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Potency *Syzygium cumini* L as adjuvant therapy on mice model malaria

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Abstract

The objective of the study was to prove the potential extract of *Syzygium cumini* L leaf and stem bark as an adjuvant to malaria modelling mice. Antimalarial effects were assessed by the percentage of parasitemia, growth inhibition, 50% dose level (ED50), Parasite Clearance Time (PCT), Recrudescence Time (RT) of *Plasmodium berghei*. Male albino Swiss mice infected with 1×10^5 *P. berghei* parasite in 0.2 ml intraperitoneally. Treatment with chloroquine 25 mg/kg body weight, chloroquine combination 25 mg/kg body weight with leaf and stem bark extract of *Syzygium cumini* L dose 600 mg / kg body weight for 4 days and 24 hours after infection, and then its activity as antimalarial and adjuvant therapy were observed. The results showed that the extract of *Syzygium cumini* L leaf combined with chloroquine gives highly significant result in inhibiting the growth of parasites than the chloroquine alone and the extract of *Syzygium cumini* L leaf combined with chloroquine gives the Parasite Clearance Time faster and Recrudescence Time (RT) longer than the other treatment.

Keywords: Syzygium, Plasmodium, Adjuvant

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كفاءة البامبوزيا كعامل مساعد في علاج الملاريا نموذج الفئران

ليلاك مشلاخ و رحيمي سوجارتوتي

فرع العلوم البيطرية الأساسية، كلية الطب البيطري، جامعة اريلنكا، سراييا، اندونيسيا

الخلاصة

هدفت الدراسة إثبات كفاءة المستخلص لورقة *Syzygium cumini* L البامبوزيا واللحاء كعامل مساعد لفئران النمذجة الخاصة بالملاريا. تم تقييم التأثيرات المضادة للملاريا من خلال النسبة المئوية لتطفل الدم، وتثبيط النمو، ومستوى الجرعة 50% (ED50)، وفترة تصفية الطفيليات (PCT)، وفترة الاسترجاع (RT) من بلازموديوم بيرغي. ذكور الفئران السويسرية خمجت بطفيلي *P. berghei* بمقدار 1×10^5 في 0.2 مل حقنت داخل الخلب. المعالجة بالكلوروكين 25 ملغم/كم من وزن الجسم، تركيبة الكلوروكين 25 ملغم / كغم من وزن الجسم مع مستخلص لحاء الأوراق واللقاح من *Syzygium cumini* L جرعة 600 ملغم / كغم من وزن الجسم لمدة 4 أيام و 24 ساعة بعد الإصابة، ومن ثم نشاطها كمضاد للملاريا وقد لوحظ العلاج المساعد. أظهرت النتائج أن مستخلص أوراق سيزيجيوم كميني لتر مع الكلوروكين يعطي نتائج ذات دلالة إحصائية في تثبيط نمو الطفيليات مقارنة مع الكلوروكين وحده، ويمتدح مستخلص نبات سيزيجيوم كميني لتر مع الكلوروكين وقت إزالة الطفيليات بشكل أسرع ووقت الاسترجاع (RT). أطول من العلاج الآخر.

Introduction

Malaria is one of infectious diseases caused by Plasmodium which is occurring especially in tropical countries. Every year, approximately 300-500 million people in the world are infected with malaria, and 1-3

million people die. The greatest deaths occur in Africa and the Sahara, 90% of deaths occurs in children under 5 years old. This disease is still a public health problem in 107 countries because malaria is still the fifth cause of death due to infectious diseases in the world (1).

During malaria infection, antigens will stimulate immune system activity that results the formation of Reactive oxygen species (ROS) by inflammatory cells. In trophozoite malaria infections, *Plasmodium* infects erythrocytes and producing H_2O_2 radical and OH which is twice larger than normal erythrocytes, excessive H_2O_2 can lead to breakdown of heme and release of free Fe ion which will form OH radicals again by Fenton reaction. All of these factors that cause lipid peroxide increase which be able to cause oxidative stress (2,3).

