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The potency of butanol fraction *Syzygium cumini* fruit as antimalarial on *Plasmodium berghei* infected mice

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ABSTRACT

Plasmodium berghei is the agent responsible for malaria in rodents. Medicinal plants significantly aid in malaria treatment, with *Syzygium cumini* fruit showing potential as an antimalarial. This study aimed to investigate the potency of the butanol fraction of *Syzygium cumini* fruit as an antimalarial on *Plasmodium berghei*-infected mice, contributing to good health and well-being. Mice were divided into six groups: groups 1 to 4 were treated with the butanol fraction of *Syzygium cumini* fruit at doses of 100, 200, 400, and 600 mg/kgBW, respectively, while distilled water served as a negative control and chloroquine at 25 mg/kgBW as a positive control. Each mouse had a thin blood smear sample taken, and after five days, the parasitemia levels were compared to those in untreated mice. The ED_{50} was calculated using probit analysis to determine the inhibition level over the five days. The results showed a significant difference ($P<0.05$) in parasitemia percentage and inhibition at all doses compared to the negative control. The ED_{50} of the butanol fraction was determined to be 450 mg/kgBW, classifying it as a medium antimalarial. This study concluded that the butanol fraction of *Syzygium cumini* fruit has antimalarial potential against *Plasmodium berghei* in mice, promoting health and well-being.

Introduction

Malaria remains a leading cause of mortality in children and adults, particularly in tropical and subtropical regions. These areas include Sub-Saharan Africa, Central and South America, the Middle East, India, Southeast Asia, and Oceania. The statistics were staggering in 2017, there were 219 million reported cases of malaria, resulting in 435,000 deaths globally. The highest number of fatalities occurred in Africa, highlighting the region's acute vulnerability to this disease (WHO, 2020).

In Indonesia, malaria represents a significant health challenge, especially in regions outside of Java and Bali. The country comprises 33 provinces, 15 of which exhibit malaria prevalence rates exceeding the national average, with most of these high-prevalence areas located in Eastern Indonesia (UNICEF, 2022).

Malaria in humans is caused by several *Plasmodium* species, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* (Sato, 2021). Additionally, *Plasmodium* parasites can also induce malaria in rodents, specifically through *Plasmodium berghei*, which is transmitted by female Anopheles mosquitoes (Orfano *et al*., 2016). The morphology and life cycle of *Plasmodium berghei* closely resembles those of *Plasmodium* species that infect humans, making it a valuable model for studying the disease (De Niz *et al*., 2016).

Rodents infected with *Plasmodium berghei* exhibit symptoms akin to those observed in human malaria cases, including fever, anemia, weakness, hepatomegaly, splenomegaly, and complications such as cerebral malaria, coma, and death (Olatunde *et al*., 2022). The treatment of malaria infections largely relies on antimalarial drugs like chloroquine, which has historically served as the first line of defense against the disease (Zhou *et al*., 2020). However, the emergence of *Plasmodium* resistance to antimalarial drugs poses a significant challenge. This resistance can lead to treatment failures and increase the risk of mortality (Charlotte *et al*., 2022).

Given the escalating problem of drug resistance and the absence of an effective vaccine, there is a pressing need to discover new antimalarial agents derived from natural products. One promising candidate is the *Syzygium cumini* fruit (Taek *et al*., 2018), commonly known as jamun or juwet. *Syzygium cumini* is rich in bioactive compounds, including alkaloids, flavonoids, tannins, polyphenols, saponins, vitamin C, vitamin E, and triterpenoids (Chagas *et al*., 2015). Among these, flavonoids and polyphenols exhibit potent antioxidant properties, while flavonoids and alkaloids have shown significant antimalarial activity. These compounds inhibit hemoglobin degradation and heme detoxification within the parasite's food vacuoles, thereby impeding parasite growth (Maslachah and Purwitasari, 2023).

The extraction of polar compounds such as flavonoids, tannins, and alkaloids from *Syzygium cumini* can be achieved through fractionation, a process that separates substances based on their polarity (Lestario *et al*., 2004). Butanol, a polar solvent, is particularly effective in extracting flavonoids, phenols, tannins, alkaloids, and saponins from natural products (Rebaya *et al*., 2014; Maslachah and Sugiharturi 2018). Previous research by Maslachah *et al*. (2020) has demonstrated that nanoparticles of *Syzygium cumini* extract, when used as adjuvant therapy with chloroquine, enhanced resistance and reduced parasitemia levels in mice infected with *Plasmodium berghei*.

