JMU
Jurnal Medika Udayana
ISSN: 2597-8012 JURNAL MEDIKA UDAYANA, VOL. 13 NO.03, MARET, 2024
DOAJ DIRECTORY OF OPEN ACCESS JOURNALS
Accredited SINTA 3
Received: 2023-08-16 Revision: 2023-12-28 Accepted: 25-02-2024

THE EFFECT OF JOHAR PLANT FLOWER EXTRACT *"CASSIA SIAMEA"* ON THE DEGREE OF *PLASMODIUM BERGHEI* PARASITEMIA *IN VIVO*

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ABSTRACT

Malaria is a disease caused by a parasite of the genus *Plasmodium sp.* that spreads through the bite of the female *Anopheles* mosquito. Malaria is still a global problem because almost half of the world's population is at risk of malaria which is endemic to 86 tropical and subtropical countries. In addition, resistance to antimalarial drugs that occur in some places makes many researchers try to find antimalarial drugs made from natural ingredients so that they can reduce the risk of resistance with minimal effects. One natural ingredient that is thought to be used as an antimalarial drug is the johar flower *(Cassia siamea).* The objective of this study is to determine the effect of johar flower extract *(Cassia siamea)* at doses of 10 mg/kgBW, 100 mg/kgBW, and 1000 mg/kgBW on the degree of parasitemia in male mice Balb/c strain infected with *Plasmodium berghei.* This study is an experimental study in vivo using the design of the Randomized Post-test Only Controlled Group method using 24 samples of mice which were divided into four groups, one negative control group and three treatment groups. The average degree of parasitemia was obtained in the control group of 24.9%, the 10 mg/kgBW dose treatment group of 13.4%, the 100 mg/kgBW dose treatment group of 10.3% and the 1000 mg/kgBW dose treatment group of 12.2%. Based on the results of the analysis with One-way ANOVA, an average difference was found between the control and treatment groups with a *p-value* <0.001. The result showed that giving johar flower extract *(Cassia siamea)* had an influence on antimalarial activity in reducing the percentage of parasitemia of mice infected with *Plasmodium berghei* by looking at the average difference between the control group and the treatment and obtained the average results of the treatment group of 100 mg/kgBW and 1000 mg/kgBW had the highest influence in reducing the degree of parasitemia.

Keywords: *Cassia siamea.,* Antimalaria., *Plasmodium berghei.,* Degree of Parasitemia

INTRODUCTION

Malaria is a disease caused by parasites from the genus *Plasmodium sp.* which is spread through mosquitoes. This disease can be a chronic disease or an acute disease that attacks the erythrocytes of the host cell so that it can cause an inflammatory reaction so that sufferers will experience fever, chills, and flu-like illness.[1,2] The World Health Organization (WHO) states that in 2020 it is estimated that 241 million cases of malaria occurred worldwide and 627,000 people died, most of them children in sub-Saharan Africa. The proportion of malaria cases caused in the African region is more than 99%, then the problem in the Western Pacific region is 71.8%, the Eastern Mediterranean is 69% and the Southeast Asian region is 62.8%.[3]

Malaria in Indonesia is still a threat that must be watched out for, especially for people living in eastern Indonesia such as Papua, West Papua, East Nusa Tenggara, and East Kalimantan.[4] Treatment with ACT is the first and second-line treatment for malaria throughout the world, however, The use of ACT can carry the risk of resistance, namely delayed clearance of malaria parasites. Resistance to ACT has been proven by several studies using in vitro and in vivo methods.[5] In addition, climate change which has become increasingly extreme in recent years has influenced on the growth of mosquitoes and the spread of mosquitoes that carry malaria parasites, thereby increasing malaria cases.[6] Based on the problem This is why many researchers are trying to find antimalarial drugs made from natural ingredients so that they can reduce the risk of resistance with minimal side effects. One of the natural ingredients that is thought to be able to be used as an antimalarial drug is *Cassia siamea* commonly referred to as johar leaves by the public. Several studies state that extracts of leaves stems, flowers, seeds, and even roots from Cassia siamea can treat several diseases, especially malaria, by extracting them using solvents such as ethanol, methanol, chloroform, or hexane.[7]