The potential effects of damage from normal oxidative stress can be eliminated by the antioxidants present in the body, but in malaria infection there is a decrease levels of antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase and other antioxidants such as albumin, glutathione and ascorbate. When the imbalance of ROS formation in malaria infections can not compensated with cytoprotective enzymes and antioxidants in the body, it can cause oxidative damage (4). Oxidative damage will be increasing the tissue and organ damage (liver, kidney, lung and brain), increasing cerebral or severe malaria that may cause death or decrease in survival time (5).

The presence of multidrug resistant of malaria and decreased efficacy of antimalarial drugs make it essential for new therapy which is adjuvant therapy (additional therapy that may affect on the pathophysiology of the cause of malaria). Adjuvant therapy that has been used in severe malaria is immune system modulator, antioxidants, anticoagulants and agents that have antiseizure activities (6).

One of the medicinal plants that can be found in Indonesia is *Syzygium cumini*. In India, this plant is used by brewed with hot water traditionally. This study used leaf and stem bark extract of *Syzygium cumini*. This plant has a high antioxidant activity because the content of anthocyanin, ellagitannin, ellegic acid, phenolic, flavonoids, and vitamins (7,8). The results of research showed that *Syzygium cumini* has radical scavenging activity and strong antioxidants (8). The use of medicinal plants as antimalarials drugs has been widely utilized, but *Syzygium cumini* which has high potency of strong antioxidant as antimalarial drug had never been reported, so this study would like to know the potential of antioxidant *Syzygium cumini* L as adjuvant therapy in mice as malaria model. This study used *Plasmodium berghei* rodent malaria as an in vivo model by infecting that to the mice (9).

Materials and methods

Experimental animals

This study used 2,5 months male albino Swiss strain of mice weighing 20g - 30g from Pusat Veterinaria Farma (Pusvetma) Surabaya. Every mice were kept in a cage.

Food and drink were given *ad libitum*. This study has got approval with certificate No. 722-KE from Animal Care and Use Committee on Veterinary Medicine Airlangga University Surabaya Indonesia.

Inoculation of parasites in experimental animals

The parasite that is used to infect mice is *Plasmodium berghei* ANKA strain. Mice are infected with red blood cells containing 1×10^5 *P. berghei* parasites in 0.2 ml intraperitoneally. In order to find out the infection has occurred, microscopic examination of erythrocytes of mice is daily taken by a thin blood smear which is taken from the vein of the tail and stained with Giemsa 20% (10).

Drugs and plant materials

Chloroquine that is used from Sigma Chemical Co. Leaf and stem bark of *Syzygium cumini* is got from Kediri city, East Java, Indonesian, and identified on plant laboratory Kebun Raya Purwodadi Pasuruan. Chloroquine dose that is used is 25 mg/Kg and given daily for 4 days (11). *Syzygium cumini* dose is 600mg/kg (12).

Preparation of leaf and stem bark extract of *Syzygium cumini*

The leaves and stem bark of *Syzygium cumini* are dried in the room temperature until dried after that, it was smoothed into smooth simplicia. Simplicia *Syzygium cumini* are macerated with methanol 3x24 hours then filtered. The filtrate was evaporated using a Rotary Evaporator at a temperature of 40-50°C with low pressure. The result of extraction is saved on -8°C until ready to use (4).

Research design

Thirty mice were randomly divided into 6 groups, 5 mice for each. K1: Control group that is infected with *P. berghei* 1×10^5 intraperitoneally and did not get any treatment. K2: Control group that is infected with *P. berghei* 1×10^5 intraperitoneally and given chloroquine 25 mg/kg. P1: Treatment group that is infected with *P. berghei* 1×10^5 intraperitoneally and given leaf extract of *Syzygium cumini* 600 mg/kg. P2: Treatment group that is infected with *P. berghei* 1×10^5 intraperitoneally and given chloroquine, which 25 mg/kg and leaf extract of *Syzygium cumini* 600 mg/kg. P3: Treatment group that is infected with *P. berghei* 1×10^5 intraperitoneally and given stem bark extract of *Syzygium cumini* 600 mg/kg. P4: Treatment group that is infected with *P. berghei* 1×10^5 intraperitoneally and given chloroquine 25 mg/kg and stem bark extract of *Syzygium cumini* 600 mg/kg. Therapy is given for 4 days, 24 hours post infection. Every day, thin blood smear is taken from the tail and stained with giemsa 20% until 21 days post infection in order to see the percentage of parasitemia, growth inhibition, 50% effective

dose level, Parasite Clearance Time (PCT), Recrudescence Time (RT).