This study aimed to further explore the antimalarial potential of the butanol fraction of *Syzygium cumini* in mice infected with *Plasmodium berghei*, contributing to good health and well-being. The novelty of this research lies in its approach to leveraging the bioactive compounds in *Syzygium cumini* to provide an alternative solution to the growing problem of antimalarial drug resistance, ultimately aiming to improve malaria treatment outcomes and reduce mortality rates. The research focused on evaluating the efficacy of this natural extract in combating malaria and addressing the issue of drug resistance. By investigating the therapeutic effects of *Syzygium cumini*, this study sought to contribute to the development of new, effective antimalarial treatments derived from natural sources.

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Materials and methods

Ethical approval

This study was conducted after obtaining approval with certificate number No. 2.KE.039.05.2020 from the Animal Ethics Committees of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia.

Research design and experimental animals, parasites, drugs, and materials used

This research is an experimental study using a post-test-only control group random design. The experimental animals used in this research were male mice (*Mus musculus*) aged 6-8 weeks weighing 20-30 grams from the Pusat Veterineria Farma (Pusvetma) Surabaya, the parasite used was *Plasmodium berghei* strain ANKA was obtained from the Tropical Disease Center (TDC) Universitas Airlangga. Pro-analytical chloroquine from Sigma, the dose of chloroquine given to mice was 25 mg/kg BW and juwet fruit from the city of Lumajang, East Java. Mice are reared using plastic cages with sawdust as a base. The cage is closed using a wire cover. The mice were fed using BR 511 chicken feed. Food and drink were given ad libitum (without limits).

Place and time of research

Drying and making juwet fruit (*Syzygium cumini*) Simplicia was carried out at the Animal Feed Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga. Juwet fruit extract and fractionation were made at the Phytochemistry Laboratory, Faculty of Pharmacy, Universitas Airlangga, and the preparation of medicinal preparations was carried out at the Veterinary Pharmaceutical Science Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga. Care and treatment of mice was carried out at the Experimental Animal Cage, Universitas Airlangga, Surabaya in 2020.

Research procedure

Preparation of juwet fruit extract (*Syzygium cumini*)

The finely ground Simplicia of juwet fruit (*Syzygium cumini*) was macerated with 96% pro analytical ethanol (Merck KGaA, Darmstadt, Germany) for 4×24 hours, with filtration conducted every 24 hours. Maceration continued until the filtrate was colorless. The resulting filtrate was concentrated using a Rotary Evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 50ºC. The concentrated extract was then dried using a Freeze-Dryer (Labconco Corporation, Kansas City, MO) to produce a thick extract.

Preparation of *Syzygium cumini* butanol fractionation

Fractionation was performed using a sequential method. A total of 100 grams of *Syzygium cumini* extract was mixed with 200 mL of Butanol (Fisher Scientific, Pittsburgh, PA) and 200 mL of distilled water, then shaken until homogeneous in a Separating Funnel (Pyrex, Corning Inc., Corning, NY). The filtrate from the separation of the water and butanol phases was collected and concentrated using a Rotary Evaporator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 50ºC, followed by drying with a Freeze-Dryer (Labconco Corporation, Kansas City, MO). The resulting dry butanol fraction was stored in a Glass Bottle (Wheaton Industries, Millville, NJ) and kept in a refrigerator.

Plasmodium berghei infection dose

Erythrocytes were collected from blood donors containing *Plasmodium berghei*-infected mice. Infection in mice was induced by intraperitoneal injection of 0.2 mL of blood containing 1×10^6 parasites. Parasitemia was checked using thin blood smears 24 hours post-infection. Blood from the caudal vein was smeared, fixed with Methanol (Sigma-Aldrich, St. Louis, MO), stained with 25% Giemsa solution (Merck KGaA, Darmstadt, Germany) for 30 minutes, rinsed with running water, and dried. Blood smears were observed under a Light Microscope (Nikon Instruments Inc., Melville, NY) at 400× magnification using oil immersion.