Specific research regarding the effect of johar flower extract on the degree of parasitemia of mice infected with *Plasmodium berghei* has not been carried out in Indonesia. However, there has been research discussing the effectiveness of johar leaves as an antimalarial, such as research conducted by Wiwied Ekasari and friends in 2003 where they made an extract. Johar leaf ethanol was then given orally to mice infected with Plasmodium berghei and the ED50 results from *Cassia siamea* leaves were 8.43 kg/BW.[8]

In the process of researching the effect of johar flower extract *(Cassia Siamea)* on male Balb/c mice infected with Plasmodium berghei, the researchers hypothesized that there would be a reduction in the degree of parasitemia on the growth of *Plasmodium berghei* when given johar flower *(Cassia Siamea)* extract at a dose of 10 mg/kg BW, 100 mg/kg BW and 1000 mg/kg BW. The research aimed to determine the effect of johar plant flower extract *(Cassia siamea)* at doses of 10 mg/kgBW, 100 mg/kgBW, and 1000 mg/kgBW on the degree of parasitemia in male Balb/c mice infected with *Plasmodium berghei*.

HEADING

Malaria

Malaria is a disease caused by a parasite that is spread via the *Anopheles* mosquito. This disease can be a chronic disease or an acute disease that attacks the erythrocytes of the host cells and causes several inflammatory reactions in the body such as fever caused by a parasite originating from the genus *Plasmodium*.[2] Malaria can cause several complications, but the most frequent is cerebral malaria which can cause swelling of the brain so that sufferers will experience seizures and coma.[9] Malaria is often found in tropical countries, one of which is Indonesia, especially the eastern region of Indonesia.[4] The diagnosis of malaria is from anamnesis where the main complaints are fever, chills, muscle aches, and several gastrointestinal symptoms, then followed by a physical examination, namely by examining vital signs, the temperature is found to be elevated and for the gold standard malaria examination is examination of a thin blood smear which is then carried out with *Giemsa* staining and observation under a microscope to see the morphology of the *Plasmodium sp* parasite.[2] Treatment for malaria is using Artermisinin-Based Combination Therapy (ACT), but ACT also has a risk of resistance.[10]

Plasmodium Berghei

Plasmodium berghei is a parasite that infects rodents and is widely used in malaria research because its morphology is the same as *Plasmodium falciparum* compared to other *Plasmodium*.[11] If a thin blood smear is examined, young trophozoites will be found in the form of commas and open rings, gametocytes are banana-shaped, and there are spots. on red blood cells.[12]

Johar Plant *(Cassia Siamea)*

The *Cassia siamea* plant is a native plant that lives and grows in the Southeast Asia region, starting from Indonesia to Sri Lanka. This plant is usually called the Johar plant by ordinary people. This plant has the scientific name *Cassia siamea* which refers to its country of origin, namely Siam or Thailand.[2] The contents contained in this plant have been proven to have good benefits for treating several diseases including malaria. This is due to the content of different groups of chemicals in the plant. This plant is very useful as a natural antioxidant including phenolics, flavonoids, anthraquinones, alkaloids, steroids, saponins, carotenoids, antinutrients, reducing sugars, vitamins, and enzymes.[13] Natural antioxidants from this plant can be found in the leaves, flowers, seeds, stems, and even roots which work in different ways on the body and cause different effects so it can be useful for the treatment of many diseases.[7]

