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Original Research Article

Malaricidal efficacy of the ethyl acetate fraction of *Syzygium cumini* fruit on spleen index, hemoglobin levels and mean survival time in mice (*Mus musculus*) infected with *Plasmodium berghei*

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Abstract

Purpose: *To determine the effectiveness of ethyl acetate fraction (EAF) of Syzygium cumini (Juwet fruit) on spleen index, hemoglobin levels and mean survival time of mice (Mus musculus) infected with Plasmodium berghei.*

Methods: *A total of 60 mice were divided into 6 treatment groups of 10 mice each, namely N (not infected), K- (infected and untreated), K+ (infected, treated with 25 mg/kg chloroquine), P1 (infected, treated with 200 mg/kg ethyl acetate fraction), P2 (infected, treated with 400 mg/kg ethyl acetate fraction) and P3 (infected, treated with 600 mg/kg ethyl acetate fraction). The mice were each infected with 0.2 mL of 1 x 10^6 parasitized red blood cells (PRBCs) of P. berghei. Treatment was administered for 9 days after which spleen indices, hemoglobin levels and mean survival time were assessed.*

Results: *The results showed significant difference in spleen index ($p < 0.05$) between N, K+, and P2. However, no significant difference ($p > 0.05$) was observed between K-, K+, P1 and P3, as well as P2, K-, P1 and P3. The hemoglobin levels significantly differed ($p < 0.05$) among N, K- and K+. There was also no significant difference ($p > 0.05$) in the hemoglobin levels between P1, K+, P2 and P3 as well as P2, K-, P1 and P3. Furthermore, there was a significant difference ($p < 0.05$) in the mean survival time between N, K- and P3, but no significant differences ($p > 0.05$) between K-, P1, P2 and P3, as well as N, K+, P1 and P2.*

Conclusion: *The ethyl acetate fraction of S. cumini fruit neither reduce the spleen index nor increase the mean survival time of mice infected with P. berghei. However, the administration-maintains hemoglobin levels within the normal range of infected mice.*

Keywords: *Ethyl acetate fraction, Hemoglobin, Spleen index, Malaria infection, Mean survival time, Syzygium cumini*

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INTRODUCTION

Malaria is an infectious disease and a health problem throughout the world, especially in many parts of Africa and Southeast Asia [1]. The clinical symptoms depend on the immunity of the patient and the high transmission of the infection. Malaria has 3 main symptoms, namely paroxysmal fever, anemia and splenomegaly [2].

The spleen is a complex secondary lymphoid organ that filters and disposes damaged and malaria-infected erythrocytes and infectious microorganisms [3]. Splenomegaly is caused by a large number of infected erythrocytes, lymphocytes and macrophages deposited in the organ. Uninfected erythrocytes are removed due to increased macrophage activity during malaria infection and this process leads to anemia. Anemia arising from a malarial infection depends on the severity of the disease and the duration before medical intervention. The World Health Organization (WHO) recommends anemia as an additional indicator in determining the severity of malaria and measuring hemoglobin levels [1].

Resistance of plasmodium parasite to several antimalarial drugs, such as artemisinin, chloroquine and artesunate-mefloquine combination, results in treatment difficulty, resulting in poorly treated malaria and sometimes death [3]. Several studies using plants and fruits as antimalarial drugs have been carried out [4-7]. Juwet fruit (*Syzygium cumini*) has been used as an antimalarial drug because the extract reduces parasitemia, increases parasite growth inhibition, enhances PCT (Parasite Clearance Time) and extends parasites' recrudescence time [4]. *Plasmodium* spp. infection leads to increased ROS (Reactive Oxygen Species) production due to host neutrophil activation and hemoglobin degradation. Therefore, external or exogenous antioxidants are required due to decrease in their levels *in vivo*. Exogenous antioxidants are obtained from natural ingredients, such as anthocyanins and flavonoids from *Syzygium cumini* [5]. Flavonoid compounds also have the potential to function as antimalarials. In this context, ethyl acetate is a semipolar solvent used to extract secondary metabolites such as flavonoids [6]. This fraction of *Syzygium cumini* has a strong antioxidant potential as reported by the radical scavenger ability with an IC_{50} value of 13.62 µg/mL [7]. The research aimed to analyze the impact of the ethyl acetate fraction of *Syzygium cumini* on spleen index, hemoglobin levels, and Mean Survival Time (MST) in mice (*Mus musculus*) infected with *Plasmodium berghei*.