Treatment of experimental animals

Syzygium cumini butanol fraction test material, doses used in treatments were 100, 200, 400, and 600 mg/kg BW. The volume of administration is 0.5 ml once a day orally. Mice were randomly divided into 6 treatment groups. Consisting of 5 mice in each group, with details of treatment groups as follows: Negative control (K-): Group of mice infected and given distilled water, Positive control (K+): Group of mice infected and given chloroquine 25 mg/kg BW, (P1): The group of infected mice was given 100 mg/kg BW of *Syzygium cumini* butanol fraction. (P2): Groups of infected mice were given *Syzygium cumini* butanol fraction 200 mg/kg BW, (P3): Groups of infected mice were given *Syzygium cumini* butanol fraction 400 mg/kg BW. (P4): A group of mice were infected and given 600 mg/kg BW of *Syzygium cumini* butanol fraction. After 24 hours post-infection therapy is given once a day for 4 days.

Making blood preparations and calculating the number of parasites

Peripheral blood from the mice's tail was used to prepare thin blood smears, which were then air-dried and fixed with Absolute Methanol (Sigma-Aldrich, St. Louis, MO) for 1–2 minutes. The smears were stained with 20–30% Giemsa Stain (Merck KGaA, Darmstadt, Germany) for 20–30 minutes, rinsed gently with Distilled Water (Thermo Fisher Scientific, Waltham, MA), and air-dried. Immersion Oil (Cargille Laboratories, Cedar Grove, NJ) was applied, and the smears were examined under a Light Microscope (Nikon Instruments Inc., Melville, NY) at 1000× magnification.

Calculation of the percentage of parasitemia

Blood smear preparations were carried out to observe the number of erythrocytes infected with the *Plasmodium berghei* parasite per 1000 erythrocytes, and subsequently determined the growth, growth inhibition, and parasitemia percentages. To compute the proportion of parasitemia, the formula from Ljungstrom *et al*. (2004) and Garcia (2007)

% Parasitemia =(∑ infected erythrocytes) / (1000 erythrocytes) x 100

Percent parasitemia data, the percentage of parasite growth is calculated using the following formula:

% Growth= % Parasitemia D4 - % Parasitemia D0 x 100

Information:

D4= percent parasitemia on day 5 (the day after the last administration of test material)

D0= percent parasitemia on day 0 (before administering the test material)

Obstacle percentage data is calculated using the formula from Ljungstrom *et al* (2004):

% Resistance = 100%- Xp/Xk x 100

Information:

Xp = Parasitemia treatment

Xk = Parasitemia control

Data analysis

Data on the percentage of parasitemia, parasite growth, and parasite inhibition for 5 days (D0-D4) on the dose of *Syzygium cumini* butanol fraction obtained were then analyzed using probit analysis on IBM SPSS statistics 26 to get the ED_{50} value of the butanol fraction *Syzygium cumini*. The ED_{50} value indicates the dose of the test substance that can inhibit

50% of the growth of P *Plasmodium berghei*.

Results

The study examined the percentage of parasitemia in mice (Mus musculus) infected with *Plasmodium berghei* across different groups, including control and treatment groups, over a period of five days. The results are summarized in Table 1. The results of the percentage of parasitemia on day 5 using the ANOVA test for each group showed that there was a significant difference (P<0.05). The negative control group (K-) had the highest percentage of parasitemia at 4.34%, while the positive control group (K+) had the lowest at 0.68%. Among the treatment groups, the group receiving the highest dose of *Syzygium cumini* butanol fraction (P4, 600 mg/kg BW) exhibited the lowest parasitemia at 1.32%, whereas the group receiving the lowest dose (P1, 100 mg/kg BW) showed the highest parasitemia at 2.64%. Figure 1 illustrates that parasitemia increased daily across all groups. The negative control group exhibited the highest percentage of parasitemia, while all treatment groups showed lower parasitemia levels compared to the negative control. The group treated with 600 mg/kg BW of *Syzygium cumini* butanol fraction had the lowest parasitemia levels among the treatment groups, indicating the dose-dependent antimalarial activity of the extract.

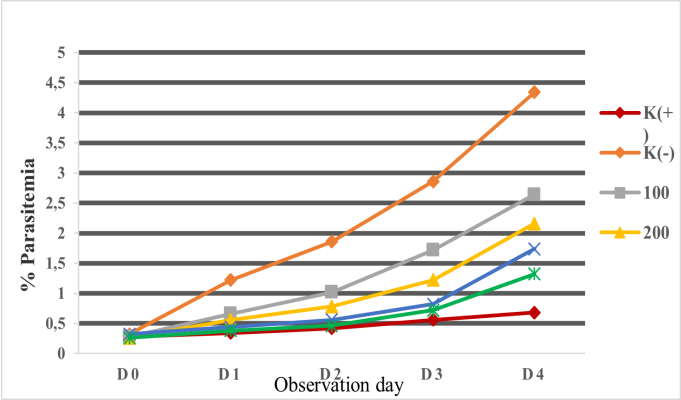

Fig. 1. Graph of the relationship between the percentage level of parasitemia and days of observation in the control group and treatment group.

