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Thank you

Best Regard,

Dr.Lilik Maslachah., DVM
Coresponding Author,
Veterinary Pharmacy Laboratory, Departement of Basic Medicine Veterinary Medicine Faculty of Airlangga University Surabaya-Indonesia

Short description of each individual's contribution to the research and its publication, e.g. designed study, analysed data, drafted paper. (In prose style and not in a point by point manner.)

Lilik Maslachah : Research project leader and coordinating research, Designed study, analysed data and corresponding author

Thomas Valentinus Widiyatno: Examination of Parasite Clearance Time and Recrudescence Time and drafted paper

Lita Rakhma Yustinasari: Processing of blood for morphological stadium observation

Hani Plumeriastuti : Processing of blood for Transmission Electron Microscope (TEM)

Hematologic Changes and Limpha Index on Mice Model Malaria Given *Syzygium cumini* Extract as Adjuvant Therapy

Abstract

Aims: to determine the efficacy of *syzygium cumini L* as adjuvant therapy on blood changes and limpha index of mice model malaria

Materials and Methods: Mice was infected with red blood cell that contain $1x10^6$ *Plasmodium berghei* parasite in 0,2 ml intraperitoneally. Thirty five mices were divided into 7 treatment groups. Group (K0): Mice was not infected. (K1): Mice was infected. (K2): Mice was infected and given chloroquine (P1): Mice was infected and given *Syzygium cumini* leaf extract. (P2) : Mice was infected and given chloroquine and also *Syzygium cumini* leaf extract. (P3) : Mice was infected and given *Syzygium cumini* stem bark extract. (P4) : Mice was infected and given chloroquine and *Syzygium cumini* stem bark extract. Treatment was given for 4 days 24 hours after per oral infection. 21st day post infection, blood was taken from the heart for hematological examination, limpha was taken for index limpha examination, weight and length of limpha. Hematological data and limpha index were analyzed by *Analysis of Varian* test and if there is a difference, the test is continued by *Duncan Multiple Range Test* with 5% level.

Results: The hemoglobin, RBC and hematocrit K0 values are still in the normal range were significantly different ($p < 0.05$) with all groups. At K1 the values of hemoglobin, RBC and hematocrit are the lowest from the normal range and were significantly different ($p < 0.05$) compared to the other groups. MCV and MCH values showed a decreasing in the K2 group and treatment. The number of leucocytes, lymphocytes, monocytes were increasing in K1 and were significantly different ($P < 0.05$) with K2 and treatment group. The length, width, weight and index of limpha at K1 of limpha organ were significantly different ($p < 0.05$) with group K0. At K2 and treatment showed length and width of limpha were significantly different ($p < 0.05$) with K1.

Conclusion: The combination of chloroquin with leaf and stem bark extract of *Syzygium cumini* as adjuvant therapy may increase the amount of erythosit, decrease the number of leukocytes, lymphocytes and monocytes and decrease the length, width and index of limpha mice model malaria.

Keywords: Plasmodium berghei, Hematology, limpha index, Syzygium cumini

Introduction

Malaria is still a public health problem in 107 countries until right now, because malaria is still the fifth cause of infectious diseases in the world [1]. The control and treatment of malaria in recent years is more difficult because malaria parasites have been resistant to drugs and also the mosquitoes is resistant to insecticides. The development of treatment, prevention and control of malaria is one of the substantial

problems in the world. In 2012, approximately 600,000 children died, mostly from sub-Saharan Africa [2].

The cause of death due to malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly and liver dysfunction. Hematologic abnormalities during malarial infection are caused by high parasitemia in the patient, hematologic abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of limpha is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic stages of malaria infection, limpha is an important organ in the immune response and elimination of infected erythrocytes by malaria parasite (iRBC) through modulation of immune response and limpha remodeling, resulting in an immune response imbalance that can lead to severe malaria [5].

The World Health Organization (WHO) recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and ACT combination in Cambodia in clinical cases [6]. In addition, there is a decrease in efficacy in antimalarial drugs currently used, so it is important to develop adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria, adjuvant therapy used in severe malaria, among others by the use of immune system modulator preparations, antioxidants, anticoagulants and agents having antiseizure activity [7]. The results showed that the use of MMP inhibitor was able to increase the survival of mice in cerebral malaria, dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, TNFα and IL 1B [8,9,10].

Syzygium cumini has a very high antioxidant activity potential due to anthocyanin content, ellagitannin, ellegic acid, phenolic, flavonoids and vitamins. This plant is one of the many medicinal plants found in Indonesia [11,12]. Results of research conducted by Zhang et al, 2009 that *Syzygium cumini* has radical scavenging activity and strong antioxidant so that this research was aimed to determine

the efficacy of *syzygium cumini L* as adjuvant therapy on hematological changes and limpha index in mice model malaria.

Materials and Methods

Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine Airlangga University Surabaya Indonesia

Parasite, host and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium bergei* ANKA strain. The mice used were male Swiss albino mice weighing 20g -30 g 2.5 months old obtained from Veterinary Farma Surabaya (Pusvetma) Center. Chloroquine used was Chloroquine Pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used was a therapeutic dose in mice of 25 mg /Kgbw and was administered daily for 4 days [13]. The leaves and stem bark of *Syzygium cumini* are obtained from Kediri city of East Java Indonesia and identified in the laboratory of Purwodadi botanical garden Pasuruan. *Syzygium cumini* dose was 600mg /kgbw [14].

Dose of *Plasmodium berghei* infection in mice

Mice infected with red blood cells containing *Plasmodium berghei* parasites $1x10^6$ in 0.2 ml intraperitoneally. In order to find out the infection has occurred in mice, microscopic examination of erythrocyte was daily done with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [15]. Calculation of the dose of a parasitic infection was determined by counting the number of parasites from the thin blood that stained with Giemsa then calculated the number of parasites per number of erythrocytes. The next stage is the calculation of the amount of erythrocytes by blood is diluted by using PBS solution in effendorf 0.5 ml. Erythrocytes that diluted with PBS were calculated using the

Improved Neubauer Counting Chamber. The number of parasitic doses is obtained by multiplying the number of parasites with the amount of erythrocytes that have been calculated and converted to per ml.

Preparation of leaf and stem bark of *Syzygium cumini*

The leaves and stem bark of *Syzygium cumini* are dried, after that, it were crushed into soft simplicia. Extracted with PA methanol. Maseration for 3x24 hours. The filtrate was evaporated using a Rotary Evaporator at a temperature of 40-50°C with low pressure. The extraction results are stored on the desiccator until they are ready for use [16].

Treatment of the experimental animals

Thirty five mices were randomly divided into 7 treatment groups and each group consisting of 5 mices. Details of each group as follows

Group (K0): Mice was only given drug solvent, and not infected. (K1): Mice was infected and given drug solvent. (K2): Mice was infected and given 25 mg/kg body weight. (P1): Mice was infected and given 600 mg/kg body weight of *Syzygium cumini* leaf extract. (P2) : Mice was infected and given 25 mg/kg body weight and also given 600 mg/kg body weight of *Syzygium cumini* leaf extract. (P3) : Mice was infected and given 600 mg/kg body weight of *Syzygium cumini* stem bark extract. (P4) : Mice was infected and given 25 mg/kg body weight and also given 600 mg/kg body weight of *Syzygium cumini* stem bark extract.

Treatment is given for 4 days 24 hours after per oral infection. After 21 days post infection, mice was anesthetized with ketamine (sigma), then thoracotomy, blood taken from the heart (1 ml) using a tuberculin syringe inserted in a vial that has been given anticoagulation for hematologic examination with SYSMEX XT 4000i automated blood analyzer, limpha organ taken for examination of limpha index and examination of weight and length of limpha.

Examination of weight, length and index of limpha.

Previously, the mice were weighed, after that, the mice were injected with ketamine, the abdominal cavity was opened and the limpha organ was taken and weighed using an analytical scale, then measured the length and width of the limpha using a ruler with a mm scale. According to [17]. The index value of limpha (spleenic index) was calculated using the limpha Index equation = (weight of spleen organ of mice) / body weight of mice.

Data analysis

The data of hematotology observation of blood change and limpha index were processed by using Analysis of Varian (ANOVA) using SPSS System 17.0 then followed by Duncan Multiple Range Test test with 5% level.

Results

Hematological results of RBC

The results of statistical tests of mean hemoglobin (HGB), red blood cells (RBC), hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Copuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) can be seen in Table 1. The mean value of hemoglobin in the non-infected control group (K0) was normal (15.45 g / dl; the normal range is 13.4-15.8 g / dl) which was significantly different ($p < 0.05$) with all the groups in the infected group *Plasmodium berghei* and not given treatment (K1) and groups that treated with chloroquine (K2) as well as the treatment group that given leaf extract, stem bark extract and a combination of leaf, stem bark extract of *Syzygium cumini,* and also chloroquine (P1, P2, P3 and P4). In the K1 group showed the lowest hemoglobin below the normal range was significantly different ($p < 0.05$) compared to the other group, whereas in K2 the mean hemoglobin was significantly different ($p < 0.05$) with P1 and P4 and was not significantly different with P2 and P3. In the treatment group between P1 and P4 were not significantly different but significantly

different with P3. The mean value of red blood cells (RBC) in K1 showed the lowest decrease under normal 8.15-9.75 (10^6 / mm^3) which was significantly different with all treatment groups ($p < 0.05$), whereas in group K2 was significantly different with P2 and not significantly different with P1, P3 and P4. At treatment P1, P2, P3 and P4 did not show any significant difference.

The mean of hematocrit (HCT / PCV) results showed K0 in the normal range (44.4-50.4%) which was different with all treatment groups. In group K1 showed the lowest hematocrit value and did not differ significantly with K2, P3 and significantly different with P1, P2 and P4. P1, P2 and P4 were not significantly different. MCV and MCH values show a decrease in groups of K2 and P1, P2, P3 and P4.

Hematology result of white blood cells

The mean number of leukocytes (WBC) increased in the K1 group infected with *Plasmodium berghei* significantly different ($P < 0.05$) compared with the infected group treated with chloroquine K2 and the group treated with the combination of *Syzygium cumini* leaf and stem bark extract with chloroquine P2 and P4. The average number of leukocytes in K2, P1, P2, P3 and P4 is still within normal range between 8.00-11.8 (103 / m^3). The average platelet value (PLT) is still within normal limits. The highest lymphocyte values is in the K1 group were significantly different from K0, P2 and P4 and did not differ significantly with K2, P1 and P3 with normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p < 0.05$) with K0, K2 and P4 and was not significantly different ($p > 0.05$) with P1, P2 and P3. The average number of neutrophils is in the normal range of 6.20-42.6 in the K0 and P2 groups was significantly different ($p < 0.05$) with K1, K2, P1, and P3 and not significantly different ($p > 0.05$) with P2 and P4. Hematologic data of white blood cells are shown in Table 2.

Measurement results and limpha index

The result of length, width, weight and index of limpha measurement in treatment group infected with *Plasmodium berghei* showed an enlarged limpha organ and significantly difference ($p < 0.05$) with uninfected control group K0. In the infected and treated group with chloroquin K2 showed the length and width of limpha is significantly different ($p < 0.05$) with K1 group, while leaf, stem bark extract of

Syzygium cumini and combination of extract with chloroquine (P1, P2, P3 and P4) showed no significant difference ($p > 0.05$) with group K2. Limpha data showed in table 3.

Discussion

Red blood cell hematology

The mean hemoglobin value in non-infected control group *Plasmodium berghei* is15.45 g/dl, erythrocytes cell count 10.56 (10^6 / mm3), average hematocrit 51.40% in normal range. This is due to the mice under normal circumstances and the erythrocytes is not damaged. In the infected group *Plasmodium berghei* and was not treated and the infected group treated with chloroquine, leaf, stem bark of *Syzygium cumini* and the combination of hemoglobin values, erythritrositic cell count, mean hematocrit and MCV and MCH values showed a decrease, below the normal range, the lowest in K1 which is different from the other treatment groups, followed by K2 and PI, P3 and P4 but at P2 the number of erythrocytes in normal range.

This suggests that Plasmodium infections causing erythrocyte haemolysis, the removal of erythrocyte that infected with parasites and uninfected cause erytropoiesis in the body becomes ineffective, this is caused by the abnormally high levels of TNF that have an effect on ineffective erytropoiesis [18].

In vitro and in vivo studies show proinflamatory cytokines including interferon y, tumor necrosis factor α and macrophage migration inhibitory (MIF), as well as plasmodium products that hemozoin plays a role in the pathogenesis of malarial anemia [19]. Hemozoin produced from hemoglobin digestion by Plasmodium, induces macrophage for the secretion of proinflamatory cytokines and other mediators that inhibit the effects of erythropoiesis [20,21]. Plasmodium hemozoin products play a role in erythropoiesis resistance, low reticulocytosis and malarial anemia by inhibiting Epo induced proliferation from erythroid precursors [22].

Malarial anemia due to Plasmodium infection may increase IgG autoantibodies levels against non infected red blood cells (nRBCs) and its deposition on the surface of nRBCs can decrease red cell

deformability and improve erythrophagocytosis [23]. The results of this study in the treatment group Hb levels, the number of erythrocytes and levels of PVC are below normal, its due to hypochromic micrositic anemia because MCV and MCH values below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum* 71% of anemic patients having hypochromic microcytic anemia [18].

In the P2 group infected with *Plasmodium berghei* treated with a combination of chloroquine and *Syzygium cumini* leaf extract the number of erythrocytes cells within normal limits. The antimalarial effects of chloroquine and antioxidants contained in *Syzygium cumini* leaf extracts on infection by *Plasmodium berghei* cause the proliferative resistance of *Plasmodium berghei* through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of hemoglobin [24]. Decreased proliferation in *Plasmodium berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [25]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [26]. Results of research conducted by Haroon 2015 states that leaves, stems and fruit *Syzygium cumini* has antioxidant and antiinflammatory activity. The value of IC50 leaves of Syzygium cumini is 12.84 ppm so it is potentially developed as an antioxidant [27]. The results of Ruan et al., 2008 showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *Syzygium cumini* leaf extract. [28].

White blood cell hematology

Increased WBC, the number of lymphocytes and the number of monocytes in the K1 group infected with *Plasmodium berghei* were not treated. Decreased WBC, number of lymphocytes and number of monocytes in the infected K2 group and treated with chloroquine, as well as groups treated with a combination of Syzygium cumini leaf and stem bark extract with chloroquine P2 and P4. These results suggest that WBC elevation in infection by plasmodium may stimulate the immune system as a physiological response of the body to malaria infection, because the WBC has a role in fighting infection,

as the body's defense against infection through phagocytosis against the invasion of foreign organisms and producing antibodies as an immune response. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment provided was able to fight infection [29]

Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *Plasmodium berghei* infection [29]. Monocytes are phagocytes. An increase in the number of monocytes suggests a body's immune response to accelerate the activity of plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization and regulation of immune-effector cells, also contributing to infection elimination [30]. The reduction of lymphocytes and monocytes in the treatment of antimalarials and combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids and ellagic acid in *Syzygium cumini* stem bark extracts have antioxidant effects. The antioxidant effects of phitochemicals in mice infected with *Plasmodium berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehide (MDA) to improve hematologic parameters [31].

Measurement and limpha index

On the result of measurement of length, width, weight and index of limpha in treatment group infected with *Plasmodium berghei.* shows the enlargement of limpha organs. Limpha works as an effector against malaria infection especially in protective immunity against infections of the blood [32]. The total number of limpha cells increased during high parasitemia and then decreased at lower levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, this reaction due to the increase in hematopoietic and an increase in the number of macrophages. Increased macrophage occurs due to the process of erythrophagocytosis. Macrophage in the red pulp limpha plays an important role in removing Plasmodium that infects red blood cells from circulation [33].

Plasmodium infections in red blood cells may lead to a complex pathophysiology, rapid growth

of Plasmodium will increase the production of reactive oxygen species (ROS) causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [34]. The administration of chloroquin antimalarial drug and leaf and stem bark extract of *Syzygium cumini* and its combination can decrease the length, width and index of limpha. This shows the effects of antimalarials and antioxidant content in leaf, stem bark extract of *Syzygium cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, elagat acid, ferulic acid and gallic acid. The leaves are also contained tannins and essential oil terpenes. [35]. *Syzygium cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaemferol, myricetin, flavonol, glycosides, saponins, triterpenoids and anthocyanins [36].

Research conducted by Sami (2016) [37]. stated the value of IC50 extract ethanol bark *Syzygium cumini* is 164,3 ppm. Activity as an antioxidant is thought to be due to the presence of flavonoids and polyphenols in the plant [38]. The combination of desferroxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in Plasmodium yoelii infection produces an antimalarial additive effect [39]. Decreased lesions are reversible in limpha following a decrease in parasitemia through apoptosis as a defense mechanism of immune limpha against malaria infection [32].

Conclusion

The combination of chloroquin with leaf and stem bark extract of *Syzygium cumini* as adjuvant therapy may increase the amount of erythosit, decrease the number of leukocytes, lymphocytes and monocytes and decrease the length, width and index of limpha mice model malaria.

Authors´Contributions

LL as the project's leader designing the research, supervising the research and compiling the manuscript, RS and RSW helps analyze blood and limpha data. All authors read the final manuscript.