MATERIALS AND METHODS

This research is an in vivo experimental research using a Post Test Only Control Group Design research design. Samples will be taken randomly and measurements will be taken after administering the intervention. The research was carried out at the Parasitology and Integrated Biomedical Experimental Animal Laboratory at Udayana University with a time allocation from January to July 2023. The samples used were male mice of the Balb/c strain obtained from the Bikul Bali pet shop. Samples were selected according to the inclusion criteria, healthy male Balb/c mice with a body weight of 25-30 grams and 2-3 months old. The exclusion criteria were male Balb/c mice that did not want to eat, were lost, or died. The samples used in this study were divided into 4 groups consisting of 1 negative control group (K-) and 3 experimental groups, namely a treatment dose of 10 mg/kgBW (P1), a treatment dose of 100 mg/kgBW (P2) and a treatment dose of 1000 mg /kgBW (P3). The independent variable in this study was the ethanol extract of johar flowers *(Cassia Siamea*) with different doses which were divided into three different doses, namely 10 mg/kgBW, 100 mg/kgBW, and 1000 mg/kgBW. The dependent variable in this study was the degree of parasitemia in the bodies of male Balb/c mice after being given johar flower extract *(Cassia Siamea).* The control variables in this study were the age of the mice, the body weight of the mice, the sex of the mice, environmental conditions, and the mice's food.

The tools used to prepare the mice were a cage measuring 40 x 30 x 18 cm with a lid, a food sack, a drinking bowl, and a sonde used to administer johar flower extract, the Laboratory has provided all these tools for Drug Development and Biomedical Testing Animals. The tools used to make johar flower extract are a glass beaker (Pyrex, America), a stirring rod (Pyrex, America), a set of 1L rotary evaporators (Eyela, Japan), a soundproof glass jars (Pyrex, America), a blender (Waring type 8010 BU, America). The tools used for taking blood and making blood smears from mice were EDTA tubes (One Med, Indonesia), 1 cc syringe (One Med, Indonesia), object and cover glass (One Med, Indonesia), handscone (One Med, Indonesia), micropipette (Research plus, Germany), dropper pipette (Biologix, Indonesia). Materials used were Plasmodium berghei, Giemsa dye (Indoreagen, Indonesia), distilled water (Smart-

Lab A-1078, Indonesia), immersion oil (Indonesia), alcohol (Indonesia), mouse feed (AD II pellets, Indonesia).

Preparation of johar flower extract (Cassia siamea)

The first procedure is making a Johar flower extract. Fresh johar flowers will be collected from johar plantations in East Sumba, washed with running water, and then dried. The drying process is carried out by placing the johar flowers in a drying cabinet at a temperature of 400C. The dried johar flowers will be blended until they form a dry powder. Extracted samples of 48.8 grams of johar flowers were put into a container, then 600 ml of 96% ethanol was added, then stored for 3 days then filtered using a vacuum filter. The resulting filtrate was separated using a rotary vacuum evaporator under a reduced pressure of 520 C to obtain a thick johar flower extract. 1 gram of thick johar flower extract was weighed and dissolved in 100 ml of ethanol and a solution of johar flower extract was obtained with a concentration of 10,000 ppm or the equivalent of 10,000 mg.[14]

Preparation of experimental animals

Preparation of experimental animals begins with ensuring that the mice are healthy and meet the intrinsic research factors, then the mice will be placed in cages measuring 40 x 30 x 18 cm with each cage occupied by 7 mice. The environmental humidity around the mice will be set at around 40-70% with an environmental temperature ranging from 18-260C, then for bedding or cage mats the mice will use sterilized rice husks while still ensuring the mice's comfort. During the research process, the mice will be placed in a room with lighting. as low as 40 lux and adequate ventilation to provide adequate air quality and oxygen supply to avoid environmental stress.[15] The test animals used were 28 male Balb/c strain mice weighing 25-30 grams. The test animals were divided into 4 groups, namely the negative control group (K-) and 3 treatment groups (P1, P2, P3). Each group consisted of 7 mice. Before treatment was carried out on mice, an acclimatization process was carried out for 7 days by providing standard feed and distilled water.