EXPERIMENTAL

Parasites, animals, drugs, and materials used

Plasmodium berghei ANKA strain was obtained from the Tropical Disease Center (TDC), Universitas Airlangga. The experimental male mice were 2.5 months old, weighing between 20 - 30 g and were obtained from the Surabaya Veterinary Farma Center (Pusvetma). The mice were housed in a box made from wire mesh and were allowed free access to food and water *ad libitum*, in line with welfare standards. Chloroquine, obtained from Sigma, was used as the pro-analytical compound, and the dose administered was 25 mg/kg. *Syzygium cumini*, sourced from Lumajang, East Java, was authenticated by the Herbal Laboratory, Materia Medica Batu.

Ethical approval

Ethical approval for the study was obtained from the Animal Ethics Committees of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia (approval no. 2.KE.039.05.2020) and the study complied with international guidelines for animal studies.

Plasmodium berghei infection

Mice were infected with erythrocytes containing *Plasmodium berghei* from donor blood. The infection was carried out by intraperitoneally injecting 0.2 mL of blood containing 1 x 10^6 parasites. Examination of parasites in the blood was done using thin smears 24 hours after infection. Subsequently, the blood smear from the caudal vein was fixed with methanol and stained using 25 % Giemsa solution for 30 minutes before rinsing in running water. Observation of the blood smear was conducted under a light microscope (Olympus CX21 Tokyo, Japan) with 400x magnification using oil immersion.

Extraction of ethyl acetate fraction of *Syzygium cumini*

Syzygium cumini fruits were cleaned with running water to remove dirt. They were dried in an open space safe from direct sunlight and ground into a fine powder. Thereafter, 1,000 g of the fine powder was macerated in 7500 mL ethanol (96 %) for 24 h and the process was repeated three times. The solvent was filtered every 24 h to produce a clear filtrate which was filtered using a Buchner funnel, concentrated with a rotary evaporator bath at 50 ^oC and freeze-dried to obtain a concentrated ethanol extract. The ethyl acetate fraction was prepared by a multilevel fractionation method using ethyl acetate solvent. For this, 100 g of the concentrated extract of *Syzygium cumini* was put into a separating funnel and 200 mL each of distilled water and ethyl acetate were added. After shaking, the ethyl acetate layer was removed and concentrated.

Treatment of animals

The experimental animals were divided into 6 groups, namely: Control (K-) group made up of mice infected with *P. berghei* and given aquadest drug solvent, a normal control (N) comprising mice not infected with *P. berghei* but given only aquadest drug solvent, a control (K+) group infected with *P. berghei* (1×10^6) parasitized red blood cells (PRBCs) and treated orally with 25 mg/kg BW of chloroquine, a treatment (P1) group composed of *P. berghei*-infected mice and treated orally with 200 mg/kg BW EAF, a second (P2) and third (P3) treatment groups made up of mice infected with *P. berghei* and treated orally with EAF of *Syzygium cumini* fruit extract at 400 and 600 mg/kg BW, respectively.

Blood sampling, necropsy, and organ sampling

After the 9th day of treatment, the experimental animals were anesthetized with ketamine at a dose of 40 mg/kg intramuscularly. Blood was withdrawn intracardially to evaluate hemoglobin levels and the mice were necropsied to take samples of spleen before calculating the index.

Spleen index measurement

The spleen obtained from the animals was directly weighed with digital scales. The length and width were measured with vernier calipers and recorded for each group to calculate the index.

Calculation of mean survival time

Mice fatality was observed daily and recorded to calculate Mean Survival Time (MST) on the 9th day. Subsequently, the MST for each group was calculated.

Testing hemoglobin levels

Blood was taken intracardially with a 1 mL syringe after anesthetizing the mice. The blood was dispensed in an EDTA tube and examined using a hematology analyzer.

