-10 production by T cells. Although IL-10 aims to limit tissue damage, it compromises microbial control and results in severe lung disease. Treatment with the antibiotic linezolid prevents MA-ARDS-related deaths by clearing bacterial infections. These findings underscore IL-10's pivotal role in MA-ARDS, highlighting the need to balance its anti-inflammatory effects with effective microbial control to manage severe respiratory complications in malaria. A comparative analysis reveals a sharp decline in the AEAML treated group, whereas those receiving standard ACT or the AEAML-ACT combination show longer survival durations. Songga wood extract (SWE) intervention is associated with increase IL-10 produced by spleen of mice on day 7 PbA-infection.[26] Spleen-IL-10-production increase in the recovery phase of ACT treated malaria mouse model, and the SWE associated with reduce IL-10 production by ACT treated mice. It is necessary to study whether AEAML intervention induces MA-ARDS through increased IL-10 during uncontrolled PbA infection, and whether the AEAML-ACT combination can prevent MA-ARDS in an IL-10-dependent manner. The superior effect of the AEAML-ACT combination over AEAML alone was evident in terms of reduced parasite load and improved survival rates. However, this significant difference was not observed when comparing the AEAML-ACT combination to ACT alone. Therefore, it is essential to investigate other protective biomarkers in this study. It can be concluded that *Annona muricata* significantly enhances both survival rates and parasite load reduction in ACT-treated malaria compared to those not receiving ACT.

CONCLUSION

Annona muricata demonstrates a more significant impact on both the survival-rate and parasite-load of ACT-treated-malaria compared to those not receiving ACT.

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