The first treatment on mice was culturing Plasmodium berghei into donor mice. Isolates containing the blood of experimental animals infected with P.berghei from frozen storage were thawed at room temperature. Isolates were obtained at the Parasitology Laboratory, Faculty of Medicine, Udayana University. The liquid isolate was centrifuged at 2000 rpm for 5 minutes. Then two layers will be formed, namely the supernatant and pellet layers. The supernatant layer will be taken away so that only the pellet layer is left. The pellet layer in Eppendorf was washed with PBS and centrifuged again for 5 minutes. Then the supranatant layer is taken back so that what is left is only the pellet layer. The volume of the pellet layer in the Eppendorf was measured and mixed with the same volume of the complete cell culture medium. This preparation was then injected into mice as much as 0.2 mL intraperitoneally. Then the degree of parasitemia will be checked every day by taking blood from the tails of donor mice until the degree of parasitemia reaches ≥ 10% and it is considered good for inoculation into other mice because the possibility of being positive is greater. Donor mice's blood of ≥ 10% was taken intracardially then stored as isolate stock and placed in a refrigerator at a temperature of -800 C.[7,16]

This was followed by intraperitoneal inoculation of the test animals with the degree of parasitemia in the mice reaching ≥ 10%. Infected blood: 0.75 cc of infected blood is taken with a 1 cc syringe and then put into an EDTA tube. Then 10 µL of blood was taken from EDTA using a micropipette and put into the first eppendorf containing PBS 990 µL. Then the mixture of blood and PBS in the first Eppendorf was suspended, after which 10 µL was taken back to be put into the second Eppendorf which contained PBS 990 µL. Then the number of erythrocytes will be calculated by dripping the liquid in the second Eppendorf onto the hemocytometer. Then you will look under a microscope to count the number of erythrocytes in all 25 boxes, limited by 3 lines in each box. After obtaining the required amount of blood, the amount of PBS that will be required for the blood mixture that will be used for induction is obtained by calculating the total volume of the mixture that will be injected according to the number of mice tested minus the amount of blood required. Donor mouse blood and PBS were added and mixed slowly into a falcon tube to avoid hemolysis. Then the mixture will be injected into mice using a 0.2 mL 1 cc syringe intraperitoneally.[7,16]

Suppose *plasmodium berghei* has been induced in test animals and shows positive results for malaria in mice, then johar flower extract *(Cassia Siamea)* will be given at a dose of 2.5 mg/25 gBW, 25 mg/25 gBW, and 250 mg/20 gBW in the treatment group. The negative control group was not given johar flower extract therapy but was only PBS 0.2 ml as solvent control. Administration was carried out during the days from D0 – H3 after which treatment was not given again until the eighth day.[7,16]

Making Blood Smears for Thin Blood Drops

Thin blood smears were made at the Parasitology Laboratory, Udayana Medical Faculty. The blood used for blood smears is blood taken from the tails of mice. A small amount of blood was taken from the mice and placed on a glass object spread flat with a covered glass dried in air fixed in methanol for 3 minutes and dried again. After that, staining was carried out with 10% *Giemsa* for 15 minutes.[7]

Observation of the Degree of Parasitemia

The next stage is that a thin blood smear that has been made and stained with *Giemsa* 10% for 15 minutes will be used to observe the degree of parasitemia under a microscope. Observation of the degree of parasitemia using a light microscope with a magnification of 1000 times to count the number of Plasmodium berghei parasites. Calculation of the number of parasites is carried out on around 1000 erythrocytes and a calculation of the number of erythrocytes infected by parasites will be obtained which will then be converted into a level of parasitemia in percent and the percentage of substances inhibiting parasite growth.[7]

Data analysis

Data analysis is carried out using descriptive analysis methods, normality tests, and homogeneity tests. data. The test was carried out using the SPSS 26.0 software application using the One Way Anova test with a p-value <0.05 if the data was normally distributed and homogeneous. If the data is not normally distributed, the Kruskal-Wallis test will be continued. This research has received ethical permission from the ethics commission of Udayana University with the number of ethics exemption letters: 914/UN14.2.2.VII.14/LT/2023