Data analysis

Data analyses were performed using SPSS 25.0 statistical software. The differences in Spleen index, hemoglobin level and MST were examined using the Analysis of Variants (ANOVA) and Duncan's Multiple Range Test. Values of p less than 0.05 were considered statistically significant.

RESULTS

Spleen index

The highest and lowest spleen indexes were seen in the P2 treatment and N groups, respectively. The results show a significant difference ($p < 0.05$) in the average spleen index between N group and other groups. Meanwhile, no significant difference ($p > 0.05$) between treatment groups K-, K+, P1, P2 and P3 was observed. The results of the spleen indices are presented in Table 1.

Hemoglobin levels

The highest and lowest hemoglobin levels were seen in N and K- groups, respectively. Hemoglobin levels in N group were significantly different ($p < 0.05$) from all other groups. Furthermore, K- treatment group was significantly different from N, K+, P1 and P3 ($p < 0.05$), but not significantly different from P2 ($p > 0.05$). Meanwhile, K+ treatment group was significantly different from N, K- and P2 ($p < 0.05$), and showed no difference with P1 and P3 ($p > 0.05$). The comparison of P1, P2 and P3 groups reported no significant differences ($p > 0.05$) in the hemoglobin levels as seen in Table 2.

Mean survival time (MST)

Calculations of the percentage of live mice and MST were obtained from mortality data observed and recorded daily. The highest mortality was seen in P3 while N and K+ had the lowest. The highest MST was in N and K+ treatment groups while the lowest was in K-. With respect to MST, the N group was significantly different from K- and P3 ($p < 0.05$), but not significantly different from K+, P1 and P2 ($p > 0.05$). Control (K-) treatment group showed significant differences compared to N and K+ treatment ($p < 0.05$), but not no significant difference when compared to P1, P2, and P3 ($p > 0.05$). However, K+ treatment group was significantly different from K- and P3 ($p < 0.05$), but no significant difference was seen when compared with N, P1 and P2 ($p > 0.05$). Furthermore, no significant differences were observed between P1, P2 and P3 treatment groups ($p > 0.05$; Table 3).

DISCUSSION

The spleens of mice in normal groups, as well as those treated with ethyl acetate fraction of *Syzygium cumini* and chloroquine, experienced splenomegaly.

Table 1: Spleen index of control and treatment group mice infected with *P. berghei* for 9 days

Group	Mean body weight (g)	Mean spleen weight (g)	Spleen size		Spleen color	Mean spleen index
			Mean length (cm)	*Mean width (cm)*		
N	25.94±0.96	0.11±0.04	1.57±0.39	0.50±0.07	Fresh red	0.43±0.16[a]
K-	35.15±0.92	0.54±0.11	2.94±0.37	0.90±0.09	Dark brown	1.53±0.33[bc]
K+	32.95±0.55	0.41±0.13	2.77±0.47	0.86±0.11	Fresh red	1.25±0.38[b]
P1	30.17±0.57	0.56±0.10	2.91±0.27	0.92±0.11	Black	1.87±0.45[bc]
P2	27.97±0.89	0.54±0.11	3.01±0.28	0.91±0.13	Dark brown	1.95±0.47[c]
P3	28.21±0.63	0.52±0.22	2.81±0.51	0.88±0.10	Black	1.83±0.75[bc]

Note: Different superscripts ([a, b, c]) in the same column indicate significant differences $p \leq 0.05$. (N) not infected, (K-) infected untreated, (K^+) infected and treated 25 mg/kg chloroquine, (P1) infected and treated 200 mg/kg EAF, (P2) infected and treated 400 mg/kg EAF, (P3) infected and treated 600 mg/kg EAF

Table 2: Hemoglobin levels of mice infected with *P. berghei*

Group	Hemoglobin levels (g/dL)
N	14.34±0.32[d]
K-	11.68±0.64[a]
K+	13.15±0.64[c]
P1	12.92±0.92[bc]
P2	12.38±0.13[ab]
P3	13.05±0.39[bc]

Note: (a, b, c) different superscripts in the same column show significant differences $p \leq 0.05$. (N) not infected, (K-) infected untreated, (K^+) infected and treated 25 mg/kg chloroquine, (P1) infected and treated 200 mg/kg EAF, (P2) infected and treated 400 mg/kg EAF, (P3) infected and treated 600 mg/kg EAF