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Competing Interest

The author declare that they have no competing interests

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Table 1 Mean and standard deviation of red blood cell hematology in control and treatment groups

Mice Groups	Parameter of Red Blood Cell Hematology						
	HGB (g/dL)	RBC (10^6/µL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW-CV (%)
K0	15.45± 0.50^{d}	10.56 ± 0.27^{d}	51.40 ± 2.55^{c}	48.10 ± 3.39^{a}	14.60 ± 0.53^{b}	30.30 ± 0.49^{a}	23.42 ± 0.70^{a}
K1	11.12± 0.64^{a}	8.09 ± 0.33^{a}	36.80 ± 3.88^{a}	45.52 ± 4.95^{a}	13.75 ± 0.71^{ab}	30.37 ± 1.80 ^{a}	25.92 ± 3.51^{b}
K2	12.25± 0.12^{b}	8.90 ± 0.89^{b}	39.70± 0.98^{ab}	45.37 ± 4.91^{a}	13.87 ± 1.07^{ab}	30.97 ± 1.06 ^{a}	25.85 ± 0.23^{b}
P1	13.12± 0.41^{c}	9.14 ± 0.36^{bc}	42.57 ±2.13^{b}	46.97 ± 2.57^{a}	14.40 ± 0.57^{ab}	30.67 ± 0.45 ^{a}	21.80 ± 0.66^{a}
P2	12.80± 0.29^{bc}	9.75 ± 0.36^{c}	42.55 ±2.13^{b}	43.72 ± 1.31^{a}	13.25 ± 0.36^{a}	30.20 ± 0.87 ^{a}	23.97 ± 0.59^{a}
P3	12.32± 0.45^{b}	9.15 ± 0.53^{bc}	40.15 3.70^{ab}	43.82 ± 2.47^{a}	13.52 ± 0.67^{ab}	30.85 ± 1.88 ^{a}	24.67 ± 3.51^{ab}
P4	13.07± 0.36^{c}	9.14 ± 0.62^{bc}	41.26 ±1.03^{b}	45.42 ± 3.39^{a}	14.37 ± 0.77^{ab}	31.67 ± 0.65 ^{a}	23.30 ± 126^{ab}

Note: Different superscript on same column shows significant difference at significant level at 0.05%

Table 2 Mean and standard deviation of white blood cell hematology in control and treatment groups

Mice Group	Parameter of White Blood Cell Hematology							
	WBC	PLT	Lymph	Lymph (%)	Mono	Mono (%)	Neut	Neut (%)
K0	4.33 ± 0.17^{a}	1322.25±3 5.34^{ab}	3.60 ± 0.11^{a}	83.40 ± 0.66^{abc}	0.27 ± 0.02 ^{a}	6.20 ± 0.29 ^{a}	0.35 ± 0.04^{a}	8.07±. 0.17^{b}
K1	13.22 ± 6.35^{c}	1551.50±4 51.38^{b}	10.33 ± 4.83^{c}	79.87 ± 6.95^{ab}	2.24±2. 02^{c}	16.75 ± 8.83^{c}	0.46 ± 0.44^{a}	1.95±3 .90^{a}
K2	8.41 ± 0.56^{ab}	1432.75± 21.09^{ab}	7.08 ± 0.20^{bc}	88.37 ± 2.00^{c}	0.76 ± 0.06^{ab}	9.22 ± 0.51^{ab}	0.35 ± 0.04^{a}	2.07±2 .39^{a}
P1	10.59 ± 1.14^{bc}	1245.50±9 9.37^{ab}	8.29 ± 0.74^{bc}	79.37 ± 1.82^{ab}	1.70 ± 0.51^{bc}	16.15 ± 4.10^{bc}	0.35 ± 0.30^{a}	3.22±3 .80^{a}
P2	8.17 ± 0.30^{ab}	1127.50±1 57.55^{a}	6.17 ± 0.33^{ab}	76.07 ± 2.36^{a}	1.36 ± 0.19^{abc}	16.22 ± 2.39^{bc}	0.55 ± 0.13^{a}	6.90±0 .90^{ab}
P3	11.03 ± 0.51^{bc}	1097.25±2 95.11^{a}	8.83 ± 0.41^{bc}	80.92 ± 3.84^{abc}	1.70 ± 0.59^{bc}	15.12 ± 4.66^{bc}	0.36 ± 0.26^{a}	2.52±3 .17^{a}
P4	7.87 ± 2.48^{ab}	1156.75±2 13.69^{a}	6.62 ± 2.28^{b}	85.15 ± 9.55^{bc}	0.46 ± 0.20^{ab}	6.63 ± 4.18^{a}	0.66 ± 0.36^{a}	5.92±4 .31^{ab}

Note: Different superscript on same column shows significant difference at significant level at 0.05%

Table 3 Mean and standard deviations of length, width, weight and index of limpha mice in the control and treatment groups

Mice Group	Length (cm)	Width (cm)	Weight (g)	Limpha index
K0	0.82 ± 0,14[a]	0.22 ± 0.12[a]	0.21 ± 0.11[a]	0.008 ± 0.002[a]
K1	3.07 ± 0.22[c]	0.65 ± 0 .10[c]	0.44 ± 0.14[c]	0.016 ± 0.003[c]
K2	2.20 ± 0.62[b]	0.45 ± 0.19[b]	0.30 ± 0.10[bc]	0.011 ±0.001[bc]
P1	2,65 ± 0.26[bc]	0.57 ± 0.09[bc]	0.37 ± 0.08[bc]	0.013 ±0.001[c]
P2	2,42 ± 0.38[bc]	0.50 ± 0.11[bc]	0.34 ± 0.10[bc]	0.013 ±0.003[bc]
P3	2,37 ± 0.47[b]	0.60 ± 0.08[bc]	0.31 ± 0.04[bc]	0.013 ±0.002[bc]
P4	2,47 ± 0.30[bc]	0.55 ± 0.10[bc]	0.27 ± 0.07[b]	0.009 ±0.002[b]

Note: Different superscript on same column shows significant difference at significant level at 0.05%

Article Revision Letter for Authors - (VETWORLD-2018-04-187)

Yahoo/Email M...

Veterinary World <vetworld@ejmanager.com>

Kepada: lilik.maslachah@yahoo.com

Sab, 10 Nov 2018 jam 12.33

Dear Lilik Maslachah,

Your manuscript entitled "Hematologic Changes and Limpha Index on Mice Model Malaria Given Syzygium cumini Extract as Adjuvant Therapy" (Ms.Nr. VETWORLD-2018-04-187) was reviewed by reviewers of the Veterinary World. As initial decision, your manuscript was found interesting but some revisions have to be made before it can reach a publishable value. Please refer comments given at bottom.

You should send your revised manuscript via the online system of ScopeMed on my.ejmanager.com.

Sincerely yours,

Dr. Anjum Sherasiya
Editor-Veterinary World
Star, Gulshan Park,
NH-8A, Chandrapur Road, Wankaner 363621
Dist. Morbi (Gujarat) INDIA

COMMENTS for Authors:

EDITORIAL COMMENTS:

- Highlight all corrections/additions in red color font in revised manuscript.
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- Include all authors name, affiliation and email address in revised Word file as per format and style of Veterinary World. Please check latest article from www.veterinaryworld.org for format of this section.
- All reference no. in the text must be in continuous no. as per style of Veterinary World and amend the reference section accordingly if you have not done it.
- Please divide the introduction into 3 paragraphs if you have already not done. Introduction must be divided into 3 paragraphs i.e., 1. introduction 2. significance of the study and 3. aim of the study.
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Manuscript entitled "Hematologic Changes and Limpha Index on Mice Model Malaria Given Syzygium cumini Extract as Adjuvant Therapy" is interesting but there are grammatical error in manuscript writing.
It could be accepted after a major revision of English language.

Lilik Maslachah
To: Veterinary World - Publisher

To : Dr. Anjum Sherasiya

Editor-Veterinary World

Star-Gulshan Park

NH-8A, Chandrapur Road, Wankaner 363621

Dist. Morbi (Gujarat) INDIA

We would like to say thank you for the revision and suggestion of my proof for correction entitled "**Hematologic Changes and Splenic Index on Malaria Mice Models Given Syzygium cumini Extract as Adjuvant Therapy**"

We have revised our proof for corrections as per suggestion by editor

1. Our revised proof for correction highlight in red colour
2. We added all authors name, affiliation and e-mail address.
3. We added References, acknowledgment, were already available in our proof for correction
4. All of grammatically error have been revised in proof for correction (red colour font)

Best regard,

Dr. Lilik Maslachah

Hematologic Changes and Limpha Index on Malaria Mice Models Given *Syzygium cumini* Extract as Adjuvant Therapy

Lilik Maslachah[1], Rahmi Sugihartuti[1], Retno Sri Wahyuni[2]

[1]Departement of Basic Veterinary Medicine, Veterinary Pharmacy Laboratory [2] Departement of Basic Veterinary Medicine, Clinical Pathology Laboratory, Faculty of Veterinary Medicine Airlangga University Surabaya Indonesia

[*]Corresponding author : Lilik Maslachah, e-mail : lilik.maslachah@yahoo.com

Co-authors:RS: imharedi@yahoo.co.id, RSW: wahyuniretno@yahoo.com

Abstract

Aims: This research aim to determine the efficacy of *Syzygium cumini L* as adjuvant therapy on blood changes and limpha index of mice model malaria

Materials and Methods: Mice was infected intraperitoneally with 0,2 ml red blood cell that contain $1x10^6$ *Plasmodium berghei*. Thirty five mices were divided into 7 treatment groups. Group (K0): Mice was not infected. (K1): Mice was infected. (K2): Mice was infected and given chloroquine (P1): Mice was infected and given *Syzygium cumini* leaf extract. (P2) : Mice was infected and given chloroquine and also *Syzygium cumini* leaf extract. (P3) : Mice was infected and given *Syzygium cumini* stem bark extract. (P4) : Mice was infected and given chloroquine and *Syzygium cumini* stem bark extract. Treatment was given for 4 days 24 hours post *Plasmodium berghei* infection. Twenty first day post *Plasmodium berghei* infection, blood was taken from the heart for hematological examination, limpha was taken to examinate the limpha index, and also to measure the weight and length of limpha. Hematological data and limpha index were analyzed by *Analysis of Varian* test and if there is a difference, the test is continued by *Duncan Multiple Range Test* with 5% level.

Results: The K0 group have normal hemoglobin, RBC, hematocrit and significantly different ($p <0.05$) than other groups. Hemoglobin, RBC and hematocrit of K1 group was under normal range, lowest and significantly different ($p <0.05$) than other groups. MCV and MCH values of K2 groups showed a decreasing. The number of leucocytes, lymphocytes, monocytes of K1 groups were increasing and significantly different ($P <0.05$) with K2 and treatment group. The length, width, weight and index limpha of K1 group were significantly different ($p <0.05$) with K0 group. K2 and treatment groups showed that the length and width of limpha were significantly different ($p <0.05$) with K1.

Conclusion: The combination of chloroquine with leaf and chloroquine with stem bark extract of *Syzygium cumini* as adjuvant therapy may increase the amount of erythosit, decrease the number of leukocytes, lymphocytes and monocytes and decrease the length, width and index of limpha on malaria mice models.

Keywords: *Plasmodium berghei,* Hematology, limpha index, *Syzygium cumini*

Introduction

Malaria is still be a public health problem in 107 countries until right now, because malaria is still the fifth rank of infectious diseases in the world [1]. The control and treatment of malaria is more difficult recently because malaria parasites have been resistant to drugs and also the mosquitoes is resistant to insecticides. The development of treatment, prevention and control of malaria is one of the substantial problems in the world. In 2012, approximately 600,000 children died, mostly from sub-Saharan Africa [2].

The death of malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly and liver dysfunction. Hematological abnormalities during malarial infection are caused by high parasitemia in the patient, hematological abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of limpha is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic stages of malaria infection, limpha is an important organ in the immune respons. Elimination of infected erythrocytes through modulating the immune response and limpha remodeling, resulting an immune response imbalance that can lead to severe malaria [5].

The World Health Organization (WHO) recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and ACT combination on clinical cases in Cambodia [6]. In addition, there is a decrease in efficacy of antimalarial drugs that currently used, so it is important to develop an adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria. Adjuvant therapy that can be used in severe malaria are immune system modulator preparations, antioxidants, anticoagulants and agents that have antiseizure activity [7]. The results showed that the use of MMP inhibitor was able to increase the

survival of mice in cerebral malaria, dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, TNFα and IL 1B [8,9,10]. *Syzygium cumini* contains anthocyanin, ellagitannin, ellegic acid, phenolic, flavonoids and vitamins so that it has a high antioxidant activity. This plant is one of medicinal plants which is easy to be found in Indonesia [11,12]. Results of research conducted by Zhang et al, 2009 showed that *Syzygium cumini* has radical scavenging activity and strong antioxidant.

The research was aimed to determine the efficacy of *Syzygium cumini L* as adjuvant therapy on hematological changes (Red blood cells and white blood cells) and limpha index in mice model malaria.

Materials and Methods

Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine Airlangga University Surabaya Indonesia

Parasite, host and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium bergei* ANKA strain. The mice used were male Swiss albino mice with 20g -30g weight, 2.5 months old, and obtained from Veterinary Farma Surabaya (Pusvetma) Center. Antimalarial drug used Chloroquine Pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used 25 mg /kg bw mice as a therapeutic dose. This drug was administered daily for 4 days [13]. The leaves and stem bark of *Syzygium cumini* are obtained from Kediri city of East Java Indonesia and identified in the laboratory of Purwodadi botanical garden Pasuruan. *Syzygium cumini* dose was 600mg /kgbw [14].

Infection Dose of *Plasmodium berghei* in mice

Mice was infected with 0,2 ml red blood cells containing $1x10^6$ *Plasmodium berghei* parasites intraperitoneally. In order to find out the infection has occurred in mice, daily microscopic examination of erythrocyte was done with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [15]. Calculation of the dose of a parasitic infection was determined by counting the number of parasites from the thin blood smear that stained with Giemsa then calculated the number of parasites per number of erythrocytes. The next step is calculating the amount of erythrocytes by diluting the blood using PBS solution in effendorf 0.5 ml. Then, this diluted blood erythrocytes were calculated using the *Improved Neubauer Counting Chamber*. The number of parasitic doses are obtained from multiplying by the number of parasites with the amount of erythrocytes that have been calculated and converted to per ml.

Preparation of leaf and stem bark of *Syzygium cumini*

The leaves and stem bark of *Syzygium cumini* are dried, after that, it were crushed into small pieces (simplicia). Simplicia was extracted with PA methanol and maseration for 3x24 hours. The filtrate was evaporated using a Rotary Evaporator at 40-50^{0}C with low pressure. The extraction results are stored on the desiccator until ready for use [16].

Treatment of the experimental animals

Thirty five mices were randomly divided into 7 treatment groups and each group consisting of 5 mices. Details of each group as follows

Group (K0): Mice was only given drug solvent, and not infected. (K1): Mice was infected and given drug solvent. (K2): Mice was infected and given chloroquine 25 mg/kg body weight (P1): Mice was infected and given *Syzygium cumini* leaf extract 600 mg/kg body weight. (P2) : Mice was infected and given chloroquine 25 mg/kg body weight and also given *Syzygium cumini* leaf extract 600 mg/kg body weight. (P3) : Mice was infected and given *Syzygium cumini* stem bark extract 600 mg/kg body weight. (P4) :

Mice was infected and given chloroquine 25 mg/kg body weight and also given *Syzygium cumini* stem bark extract 600 mg/kg body weight.

Treatment was given for 4 days since 24 hours after per oral infection. After 21 days post infection, mice was anesthetized with ketamine (sigma), then thoracotomy, blood samples were taken from the heart (1 ml) using a tuberculin syringe and collected in a vial that has been given anticoagulation for hematologic examination using automated blood analyzer SYSMEX XT 4000i, limpha organ were taken to examinate the limpha index and also measure the weight and length of limpha.

Examination of weight, length and index of limpha.

Previously, the weight of the mice was measured, after that, the mice were injected with ketamine IM. The abdominal cavity was opened and the limpha organ was taken and weighed using an analytical scale, then measured the length and width of the limpha using a ruler in a mm scale. According to [17], the index value of limpha (spleenic index) was calculated using the limpha Index equation = (weight of spleen organ of mice) / body weight of mice.

Data analysis

The data of hematology observation of blood change and limpha index were processed by using Analysis of Varian (ANOVA) using SPSS System 17.0 then followed by Duncan Multiple Range Test test with 5% level.

Results

Hematological results of RBC

The results of statistical tests of mean hemoglobin (HGB), red blood cells (RBC), hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Copuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) can be seen in Table 1. The mean value of hemoglobin in the non-infected control group (K0) was normal (15.45 g / dl; the normal range is 13.4-15.8 g / dl) which was

significantly different ($p < 0.05$) with all the groups in the infected group and not given treatment (K1) and groups that treated with chloroquine (K2) as well as the treatment group that given leaf extract, stem bark extract and a combination of leaf, stem bark extract of *Syzygium cumini,* and also chloroquine (P1, P2, P3 and P4). In the K1 group showed the lowest hemoglobin below the normal range that was significantly different ($p < 0.05$) when compared to the other group. In K2 group, the mean hemoglobin was significantly different ($p < 0.05$) with P1 and P4 and was not significantly different with P2 and P3. In the treatment group between P1 and P4 were not significantly different but significantly different with P3. The mean value of red blood cells (RBC) in K1 showed the lowest decrease under normal 8.15-9.75 (10^6 / mm^3) which was significantly different with all treatment groups ($p < 0.05$). In K2 group was significantly different with P2 and not significantly different with P1, P3 and P4. At P1, P2, P3 and P4 group did not show any significant difference.

The mean of hematocrit (HCT / PCV) showed that K0 is in the normal range (44.4-50.4%) which was different with all treatment groups. In K1 group showed the lowest hematocrit value and did not differ significantly with K2, P3. K1 group was significantly different with P1, P2 and P4. However, P1, P2 and P4 were not significantly different. MCV and MCH values show a decrease in K2, P1, P2, P3 and P4.

Hematology result of white blood cells

The mean number of leukocytes (WBC) increased in the K1 group infected with *Plasmodium berghei* and was significantly different ($P < 0.05$) when compared with the infected group treated with chloroquine (K2 group) and the group treated with the combination of *Syzygium cumini* leaf and stem bark extract with chloroquine (P2 and P4 groups). The average number of leukocytes in K2, P1, P2, P3 and P4 were still within normal range between 8.00-11.8 (103 / m^3). The average platelet value (PLT) was still within normal limits. The highest lymphocyte values is in the K1 group and were significantly different from K0, P2 and P4 and did not differ significantly with K2, P1 and P3 with normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p < 0.05$) with K0, K2 and P4 and was not significantly different ($p > 0.05$) with P1, P2 and P3. The average number of neutrophils is in the normal range of 6.20-42.6 in the K0 and P2 groups were significantly different (p

<0.05) with K1, K2, P1, and P3 and not significantly different (p> 0.05) with P2 and P4. Hematologic data of white blood cells are shown in Table 2.

Measurement results and limpha index

The result of length, width, weight and index of limpha measurement in treatment group infected with *Plasmodium berghei* showed an enlarged limpha organ and significantly different (p <0.05) with uninfected control group (K0). In the infected and treated group with chloroquine (K2) showed the length and width of limpha which is significantly different (p <0.05) with K1 group. However, P1, P2, P3 and P4 showed no significant different (p> 0.05) with K2. Limpha data showed in table 3.