RESULTS

Results of Mice Blood Smear Examination

Examination of mouse blood smears was carried out by looking under a microscope at 1000x magnification to determine the degree of parasitemia of each experimental animal. Blood smears were made from 6 mice in each control and treatment group and then observed in five fields of view. The following are the results of blood smears in the negative control group (K-) and treatment group 2 (P2) seen through a microscope with arrows indicating the red blood cells of mice infected with *Plasmodium berghei*. The results of the blood smear from mice can be seen in figure 1.

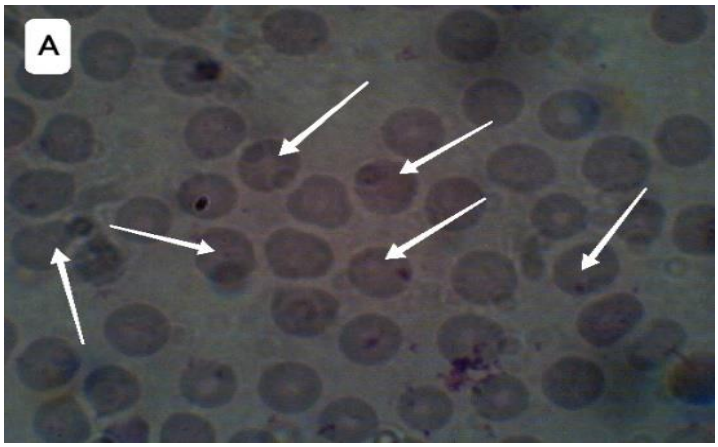

Figure 1A. Mice blood smear of negative control

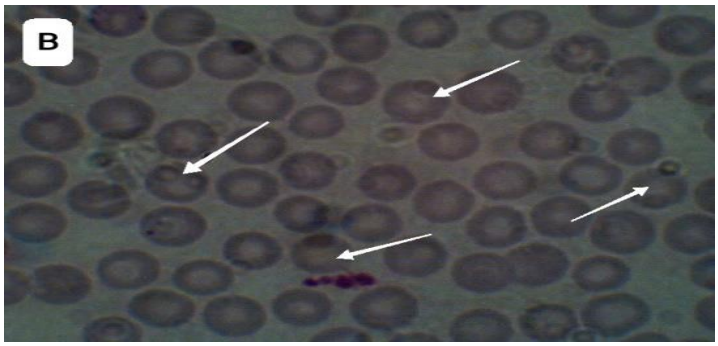

Figure 1B. Mice blood smear of treatment group 2

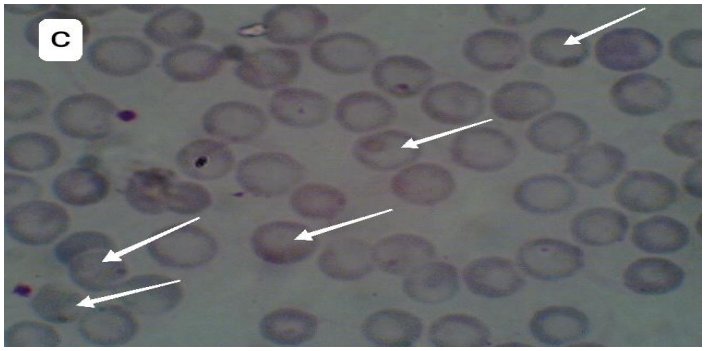

Figure 1C. Mice blood smear of treatment group 1

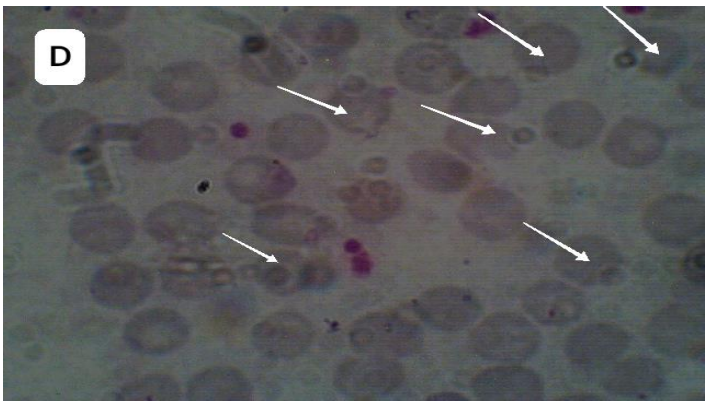

Figure 1D. Mice blood smear of treatment group 3

Antimalarial Activity Test

Antimalarial activity can be observed by measuring the degree of parasitemia in each treatment group. From the results of the descriptive tests carried out, it was found that the highest mean degree of parasitemia was experienced in mice in the negative control group (K-), namely 24.9%, while the lowest mean degree of parasitemia was experienced in mice in treatment group two (P2), namely 10.3%. The results of the malaria activity test can be seen in table 1.

Table 1. Antimalarial activity of male mice strain balb/c

Group	Sample (n)	Statistics	df	*p-value*
Control (-)	6	0,902	6	0,389
Treatment 1	6	0,822	6	0,091
Treatment 2	6	0,866	6	0,212
Treatment 3	6	0,800	6	0,059

Parasitemia Degree Normality Test

This normality test was carried out to know whether the parasitemia degree data obtained was normally distributed or not. The normality test was carried out using the Shapiro-Wilk test and obtained a p-value> 0.05 which shows that the data obtained was distributed normally. The normality test shows that the data is normally distributed, so parametric tests can be carried out. The results of the normality test for the degree of parasitemia can be seen in table 2.