Calculation of parasitemia and growth inhibition

The calculation of parasitemia percentage and growth inhibition of *Plasmodium berghei* on the mice is done on the first day post infection by counting the number of erythrocytes that infected in every 1000 cells of erythrocytes under the light microscope with 1000x magnification. Percentage of parasitemia and growth inhibition is calculated by using formulas from (13,14).

Measurement of 50% effective dose level

Measurement 50 % effective dose level in every treatment after 24 hours, post infection is using formula $(A-B)/A \times 100$. Where A is the control group of parasitemia and B is the treatment group of parasitemia. ED_{50} is determined by using linear regression program (15).

Parasite Clearance Time (PCT) and Recrudescence Time (RT) *Plasmodium berghei*

Parasite Clearance Time and Recrudescence Time (RT) *Plasmodium berghei* is done by examination of parasite growth from day 1 (D1) post infection to day 20 (D20) post infection and showed by the absence of parasite on the thin blood smear that taken from vein of the tail and stained Giemsa 20% for 20 minutes and that examined using light microscope with 1000x magnification. That examination is done until the parasites that can grow back is found (16,17).

Data Analysis

The result of observation of % parasitemia, % growth inhibition, is processed by *Analysis of Variance* (ANOVA), then continued with *Duncan Multiple Range Test* with level 5% in order to know the differences of treatment that given. 50 % effective dose level Parasite Clearance Time (PCT) and Recrudescence Time (RT) are processed by linear regression using *SPSS System 17.0*.

Results

Result of *Plasmodium berghei* parasitemia percentage in control group and treatment group that given leaf and steam bark extract of *Syzygium cumini*

The test using analysis of variance (Anova) proved that *Plasmodium berghei* parasitemia percentage in control group (K1,K2) and treatment group (P1, P2, P3, P4) given leaf and stem extract of *Syzygium cumini* showed that there is high significance difference ($P < 0.05$). Then, the test is continued with the difference test between the treatment groups, the results showed that the control group that infected without treatment (K1) with the control group that infected and given chloroquine (K2), treatment group that infected and given leaf and steam bark extract of *Syzygium*

cumini (P1, P2, P3, P4) showed that there is a high significance difference ($P < 0.05$). Whereas between control group (K2) and treatment group (P1, P2, P3, P4), also between treatment group (P1, P2, P3, P4) did not significance difference, ($P > 0.05$) (Figure 1 and table 1).

Inhibition percentage of *Plasmodium berghei* growth on control group and treatment group that given leaf and steam bark extract of *Syzygium cumini*

The result of growth inhibition of *Plasmodium berghei* on control group is difference than treatment group that given leaf and steam bark extract of *Syzygium cumini* ($P < 0.05$). The test is continued with the difference test between the treatment groups, the results showed that the control group that infected without treatment (K1) with the control group that infected and given chloroquine (K2), treatment group that infected and given leaf and steam bark extract of *Syzygium cumini* (P1, P2, P3, P4) showed difference ($P < 0.05$), whereas between group (K2) and group (P1, P2, P3, P4) did not show any difference, also between treatment group (P1, P2, P3, P4) did not show difference at $\alpha 0.05$ ($P > 0.05$) (Figure 2 and table 2).

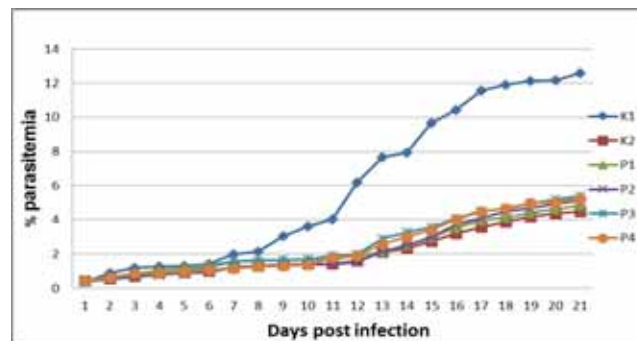


Figure 1: Graph of Percentage of *Plasmodium berghei* Parasitemia K1: infected untreated. K2: infected treated chloroquine. P1: infected treated leaf extract. P2: infected treated chloroquine and leaf extract. P3: infected treated stem bark extract P4: infected treated chloroquine and stem bark extract.