Discussion

Red blood cell hematology

The normal range of hemoglobine, erythrocytes and hematocrit in non infected control group due to the mice under normal circumstances and the erythrocytes is not damaged. In the group that infected by *Plasmodium berghei* and was not treated and the infected group that treated with chloroquine, leaf, stem bark of *Syzygium cumini* and its combination, the hemoglobin values, erythritrositic cell count, hematocrit mean, MCV and MCH values showed a decrease below the normal range, the lowest is in K1 which was different from the other treatment groups, followed by K2 and P1, P3 and P4 but at P2, the number of erythrocytes is in normal range.

This suggests that Plasmodium infections cause erythrocyte haemolysis. The removal of infected and uninfected erythrocyte cause erytropoiesis in the body becomes ineffective, this is caused by the abnormally high levels of TNF that have effect on ineffective erytropoiesis [18].

In vitro and in vivo studies show proinflamatory cytokines including interferon y, tumor necrosis factor α and macrophage migration inhibitory (MIF), as well as plasmodium products (hemozoin) plays a role in the pathogenesis of malarial anemia [19]. Hemozoin was produced from hemoglobin digestion by Plasmodium, induces macrophage for the secretion of proinflamatory cytokines and other mediators that inhibit the effects of erythropoiesis [20,21]. Plasmodium hemozoin products play a role in erythropoiesis

resistance, low reticulocytosis and malarial anemia by inhibiting Epo induced proliferation from erythroid precursors [22].

Malarial anemia due to Plasmodium infection may increase IgG autoantibodies levels against non infected red blood cells (nRBCs) and its deposition on the surface of nRBCs can decrease red cell deformability and improve erythrophagocytosis [23]. The results of this study in the treatment group, Hb levels, the number of erythrocytes and levels of PVC are below normal, its due to hypochromic micrositic anemia because MCV and MCH values below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum* 71% of anemic patients having hypochromic microcytic anemia [18].

In the P2 group infected with *Plasmodium berghei* treated with a combination of chloroquine and *Syzygium cumini* leaf extract, the number of erythrocytes cells within normal limits. The antimalarial effects of chloroquine and antioxidants containing *Syzygium cumini* leaf extracts cause proliferative resistance of *Plasmodium berghei.* It happened through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of hemoglobin [24]. The decreased of proliferation in *Plasmodium berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [25]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [26]. Results of research conducted by Haroon 2015 states that leaves, stems and fruit *Syzygium cumini* has antioxidant and antiinflammatory activity. The value of IC50 leaves of *Syzygium cumini* is 12.84 ppm so it is potentially developed as an antioxidant [27]. The results of Ruan et al., 2008 showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *Syzygium cumini* leaf extract. [28].

White blood cell hematology

Increased WBC, the number of lymphocytes and the number of monocytes in the K1 group infected with *Plasmodium berghei* were not treated. Decreased WBC, number of lymphocytes and number of monocytes in the infected K2 group and treated with chloroquine, as well as groups treated

with a combination of *Syzygium cumini* leaf and stem bark extract with chloroquine P2 and P4. These results suggest that WBC elevation in infection by plasmodium may stimulate the immune system as a physiological response of the body to malaria infection, because the WBC has a role in fighting infection, as the body's defense against infection through phagocytosis against the invasion of foreign organisms and producing antibodies as an immune response. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment was able to fight infection [29]

Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *Plasmodium berghei* infection [29]. Monocytes are phagocytes. The increase in the number of monocytes suggests a body's immune response to accelerate the activity of plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization and regulation of immune-effector cells, also contributing to infection elimination [30]. The reduction of lymphocytes and monocytes in the treatment of antimalarials and its combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids and ellagic acid in *Syzygium cumini* stem bark extracts have antioxidant effects. The antioxidant effects of phitochemicals in mice infected with *Plasmodium berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehide (MDA) to improve hematologic parameters [31].

Measurement and limpha index

The result of measurement of length, width, weight and index of limpha in treatment group infected with *Plasmodium berghei.* shows the enlargement of limpha organs. Limpha works as an effector against malaria infection especially in protective immunity against infections of the blood [32]. The total number of limpha cells increased during high parasitemia and then decreased at lower levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, this reaction due to the increase in hematopoietic and an increase in the number of macrophages. Increased macrophage occurs due to the process of erythrophagocytosis.

Macrophage in the red pulp limpha plays an important role in removing Plasmodium that infects red blood cells from circulation [33].

Plasmodium infections in red blood cells may lead to a complex pathophysiology, rapid growth of Plasmodium will increase the production of reactive oxygen species (ROS) causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [34]. The administration of chloroquine antimalarial drug and leaf and stem bark extract of *Syzygium cumini* and its combination can decrease the length, width and index of limpha. This shows the effects of antimalarials and antioxidant content in leaf, stem bark extract of *Syzygium cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, elagat acid, ferulic acid and gallic acid. The leaves are also contained tannins and essential oil terpenes. [35]. *Syzygium cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaemferol, myricetin, flavonol, glycosides, saponins, triterpenoids and anthocyanins [36].

Research conducted by Jayachandra et al, (2012) showed that the total phenolic content was 580.23 ± 3.03 mg/g, tannin content was 534 ± 4.03 mg/ g while the flavonoid content was 315.42 ± 4.52 mg/g. The methanolic and aqueous extracts of bark were screened for antioxidant activity using Nitric oxide scavenging activity method [37]. Activity as an antioxidant due to the presence of flavonoids and polyphenols in the plant [38]. The combination of desferroxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in *Plasmodium yoelii* infection produces an antimalarial additive effect [39]. Decreased lesions are reversible in limpha following a decrease in parasitemia through apoptosis as a defense mechanism of immune limpha against malaria infection [32].

Conclusion

The combination of chloroquine with leaf and stem bark extract of *Syzygium cumini* as adjuvant therapy may increase the amount of erythrocytes, decrease the number of leukocytes, lymphocytes and monocytes and decrease the length, width and index of limpha malaria mice models.

Authors´Contributions

LL as the project's leader designing the research, supervising the research and compiling the manuscript,

RS and RSW helps analyze blood and limpha data. All authors have proof read the final manuscript.

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Competing Interest

The author declare that they have no competing interests

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Table 1 Mean and standard deviation of red blood cell hematology in control and treatment groups

Mice Groups	Parameter of Red Blood Cell Hematology						
	HGB (g/dL)	RBC (10^6/µL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW-CV (%)
K0	15.45 ± 0.50^d	10.56 ± 0.27^d	51.40 ± 2.55^c	48.10 ± 3.39^a	14.60 ± 0.53^b	30.30 ± 0.49^a	23.42 ± 0.70^a
K1	11.12 ± 0.64^a	8.09 ± 0.33^a	36.80 ± 3.88^a	45.52 ± 4.95^a	13.75 ± 0.71^{ab}	30.37 ± 1.80^a	25.92 ± 3.51^b
K2	12.25 ± 0.12^b	8.90 ± 0.89^b	39.70 ± 0.98^{ab}	45.37 ± 4.91^a	13.87 ± 1.07^{ab}	30.97 ± 1.06^a	25.85 ± 0.23^b
P1	13.12 ± 0.41^c	9.14 ± 0.36^{bc}	42.57 ± 2.13^b	46.97 ± 2.57^a	14.40 ± 0.57^{ab}	30.67 ± 0.45^a	21.80 ± 0.66^a
P2	12.80 ± 0.29^{bc}	9.75 ± 0.36^c	42.55 ± 2.13^b	43.72 ± 1.31^a	13.25 ± 0.36^a	30.20 ± 0.87^a	23.97 ± 0.59^a
P3	12.32 ± 0.45^b	9.15 ± 0.53^{bc}	40.15 3.70^{ab}	43.82 ± 2.47^a	13.52 ± 0.67^{ab}	30.85 ± 1.88^a	24.67 ± 3.51^{ab}
P4	13.07 ± 0.36^c	9.14 ± 0.62^{bc}	41.26 ± 1.03^b	45.42 ± 3.39^a	14.37 ± 0.77^{ab}	31.67 ± 0.65^a	23.30 ± 126^{ab}

Note: Different superscript on same column shows significant difference at significant level at 0.05%

Table 2 Mean and standard deviation of white blood cell hematology in control and treatment groups

Mice Group	Parameter of White Blood Cell Hematology							
	WBC	PLT	Lymph	Lymph (%)	Mono	Mono (%)	Neut	Neut (%)
K0	4.33 ± 0.17^{a}	1322.25±3 5.34^{ab}	3.60 ± 0.11^{a}	83.40 ± 0.66^{abc}	0.27 ± 0.02 ^{a}	6.20 ± 0.29 ^{a}	0.35 ± 0.04^{a}	8.07±. 0.17^{b}
K1	13.22 ± 6.35^{c}	1551.50±4 51.38^{b}	10.33 ± 4.83^{c}	79.87 ± 6.95^{ab}	2.24±2. 02^{c}	16.75 ± 8.83^{c}	0.46 ± 0.44^{a}	1.95±3 .90^{a}
K2	8.41 ± 0.56^{ab}	1432.75± 21.09^{ab}	7.08 ± 0.20^{bc}	88.37 ± 2.00^{c}	0.76 ± 0.06^{ab}	9.22 ± 0.51^{ab}	0.35 ± 0.04^{a}	2.07±2 .39^{a}
P1	10.59 ± 1.14^{bc}	1245.50±9 9.37^{ab}	8.29 ± 0.74^{bc}	79.37 ± 1.82^{ab}	1.70 ± 0.51^{bc}	16.15 ± 4.10^{bc}	0.35 ± 0.30^{a}	3.22±3 .80^{a}
P2	8.17 ± 0.30^{ab}	1127.50±1 57.55^{a}	6.17 ± 0.33^{ab}	76.07 ± 2.36^{a}	1.36 ± 0.19^{abc}	16.22 ± 2.39^{bc}	0.55 ± 0.13^{a}	6.90±0 .90^{ab}
P3	11.03 ± 0.51^{bc}	1097.25±2 95.11^{a}	8.83 ± 0.41^{bc}	80.92 ± 3.84^{abc}	1.70 ± 0.59^{bc}	15.12 ± 4.66^{bc}	0.36 ± 0.26^{a}	2.52±3 .17^{a}
P4	7.87 ± 2.48^{ab}	1156.75±2 13.69^{a}	6.62 ± 2.28^{b}	85.15 ± 9.55^{bc}	0.46 ± 0.20^{ab}	6.63 ± 4.18^{a}	0.66 ± 0.36^{a}	5.92±4 .31^{ab}

Note: Different superscript on same column shows significant difference at significant level at 0.05%

Table 3 Mean and standard deviations of length, width, weight and index of limpha mice in the control and treatment groups

Mice Group	Length (cm)	Width (cm)	Weight (g)	Limpha index
K0	0.82 ± 0,14^{a}	0.22 ± 0.12^{a}	0.21 ± 0.11^{a}	0.008 ± 0.002^{a}
K1	3.07 ± 0.22^{c}	0.65 ± 0 .10^{c}	0.44 ± 0.14^{c}	0.016 ± 0.003^{c}
K2	2.20 ± 0.62^{b}	0.45 ± 0.19^{b}	0.30 ± 0.10^{bc}	0.011 ±0.001^{bc}
P1	2,65 ± 0.26^{bc}	0.57 ± 0.09^{bc}	0.37 ± 0.08^{bc}	0.013 ±0.001^{c}
P2	2,42 ± 0.38^{bc}	0.50 ± 0.11^{bc}	0.34 ± 0.10^{bc}	0.013 ±0.003^{bc}
P3	2,37 ± 0.47^{b}	0.60 ± 0.08^{bc}	0.31 ± 0.04^{bc}	0.013 ±0.002^{bc}
P4	2,47 ± 0.30^{bc}	0.55 ± 0.10^{bc}	0.27 ± 0.07^{b}	0.009 ±0.002^{b}

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RESEARCH ARTICLE

Hematologic changes and lymph index on malaria mice models given *Syzygium cumini* extract as adjuvant therapy

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I think it should be lymph instead of limpha

Lilik Maslachah[1], Rahmi Sugihartuti[1] and Retno Sri Wahyuni[2]

1. Department of Basic Veterinary Medicine, Veterinary Pharmacy Laboratory, Airlangga University, Surabaya, Indonesia; 2. Department of Basic Veterinary Medicine, Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia.

Corresponding author: Lilik Maslachah, e-mail: lilik.maslachah@yahoo.com

Co-authors: RS: imharedi@yahoo.co.id, RSW: wahyuniretno@yahoo.com

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Abstract

Aims: This research aimed to determine the efficacy of *Syzygium cumini* L. as adjuvant therapy on blood changes and lymph index of mice model malaria.

Materials and Methods: Mice were infected intraperitoneally with 0.2 ml red blood cell (RBC)

that contains 1×10^6 *Plasmodium berghei*. 35 mice were divided into seven treatment groups: Group K0: Mice were not infected; K1: Mice were infected; K2: Mice were infected and given chloroquine; P1: Mice were infected and given *S. cumini* leaf extract; P2: Mice were infected and given chloroquine and also *S. cumini* leaf extract; P3: Mice was infected and given *S. cumini* stem bark extract; and P4: Mice were infected and given chloroquine and *S. cumini* stem bark extract. Treatment was given for 4 days 24 h post-*P. berghei* infection. 21st day post-*P. berghei* infection, blood was taken from the heart for hematological examination, and the lymph was taken to examine the lymph index and also to measure the weight and length of the lymph. Hematological data and lymph index were analyzed by analysis of variance test, and if there is a difference, the test is continued by Duncan's multiple range test with 5% level.

Results: The K0 group has normal hemoglobin (HGB), RBC, and hematocrit (HCT) and significantly different ($p<0.05$) than other groups. HGB, RBC, and HCT of K1 group were under normal range, lowest, and significantly different ($p<0.05$) than other groups. Mean corpuscular volume and mean corpuscular HGB values of K2 groups showed a decrease. The number of leukocytes, lymphocytes, and monocytes of K1 groups was increasing and significantly different ($p<0.05$) with K2 and treatment group. The length, width, weight, and lymph index of K1 group were significantly different ($p<0.05$) with K0 group. K2 and treatment groups showed that the length and width of lymph were significantly different ($p<0.05$) with K1.

Conclusion: The combination of chloroquine with leaf and chloroquine with stem bark extract

of *S. cumini* as adjuvant therapy may increase the amount of erythrocyte; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and index of lymph on malaria mice models.

Keywords: hematology, lymph index, *Syzygium cumini*, *Plasmodium berghei*.

<H1>Introduction

Malaria is still be a public health problem in 107 countries until right now because malaria is still the fifth rank of infectious diseases in the world [1]. The control and treatment of malaria are more difficult recently because malaria parasites have been resistant to drugs and also the mosquitoes are resistant to insecticides. The development of treatment, prevention, and control of malaria is one of the substantial problems in the world. In 2012, approximately 600,000 children died, mostly from Sub-Saharan Africa [2].

The death of malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly, and liver dysfunction. Hematological abnormalities during malarial infection are caused by high parasitemia in the patient; hematological abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors, and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of lymph is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic stages of malaria infection, lymph is an important organ in the immune response. Elimination of infected

erythrocytes through modulating the immune response and lymph remodeling, resulting an immune response imbalance that can lead to severe malaria [5].

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The World Health Organization recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and artemisinin-based combination therapies combination on clinical cases in Cambodia [6]. In addition, there is a decrease in efficacy of antimalarial drugs that currently used, so it is important to develop an adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria. Adjuvant therapy that can be used in severe malaria is immune system modulator preparations, antioxidants, anticoagulants, and agents that have anti-seizure activity [7]. The results showed that the use of matrix metalloproteinase (MMP) inhibitor was able to increase the survival of mice in cerebral malaria, and dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, tumor necrosis factor-alpha (TNF-α), and interleukin-1beta [8-10]. *Syzygium cumini* contains anthocyanin, ellagitannin, ellagic acid, phenolic, flavonoids, and vitamins so that it has a high antioxidant activity. This plant is one of the medicinal plants which is easy to be found in Indonesia [11,12]. Results of research conducted by Zhang *et al*., 2009, showed that *S. cumini* has radical scavenging activity and strong antioxidant.

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The research was aimed to determine the efficacy of *S. cumini* L. as adjuvant therapy on hematological changes (red blood cells [RBC] and white blood cells [WBC]) and lymph index in mice model malaria.

<H1>Materials and Methods

<H2>Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine, Airlangga University, Surabaya, Indonesia.

<H2>Parasite, host, and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium berghei* ANKA strain. The mice used were male Swiss albino mice with 20-30 g weight and 2.5 months old, and obtained from Veterinary Farma Surabaya (Pusvetma) Center. Antimalarial drug used chloroquine pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used 25 mg/kg body weight (bw) mice as a therapeutic dose. This drug was administered daily for 4 days [13]. The leaves and stem bark of *S. cumini* are obtained from Kediri city of East Java, Indonesia, and identified in the laboratory of Purwodadi Botanical Garden, Pasuruan. *S. cumini* dose was 600 mg/kg bw [14].

<H2>Infection dose of *P. berghei* in mice

Mice were infected with 0.2 ml RBC containing 1×10^6 *P. berghei* parasites intraperitoneally. To find out the infection which has occurred in mice, daily microscopic examination of erythrocyte

was performed with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [15]. Calculation of the dose of a parasitic infection was determined by counting the number of parasites from the thin blood smear that stained with Giemsa and then calculated the number of parasites per number of erythrocytes. The next step is calculating the amount of erythrocytes by diluting the blood using PBS solution in Eppendorf 0.5 ml. Then, this diluted blood erythrocytes were calculated using the improved Neubauer counting chamber. The number of parasitic doses is obtained from multiplying by the number of parasites with the amount of erythrocytes that have been calculated and converted to per ml.

<H2>Preparation of leaf and stem bark of *S. cumini*

The leaves and stem bark of *S. cumini* are dried, after that it was crushed into small pieces (simplicia). Simplicia was extracted with PA methanol and maceration for 3×24 h. The filtrate was evaporated using a Rotary Evaporator at 40-50°C with low pressure. The extraction results are stored on the desiccator until ready for use [16].