Table 3: Mean survival time in control and treatment groups infected with *Plasmodium berghei* for 9 days

Group	Live mice presentation (%)	Mean survival time (days)
N	100 (10/10)	9.00±0.00[b]
K-	60 (6/10)	8.20±1.03[a]
K+	100 (10/10)	9.00±0.00[b]
P1	60 (6/10)	8.60±0.52[ab]
P2	40 (4/10)	8.70±0.67[ab]
P3	20 (2/10)	8.30±0.82[a]

Note: (a, b) different superscripts in the same column show significant differences $p \leq 0.05$. (N) not infected, (K-) infected untreated, (K^+) infected and treated 25 mg/kg chloroquine, (P1) infected and treated 200 mg/kg EAF, (P2) infected and treated 400 mg/kg EAF, (P3) infected and treated 600 mg/kg EAF

However, mice infected with *P. berghei* and treated with chloroquine had a lower splenic index compared to those infected with *P. berghei* without treatment and ethyl acetate fraction of *Syzygium cumini*. This suggests that splenomegaly in malaria sufferers is related to the white and red pulp area expansion as a result of larger follicle size caused by hematopoietic reaction. Meanwhile, the increase in macrophages occurs because of erythrophagocytosis. Macrophages in the spleen red pulp play an important role in removing *Plasmodium*-infected erythrocytes from blood circulation [8]. The spleen induces several pro-inflammatory cytokines such as interleukin (IL)-1β, IL6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, as well as nitric oxide (NO) synthesis. These substances play a role in eliminating parasites [9]. Expansion of the pulp area is the result of spleen cell hyperplasia caused by increased work of the spleen to eliminate infected erythrocytes. In this context, hyperplasia occurs until the causative factors disappear. Splenomegaly decreases and is reversible after reducing parasitemia through the process of apoptosis [10].

The administration of chloroquine therapy to positive controls reduces the weight, length, width and index of spleen. In malaria, spleen functions directly in destroying parasites contained in the erythrocytes. The organ also induces several pro-inflammatory cytokines to eliminate parasites in the body. Chloroquine acts through the inhibition of nucleic acids, nucleoproteins, as well as DNA and RNA polymerase enzymes in the parasites. Activity of enzyme plasmepsin is also inhibited to prevent the conversion of heme into hemozoin. Increased free heme in erythrocytes is toxic to parasites, causing lysis of membrane and triggering ROS to inhibit parasite metabolism. Chloroquine also affects parasite's protease enzyme used to digest hemoglobin. Food vacuole of the parasite is acidic in the presence of chloroquine which combines with proteins to cause alkalization of organelles.

This situation allows the parasite to digest erythrocytes needed for metabolism [11]. A decrease in infected erythrocytes on chloroquine therapy subjects cells to remodeling. Therefore, spleen index and hemoglobin levels are decreased due to absence of hemolysis in erythrocytes. Treatment with ethyl acetate fraction of *S. cumini* at doses of 200, 400 and 600 mg/kg BW showed the same effect as chloroquine on decreasing spleen index. This can be attributed to flavonoids in ethyl acetate fraction of *Syzygium cumini* with antimalarial activity. Flavonoids inhibit parasite growth by reducing transport of nutrients, process of

hemoglobin degradation and heme detoxification in parasite-feeding vacuoles [12].

Hemoglobin levels after treatment with ethyl acetate fraction of *S. cumini* at doses of 200, 400 and 600 mg/kg was within the normal range. Flavonoids also exert an effect by interfering with fatty acid biosynthesis (FAS II) in parasites as well as biosynthesis of L-glutamine and myoinositol in infected erythrocytes. The target is also on functional biomolecules such as proteins, enzymes and DNA important for the survival of parasites. Flavonoids have an acidic character due to the phenolic group which is easily converted into stable phenoxy radical anions (semiquinones) in the host body causing oxidative damage to cellular components of parasite or tissue damage. This is due to interaction of irreversible covalent bonds with protein structures or DNA of the parasite [13]. The decreased parasitemia in groups treated with ethyl acetate implied maintenance of hemoglobin levels within the normal range. However, this was not followed by a significant decrease in the spleen index because of some infected erythrocytes necessitating the spleen to function more. Cell hyperplasia continues to occur because the causative factor is within the erythrocytes. Splenomegaly decreases and becomes reversible following the reduction of parasitemia through apoptosis as a defense mechanism [10]. Compensatory mechanisms continue the process of maturation and formation of blood cells in maintaining erythrocytes within normal limits. According to Maslachah *et al* [4], treatment with the leaf and stem extracts as well as the methanol fraction of *S. cumini* showed no difference from those treated with chloroquine.