In Table 2, the results of the ANOVA test on the percentage of growth for each group show that there are significant differences (P<0.05). The P1 group with a dose of 100 mg/kgBW had the highest growth percentage after the K- group with an average growth of 2.36% and the P4 group with a dose of 600 mg/kgBW had the lowest growth percentage after the K+ group with an average growth of 1.06 %.

The results of the ANOVA test on the percentage of inhibition for each group showed significant differences (p<0.05). The P1 group with a dose of 100 mg/kgBW had the lowest percentage level of inhibition compared to other treatment groups, an average of 39.17%, while the P4 group with a dose of 600 mg/kgBW had the highest percentage level of inhibition after K+, an average of 69.59%. Table 2 shows that the greater the dose of the butanol fraction *Syzygium cumini* given, the higher the percentage of inhibition can suppress the growth of parasites.

Table 1. Average percentage of parasitemia mice in the control group and treatment group from day 1 to day 5.

Test Group (mg/kg BW)	Mean percentage of parasitemia (%) ± SD				
	D1	D2	D3	D4	D5
K(-)	0.32 ± 0.08	1.22a ± 0.16	1.86a ± 0.32	2.86a ± 0.38	4.34a ± 0.25
K(+)	0.28 ± 0.08	0.34b ± 0.05	0.42b ± 0.04	0.56b ± 0.05	0.68b ± 0.08
P1	0.28 ± 0.08	0.66cd ± 0.05	0.98c ± 0.08	1.72c ± 0.13	2.64c ± 0.11
P2	0.26 ± 0.05	0.56de ± 0.09	0.78d ± 0.08	1.22d ±0.08	2.16d ± 0.09
P3	0.32 ± 0.08	0.44be ± 0.05	0.56b ± 0.05	0.82ef ± 0.08	1.74e ± 0.11
P4	0.26 ± 0.11	0.38bef ± 0.08	0.46b ± 0.05	0.72bf ± 0.11	1.32f ± 0.08

Different notations in the same column indicate significant differences at the confidence level of P<0.05.

Table 2 Average percentage of growth and parasite inhibition in the control group and treatment group on day 5.

Test group (mg/kg BW)	% Parasite growth ± SD	% Parasite inhibition ± SD
K(-)	4.02a ± 0.26	0.00a ± 0.00
K(+)	0.4b ± 0.07	84.33b ± 1.93
P1	2.36c ± 0.13	39.17c ± 2.63
P2	1.92d ± 0.16	50.23d ± 2.06
P3	1.38e ± 0.11	61.29e ± 1.92
P4	1.06f ± 0.05	69.59f ± 1.93

Different notations in the same column indicate significant differences at the confidence level of P<0.05.

Discussion

The effect of increasing the percentage of inhibition that occurs with increasing doses is caused by the active compound content in the test material from the *Syzygium cumini* fraction which has antimalarial activity (Fentahun *et al*., 2017). The higher the dose of butanol fraction *Syzygium cumini* the higher the percentage level of inhibition of the growth of the *Plasmodium* parasite. According to the results of research conducted by Misganaw *et al*., (2019), flavonoids contained in the butanol fraction of Hypoestes forskalei can increase the percentage of parasite resistance by inhibiting the entry of myoinositol and L-glutamine into infected erythrocytes so that *Plasmodium* does not get nutrients which cause death of the parasite. The results of research by Maslachah and Purwitasari (2023) in vitro also show that the butanol fraction *Syzygium cumini* can increase the percentage of inhibition so that it can suppress the growth of *Plasmodium* parasites.

The positive control treated with chloroquine showed a high percentage of parasitemia inhibition from the results of thin blood smears indicating minimal invasion of *Plasmodium* in erythrocytes. This is because chloroquine as an antimalarial works against parasites in erythrocytes by inhibiting the activity of heme polymerase which *Plasmodium* uses to detoxify heme ferriprotoporphyrin IX. This heme is a compound that is membranolytic, an increase in heme in the parasite will cause the parasite membrane to lyse and cause the death of the parasite so that *Plasmodium* invasion can be stopped (Echeverry *et al*., 2019).