<H2>Treatment of the experimental animals

A total of 35 mice were randomly divided into seven treatment groups, and each group consists of 5 mice. Details of each group are as follows:

Group K0: Mice were only given drug solvent and not infected; K1: Mice were infected and given drug solvent; K2: Mice were infected and given chloroquine 25 mg/kg bw; P1: Mice were

infected and given *S. cumini* leaf extract 600 mg/kg bw; (P2): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* leaf extract 600 mg/kg bw; (P3): Mice were infected and given *S. cumini* stem bark extract 600 mg/kg bw; and (P4): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* stem bark extract 600 mg/kg bw.

Treatment was given for 4 days since 24 h after per oral infection. After 21 days post-infection, mice were anesthetized with ketamine (Sigma) and then thoracotomy, blood samples were taken from the heart (1 ml) using a Tuberculin Syringe and collected in a vial that has been given anticoagulation for hematologic examination using automated blood analyzer SYSMEX XT 4000i, and lymph organ was taken to examine the lymph index and also measure the weight and length of lymph.

<H2>Examination of weight, length, and index of lymph

Previously, the weight of the mice was measured, after that the mice were injected with ketamine IM. The abdominal cavity was opened, and the lymph organ was taken and weighed using an analytical scale and then measured the length and width of the lymph using a ruler in a millimeter scale. According to Gluhcheva *et al*. [17], the index value of lymph (splenic index) was calculated using the lymph index equation = (weight of spleen organ of mice)/bw of mice.

<H2>Statistical analysis

The data of hematology observation of blood change and lymph index were processed using analysis of variance using SPSS System 17.0 then followed by Duncan's multiple range test with 5% level.

<H1>Results

<H2>Hematological results of RBC

The results of statistical tests of mean hemoglobin (HGB), RBC, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), and MCHGB concentration is presented in Table-1. The mean value of HGB in the non-infected control group (K0) was normal (15.45 g/dl; the normal range is 13.4-15.8 g/dl) which was significantly different ($p<0.05$) with all the groups in the infected group and not given treatment (K1) and groups that treated with chloroquine (K2) as well as the treatment group that given leaf extract, stem bark extract and a combination of leaf, stem bark extract of *S. cumini,* and also chloroquine (P1, P2, P3 and P4). The K1 group showed the lowest HGB below the normal range that was significantly different ($p<0.05$) when compared to the other group. In K2 group, the mean HGB was significantly different ($p<0.05$) with P1 and P4 and was not significantly different with P2 and P3. The treatment group between P1 and P4 was not significantly different but significantly different with P3. The mean value of RBCs in K1 showed the lowest decrease under normal 8.15-9.75 ($10^6/mm^3$) which was significantly different with all treatment groups ($p<0.05$). K2 group was significantly different with P2 and not significantly different with P1, P3, and P4. P1,

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P2, P3, and P4 groups did not show any significant difference.

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The mean of HCT (HCT/PCV) showed that K0 is in the normal range (44.4-50.4%) which was different with all treatment groups. K1 group showed the lowest HCT value and did not differ significantly with K2 and P3. K1 group was significantly different with P1, P2, and P4. However, P1, P2, and P4 were not significantly different. MCV and MCH values show a decrease in K2, P1, P2, P3, and P4.

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<H2>Hematology result of WBCs

The mean number of leukocytes (WBC) increased in the K1 group infected with *P. berghei* and was significantly different ($p<0.05$) when compared with the infected group treated with chloroquine (K2 group) and the group treated with the combination of *S. cumini* leaf and stem bark extract with chloroquine (P2 and P4 groups). The average number of leukocytes in K2, P1, P2, P3, and P4 was still within normal range between 8.00 and 11.8 ($103/m^3$). The average platelet value was still within normal limits. The highest lymphocyte values are in the K1 group and were significantly different from K0, P2, and P4 and did not differ significantly with K2, P1, and P3 with normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p<0.05$) with K0, K2, and P4 and was not significantly different ($p>0.05$) with P1, P2, and P3. The average number of neutrophils is in the normal range of 6.20-42.6 in the K0 and P2 groups were significantly different ($p<0.05$) with K1, K2, P1, and P3 and not significantly different ($p>0.05$) with P2 and P4. Hematologic data of WBC are shown

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in Table-2.

<H2>Measurement results and lymph index

The result of length, width, weight, and index of lymph measurement in treatment group infected with *P. berghei* showed an enlarged lymph organ and significantly different ($p<0.05$) with uninfected control group (K0). In the infected and treated group with chloroquine (K2) showed the length and width of lymph which is significantly different ($p<0.05$) with K1 group. However, P1, P2, P3, and P4 showed no significant different ($p>0.05$) with K2. Lymph data are shown in Table-3.

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<H1>Discussion

<H2>RBC hematology

The normal range of HBG, erythrocytes, and HCT in non-infected control group due to the mice under normal circumstances and the erythrocytes is not damaged. In the group that infected by *P. berghei* and was not treated and the infected group that treated with chloroquine, leaf, stem bark of *S. cumini*, and its combination, the HGB values, erythrocytic cell count, HCT mean, MCV, and MCH values showed a decrease below the normal range, and the lowest is in K1 which was different from the other treatment groups, followed by K2 and P1, P3 and P4 but at P2, the number of erythrocytes is in normal range.

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This suggests that plasmodium infections cause erythrocyte hemolysis. The removal of infected and uninfected erythrocyte cause erythropoiesis in the body which becomes ineffective;, this is

caused by the abnormally high levels of TNF that has effect on ineffective erythropoiesis [18].

In vitro and *in vivo* studies show pro-inflammatory cytokines including interferon y, TNF-α, and macrophage migration inhibitory factor, as well as plasmodium products (hemozoin) which play a role in the pathogenesis of malarial anemia [19]. Hemozoin was produced from HGB digestion by plasmodium and induces macrophage for the secretion of pro-inflammatory cytokines and other mediators that inhibit the effects of erythropoiesis [20,21]. Plasmodium hemozoin products play a role in erythropoiesis resistance, low reticulocytosis, and malarial anemia by inhibiting Epo-induced proliferation from erythroid precursors [22].

Malarial anemia due to plasmodium infection may increase immunoglobulin G autoantibodies levels against non-infected RBC (nRBCs) and its deposition on the surface of nRBCs can decrease red cell deformability and improve erythrophagocytosis [23]. The results of this study in the treatment group, Hb levels, the number of erythrocytes, and levels of premature ventricular contractions are below normal, and it is due to hypochromic microcytic anemia because MCV and MCH values are below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum* 71% of anemic patients having hypochromic microcytic anemia [18].

In the P2 group infected with *P. berghei* treated with a combination of chloroquine and *S. cumini* leaf extract, the number of erythrocytes cells within normal limits. The antimalarial effects of chloroquine and antioxidants containing *S. cumini* leaf extracts cause proliferative resistance of

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P. berghei. It happened through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of HGB [24]. The decrease of proliferation in *P. berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [25]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [26]. Results of research conducted by Haroon *et al*. 2015 state that leaves, stems, and fruit of *S. cumini* have antioxidant and anti-inflammatory activity. The value of IC50 leaves of *S. cumini* is 12.84 ppm, so it is potentially developed as an antioxidant [27]. The results of Ruan *et al*. [28] showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *S. cumini* leaf extract.

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<H2>WBC hematology

Increased WBC, the number of lymphocytes, and the number of monocytes in the K1 group infected with *P. berghei* were not treated. Decreased WBC, number of lymphocytes and number of monocytes in the infected K2 group and treated with chloroquine, as well as groups treated with a combination of *S. cumini* leaf and stem bark extract with chloroquine P2 and P4. These results suggest that WBC elevation in infection by plasmodium may stimulate the immune system as a physiological response of the body to malaria infection because the WBC has a role in fighting infection, as the body's defense against infection through phagocytosis against the

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invasion of foreign organisms and producing antibodies as an immune response. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment was able to fight infection [29].

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Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *P. berghei* infection [29]. Monocytes are phagocytes. The increase in the number of monocytes suggests a body's immune response to accelerate the activity of plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization, and regulation of immune-effector cells, also contributing to infection elimination [30]. The reduction of lymphocytes and monocytes in the treatment of antimalarials and its combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids, and ellagic acid in *S. cumini* stem bark extracts have antioxidant effects. The antioxidant effects of phytochemicals in mice infected with *P. berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehyde to improve hematologic parameters [31].

<H2>Measurement and lymph index

The result of measurement of length, width, weight, and index of lymph in treatment group infected with *P. berghei* shows the enlargement of lymph organs. Lymph works as an effector against malaria infection, especially in protective immunity against infections of the blood [32]. The total number of lymph cells increased during high parasitemia and then decreased at lower

levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, and this reaction is due to an increase in hematopoietic and an increase in the number of macrophages. Increased macrophage occurs due to the process of erythrophagocytosis. Macrophage in the red pulp lymph plays an important role in removing plasmodium that infects RBC from circulation [33].

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Plasmodium infections in RBC may lead to a complex pathophysiology, and a rapid growth of plasmodium will increase the production of reactive oxygen species causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [34]. The administration of chloroquine antimalarial drug and leaf and stem bark extract of *S. cumini* and its combination can decrease the length, width, and index of lymph. This shows the effects of antimalarials and antioxidant content in leaf and stem bark extract of *S. cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, elagat acid, ferulic acid, and gallic acid. The leaves are also contained tannins and essential oil terpenes [35]. *S. cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids, and anthocyanins [36].

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Research conducted by Jayachandra *et al*. [37] showed that the total phenolic content was 580.23±3.03 mg/g, tannin content was 534±4.03 mg/g, while the flavonoid content was 315.42±4.52 mg/g. The methanolic and aqueous extracts of bark were screened for antioxidant

activity using nitric oxide scavenging activity method. Activity as an antioxidant due to the presence of flavonoids and polyphenols in the plant [38]. The combination of deferoxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in *Plasmodium yoelii* infection produces an antimalarial additive effect [39]. Decreased lesions are reversible in the lymph following a decrease in parasitemia through apoptosis as a defense mechanism of immune lymph against malaria infection [32].

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<H1>Conclusion

The combination of chloroquine with leaf and stem bark extract of *S. cumini* as adjuvant therapy may increase the amount of erythrocytes; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and index of lymph malaria mice models.

<H1>Authors' Contributions

LL designed the research, supervised the research, and compiled the manuscript; RS and RSW helped to analyze blood and lymph data. All authors have read and approved the final manuscript.

<H1>Acknowledgments

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<H1>Competing Interests

The authors declare that they have no competing interests.

<H1>Publisher's Note

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Tables

Table-1: Mean and standard deviation of RBC hematology in control and treatment groups.

Mice groups	**Parameter of RBC hematology**						
	HGB (g/dL)	**RBC (10^6/µL)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**	**RDW-CV (%)**
K0	15.45±0.50^{d}	10.56±0.27^{d}	51.40±2.55^{c}	48.10±3.39^{a}	14.60±0.53^{b}	30.30±0.49^{a}	23.42±0.70^{a}
K1	11.12±0.64^{a}	8.09±0.33^{a}	36.80±3.88^{a}	45.52±4.95^{a}	13.75±0.71^{ab}	30.37±1.80^{a}	25.92±3.51^{b}
K2	12.25±0.12^{b}	8.90±0.89^{b}	39.70±0.98^{ab}	45.37±4.91^{a}	13.87±1.07^{ab}	30.97±1.06^{a}	25.85±0.23^{b}
P1	13.12±0.41^{c}	9.14±0.36^{bc}	42.57±2.13^{b}	46.97±2.57^{a}	14.40±0.57^{ab}	30.67±0.45^{a}	21.80±0.66^{a}
P2	12.80±0.29^{bc}	9.75±0.36^{c}	42.55±2.13^{b}	43.72±1.31^{a}	13.25±0.36^{a}	30.20±0.87^{a}	23.97±0.59^{a}

P3	12.32±0.45[b]	9.15±0.53[bc]	40.15±3.70[ab]	43.82±2.47[a]	13.52±0.67[ab]	30.85±1.88[a]	24.67±3.51[ab]
P4	13.07±0.36[c]	9.14±0.62[bc]	41.26±1.03[b]	45.42±3.39[a]	14.37±0.77[ab]	31.67±0.65[a]	23.30±126[ab]

Different superscripts on the same column show a significant difference at significant level at 0.05%, RBC=Red blood cell, HGB=Hemoglobin, HCT=Hematocrit, MCH=Mean corpuscular hemoglobin

Table-2: Mean and standard deviation of WBC hematology in control and treatment groups.

Mice group	**Parameter of WBC hematology**							
	WBC	**PLT**	**Lymph**	**Lymph (%)**	**Mono**	**Mono (%)**	**Neut**	**Neut (%)**
K0	4.33±0.17[a]	1322.25±35.34[ab]	3.60±0.11[a]	83.40±0.66[abc]	0.27±0.02[a]	6.20±0.29[a]	0.35±0.04[a]	8.07±0.17[b]

K1	13.22±6.35^{c}	1551.50±451.38^{b}	10.33±4.83^{c}	79.87±6.95^{ab}	2.24±2.02^{c}	16.75±8.83^{c}	0.46±0.44^{a}	1.95±3.90^{a}
K2	8.41±0.56^{ab}	1432.75±21.09^{ab}	7.08±0.20^{bc}	88.37±2.00^{c}	0.76±0.06^{ab}	9.22±0.51^{ab}	0.35±0.04^{a}	2.07±2.39^{a}
P1	10.59±1.14^{bc}	1245.50±99.37^{ab}	8.29±0.74^{bc}	79.37±1.82^{ab}	1.70±0.51^{bc}	16.15±4.10^{bc}	0.35±0.30^{a}	3.22±3.80^{a}
P2	8.17±0.30^{ab}	1127.50±157.55^{a}	6.17±0.33^{ab}	76.07±2.36^{a}	1.36±0.19^{abc}	16.22±2.39^{bc}	0.55±0.13^{a}	6.90±0.90^{ab}
P3	11.03±0.51^{bc}	1097.25±295.11^{a}	8.83±0.41^{bc}	80.92±3.84^{abc}	1.70±0.59^{bc}	15.12±4.66^{bc}	0.36±0.26^{a}	2.52±3.17^{a}
P4	7.87±2.48^{ab}	1156.75±213.69^{a}	6.62±2.28^{b}	85.15±9.55^{bc}	0.46±0.20^{ab}	6.63±4.18^{a}	0.66±0.36^{a}	5.92±4.31^{ab}

Different superscripts on the same column show a significant difference at significant level at 0.05%, WBC=White blood cell, PLT=Platelet

Table-3: Mean and standard deviations of length, width, weight, and index of lymph mice in the control and treatment groups.

Mice Group	Length (cm)	Width (cm)	Weight (g)	Lymph index
K0	0.82±0.14[a]	0.22±0.12[a]	0.21±0.11[a]	0.008±0.002[a]
K1	3.07±0.22[c]	0.65±0.10[c]	0.44±0.14[c]	0.016±0.003[c]
K2	2.20±0.62[b]	0.45±0.19[b]	0.30±0.10[bc]	0.011±0.001[bc]

P1	2.65 ± 0.26^{bc}	0.57 ± 0.09^{bc}	0.37 ± 0.08^{bc}	0.013 ± 0.001^{c}
P2	2.42 ± 0.38^{bc}	0.50 ± 0.11^{bc}	0.34 ± 0.10^{bc}	0.013 ± 0.003^{bc}
P3	2.37 ± 0.47^{b}	0.60 ± 0.08^{bc}	0.31 ± 0.04^{bc}	0.013 ± 0.002^{bc}
P4	2.47 ± 0.30^{bc}	0.55 ± 0.10^{bc}	0.27 ± 0.07^{b}	0.009 ± 0.002^{b}
Different superscripts on the same column show a significant difference at significant level at 0.05%				

RESEARCH ARTICLE

Hematologic changes and splenic index on malaria mice models given *Syzygium cumini* extract as adjuvant therapy

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Lilik Maslachah[1], Rahmi Sugihartuti[1] and Retno Sri Wahyuni[2]

1. Department of Basic Veterinary Medicine, Veterinary Pharmacy Laboratory, Universitas Airlangga, Surabaya, Indonesia; 2. Department of Basic Veterinary Medicine, Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Corresponding author: Lilik Maslachah, e-mail: lilik.maslachah@yahoo.com

Co-authors: RS: imharedi@yahoo.co.id, RSW: wahyuniretno@yahoo.com

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Abstract

Aims: This research aimed to determine the efficacy of *Syzygium cumini* L. as adjuvant therapy on blood changes and splenic index of mice model malaria.

Materials and Methods: Mice were infected intraperitoneally with 0.2 ml red blood cell (RBC)

that contains 1×10^6 *Plasmodium berghei*. 35 mice were divided into seven treatment groups: Group K0: Mice were not infected; K1: Mice were infected; K2: Mice were infected and given chloroquine; P1: Mice were infected and given *S. cumini* leaf extract; P2: Mice were infected and given chloroquine and also *S. cumini* leaf extract; P3: Mice was infected and given *S. cumini* stem bark extract; and P4: Mice were infected and given chloroquine and *S. cumini* stem bark extract. Treatment was given for 4 days 24 h post-*P. berghei* infection. 21st day post-*P. berghei* infection, blood was taken from the heart for hematological examination, and the spleen was taken to examine the splenic index and also to measure the weight and length of the spleens. Hematological data and splenic index were analyzed by analysis of variance test, and if there is a difference, the test is continued by Duncan's multiple range test with 5% level.

Results: The K0 group has normal hemoglobin (HGB), RBC, and hematocrit (HCT) and significantly different ($p<0.05$) than other groups. HGB, RBC, and HCT of K1 group were under normal range, lowest, and significantly different ($p<0.05$) than other groups. Mean corpuscular volume and mean corpuscular HGB values of K2 groups showed a decrease. The number of leukocytes, lymphocytes, and monocytes of K1 groups was increasing and significantly different ($p<0.05$) with K2 and treatment group. The length, width, weight, and splenic index of K1 group were significantly different ($p<0.05$) with K0 group. K2 and treatment groups showed that the length and width of spleens were significantly different ($p<0.05$) with K1.

Conclusion: The combination of chloroquine with leaf and chloroquine with stem bark extract

of *S. cumini* as adjuvant therapy may increase the amount of erythrocyte; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and splenic index on malaria mice models.