Table 1: *Plasmodium berghei* parasitemia percentage in control group and treatment group

Groups	Parasitemia percentage
K1	5.88 ± 4.58 ^a
K2	2.82 ± 1.35 ^b
P1	2.32 ± 1.39 ^b
P2	2.24 ± 1.61 ^b
P3	2.56 ± 1.67 ^b
P4	2.43 ± 1.68 ^b

Mean values with different superscripts within a column differ significantly ($P < 0.05$).

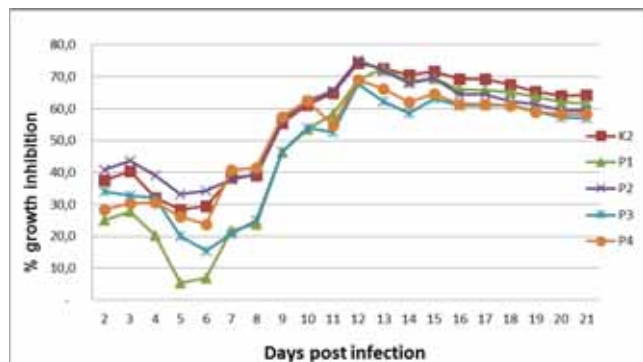


Figure 2: Graph of Percentage of Growth Inhibition of *Plasmodium berghei* K1: infected untreated. K2: infected treated chloroquine. P1: infected treated leaf extract. P2: infected treated chloroquine and leaf extract. P3: infected treated stem bark extract P4: infected treated chloroquine and stem bark extract.

Parasite Clearance Time (PCT) and Recrudescence Time (RT) of *Plasmodium berghei* on the control group and treatment group that given leaf and steam bark extract of *Syzygium cumini*

Control group that infected without treatment (K1) % parasitemia after 6th day post infection is 1.43%, whereas treatment that given for 4 days on the control group that

infected and given chloroquine (K2), leaf extract (P1), combination leaf extract and chloroquine (P2), stem bark extract of *Syzygium cumini* (P3) and combination therapy of stem bark extract and chloroquine (P4) Parasit Clearance Time (PCT) on 6th day post infection % parasitemia are K2: 1.01%, P1: 1.33%, P2: 0.94 %, P3:1.21% and P4: 1.09 %. That result showed that K2, P1, P2, P3, P4% parasitemia is lower than the control group K1. Provision of adjuvant therapy with combination of leaf extract and chloroquine showed the lowest % of parasitemia when compared with other treatment groups (K2, P1, P3 and P4). Recrudescence Time (RT) *Plasmodium berghei* is calculated after parasitemia reach 2.5 % after receive drugs for 4 days. The result of Recrudescence Time (RT) on K1, parasitemia reach 2.5 % after 6th days with regression model $Y = -2.002 + 0.717X$. On K2, after 11th days with regression model $Y = -0.233 + 0.209X$. On P1, after 10th days with regression model $Y = -0.063 + 0.217X$. On P2, after 11th days with regression model $Y = -0.485 + 0.247X$. On P3 after 10th days with regression model $Y = -0.325 + 0.262X$. On P4 after 10th days with regression model $Y = -0.434 + 0.261X$. The administration of chloroquine and leaf extract of *Syzygium cumini* showed a longer Recrudescence Time (RT) compared to the control group K1 (Figure 3).