Hemoglobin levels in untreated *P. berghei* infected mice was below average levels (12.4 g/dL – 14.6 g/dL). Meanwhile, mice infected with *P. berghei* and treated with chloroquine, as well as with different dose of ethyl fraction of *S. cumini* were within normal range. The decreased hemoglobin in negative control group was due to hemolysis. Elimination of infected and uninfected erythrocytes causes erythropoiesis in the host to become ineffective. This is caused by high levels of TNF-α interfering with erythropoiesis [14]. Malaria infection causes lysis of erythrocytes accompanied by release of toxins such as glycosylphosphatidylinositols (GPI), hemozoin and other parasite antigens including MSP-1, MSP-2 and RAP-1. The toxin stimulates macrophages and helper T lymphocytes to secrete pro-inflammatory cytokines, including TNF-α [15].

Also, the highest MST was in normal group and those infected with *P. berghei* before treating with chloroquine. On the other hand, the lowest MST was in mice infected without treatment. Similarly, mice infected with *P. berghei* and treated with ethyl acetate fraction of *S. cumini* had an MST of 8.60, 8.70 and 8.30 days at 200, 400, and 600 mg/kg, respectively.

Malaria has general clinical symptoms such as lethargy, malaise, headache, feeling cold, backaches as well as pain in the bones, low-grade fever, anorexia, stomach discomfort, and mild diarrhea. The typical symptoms are periodic fever, anemia and splenomegaly. Damaged and infected erythrocytes are destroyed and excreted from the blood vessels by the spleen. The work of the organ increases during infection, leading to hyperplasia and splenomegaly [16,17]. In this context, splenomegaly increases intrasplenic tension due to cell hyperplasia and swelling. Therefore, the spleen compresses the diaphragm muscle during physiological activity [18]. Pressure on the diaphragm muscle causes mice to have difficulty in breathing and suffocation which leads to death. Damaged erythrocytes are also destroyed and removed followed by the destruction of uninfected cells. This is due to increased macrophage function during malaria infection which leads to anemia, ultimately causing death in untreated malaria sufferers. Anemia experienced by malaria sufferers is hemolytic and erythrocytes are destroyed prematurely. In this condition, the spinal cord overcomes the shortage of erythrocytes by producing cells more quickly.

Treatment of infected mice with ethyl acetate fraction of *S. cumini* at 200, 400 and 600 mg/kg had the same effect on MST because the ethyl acetate fraction was unable to reduce the spleen index. The mice continued to experience splenomegaly even though hemoglobin levels in groups treated with ethyl acetate fraction showed a normal range. In addition, flavonoids within *S. cumini* were unable to increase MST compared to chloroquine. The MST of chloroquine- and EAF-treated groups were longer than the control.

CONCLUSION

Administration of ethyl acetate fraction of *S. cumini* does not reduce the spleen index and MST of mice but maintains the hemoglobin levels within the normal range. It will be necessary to carry out histopathological examination of the spleen organ, to support the results of this study, as well as toxicity tests to determine the duration and safety of administering fractions.

DECLARATIONS

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Ethical approval

Ethical approval for the study was obtained from the Animal Ethics Committees of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia (approval no. 2.KE.039.05.2020).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Arina Ilmayani and Lilik Maslachah designed the research and performed the experiments, Dewa Ketut Meles supervised the project, and Budi Setiawan and Rahmi Sugihartuti collected the data. Retno Sri Wahyuni analyzed and interpreted the data, and Kuncoro Puguh Santoso prepared the research and reviewed the draft. The authors read and approved the manuscript for publication.

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