Keywords: hematology, splenic index, *Syzygium cumini*, *Plasmodium berghei*.

<H1>Introduction

Malaria is still be a public health problem in 107 countries until right now because malaria is still the fifth rank of infectious diseases in the world [1]. The control and treatment of malaria are more difficult recently because malaria parasites have been resistant to drugs and also the mosquitoes are resistant to insecticides. The development of treatment, prevention, and control of malaria is one of the substantial problems in the world. In 2012, approximately 600,000 children died, mostly from Sub-Saharan Africa [2].

The death of malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly, and liver dysfunction. Hematological abnormalities during malarial infection are caused by high parasitemia in the patient; hematological abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors, and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of spleen is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic stages of malaria infection, spleen is an important organ in the immune response. Elimination of infected

erythrocytes through modulating the immune response and spleen remodeling, resulting stringent splenic retension of rings and uninfected erythrocytes reduce the risk of cerebral malaria so that severe malaria can not accur [5].

The World Health Organization recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and artemisinin-based combination therapies combination on clinical cases in Cambodia [6]. In addition, there is a decrease in efficacy of antimalarial drugs that currently used, so it is important to develop an adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria. Adjuvant therapy that can be used in severe malaria is immune system modulator preparations, antioxidants, anticoagulants, and agents that have anti-seizure activity [7]. The results showed that the use of matrix metalloproteinase (MMP) inhibitor was able to increase the survival of mice in cerebral malaria, and dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, tumor necrosis factor-alpha (TNF-α), and interleukin-1beta [8-10]. *Syzygium cumini* contains anthocyanin, ellagitannin, ellagic acid, phenolic, flavonoids, and vitamins so that it has a high antioxidant activity. This plant is one of the medicinal plants which is easy to be found in Indonesia [11,12]. Results of research conducted by Zhang *et al*. [13] showed that *S. cumini* has radical scavenging activity and strong antioxidant.

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The research was aimed to determine the efficacy of *S. cumini* L. as adjuvant therapy on hematological changes (red blood cells [RBC] and white blood cells [WBC]) and splenic index in mice model malaria.

<H1>Materials and Methods

<H2>Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

<H2>Parasite, host, and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium berghei* ANKA strain. The mice used were male Swiss albino mice with 20-30 g weight and 2.5 months old, and obtained from Veterinary Farma Surabaya (Pusvetma) Center. Antimalarial drug used chloroquine pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used 25 mg/kg body weight (bw) mice as a therapeutic dose. This drug was administered daily for 4 days [14]. The leaves and stem bark of *S. cumini* are obtained from Kediri city of East Java, Indonesia, and identified in the laboratory of Purwodadi Botanical Garden, Pasuruan. *S. cumini* dose was 600 mg/kg bw [15].

<H2>Infection dose of *P. berghei* in mice

Mice were infected with 0.2 ml RBC containing 1×10^6 *P. berghei* parasites intraperitoneally. To find out the infection which has occurred in mice, daily microscopic examination of erythrocyte

was performed with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [16]. Calculation of the dose of a parasitic infection was determined by counting the number of parasites from the thin blood smear that stained with Giemsa and then calculated the number of parasites per number of erythrocytes. The next step is calculating the amount of erythrocytes by diluting the blood using PBS solution in Eppendorf 0.5 ml. Then, this diluted blood erythrocytes were calculated using the improved Neubauer counting chamber. The number of parasitic doses is obtained from multiplying by the number of parasites with the amount of erythrocytes that have been calculated and converted to per ml.

<H2>Preparation of leaf and stem bark of *S. cumini*

The leaves and stem bark of *S. cumini* are dried, after that it was crushed into small pieces (simplicia). Simplicia was extracted with PA methanol and maceration for 3×24 h. The filtrate was evaporated using a Rotary Evaporator at 40-50°C with low pressure. The extraction results are stored on the desiccator until ready for use [17].

<H2>Treatment of the experimental animals

A total of 35 mice were randomly divided into seven treatment groups, and each group consists of 5 mice. Details of each group are as follows:

Group K0: Mice were only given drug solvent and not infected; K1: Mice were infected and given drug solvent; K2: Mice were infected and given chloroquine 25 mg/kg bw; P1: Mice were

infected and given *S. cumini* leaf extract 600 mg/kg bw; (P2): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* leaf extract 600 mg/kg bw; (P3): Mice were infected and given *S. cumini* stem bark extract 600 mg/kg bw; and (P4): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* stem bark extract 600 mg/kg bw.

Treatment was given for 4 days since 24 h after per oral infection. After 21 days post-infection, mice were anesthetized with ketamine (Sigma) and then thoracotomy, blood samples were taken from the heart (1 ml) using a Tuberculin Syringe and collected in a vial that has been given anticoagulation for hematologic examination using automated blood analyzer SYSMEX XT 4000i, and spleen organ was taken to examine the splenic index and also measure the weight and length of spleen .

<H2>Examination of weight, length, and splenic index

Previously, the weight of the mice was measured, after that the mice were injected with ketamine IM. The abdominal cavity was opened, and the spleen organ was taken and weighed using an analytical scale and then measured the length and width of the spleen using a ruler in a millimeter scale. According to Gluhcheva *et al*. [18], the index value of spleen (splenic index) was calculated using the splenic index equation = (weight of spleen organ of mice)/bw of mice.

<H2>Statistical analysis

The data of hematology observation of blood change and splenic index were processed using analysis of variance using SPSS System 17.0 then followed by Duncan's multiple range test with 5% level.

<H1>Results

<H2>Hematological results of RBC

The results of statistical tests of mean hemoglobin (HGB), RBC, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), and MCHGB concentration is presented in Table-1. The mean value of HGB in the non-infected control group (K0) was normal (15.45 g/dl; the normal range is 13.4-15.8 g/dl) which was significantly different ($p<0.05$) with infected group (K1) and infected groups and given with chloroquine (K2) as well as the infected group and given leaf extract (P1), given stem bark extract (P3) and given combination of chloroquine and leaf extract of *S.cumini* (P2), chloroquine and stem bark extract of *S. cumini,* (P4). The mean number of HGB decreased in the K1 group infected with *P.berghei* below the normal range that was significantly different ($p<0.05$) when compared to the other group (K0, K2, P1, P2, P3 and P4). In K2 group, the mean HGB was significantly different ($p<0.05$) with P1 and P4 and was not significantly different with P2 and P3. The treatment group between P1 and P4 the mean HGB was not significantly different but significantly different with P3. The mean value of RBCs in K1 showed the lowest decrease under normal 8.15-9.75 (10^6/mm^3) which was significantly different with all treatment groups ($p<0.05$). The mean

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number of RBCs K2 group was significantly different with P2 and not significantly different with P1, P3, and P4. However P1, P2, P3, and P4 groups did not show any significant difference. The mean of HCT (HCT/PCV) showed that K0 is in the normal range (44.4-50.4%) which was different with all treatment groups. The mean number of HCT decreased in the K1 group infected with *P. berghei* and did not significantly different with K2 and P3. K1 group was significantly different with P1, P2, and P4. However, P1, P2, and P4 were not significantly different. MCV and MCH values show a decrease in K2, P1, P2, P3, and P4.

<H2>Hematology result of WBCs

The mean number of leukocytes (WBC) increased in the K1 group infected with *P. berghei* and was significantly different ($p<0.05$) when compared with the infected group treated with chloroquine (K2 group) and the group treated with the combination of *S. cumini* leaf and stem bark extract with chloroquine (P2 and P4 groups). The average number of leukocytes in K2, P1, P2, P3, and P4 was still within normal range between 8.00 and 11.8 (103/m^3). The average platelet value was still within normal limits. The highest lymphocyte values are in the K1 group and were significantly different from K0, P2, and P4 and did not differ significantly with K2, P1, and P3 with normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p<0.05$) with K0, K2, and P4 and was not significantly different ($p>0.05$) with P1, P2, and P3. The normal range of neutrophils 6.20-42.6 in the K0 and P2 groups were significantly different ($p<0.05$) with K1, K2, P1. The mean number of

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neutrophils in the P3 group not significantly different ($p>0.05$) with P2 and P4. Hematologic data of WBC are shown in Table-2.

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<H2>Measurement results and splenic index

The result of length, width, weight, and splenic index measurement in treatment group infected with *P. berghei* showed an enlarged spleen organ and significantly different ($p<0.05$) with uninfected control group (K0). The mean number of length and width of spleen in the K1 group infected with *P. bergei* was significantly different ($p<0.05$) with infected and treated group given with chloroquine (K2). However, P1, P2, P3, and P4 showed no significant different ($p>0.05$) with K2. Lymph data are shown in Table-3.

<H1>Discussion

<H2>RBC hematology

The normal range of HBG, erythrocytes, and HCT in non-infected control group due to the mice under normal circumstances and the erythrocytes is not damaged. In the group that infected by *P. berghei* and was not treated and the infected group that treated with chloroquine, leaf, stem bark of *S. cumini*, and its combination, the HGB values, erythrocytic cell count, HCT mean, MCV, and MCH values showed a decrease below the normal range, and the lowest is in K1 which was different from the other treatment groups.

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This suggests that plasmodium infections cause erythrocyte hemolysis. The removal of infected and uninfected erythrocyte cause erythropoiesis in the body which becomes ineffective;, this is

caused by the abnormally high levels of TNF that has effect on ineffective erythropoiesis [19].

In vitro and *in vivo* studies show pro-inflammatory cytokines including interferon y, TNF-α, and macrophage migration inhibitory factor, as well as plasmodium products (hemozoin) which play a role in the pathogenesis of malarial anemia [20]. Hemozoin was produced from HGB digestion by plasmodium and induces macrophage for the secretion of pro-inflammatory cytokines and other mediators that inhibit the effects of erythropoiesis [21,22]. Plasmodium hemozoin products play a role in erythropoiesis resistance, low reticulocytosis, and malarial anemia by inhibiting Epo-induced proliferation from erythroid precursors [23].

Malarial anemia due to plasmodium infection may increase immunoglobulin G autoantibodies levels against non-infected RBC (nRBCs) and its deposition on the surface of nRBCs can decrease red cell deformability and improve erythrophagocytosis [24]. The results of this study in the treatment group, Hb levels, the number of erythrocytes, and levels of premature ventricular contractions are below normal, and it is due to hypochromic microcytic anemia because MCV and MCH values are below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum* 71% of anemic patients having hypochromic microcytic anemia [19].

In the P2 group infected with *P. berghei* treated with a combination of chloroquine and *S. cumini* leaf extract, the number of erythrocytes cells value was still within normal. The antimalarial effects of chloroquine and antioxidants containing *S. cumini* leaf extracts cause proliferative

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resistance of *P. berghei*. It happened through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of HGB [25]. The decrease of proliferation in *P. berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [26]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [27]. Results of research conducted by Haroon *et al*. [28] state that leaves, stems, and fruit of *S. cumini* have antioxidant and anti-inflammatory activity. The value of IC50 leaves of *S. cumini* is 12.84 ppm, so it is potentially developed as an antioxidant [29]. The results of Ruan *et al*. [30] showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *S. cumini* leaf extract.

<H2>WBC hematology

Increased WBC, the number of lymphocytes, and the number of monocytes in the K1 group infected with *P. berghei* were not treated. The number of WBC, number lymphocytes and monocytes decreased in the infected K2 group and treated with chloroquine, as well as groups treated with a combination of *S. cumini* leaf (P2) and stem bark extract with chloroquine (P4). These results suggest that WBC elevation in infection by plasmodium may stimulate the immune system as a physiological response of the body to malaria infection because the WBC has a role in fighting infection, as the body's defense against infection through phagocytosis against the

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invasion of foreign organisms and stimulate increase in the production of the cells of immune system to release antibodies as an immune response. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment was able to fight infection [31].

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Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *P. berghei* infection [31]. Monocytes are phagocytes. The increase in the number of monocytes suggests a body's immune response to accelerate the activity of plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization, and regulation of imune-effector cells, also contributing to infection elimination [32]. The reduction of lymphocytes and monocytes in the treatment of antimalarials and its combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids, and ellagic acid in *S. cumini* stem bark extracts have antioxidant effects. The antioxidant effects of phytochemicals in mice infected with *P. berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehyde to improve hematologic parameters [33].

<H2>Measurement and splenic index

The result of measurement of length, width, weight, and splenic index of spleen in treatment group infected with *P. berghei* shows the enlargement of spleen organs. Spleen works as an effector against malaria infection, especially in protective immunity against infections of the

blood [34]. The total number of spleen cells increased during high parasitemia and then decreased at lower levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, this reaction caused hematopoietic and the number of macrophages increased. In the spleen organ increased macrophage occurs due to the process of erythrophagocytosis. Macrophage in the red pulp spleen plays an important role in removing plasmodium that infects RBC from circulation [35].

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Plasmodium infections in RBC may lead to a complex pathophysiology, and a rapid growth of plasmodium will increase the production of reactive oxygen species causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [36]. The administration of chloroquine antimalarial drug and leaf and stem bark extract of *S. cumini* and its combination can decrease the length, width, and splenic index of spleen. This shows the effects of antimalarials and antioxidant content in leaf and stem bark extract of *S. cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, ellagic acid, ferulic acid, and gallic acid. The leaves are also contained tannins and essential oil terpenes [37]. *S. cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids, and anthocyanins [38].

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Research conducted by Jayachandra *et al*. [39] showed that the total phenolic content was 580.23±3.03 mg/g, tannin content was 534±4.03 mg/g, while the flavonoid content was

315.42±4.52 mg/g. The methanolic and aqueous extracts of bark were screened for antioxidant activity using nitric oxide scavenging activity method. Activity as an antioxidant due to the presence of flavonoids and polyphenols in the stem bark of the plant [40]. The combination of deferoxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in *Plasmodium yoelii* infection produces an antimalarial additive effect [41]. Decreased lesions are reversible in the spleen following a decrease in parasitemia through apoptosis as a defense mechanism of immune spleen against malaria infection [34].

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<H1>Conclusion

The combination of chloroquine with leaf and stem bark extract of *S. cumini* as adjuvant therapy may increase the amount of erythrocytes; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and splenic index of spleen malaria mice models.

<H1>Authors' Contributions

LL designed the research, supervised the research, and compiled the manuscript; RS and RSW helped to analyze blood and spleen data. All authors have read and approved the final manuscript.

<H1>Acknowledgments

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<H1>Competing Interests

The authors declare that they have no competing interests.

<H1>**Publisher's Note**

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Tables

Table-1: Mean and standard deviation of RBC hematology in control and treatment groups.

Mice groups	Parameter of RBC hematology						
	HGB (g/dL)	**RBC (10^6/µL)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**	**RDW-CV (%)**
K0	15.45±0.50[d]	10.56±0.27[d]	51.40±2.55[c]	48.10±3.39[a]	14.60±0.53[b]	30.30±0.49[a]	23.42±0.70[a]
K1	11.12±0.64[a]	8.09±0.33[a]	36.80±3.88[a]	45.52±4.95[a]	13.75±0.71[ab]	30.37±1.80[a]	25.92±3.51[b]

K2	12.25±0.12^{b}	8.90±0.89^{b}	39.70±0.98^{ab}	45.37±4.91^{a}	13.87±1.07^{ab}	30.97±1.06^{a}	25.85±0.23^{b}
P1	13.12±0.41^{c}	9.14±0.36^{bc}	42.57±2.13^{b}	46.97±2.57^{a}	14.40±0.57^{ab}	30.67±0.45^{a}	21.80±0.66^{a}
P2	12.80±0.29^{bc}	9.75±0.36^{c}	42.55±2.13^{b}	43.72±1.31^{a}	13.25±0.36^{a}	30.20±0.87^{a}	23.97±0.59^{a}
P3	12.32±0.45^{b}	9.15±0.53^{bc}	40.15±3.70^{ab}	43.82±2.47^{a}	13.52±0.67^{ab}	30.85±1.88^{a}	24.67±3.51^{ab}
P4	13.07±0.36^{c}	9.14±0.62^{bc}	41.26±1.03^{b}	45.42±3.39^{a}	14.37±0.77^{ab}	31.67±0.65^{a}	23.30±126^{ab}
Different superscripts on the same column show a significant difference at significant level at 0.05%, RBC=Red blood cell, HGB=Hemoglobin, HCT=Hematocrit, MCH=Mean corpuscular hemoglobin							

Table-2: Mean and standard deviation of WBC hematology in control and treatment groups.								
Mice group	**Parameter of WBC hematology**							
	WBC	**PLT**	**Lymph**	**Lymph (%)**	**Mono**	**Mono (%)**	**Neut**	**Neut (%)**

K0	4.33±0.17^{a}	1322.25±35.34^{ab}	3.60±0.11^{a}	83.40±0.66^{abc}	0.27±0.02^{a}	6.20±0.29^{a}	0.35±0.04^{a}	8.07±0.17^{b}
K1	13.22±6.35^{c}	1551.50±451.38^{b}	10.33±4.83^{c}	79.87±6.95^{ab}	2.24±2.02^{c}	16.75±8.83^{c}	0.46±0.44^{a}	1.95±3.90^{a}
K2	8.41±0.56^{ab}	1432.75±21.09^{ab}	7.08±0.20^{bc}	88.37±2.00^{c}	0.76±0.06^{ab}	9.22±0.51^{ab}	0.35±0.04^{a}	2.07±2.39^{a}
P1	10.59±1.14^{bc}	1245.50±99.37^{ab}	8.29±0.74^{bc}	79.37±1.82^{ab}	1.70±0.51^{bc}	16.15±4.10^{bc}	0.35±0.30^{a}	3.22±3.80^{a}
P2	8.17±0.30^{ab}	1127.50±157.55^{a}	6.17±0.33^{ab}	76.07±2.36^{a}	1.36±0.19^{abc}	16.22±2.39^{bc}	0.55±0.13^{a}	6.90±0.90^{ab}
P3	11.03±0.51^{bc}	1097.25±295.11^{a}	8.83±0.41^{bc}	80.92±3.84^{abc}	1.70±0.59^{bc}	15.12±4.66^{bc}	0.36±0.26^{a}	2.52±3.17^{a}

P4	7.87±2.48^{ab}	1156.75±213.69^{a}	6.62±2.28^{b}	85.15±9.55^{bc}	0.46±0.20^{ab}	6.63±4.18^{a}	0.66±0.36^{a}	5.92±4.31^{ab}
Different superscripts on the same column show a significant difference at significant level at 0.05%, WBC=White blood cell, PLT=Platelet								

Table-3: Mean and standard deviations of length, width, weight, and splenic index of spleen mice in the control and treatment groups.