Table 2: Mean percentage of growth inhibition *Plasmodium berghei*

Groups	Days post infection																				
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
K2	37.5	40.3	32.0	28.5	29.4	38.4	39.1	55.4	61.2	64.8	74.3	72.6	70.6	71.7	69.3	69.2	67.6	65.4	64.0	64.2	
P1	25.0	27.7	20.3	5.4	7.0	21.7	23.7	46.2	53.4	58.4	69.0	72.5	68.3	69.4	66.1	65.7	65.1	63.8	62.0	61.6	
P2	40.9	43.7	39.1	33.1	34.3	37.9	39.5	56.4	62.5	65.6	75.1	72.0	68.1	69.4	64.5	64.4	62.3	61.4	59.4	59.3	
P3	34.1	32.8	32.0	20.0	15.4	20.7	25.1	46.6	54.0	52.6	67.7	62.3	58.6	63.2	60.9	61.0	61.2	59.2	57.2	57.0	
P4	28.4	30.3	30.5	26.2	23.8	40.9	41.4	57.4	62.5	54.9	69.1	66.2	62.0	64.8	61.3	61.3	60.9	58.9	58.4	58.3	

Examination of 50 % dose level (ED₅₀) *Plasmodium berghei* on the control group and treatment group that given Leaf and Steam Bark Extract of *Syzygium cumini*

Examination of 50% dose level (ED₅₀) to determine the effective dose inhibiting 50% growth of *Plasmodium berghei*. The infected control group chloroquine (K2) showed 50% growth inhibition occurred on 9 day after infection with regression equation $Y = 29.975 + 2.243X$. Leaf extract of *Syzygium cumini* (P1) occurred on 13 days post infection with regression equation $Y = 10.881 +$

$3.194X$. Combination of leaf extract and chloroquine (P2) occurred on 9 day post infection with regression equation, $Y = 36.344 + 1.661X$. The stem bark extract of *Syzygium cumini* (P3) occurred on 13 day of post infection with regression equation $Y = 20.921 + 2.275X$ and combination therapy of stem bark extract and chloroquine (P4) occurred on 13 day post infection with regression equation $Y = 27.239 + 2.055X$. These results show that if *Syzygium cumini* leaf extract combined with chloroquin can inhibit 50 % *Plasmodium berghei* growth with shorter time (Figure 4).