The negative group who was only given distilled water showed a high percentage of parasitemia from the results of thin blood smears, there was a very severe *Plasmodium* parasite invasion, and many erythrocytes were damaged and lysed. This is because distilled water does not have antimalarial activity and only acts as a drug solvent so that the growth of *Plasmodium* parasites cannot be inhibited. The decrease in the percentage of parasitemia in the treatment group when compared to the negative control group shows the important role of the bioactive compounds contained in *Syzygium cumini*. According to El Bouzidi (2017), the results of the Withania frutescens butanol fraction which contains

flavonoids can also inhibit the growth and multiplication of *Plasmodium* parasites by inhibiting the entry of parasite nutrients at the erythrocytic stage. The alkaloid compounds contained in the butanol fraction of Hypoestes forskalei can inhibit the work of heme polymerase and cause the accumulation of heme which is toxic to parasites so that excessive *Plasmodium* invasion of erythrocytes does not occur (Abdelsattar *et al*., 2022).

The decrease in the percentage of parasitemia and the increase in the percentage of inhibition are caused by bioactive compounds that have antimalarial activity contained in *Syzygium cumini* including alkaloids, flavonoids, tannins (Katiyar *et al*., 2016; Panghal *et al*., 2019). The antimalarial effect of tannin compounds works in the asexual phase of erythrocytes by inhibiting *Plasmodium* from infecting erythrocytes, resulting in reduced erythrocyte destruction and decreased invasion of new erythrocytes which prevents increased parasitemia in mice. Erythrocyte destruction is reduced, and hemolysis is also reduced so that it can reduce the clinical effect on blood disorders such as anemia, thrombocytopenia, and hemoglobinuria to a minimum which in the end can prevent more serious complications such as cerebral malaria (Baffour *et al*., 2023).

In malaria infection, during the intraerythrocytic phase, *Plasmodium* uses Hb from erythrocytes for life by being degraded in food vacuoles into heme and globin. The presence of parasites in erythrocytes will stimulate phagocytic cells to release free radicals or reactive oxygen species (ROS) which cause an imbalance of endogenous antioxidants, causing oxidative stress, the aim of eliminating parasites or parasitic erythrocytes, but because of their non-specific nature can destroy normal erythrocytes (RBC) thereby causing hemolysis (Egwu *et al*., 2021). Antioxidant compounds such as flavonoids, alkaloids, phenolics, tannins, and anthocyanins can act as free radical scavengers, thereby helping reduce malaria infections due to oxidative stress (Misganaw *et al*., 2020).

Observations were made until the 10th day to find out more about the therapeutic effect of the test material on parasite growth. The results show that the percentage of parasitemia is getting higher and increasing every day and the results of thin blood tests show that *Plasmodium* invasion is getting higher and there are many schizont stages. This can happen because of compounds that are thought to have antimalarial activity such as flavonoids, alkaloids, and tannins contained in juwet fruit. The levels decrease because therapy is stopped, therefore after therapy is stopped the *Plasmodium*, which is still viable or alive, the growth of the parasite cannot be stopped and increases every day. This shows that the butanol fraction of *Syzygium cumini* will be optimal if combined with other antimalarial drugs or as adjuvant therapy with primary antimalarial drugs, as research conducted by Hiben *et al*., (2016) and reported that combination therapy between leaf extract Senna singueana with chloroquine, and alsoextract of Picrasma javanica (Praptiwi *et al*., 2007) reduced the degree of parasitemia in mice infected with *Plasmodium berghei* compared to mice treated only with chloroquine.

Conclusion

Butanol fraction of *Syzygium cumini*fruit possesses significant antimalarial properties against *Plasmodium berghei*. The findings revealed a substantial reduction in parasitemia and increased inhibition of parasite growth in infected mice, with an effective dose (ED_{50}) of 450 mg/kg BW, indicating moderate antimalarial activity. These results emphasize the potential of *Syzygium cumini* as a source of natural antimalarial agents, especially in addressing the growing concern of drug-resistant malaria. The study provides a strong foundation for further research into *Syzygium cumini* and other natural products as alternative therapies, contributing to global health efforts aimed at improving malaria treatment options and outcomes. The findings also contribute to the broader goal of enhancing health and well-being by providing a basis for the development of alternative antimalarial therapies.

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Conflict of interest

The authors declare that they have no conflict of interest

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