Mice Group	Length (cm)	Width (cm)	Weight (g)	Splenic index
K0	0.82±0.14^{a}	0.22±0.12^{a}	0.21±0.11^{a}	0.008±0.002^{a}
K1	3.07±0.22^{c}	0.65±0.10^{c}	0.44±0.14^{c}	0.016±0.003^{c}
K2	2.20±0.62^{b}	0.45±0.19^{b}	0.30±0.10^{bc}	0.011±0.001^{bc}
P1	2.65±0.26^{bc}	0.57±0.09^{bc}	0.37±0.08^{bc}	0.013±0.001^{c}
P2	2.42±0.38^{bc}	0.50±0.11^{bc}	0.34±0.10^{bc}	0.013±0.003^{bc}

P3	2.37±0.47^{b}	0.60±0.08^{bc}	0.31±0.04^{bc}	0.013±0.002^{bc}
P4	2.47±0.30^{bc}	0.55±0.10^{bc}	0.27±0.07^{b}	0.009 ±0.002^{b}
Different superscripts on the same column show a significant difference at significant level at 0.05%				

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RESEARCH ARTICLE

Hematologic changes and ~~lymph~~ splenic index on malaria mice models given *Syzygium cumini* extract as adjuvant therapy

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Lilik Maslachah[1], Rahmi Sugihartuti[1] and Retno Sri Wahyuni[2]

1. Department of Basic Veterinary Medicine, Veterinary Pharmacy Laboratory, ~~Airlangga University~~Universitas Airlangga, Surabaya, Indonesia; 2. Department of Basic Veterinary Medicine, Clinical Pathology Laboratory, Faculty of Veterinary Medicine, ~~Airlangga University~~Universitas Airlangga , Surabaya, Indonesia.

Corresponding author: Lilik Maslachah, e-mail: lilik.maslachah@yahoo.com

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Co-authors: RS: imharedi@yahoo.co.id, RSW: wahyuniretno@yahoo.com

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Abstract

Aims: This research aimed to determine the efficacy of *Syzygium cumini* L. as adjuvant therapy on blood changes and ~~lymph~~ splenic index of mice model malaria.

Materials and Methods: Mice were infected intraperitoneally with 0.2 ml red blood cell (RBC)

that contains 1×10^6 *Plasmodium berghei*. 35 mice were divided into seven treatment groups: Group K0: Mice were not infected; K1: Mice were infected; K2: Mice were infected and given chloroquine; P1: Mice were infected and given *S. cumini* leaf extract; P2: Mice were infected and given chloroquine and also *S. cumini* leaf extract; P3: Mice was infected and given *S. cumini* stem bark extract; and P4: Mice were infected and given chloroquine and *S. cumini* stem bark extract. Treatment was given for 4 days 24 h post-*P. berghei* infection. 21st day post-*P. berghei* infection, blood was taken from the heart for hematological examination, and the ~~lymph~~ spleen was taken to examine the ~~lymph~~ splenic index and also to measure the weight and length of the ~~lymph~~spleen. Hematological data and ~~lymph~~ splenic index were analyzed by analysis of variance test, and if there is a difference, the test is continued by Duncan's multiple range test with 5% level.

Results: The K0 group has normal hemoglobin (HGB), RBC, and hematocrit (HCT) and significantly different ($p<0.05$) than other groups. HGB, RBC, and HCT of K1 group were under normal range, lowest, and significantly different ($p<0.05$) than other groups. Mean corpuscular volume and mean corpuscular HGB values of K2 groups showed a decrease. The number of leukocytes, lymphocytes, and monocytes of K1 groups was increasing and significantly different ($p<0.05$) with K2 and treatment group. The length, width, weight, and ~~lymph~~ splenic index of K1 group were significantly different ($p<0.05$) with K0 group. K2 and treatment groups showed that the length and width of ~~lymph~~ spleens were significantly different ($p<0.05$) with K1.

Conclusion: The combination of chloroquine with leaf and chloroquine with stem bark extract of *S. cumini* as adjuvant therapy may increase the amount of erythrocyte; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and ~~index of lymph~~splenic index on malaria mice models.

Keywords: hematology, ~~lymph~~ splenic index, *Syzygium cumini*, *Plasmodium berghei*.

<H1>Introduction

Malaria is still be a public health problem in 107 countries until right now because malaria is still the fifth rank of infectious diseases in the world [1]. The control and treatment of malaria are more difficult recently because malaria parasites have been resistant to drugs and also the mosquitoes are resistant to insecticides. The development of treatment, prevention, and control of malaria is one of the substantial problems in the world. In 2012, approximately 600,000 children died, mostly from Sub-Saharan Africa [2].

The death of malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly, and liver dysfunction. Hematological abnormalities during malarial infection are caused by high parasitemia in the patient; hematological abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors, and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of ~~lymph~~ spleen is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic

stages of malaria infection, lymph spleen is an important organ in the immune response. Elimination of infected erythrocytes through modulating the immune response and lymph remodeling, resulting an immune response imbalance that can lead to severe malaria [5]. Elimination of infected erythrocytes through modulating the immune response and spleen remodeling, resulting stringent splenic retension of rings and uninfected erythrocytes reduce the risk of cerebral malaria so that severe malaria do not accur [5]

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The World Health Organization recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and artemisinin-based combination therapies combination on clinical cases in Cambodia [6]. In addition, there is a decrease in efficacy of antimalarial drugs that currently used, so it is important to develop an adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria. Adjuvant therapy that can be used in severe malaria is immune system modulator preparations, antioxidants, anticoagulants, and agents that have anti-seizure activity [7]. The results showed that the use of matrix metalloproteinase (MMP) inhibitor was able to increase the survival of mice in cerebral malaria, and dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, tumor necrosis factor-alpha (TNF-α), and interleukin-1beta [8-10]. *Syzygium cumini* contains anthocyanin, ellagitannin, ellagic acid, phenolic, flavonoids, and vitamins so that it has a high antioxidant activity. This

plant is one of the medicinal plants which is easy to be found in Indonesia [11,12]. Results of research conducted by Zhang *et al*., [13]~~2009,~~ showed that *S. cumini* has radical scavenging activity and strong antioxidant.

The research was aimed to determine the efficacy of *S. cumini* L. as adjuvant therapy on hematological changes (red blood cells [RBC] and white blood cells [WBC]) and ~~lymph~~ splenic index in mice model malaria.

<H1>Materials and Methods

<H2>Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine, ~~Airlangga University~~Universitas Airlangga, Surabaya, Indonesia.

<H2>Parasite, host, and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium berghei* ANKA strain. The mice used were male Swiss albino mice with 20-30 g weight and 2.5 months old, and obtained from Veterinary Farma Surabaya (Pusvetma) Center. Antimalarial drug used chloroquine pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used 25 mg/kg body weight (bw) mice as a therapeutic dose. This drug was administered daily for 4 days [14]~~[13]~~. The leaves and stem bark of *S. cumini* are obtained from Kediri city of East Java, Indonesia, and identified in the

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laboratory of Purwodadi Botanical Garden, Pasuruan. *S. cumini* dose was 600 mg/kg bw [15].~~[14].~~

<H2>Infection dose of *P. berghei* in mice

Mice were infected with 0.2 ml RBC containing 1×10^6 *P. berghei* parasites intraperitoneally. To find out the infection which has occurred in mice, daily microscopic examination of erythrocyte was performed with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [16]~~[15]~~. Calculation of the dose of a parasitic infection was determined by counting the number of parasites from the thin blood smear that stained with Giemsa and then calculated the number of parasites per number of erythrocytes. The next step is calculating the amount of erythrocytes by diluting the blood using PBS solution in Eppendorf 0.5 ml. Then, this diluted blood erythrocytes were calculated using the improved Neubauer counting chamber. The number of parasitic doses is obtained from multiplying by the number of parasites with the amount of erythrocytes that have been calculated and converted to per ml.

<H2>Preparation of leaf and stem bark of *S. cumini*

The leaves and stem bark of *S. cumini* are dried, after that it was crushed into small pieces (simplicia). Simplicia was extracted with PA methanol and maceration for 3×24 h. The filtrate was evaporated using a Rotary Evaporator at 40-50°C with low pressure. The extraction results are stored on the desiccator until ready for use [17]~~[16]~~.

<H2>Treatment of the experimental animals

A total of 35 mice were randomly divided into seven treatment groups, and each group consists of 5 mice. Details of each group are as follows:

Group K0: Mice were only given drug solvent and not infected; K1: Mice were infected and given drug solvent; K2: Mice were infected and given chloroquine 25 mg/kg bw; P1: Mice were infected and given *S. cumini* leaf extract 600 mg/kg bw; (P2): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* leaf extract 600 mg/kg bw; (P3): Mice were infected and given *S. cumini* stem bark extract 600 mg/kg bw; and (P4): Mice were infected and given chloroquine 25 mg/kg bw and also given *S. cumini* stem bark extract 600 mg/kg bw.

Treatment was given for 4 days since 24 h after per oral infection. After 21 days post-infection, mice were anesthetized with ketamine (Sigma) and then thoracotomy, blood samples were taken from the heart (1 ml) using a Tuberculin Syringe and collected in a vial that has been given anticoagulation for hematologic examination using automated blood analyzer SYSMEX XT 4000i, and ~~lymph organ~~spleen was taken to examine the ~~lymph~~ splenic index and also measure the weight and length of ~~lymph~~spleen.

<H2>Examination of weight, length, and ~~index of lymph~~splenic index

Previously, the weight of the mice was measured, after that the mice were injected with ketamine IM. The abdominal cavity was opened, and the ~~lymph organ~~ spleen was taken and weighed using an analytical scale and then measured the length and width of the ~~lymph~~ spleen using a ruler in a millimeter scale. According to Gluhcheva *et al*. [~~17~~18], the ~~index value of lymph (~~splenic index~~)~~ was calculated using the lymph index equation = (weight of spleen organ of mice)/bw of mice.

<H2>**Statistical analysis**

The data of hematology observation of blood change and lymph index were processed using analysis of variance using SPSS System 17.0 then followed by Duncan's multiple range test with 5% level.

<H1>**Results**

<H2>**Hematological results of RBC**

The results of statistical tests of mean hemoglobin (HGB), RBC, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), and MCHGB concentration is presented in Table-1. The mean value of HGB in the non-infected control group (K0) was normal (15.45 g/dl; the normal range is 13.4-15.8 g/dl) HGB levels in the K0 group were significantly different from the other treatment groups, namely K1, K2, P1, P2, P3 and P4 ($p<0.05$). Among these groups, there was no significant difference between the K2 group and

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P2 and P3, and between P1 and P4 ($p>0.05$)

. The amount of RBC in the K0 group was significantly different from the number of RBCs in the entire treatment group ($p <0.05$). But there was no significant difference in the number of RBCs in the treatment groups K2, P1, P3 and P4 and between the treatment groups P1, P2, P3 and P4 ($p> 0.05$).

. The average of HCT/PCV level in the K0 group was significantly different from all treatment group in this study ($p<0.05$). However, there was no significant difference between treatment groups K1, K2 and P3 and between treatment groups K2, P1, P2, P3 and P4 ($p>0.05$)which was significantly different ($p<0.05$) with all the groups in the infected group and not given treatment (K1) and groups that treated with chloroquine (K2) as well as the treatment group that given leaf extract, stem bark extract and a combination of leaf, stem bark extract of *S. cumini*, and also chloroquine (P1, P2, P3 and P4). The K1 group showed the lowest HGB below the normal range that was significantly different ($p<0.05$) when compared to the other group. In K2 group, the mean HGB was significantly different ($p<0.05$) with P1 and P4 and was not significantly different with P2 and P3. The treatment group between P1 and P4 was not significantly different but significantly different with P3. The mean value of RBCs in K1 showed the lowest decrease under normal 8.15-9.75 ($10^6/mm^3$) which was significantly different with all treatment groups ($p<0.05$). K2 group was significantly different with P2 and not significantly different with P1, P3, and P4. P1, P2, P3, and P4 groups did not show any significant difference.

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The mean of HCT (HCT/PCV) showed that K0 is in the normal range (44.4-50.4%) which was different with all treatment groups. K1 group showed the lowest HCT value and did not differ significantly with K2 and P3. K1 group was significantly different with P1, P2, and P4. However, P1, P2, and P4 were not significantly different. MCV and MCH values show a decrease in K2, P1, P2, P3, and P4.

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<H2>Hematology result of WBCs

The mean number of leukocytes (WBC) increased in the K1 group infected with *P. berghei* and was significantly different ($p<0.05$) when compared with the infected group treated with chloroquine (K2 group) and the group treated with the combination of *S. cumini* leaf and stem bark extract with chloroquine (P2 and P4 groups). The average number of leukocytes in K2, P1, P2, P3, and P4 was still within normal range between 8.00 and 11.8 ($103/m^3$). The average platelet value was still within normal limits. The highest lymphocyte values are in the K1 group and were significantly different from K0, P2, and P4 and did not differ significantly with K2, P1, and P3 with normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p<0.05$) with K0, K2, and P4 and was not significantly different ($p>0.05$) with P1, P2, and P3. The average number of neutrophils showed varied results among the treatment groups. The treatment groups K0, K2 and P4 do not show significant differences. Similarly between treatment groups K2, P1, P2 and P3, and between treatment groups K1, P1, P2 and P3 ($p>0.05$). Significant differences were seen in the K0 group with K1,

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P1, P2 and P3 (p <0.05)~~The average number of neutrophils is in the normal range of 6.20-42.6 in the K0 and P2 groups were significantly different (p<0.05) with K1, K2, P1, and P3 and not significantly different (p>0.05) with P2 and P4.~~ Hematologic data of WBC are shown in Table-2.

<H2>Measurement results and ~~lymph~~ splenic index

The result of length, width, weight, and ~~index of lymph~~splenic index measurement in treatment group infected with *P. berghei* showed an enlarged lymph organ and significantly different (p<0.05) with uninfected control group (K0). ~~In the infected and treated group with chloroquine (K2) showed the length and width of lymph which is significantly different (p<0.05) with K1 group~~. The length and width of the spleen in the K1 group which infected with *P. bergei* were significantly different from the K2 group that infected and treated with chloroquine However, P1, P2, P3, and P4 showed no significant different (p>0.05) with K2. ~~Lymph~~ spleen data are shown in Table-3.

<H1>Discussion

<H2>RBC hematology

The normal range of HBG, erythrocytes, and HCT in non-infected control group due to the mice under normal circumstances and the erythrocytes is not damaged. In the group that infected by *P. berghei* and was not treated and the infected group that treated with chloroquine, leaf, stem bark of *S. cumini*, and its combination, the HGB values, erythrocytic cell count, HCT mean, MCV, and MCH values showed a decrease below the normal range, and the lowest is in K1 which was

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different from the other treatment groups, ~~followed by K2 and P1, P3 and P4 but at P2, the number of erythrocytes is in normal range.~~

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This suggests that plasmodium infections cause erythrocyte hemolysis. The removal of infected and uninfected erythrocyte cause erythropoiesis in the body which becomes ineffective;, this is caused by the abnormally high levels of TNF that has effect on ineffective erythropoiesis [~~18~~19]. *In vitro* and *in vivo* studies show pro-inflammatory cytokines including interferon y, TNF-α, and macrophage migration inhibitory factor, as well as plasmodium products (hemozoin) which play a role in the pathogenesis of malarial anemia [~~19~~20]. Hemozoin was produced from HGB digestion by plasmodium and induces macrophage for the secretion of pro-inflammatory cytokines and other mediators that inhibit the effects of erythropoiesis [~~20~~21,~~21~~22]. Plasmodium hemozoin products play a role in erythropoiesis resistance, low reticulocytosis, and malarial anemia by inhibiting Epo-induced proliferation from erythroid precursors [~~22~~23].

Malarial anemia due to plasmodium infection may increase immunoglobulin G autoantibodies levels against non-infected RBC (nRBCs) and its deposition on the surface of nRBCs can decrease red cell deformability and improve erythrophagocytosis [~~23~~24]. The results of this study in the treatment group, Hb levels, the number of erythrocytes, and levels of premature ventricular contractions are below normal, and it is due to hypochromic microcytic anemia because MCV and MCH values are below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum*

71% of anemic patients having hypochromic microcytic anemia [~~18~~19].

~~In the P2 group infected with *P. berghei* treated with a combination of chloroquine and *S. cumini* leaf extract, the number of erythrocytes cells within normal limits.~~ In the treatment group (P1, P2, P3 and P4), the number of erythrocytes were within normal range. The antimalarial effects of chloroquine and antioxidants containing *S. cumini* leaf extracts cause proliferative resistance of *P. berghei.* It happened through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of HGB [~~24~~25]. The decrease of proliferation in *P. berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [~~25~~26]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [~~26~~27]. ~~Results of research conducted by Haroon *et al.* 2015~~Previous research state that leaves, stems, and fruit of *S. cumini* have antioxidant and anti-inflammatory activity[28]. The value of IC50 leaves of *S. cumini* is 12.84 ppm, so it is potentially developed as an antioxidant [~~27~~29]. ~~The results of Ruan *et al.* [28]~~Another research showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *S. cumini* leaf extract[30].

<H2>WBC hematology

Increased WBC, the number of lymphocytes, and the number of monocytes in the K1 group infected with *P. berghei* were not treated. ~~Decreased WBC, number of lymphocytes and number~~

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~~of monocytes in the infected K2 group and treated with chloroquine, as well as groups treated with a combination of *S. cumini* leaf and stem bark extract with chloroquine P2 and P4~~. The number of WBC, lymphocytes, and monocyte were decreased in the K2 group as well as in P2 and P4 group. These results suggest that WBC elevation in infection by plasmodium may stimulate the immune system as a physiological response of the body to malaria infection because the WBC has a role in ~~fighting~~ against infection~~, as the body's defense against infection through phagocytosis against the invasion of foreign organisms and producing antibodies as an immune response.~~ When an infection occurs, the body will respond by phagositosis of infectious agent and stimulate increased production of immune cells to produce antibodies. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment was able to fight infection [~~29~~31].

Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *P. berghei* infection [~~29~~31]. Monocytes are phagocytes. The increase in the number of monocytes suggests a body's immune response to accelerate the activity of plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization, and regulation of immune-effector cells, also contributing to infection elimination [~~30~~32]. The reduction of lymphocytes and monocytes in the treatment of antimalarials and its combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids, and ellagic acid in *S. cumini* stem bark extracts

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have antioxidant effects. The antioxidant effects of phytochemicals in mice infected with *P. berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehyde to improve hematologic parameters [~~31~~33].

<H2>Measurement and ~~lymph~~ splenic index

The result of measurement of length, width, weight, and ~~index of lymph~~ splenic index in treatment group infected with *P. berghei* shows the enlargement of lymph organs. Lymph works as an effector against malaria infection, especially in protective immunity against infections of the blood [~~32~~34]. The total number of ~~lymph~~ spleen cells increased during high parasitemia and then decreased at lower levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, ~~and this reaction is due to an increase in hematopoietic and an increase in the number of macrophages~~ that caused by hematopoietic reaction. Increased macrophage occurs due to the process of erythrophagocytosis. Macrophage in the red pulp lymph plays an important role in removing plasmodium that infects RBC from circulation [~~33~~35].

Plasmodium infections in RBC may lead to a complex pathophysiology, and a rapid growth of plasmodium will increase the production of reactive oxygen species causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [~~34~~36]. The administration of chloroquine antimalarial drug and leaf and stem bark extract of *S. cumini* and its combination can decrease the length, width, and ~~index of lymph~~splenic index. This shows the

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effects of antimalarials and antioxidant content in leaf and stem bark extract of *S. cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, ~~elagat acid,~~ellagic acid, ferulic acid, and gallic acid. The leaves are also contained tannins and essential oil terpenes [~~35~~37]. *S. cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids, and anthocyanins [~~36~~38].

Research conducted by Jayachandra ~~*et al.*~~and colleague [~~37~~39] showed that the total phenolic content was 580.23±3.03 mg/g, tannin content was 534±4.03 mg/g, while the flavonoid content was 315.42±4.52 mg/g. The methanolic and aqueous extracts of bark were screened for antioxidant activity using nitric oxide scavenging activity method. ~~Activity as an antioxidant due to the presence of flavonoids and polyphenols in the plan~~ Antioxidant activity is caused by the flavonoid and polyphenol content ~~t~~ [~~38~~40]. The combination of deferoxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in *Plasmodium yoelii* infection produces an antimalarial additive effect [~~39~~41]. Decreased lesions are reversible in the ~~lymph~~ spleen following a decrease in parasitemia through apoptosis as a defense mechanism of ~~immune lymph against~~spleen against malaria infection [~~32~~34].

<H1>Conclusion

The combination of chloroquine with leaf and stem bark extract of *S. cumini* as adjuvant therapy may increase the amount of erythrocytes; decrease the number of leukocytes, lymphocytes, and

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monocytes; and decrease the length, width, and ~~index of lymph~~ splenic index ~~malaria~~ of malaria in mice models.

<H1>Authors' Contributions

LL designed the research, supervised the research, and compiled the manuscript; RS and RSW helped to analyze blood and ~~lymph~~ spleen data. All authors have read and approved the final manuscript.

<H1>~~Acknowledgments~~Acknowledgement

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Thank you to the Dean of the Faculty of Veterinary Medicine of ~~Airlangga University~~ Universitas Airlangga, Indonesia for RKAT funding support for this research with No SK 58/UN3.1.6/2017.

<H1>Competing Interests

The authors declare that they have no competing interests.

<H1>Publisher's Note

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Tables

Table-1: Mean and standard deviation of RBC hematology in control and treatment groups.

Mice groups	**Parameter of RBC hematology**						
	HGB (g/dL)	**RBC (10^6/µL)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dL)**	**RDW-CV (%)**
K0	15.45±0.50^{d}	10.56±0.27^{d}	51.40±2.55^{c}	48.10±3.39^{a}	14.60±0.53^{b}	30.30±0.49^{a}	23.42±0.70^{a}
K1	11.12±0.64^{a}	8.09±0.33^{a}	36.80±3.88^{a}	45.52±4.95^{a}	13.75±0.71^{ab}	30.37±1.80^{a}	25.92±3.51^{b}
K2	12.25±0.12^{b}	8.90±0.89^{b}	39.70±0.98^{ab}	45.37±4.91^{a}	13.87±1.07^{ab}	30.97±1.06^{a}	25.85±0.23^{b}

P1	13.12±0.41^{c}	9.14±0.36^{bc}	42.57±2.13^{b}	46.97±2.57^{a}	14.40±0.57^{ab}	30.67±0.45^{a}	21.80±0.66^{a}
P2	12.80±0.29^{bc}	9.75±0.36^{c}	42.55±2.13^{b}	43.72±1.31^{a}	13.25±0.36^{a}	30.20±0.87^{a}	23.97±0.59^{a}
P3	12.32±0.45^{b}	9.15±0.53^{bc}	40.15±3.70^{ab}	43.82±2.47^{a}	13.52±0.67^{ab}	30.85±1.88^{a}	24.67±3.51^{ab}
P4	13.07±0.36^{c}	9.14±0.62^{bc}	41.26±1.03^{b}	45.42±3.39^{a}	14.37±0.77^{ab}	31.67±0.65^{a}	23.30±126^{ab}

Different superscripts on the same column show a significant difference at significant level at 0.05%, RBC=Red blood cell, HGB=Hemoglobin, HCT=Hematocrit, MCH=Mean corpuscular hemoglobin

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Table-2: Mean and standard deviation of WBC hematology in control and treatment groups.

Mice group	**Parameter of WBC hematology**							
	WBC	**PLT**	**Lymp**	**Lymp**	**Mono**	**Mono**	**Neut**	**Neut (%)**

			h	**h (%)**		**(%)**		
K0	4.33±0.17^{a}	1322.25±35.34^{ab}	3.60±0.11^{a}	83.40±0.66^{abc}	0.27±0.02^{a}	6.20±0.29^{a}	0.35±0.04^{a}	8.07±0.17^{b}
K1	13.22±6.35^{c}	1551.50±451.38^{b}	10.33±4.83^{c}	79.87±6.95^{ab}	2.24±2.02^{c}	16.75±8.83^{c}	0.46±0.44^{a}	1.95±3.90^{a}
K2	8.41±0.56^{ab}	1432.75±21.09^{ab}	7.08±0.20^{bc}	88.37±2.00^{c}	0.76±0.06^{ab}	9.22±0.51^{ab}	0.35±0.04^{a}	2.07±2.39^{a}
P1	10.59±1.14^{bc}	1245.50±99.37^{ab}	8.29±0.74^{bc}	79.37±1.82^{ab}	1.70±0.51^{bc}	16.15±4.10^{bc}	0.35±0.30^{a}	3.22±3.80^{a}
P2	8.17±0.30^{ab}	1127.50±157.55^{a}	6.17±0.33^{ab}	76.07±2.36^{a}	1.36±0.19^{abc}	16.22±2.39^{bc}	0.55±0.13^{a}	6.90±0.90^{ab}
P3	11.03±0.51^{b}	1097.25±295.11^{a}	8.83±0.4	80.92±3.8	1.70±0.59^{bc}	15.12±4.	0.36±0.2	2.52±3.17^{a}

	^{c}		1^{bc}	4^{abc}		66^{bc}	6^{a}	
P4	7.87± 2.48^{ab}	1156.75±213 .69^{a}	6.62 ±2.2 8^{b}	85.15 ±9.5 5^{bc}	0.46±0. 20^{ab}	6.63 ±4.1 8^{a}	0.66 ±0.3 6^{a}	5.92±4.3 1^{ab}
Different superscripts on the same column show a significant difference at significant level at 0.05%, WBC=White blood cell, PLT=Platelet								

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Table-3: Mean and standard deviations of length, width, weight, and ~~index of lymph~~splenic index of mice in the control and treatment groups.				
Mice Group	**Length (cm)**	**Width (cm)**	**Weight (g)**	~~**Lymph**~~ **splenic**

				index
K0	0.82±0.14[a]	0.22±0.12[a]	0.21±0.11[a]	0.008±0.002[a]
K1	3.07±0.22[c]	0.65±0.10[c]	0.44±0.14[c]	0.016±0.003[c]
K2	2.20±0.62[b]	0.45±0.19[b]	0.30±0.10[bc]	0.011±0.001[bc]
P1	2.65±0.26[bc]	0.57±0.09[bc]	0.37±0.08[bc]	0.013±0.001[c]
P2	2.42±0.38[bc]	0.50±0.11[bc]	0.34±0.10[bc]	0.013±0.003[bc]
P3	2.37±0.47[b]	0.60±0.08[bc]	0.31±0.04[bc]	0.013±0.002[bc]
P4	2.47±0.30[bc]	0.55±0.10[bc]	0.27±0.07[b]	0.009 ±0.002[b]
Different superscripts on the same column show a significant difference at significant level at 0.05%				

Re: revised article Lilik Maslachah

Yahoo/Inbox

Veterinary World <editorveterinaryworld@gmail.com>

To: Lilik Maslachah

Cc: Rahmi Sugihartuti, Retno Sri Wahyuni Drh. MS, Veterinary World - Publisher

Wed, Jan 9, 2019 at 12:52 PM

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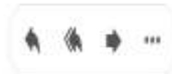

Decision Letter to Authors - Acceptance - (VETWORLD-2018-04-187)

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Kepada: lilik.maslachah@yahoo.com

Rab, 28 Nov 2018 jam 18.08

Dear Lilik - Maslachah, Rahmi - Sugihartuti, Retno Sri Wahyuni

I am pleased to inform you that your manuscript titled as "Hematologic Changes and Limpha Index on Malaria Mice Models Given Syzygium cumini Extract as Adjuvant Therapy" (Manuscript Number: VETWORLD-2018-04-187 is accepted for publication in the Veterinary World.

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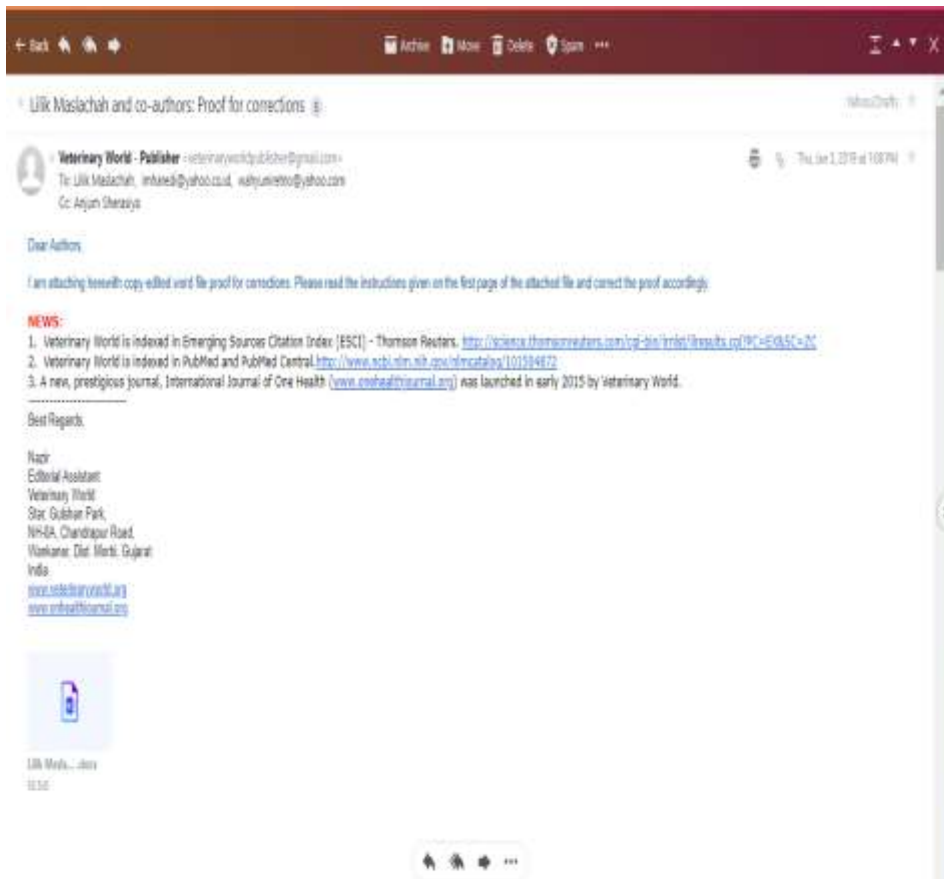

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RESEARCH ARTICLE
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Hematologic changes and splenic index on malaria mice models given *Syzygium cumini* extract as an adjuvant therapy

Lilik Maslachah[1], Rahmi Sugihartuti[1] and Retno Sri Wahyuni[2]

1. Department of Basic Veterinary Medicine, Veterinary Pharmacy Laboratory, Universitas Airlangga, Surabaya, Indonesia; 2. Department of Basic Veterinary Medicine, Clinical Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.
Corresponding author: Lilik Maslachah, e-mail: lilik.maslachah@yahoo.com
Co-authors: RS: imharedi@yahoo.co.id, RSW: wahyuniretno@yahoo.com
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Abstract

Aims: This research aimed to determine the efficacy of *Syzygium cumini* L. as an adjuvant therapy on blood changes and splenic index of mice model malaria.

Materials and Methods: Mice were infected intraperitoneally with 0.2 ml red blood cell (RBC) that contains 1×10^6 *Plasmodium berghei*. 35 mice were divided into seven treatment groups: Group K0: Mice were not infected; K1: Mice were infected; K2: Mice were infected and given chloroquine; P1: Mice were infected and given *S. cumini* leaf extract; P2: Mice were infected and given chloroquine and also *S. cumini* leaf extract; P3: Mice was infected and given *S. cumini* stem bark extract; and P4: Mice were infected and given chloroquine and *S. cumini* stem bark extract. Treatment was given for 4 days 24 h post-*P. berghei* infection. 21st day post-*P. berghei* infection, blood was taken from the heart for hematological examination, and the spleen was taken to examine the splenic index and also to measure the weight and length of the spleen. Hematological data and splenic index were analyzed by analysis of variance test, and if there is a difference, the test is continued by Duncan's multiple range test with 5% level.

Results: The K0 group has normal hemoglobin (HGB), RBC, and hematocrit (HCT) and significantly different ($p<0.05$) than other groups. HGB, RBC, and HCT of K1 group were under normal range, lowest, and significantly different ($p<0.05$) than other groups. Mean corpuscular volume and mean corpuscular HGB values of K2 groups showed a decrease. The number of leukocytes, lymphocytes, and monocytes of K1 groups was increasing and significantly different ($p<0.05$) with K2 and treatment group. The length, width, weight, and splenic index of K1 group were significantly different ($p<0.05$) with K0 group. K2 and treatment groups showed that the length and width of spleens were significantly different ($p<0.05$) with K1.

Conclusion: The combination of chloroquine with leaf and chloroquine with stem bark extract of *S. cumini* as adjuvant therapy may increase the amount of erythrocyte; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and splenic index on malaria mice models.

Keywords: hematology, splenic index, *Syzygium cumini*, *Plasmodium berghei*.

Introduction

Malaria is still a public health problem in 107 countries until right now because malaria is still the fifth rank of infectious diseases in the world [1]. The control and treatment of malaria are more difficult recently because malaria parasites have been resistant to drugs and also the mosquitoes are resistant to insecticides. The development of treatment, prevention, and control of malaria is one of the substantial problems in the world. In 2012, approximately 600,000 children died, mostly from Sub-Saharan Africa [2].

The death of malaria infection is caused by very serious systemic complications such as hematological abnormalities, splenomegaly, and liver dysfunction. Hematological abnormalities during malarial infection are caused by high parasitemia in the patient; hematological abnormalities are also associated with endemicity, hemoglobinopathy, nutritional status, demographic factors, and immunity [3]. Malaria parasite infections may also induce splenic responses characterized by splenomegaly. The size of spleen is used as a tool for determining the intensity of malaria transmission in endemic areas [4]. During the erythrocytic stages of malaria infection, spleen is an important organ in the immune response. Elimination of infected erythrocytes through modulating the immune response and spleen remodeling, resulting in stringent splenic retention of rings and uninfected erythrocytes reduce the risk of cerebral malaria so that severe malaria do not occur [5].

The World Health Organization recommends the use of artemisinin in combination with other antimalarial drugs, but it has been reported that there was resistance to artemisinin monotherapy and

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artemisinin-based combination therapies combination on clinical cases in Cambodia [6]. In addition, there is a decrease in efficacy of antimalarial drugs that currently used, so it is important to develop an adjuvant therapy that can work on specific biologic pathways in the pathophysiology of malaria. Adjuvant therapy that can be used in severe malaria is immune system modulator preparations, antioxidants, anticoagulants, and agents that have anti-seizure activity [7]. The results showed that the use of matrix metalloproteinase (MMP) inhibitor was able to increase the survival of mice in cerebral malaria, and dexamethasone was able to decrease inflammation in murine malaria model with lung pathology. The use of quercetin flavonoids was able to block the induction of hemozoin for upregulation of MMP9, tumor necrosis factor-alpha (TNF-α), and interleukin-1beta [8-10]. *Syzygium cumini* contains anthocyanin, ellagitannin, ellagic acid, phenolic, flavonoids, and vitamins so that it has a high antioxidant activity. This plant is one of the medicinal plants which is easy to be found in Indonesia [11,12]. Results of research conducted by Zhang *et al*., [13] showed that *S. cumini* has radical scavenging activity and strong antioxidant.

The research was aimed to determine the efficacy of *S. cumini* L. as an adjuvant therapy on hematological changes (red blood cells [RBC] and white blood cells [WBC]) and splenic index in mice model malaria.

Materials and Methods

Ethical approval

This study has obtained approval by certificate no 722-KE from Animal Care and Use Committee on Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

Parasite, host, and drugs that used in this research

The parasite that is used to infect the mice is *Plasmodium berghei* ANKA strain. The mice used were male Swiss albino mice with 20-30 g weight and 2.5 months old, and obtained from Veterinary Farma Surabaya (Pusvetma) Center. Antimalarial drug used chloroquine pro analysis (PA) from Sigma Chemical Co. The chloroquine dose used 25 mg/kg body weight (BW) mice as a therapeutic dose. This drug was administered daily for 4 days [14]. The leaves and stem bark of *S. cumini* are obtained from Kediri city of East Java, Indonesia, and identified in the laboratory of Purwodadi Botanical Garden, Pasuruan. *S. cumini* dose was 600 mg/kg BW [15].