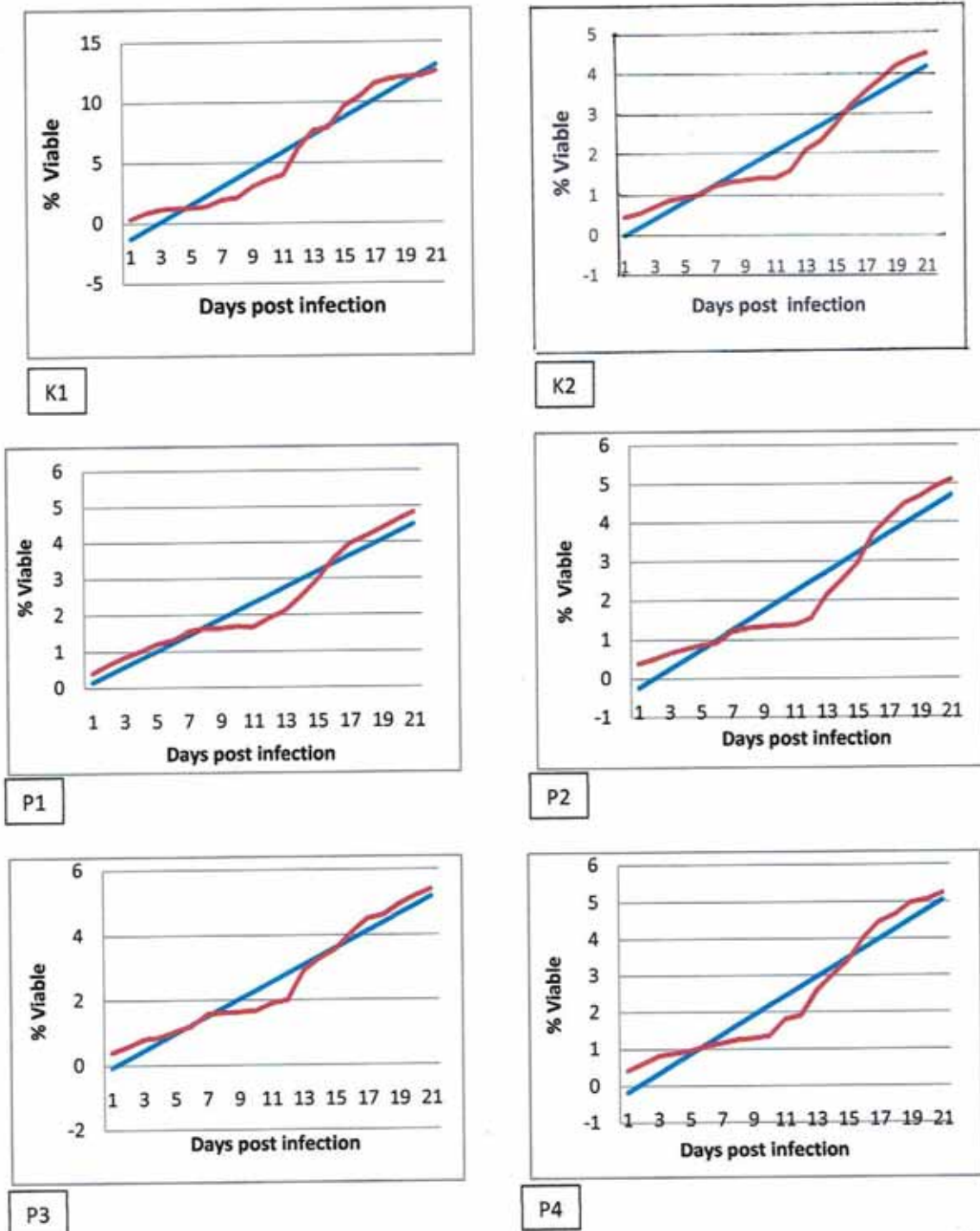


Figure 3: Graph of Parasite Clearance Time (PCT) and Recrudescence Time (RT) of *Plasmodium berghei* on the control group and treatment group that given leaf and steam bark extract of *Syzygium cumini* K1: control group infected untreated. K2: control group infected treated chloroquine. P1: infected treated leaf extract. P2: infected treated chloroquine and leaf extract. P3: infected treated stem bark extract P4: infected treated chloroquine and stem bark extract.

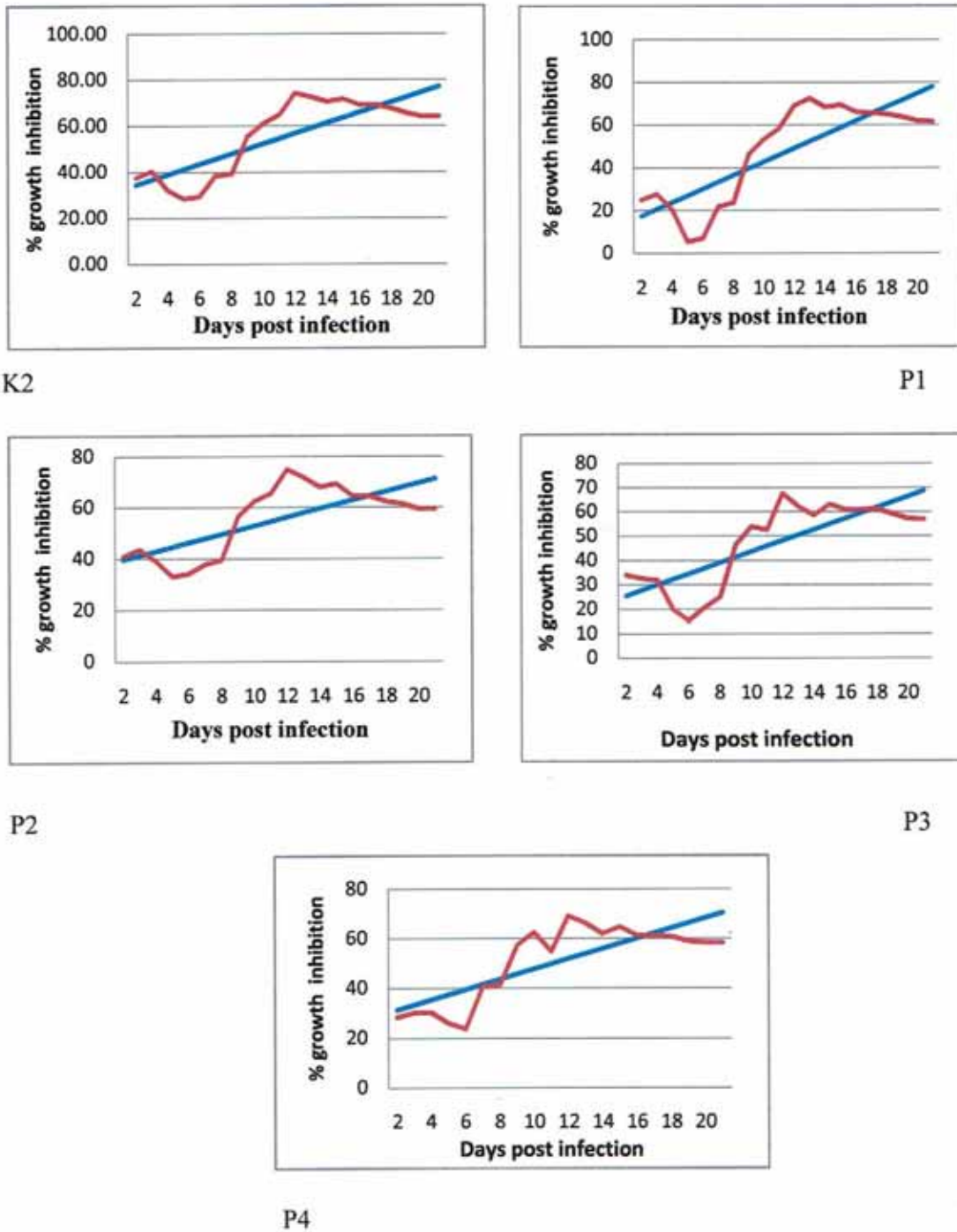


Figure 4: Graph of 50 % dose level (ED_{50}) linear regression of *Plasmodium berghei* on control group and treatment group that given Leaf and stem bark Extract of *Syzygium cumini* K2: control group infected treated chloroquine. P1: infected treated leaf extract. P2: infected treated chloroquine and leaf extract. P3: infected treated stem bark extract P4: infected treated chloroquine and stem bark extract.

Discussion

Parasitemia percentage and growth inhibition

Chloroquine as antimalarial drugs are blood schizontocides, the antimalarial is only actively working against erythrocytic stages. Chloroquine works exclusively at the intraerythrocytic cycle stage during which the active parasite degrades hemoglobin in the digestive vacuole by inhibiting free heme changes into hemozoin, the heme being toxic to the membrane. This free heme can lyse the membrane and cause peroxidative damage to lipid bilayer and protein with the formation of reactive oxygen intermediates. The accumulation of heme causes death in the parasite (18).

Leaf extract of *Syzygium cumini* combine with chloroquine (P2) also with treatment group that is infected with *Plasmodium berghei* which is given stem bark extract of *Syzygium cumini* (P3) and combination of stem bark extract of *Syzygium cumini* and chloroquine (P4) showed that leaf and stem bark extract of *Syzygium cumini* can inhibit the growth of *Plasmodium berghei*. The decreasing growth and increasing inhibition are caused by active ingredients of *Syzygium cumini* leaf and stem bark extracts containing flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids (19) Active metabolites that is found in leaves and stem bark of *Syzygium cumini* have potency as antioxidants which is shown from the research results (7,8). Results of research conducted by showed that leaves, stems and fruit of *Syzygium cumini* have antioxidant and anti-inflammatory activity (20). Activities as antioxidants are thought to be due to the presence of flavonoids and polyphenols in the plant (21).