Infection dose of *P. berghei* in mice

Mice were infected with 0.2 ml RBC containing 1×10^6 *P. berghei* parasites intraperitoneally. To find out the infection which has occurred in mice, daily microscopic examination of erythrocyte was performed with a thin blood smear taken from the vein of the tail and stained with Giemsa 20% [16]. Calculation of the dose of parasitic infection was determined by counting the number of parasites from the thin blood smear that stained with Giemsa and then calculated the number of parasites per number of erythrocytes. The next step is calculating the number of erythrocytes by diluting the blood using PBS solution in Eppendorf 0.5 ml. Then, these diluted blood erythrocytes were calculated using the improved Neubauer counting chamber. The number of parasitic doses is obtained from multiplying by the number of parasites with the number of erythrocytes that have been calculated and converted to per ml.

Preparation of leaf and stem bark of *S. cumini*

The leaves and stem bark of *S. cumini* are dried, after that, it was crushed into small pieces (simplicia). Simplicia was extracted with PA methanol and maceration for 3×24 h. The filtrate was evaporated using a Rotary Evaporator at 40-50°C with low pressure. The extraction results are stored on the desiccator until ready for use [17].

Treatment of the experimental animals

A total of 35 mice were randomly divided into seven treatment groups, and each group consists of 5 mice. Details of each group are as follows:

Group K0: Mice were only given drug solvent and not infected; K1: Mice were infected and given drug solvent; K2: Mice were infected and given chloroquine 25 mg/kg BW; P1: Mice were infected and given *S. cumini* leaf extract 600 mg/kg BW; (P2): Mice were infected and given chloroquine 25 mg/kg BW and also given *S. cumini* leaf extract 600 mg/kg BW; (P3): Mice were infected and given *S. cumini* stem bark extract 600 mg/kg BW; and (P4): Mice were infected and given chloroquine 25 mg/kg BW and also given *S. cumini* stem bark extract 600 mg/kg BW.

Treatment was given for 4 days since 24 h after per oral infection. After 21 days post-infection, mice were anesthetized with ketamine (Sigma) and then thoracotomy, blood samples were taken from the heart (1 ml) using a Tuberculin Syringe and collected in a vial that has been given anticoagulation for hematologic examination using automated blood analyzer SYSMEX XT 4000i, and spleen was taken to examine the splenic index and also measure the weight and length of spleen.

Examination of weight, length, and splenic index

Previously, the weight of the mice was measured, after that the mice were injected with ketamine IM. The abdominal cavity was opened, and the spleen was taken and weighed using an analytical scale and then measured the length and width of the spleen using a ruler on a millimeter scale. According to Gluhcheva *et al*. [18], the splenic index was calculated using the lymph index equation = (weight of spleen organ of mice)/bw of mice.

Statistical analysis

The data of hematology observation of blood change and lymph index were processed using analysis of variance using SPSS System 17.0 then followed by Duncan's multiple range test with 5% level.

Results

Hematological results of RBC

The results of statistical tests of mean hemoglobin (HGB), RBC, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular HGB (MCH), and MCHGB concentration is presented in Table-1. The mean value of HGB in the non-infected control group (K0) was normal (15.45 g/dl; the normal range is 13.4-15.8 g/dl) HGB levels in the K0 group were significantly different from the other treatment groups, namely K1, K2, P1, P2, P3 and P4 ($p<0.05$). Among these groups, there was no significant difference between the K2 group and P2 and P3, and between P1 and P4 ($p>0.05$).

The amount of RBC in the K0 group was significantly different from the number of RBCs in the entire treatment group ($p <0.05$). However, there was no significant difference in the number of RBCs in the treatment groups K2, P1, P3, and P4 and between the treatment groups P1, P2, P3 and P4 ($p> 0.05$).

The average of HCT/PCV level in the K0 group was significantly different from all treatment group in this study ($p<0.05$). However, there was no significant difference between treatment groups K1, K2 and P3 and between treatment groups K2, P1, P2, P3 and P4 ($p>0.05$).

Hematology result of WBCs

The mean number of leukocytes (WBC) increased in the K1 group infected with *P. berghei* and was significantly different ($p<0.05$) when compared with the infected group treated with chloroquine (K2 group) and the group treated with the combination of *S. cumini* leaf and stem bark extract with chloroquine (P2 and P4 groups). The average number of leukocytes in K2, P1, P2, P3, and P4 was still within a normal range between 8.00 and 11.8 ($103/m^3$). The average platelet value was still within normal limits. The highest lymphocyte values are in the K1 group and were significantly different from K0, P2, and P4 and did not differ significantly with K2, P1, and P3 with a normal range limit of 6.03-8.90. The highest average monocyte count in the K1 group was significantly different ($p<0.05$) with K0, K2, and P4 and was not significantly different ($p>0.05$) with P1, P2, and P3. The average number of neutrophils showed varied results among the treatment groups. The treatment groups K0, K2, and P4 do not show significant differences. Similarly between treatment groups K2, P1, P2 and P3, and between treatment groups K1, P1, P2 and P3 ($p>0.05$). Significant differences were seen in the K0 group with K1, P1, P2 and P3 ($p <0.05$) Hematologic data of WBC are shown in Table-2.

Measurement results and splenic index

The result of length, width, weight, and splenic index measurement in treatment group infected with *P. berghei* showed an enlarged lymph organ and significantly different ($p<0.05$) with uninfected control group (K0). The length and width of the spleen in the K1 group which infected with *P. bergei* were significantly different from the K2 group that infected and treated with chloroquine, However, P1, P2, P3, and P4 showed no significant different ($p>0.05$) with K2. spleen data are shown in Table-3.

Table-1: Mean and standard deviation of RBC hematology in control and treatment groups.

Mice groups	Parameter of RBC hematology						
	HGB (g/dL)	RBC (10^6/μL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW-CV (%)
K0	15.45 ± 0.50^{d}	10.56 ± 0.27^{d}	51.40 ± 2.55^{c}	48.10 ± 3.39^{a}	14.60 ± 0.53^{b}	30.30 ± 0.49^{a}	23.42 ± 0.70^{a}
K1	11.12 ± 0.64^{a}	8.09 ± 0.33^{a}	36.80 ± 3.88^{a}	45.52 ± 4.95^{a}	13.75 ± 0.71^{ab}	30.37 ± 1.80^{a}	25.92 ± 3.51^{b}
K2	12.25 ± 0.12^{b}	8.90 ± 0.89^{b}	39.70 ± 0.98^{ab}	45.37 ± 4.91^{a}	13.87 ± 1.07^{ab}	30.97 ± 1.06^{a}	25.85 ± 0.23^{b}
P1	13.12 ± 0.41^{c}	9.14 ± 0.36^{bc}	42.57 ± 2.13^{b}	46.97 ± 2.57^{a}	14.40 ± 0.57^{ab}	30.67 ± 0.45^{a}	21.80 ± 0.66^{a}
P2	12.80 ± 0.29^{bc}	9.75 ± 0.36^{c}	42.55 ± 2.13^{b}	43.72 ± 1.31^{a}	13.25 ± 0.36^{a}	30.20 ± 0.87^{a}	23.97 ± 0.59^{a}
P3	12.32 ± 0.45^{b}	9.15 ± 0.53^{bc}	40.15 ± 3.70^{ab}	43.82 ± 2.47^{a}	13.52 ± 0.67^{ab}	30.85 ± 1.88^{a}	24.67 ± 3.51^{ab}
P4	13.07 ± 0.36^{c}	9.14 ± 0.62^{bc}	41.26 ± 1.03^{b}	45.42 ± 3.39^{a}	14.37 ± 0.77^{ab}	31.67 ± 0.65^{a}	23.30 ± 126^{ab}

Different superscripts on the same column show a significant difference at significant level of 0.05%, RBC=Red blood cell, HGB=Hemoglobin, HCT=Hematocrit, MCH=Mean corpuscular hemoglobin

Table-2: Mean and standard deviation of WBC hematology in control and treatment groups.

Mice group	Parameter of WBC hematology							
	WBC	PLT	Lymph	Lymph (%)	Mono	Mono (%)	Neut	Neut (%)
K0	4.33 ± 0.17^{a}	1322.25 ± 35.34^{ab}	3.60 ± 0.11^{a}	83.40 ± 0.66^{abc}	0.27 ± 0.02^{a}	6.20 ± 0.29^{a}	0.35 ± 0.04^{a}	8.07 ± 0.17^{b}
K1	13.22 ± 6.35^{c}	1551.50 ± 451.38^{b}	10.33 ± 4.83^{c}	79.87 ± 6.95^{ab}	2.24 ± 2.02^{c}	16.75 ± 8.83^{c}	0.46 ± 0.44^{a}	1.95 ± 3.90^{a}
K2	8.41 ± 0.56^{ab}	1432.75 ± 21.09^{ab}	7.08 ± 0.20^{bc}	88.37 ± 2.00^{c}	0.76 ± 0.06^{ab}	9.22 ± 0.51^{ab}	0.35 ± 0.04^{a}	2.07 ± 2.39^{a}
P1	10.59 ± 1.14^{bc}	1245.50 ± 99.37^{ab}	8.29 ± 0.74^{bc}	79.37 ± 1.82^{ab}	1.70 ± 0.51^{bc}	16.15 ± 4.10^{bc}	0.35 ± 0.30^{a}	3.22 ± 3.80^{a}
P2	8.17 ± 0.30^{ab}	1127.50 ± 157.55^{a}	6.17 ± 0.33^{ab}	76.07 ± 2.36^{a}	1.36 ± 0.19^{abc}	16.22 ± 2.39^{bc}	0.55 ± 0.13^{a}	6.90 ± 0.90^{ab}
P3	11.03 ± 0.51^{bc}	1097.25 ± 295.11^{a}	8.83 ± 0.41^{bc}	80.92 ± 3.84^{abc}	1.70 ± 0.59^{bc}	15.12 ± 4.66^{bc}	0.36 ± 0.26^{a}	2.52 ± 3.17^{a}
P4	7.87 ± 2.48^{ab}	1156.75 ± 213.69^{a}	6.62 ± 2.28^{b}	85.15 ± 9.55^{bc}	0.46 ± 0.20^{ab}	6.63 ± 4.18^{a}	0.66 ± 0.36^{a}	5.92 ± 4.31^{ab}

Different superscripts on the same column show a significant difference at significant level of 0.05%, WBC=White blood cell, PLT=Platelet

Table-3: Mean and standard deviations of length, width, weight, and splenic index of mice in the control and treatment groups.

Mice group	Length (cm)	Width (cm)	Weight (g)	Splenic index
K0	0.82±0.14[a]	0.22±0.12[a]	0.21±0.11[a]	0.008±0.002[a]
K1	3.07±0.22[c]	0.65±0.10[c]	0.44±0.14[c]	0.016±0.003[c]
K2	2.20±0.62[b]	0.45±0.19[b]	0.30±0.10[bc]	0.011±0.001[bc]
P1	2.65±0.26[bc]	0.57±0.09[bc]	0.37±0.08[bc]	0.013±0.001[c]
P2	2.42±0.38[bc]	0.50±0.11[bc]	0.34±0.10[bc]	0.013±0.003[bc]
P3	2.37±0.47[b]	0.60±0.08[bc]	0.31±0.04[bc]	0.013±0.002[bc]
P4	2.47±0.30[bc]	0.55±0.10[bc]	0.27±0.07[b]	0.009±0.002[b]

Different superscripts on the same column show a significant difference at significant level of 0.05%

Discussion

RBC hematology

The normal range of HBG, erythrocytes, and HCT in non-infected control group due to the mice under normal circumstances and the erythrocytes are not damaged. In the group that infected by *P. berghei* and was not treated and the infected group that treated with chloroquine, leaf, stem bark of *S. cumini*, and its combination, the HGB values, erythrocytic cell count, HCT mean, MCV, and MCH values showed a decrease below the normal range, and the lowest is in K1 which was different from the other treatment groups.

This suggests that Plasmodium infections cause erythrocyte hemolysis. The removal of infected and uninfected erythrocyte cause erythropoiesis in the body which becomes ineffective;, this is caused by the abnormally high levels of TNF that has effect on ineffective erythropoiesis [19].

In vitro and *in vivo* studies show pro-inflammatory cytokines including interferon y, TNF-α, and macrophage migration inhibitory factor, as well as Plasmodium products (hemozoin) which play a role in the pathogenesis of malarial anemia [20]. Hemozoin was produced from HGB digestion by Plasmodium and induces macrophage for the secretion of pro-inflammatory cytokines and other mediators that inhibit the effects of erythropoiesis [21,22]. Plasmodium hemozoin products play a role in erythropoiesis resistance, low reticulocytosis, and malarial anemia by inhibiting Epo-induced proliferation from erythroid precursors [23].

Malarial anemia due to Plasmodium infection may increase immunoglobulin G autoantibodies levels against non-infected RBC (nRBCs), and its deposition on the surface of nRBCs can decrease red cell deformability and improve erythrophagocytosis [24]. The results of this study in the treatment group, Hb levels, the number of erythrocytes, and levels of premature ventricular contractions are below normal, and it is due to hypochromic microcytic anemia because MCV and MCH values are below normal. The results of this experimental animal model were consistent with the research that occurred in humans infected with *Plasmodium falciparum* 71% of anemic patients having hypochromic microcytic anemia [19].

In the treatment group (P1, P2, P3, and P4), the number of erythrocytes were within normal range. The antimalarial effects of chloroquine and antioxidants containing *S. cumini* leaf extracts cause proliferative resistance of *P. berghei.* It happened through the inhibition of hemozoin formation as well as through the parasite protease inhibition that involved in the degradation of HGB [25]. The decrease of proliferation in *P. berghei* infection in mice treated with chloroquine and combination with antioxidants can increase superoxide dismutase activity and decrease lipid peroxidation [26]. The combination of antimalarial therapy with antioxidants can counter the pathological damage due to oxidants and decrease the proliferation of parasites [27]. Previous research state that leaves, stems, and fruit of *S. cumini* have antioxidant and anti-inflammatory activity [28]. The value of IC50 leaves of *S. cumini* is 12.84 ppm, so it is potentially developed as an antioxidant [29]. Another research showed a positive association of antioxidant potential, the ability to reduce free radical and phenolic compound content on *S. cumini* leaf extract [30].

WBC hematology

Increased WBC, the number of lymphocytes, and the number of monocytes in the K1 group infected with *P. berghei* were not treated. The number of WBC, lymphocytes, and monocyte were decreased in the K2 group as well as in P2 and P4 group. These results suggest that WBC elevation in infection by Plasmodium may stimulate the immune system as a physiological response of the body to malaria infection because the WBC has a role in against infection When an infection occurs, the body will respond by phagocytosis of infectious agent and stimulate increased production of immune cells to produce antibodies. The decrease in WBC in the infected and antimalarial-treated groups and the combination with the extract showed that the treatment was able to fight infection [31].

Lymphocytes as primary effector cells have a very important role in the immune system. Increased lymphocytes show the mechanism of body defense against *P. berghei* infection [31]. Monocytes are phagocytes. The increase in the number of monocytes suggests a body's immune response to accelerating the activity of Plasmodium protozoa phagocytosis. Monocytes play an important role in the production, mobilization, and regulation of immune-effector cells, also contributing to infection elimination [32]. The reduction of lymphocytes and monocytes in the

treatment of antimalarials and its combinations with the extracts due to phytochemicals glycoside, phenol, tannin, saponins, flavonoids, and ellagic acid in *S. cumini* stem bark extracts have antioxidant effects. The antioxidant effects of phytochemicals in mice infected with *P. berghei* were able to increase the antioxidant enzyme superoxide dismutase and catalase and decrease the concentration of malondialdehyde to improve hematologic parameters [33].

Measurement and splenic index

The result of measurement of length, width, weight, and splenic index in treatment group infected with *P. berghei* shows the enlargement of lymph organs. Lymph works as an effector against malaria infection, especially in protective immunity against infections of the blood [34]. The total number of spleen cells increased during high parasitemia and then decreased at lower levels. Splenomegaly in malarial infection is associated with the expansion of the white pulp and the red pulp due to increased follicle size, that caused by hematopoietic reaction. Increased macrophage occurs due to the process of erythrophagocytosis. Macrophage in the red pulp lymph plays an important role in removing plasmodium that infects RBC from circulation [35].

Plasmodium infections in RBC may lead to complex pathophysiology, and rapid growth of Plasmodium will increase the production of reactive oxygen species causing an imbalance between plasma oxidants and host antioxidant systems leading to oxidative stress [36]. The administration of chloroquine antimalarial drug and leaf and stem bark extract of *S. cumini* and its combination can decrease the length, width, and splenic index. This shows the effects of antimalarials, and antioxidant content in leaf and stem bark extract of *S. cumini* as phenol compounds contained in the leaves include caffeine acids, chlorogenic acid, ellagic acid, ferulic acid, and gallic acid. The leaves are also contained tannins and essential oil terpenes [37]. *S. cumini* stem bark contains flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids, and anthocyanins [38].

Research conducted by Jayachandra and colleague [39] showed that the total phenolic content was 580.23±3.03 mg/g, tannin content was 534±4.03 mg/g, while the flavonoid content was 315.42±4.52 mg/g. The methanolic and aqueous extracts of bark were screened for antioxidant activity using nitric oxide scavenging activity method. Antioxidant activity is caused by the flavonoid and polyphenol content [40]. The combination of deferoxamine administration as an iron chelator that has anti-plasmodial activity with ellagic acid as an antioxidant in *Plasmodium yoelii* infection produces an antimalarial additive effect [41]. Decreased lesions are reversible in the spleen following a decrease in parasitemia through apoptosis as a defense mechanism of spleen against malaria infection [34].

Conclusion

The combination of chloroquine with leaf and stem bark extract of *S. cumini* as adjuvant therapy may increase the number of erythrocytes; decrease the number of leukocytes, lymphocytes, and monocytes; and decrease the length, width, and splenic index of malaria in mice models.

Authors' Contributions

LM designed the research, supervised the research, and compiled the manuscript; RS and RSW helped to analyze blood and spleen data. All authors have read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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