Currently work showed that there is no significant difference between parasitemia the percentage and growth inhibition on the control group that is infected *Plasmodium falciparum* and given chloroquine (K2) with control group that is infected *Plasmodium berghei* and given leaf extract of *Syzygium cumini* (P1), leaf extract of *Syzygium cumini* combine with chloroquine (P2) also with treatment group that is infected with *Plasmodium berghei* which is given stem bark extract of *Syzygium cumini* (P3) and combination of stem bark extract of *Syzygium cumini* and chloroquine (P4) showed that leaf and stem bark extract of *Syzygium cumini* have the same potential as chloroquine in inhibiting parasite growth which is indicated by the decrease of parasitemia with different working mechanisms. Chloroquine works by inhibiting the formation of hemozoin in digestive vacuoles while the active metabolite of leaf and stem bark extract of *Syzygium cumini* acts as an antioxidant. In trophozoite malaria infection, Plasmodium infects erythrocytes and produces twice larger H_2O_2 and OH^- than normal erythrocytes, excessive H_2O_2 can be also cause heme breakdown and release of free Fe ion which will form again the OH^- radical through Fenton's reaction. All of these factors lead to an increase of lipid peroxide that

causes oxidative stress in malaria infections (3). Akpotuzor *et al.* (22) showed that there is total decreasing antioxidant status in malaria patients, and the same result about imbalance between oxidants and antioxidants in pregnant women with malaria (23).

There is no significant difference between control group that is infected *Plasmodium berghei* and given leaf extract of *Syzygium cumini* (P1), leaf extract of *Syzygium cumini* combine with chloroquine (P2) also with treatment group that is infected with *Plasmodium berghei* which is given stem bark extract of *Syzygium cumini* (P3) and combination of stem bark extract of *Syzygium cumini* and chloroquine (P4) showed that *Syzygium cumini* leaf and stem bark extracts containing flavonoids, polyphenols, acetyl oleanolic acid, tannins, gallic acid, ellagic acid, quercetin, isoquercetin, kaempferol, myricetin, flavonol, glycosides, saponins, triterpenoids which have potency as antioxidant (7, 24,25).

Parasit Clearance Time (PCT) and Recrudescence Time (RT) *Plasmodium berghei*

Provision of adjuvant therapy combination with leaf extract and chloroquine showed the decrease of parasitemia than other treatment groups (K2, P1, P3 and P4). The administration of chloroquine and *Syzygium cumini* leaf and stem bark extracts also showed a longer Recrudescence Time (RT) when compared to the K1 control group. These results suggest that adjuvant therapy be able to maximize the effectiveness of the drug and accelerate the healing. Adjuvant therapy in malaria is intended to modify the pathophysiological processes caused by malaria so that the symptoms can be relieved, reduce the possibility of complications and death. Antioxidant is one of the drug which is used as adjuvant therapy (6).

Several studies have shown that adjuvant therapy with MMP inhibitors may improve survival of mice with cerebral malaria, other studies have also shown that adjuvant therapy with anti inflammation may decrease the inflammation in the lung of mouse malaria models, also addition of quercetin flavonoids, kinase inhibitors are able to suppress hemozoin induction in order to regulate MMP9, TNF alpha and IL1 beta (26,27)

Some medicinal plant extracts or natural products that have potency as antioxidants can interact by modulating cellular signaling pathways so as to accelerate the schizontocidal and antiparasitic activity by altering the balance of redox reactions on the host. Increasing of total antioxidant capacity and decreasing lipid peroxidation may decrease parasitemia and increase survival of mice, which is infected with *Plasmodium berghei* (20).

50 % dose level (ED₅₀) *Plasmodium berghei*

The results of this study showed that the use of combination *Syzygium cumini* leaf extract with chloroquin

require a shorter time in parasitic growth than the P1, P3 and P4 groups. This suggests that the effectiveness of active metabolites from *Syzygium cumini* leaf extract is better when used together with chloroquine as adjuvant therapy. These results are consistent with the Hiben *et al* study (6), using chloroquin with *S. singueana* leaf extracts that able to enhance the antimalarial effect and provide benefits when used as adjuvant therapy to support chloroquine in malaria treatment. These results were proved by all mices survived after infected with *P. barge* and then treated with combination of chloroquin with *S. singueana* leaf extract (6).

Conclusions

Administration of *Syzygium cumini* L leaf extract combined with chloroquine can give the effect of increased Clirens Time Parasite (PCT) more rapidly and Recrudescence Time (RT) longer than other treatments.

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