Table 2.Normality test for data distribution for degrees of parasitemia in male mice of the balb/c strain

	Sum of squares	df	Mean square	Significance
Between groups	769,125	3	256,375	0,000
Within groups	186,833	20	9,342	
Total	955,958	23		

Parasitemia Degree Homogeneity Test

The homogeneity test is carried out to show whether the sample data taken has the same variance or not. The homogeneity test results were obtained using the Levene test and based on the homogeneity test in this study, the results were $p < 0.001$, which indicates that the data is not homogeneous. The results of the homogeneity test for the degree of parasitemia can be seen in table 3 .

Table 3. Data homogeneity test results for the degree of parasitemia in male mice of the balb/c strain

Variable	Homogeneity test
Degree of parasitemia	0,000

One Way ANOVA Test

One Way ANOVA parametric test was carried out to determine whether there was a mean difference in the degree of parasitemia in the four sample groups. The results of data analysis showed that $p < 0.01$ showed that by administering johar flower extract, the degree of parasitemia was significantly different between the control group and the treatment group. The results of the One Way ANOVA analysis can be seen in table 4.

Table 4. Results of one-way ANOVA analysis of the mean degree of parasitemia in male mice of the balb/c strain

Group	Antimalarial activity	
	% Parasitemia ± s.b	**% Suppression**
Control (-)	24,9 ± 5,6	0,0
Treatment 1	13,4 ± 0,8	46,1
Treatment 2	10,3 ± 1,7	58,5
Treatment 3	12,2 ± 1,8	50,9

Post Hoc Test

Post hoc tests were carried out to see differences in the mean degree of parasitemia in each group.each sample group. Based on the results of the Post Hoc test, the p-value for the degree of parasitemia was <0.05. Statistically, there was a significant difference in the degree of parasitemia between the negative group and treatment group 1, treatment 2, and treatment 3, whereas treatment group 1 and treatment 2, 3 and treatment groups 2 and treatment 3 obtained insignificant results. Based on the average degree of parasitemia for each group from lowest to highest, starting from treatment group 2 (P2) at 10.3%, treatment group 3 (P3) at 12.2%, treatment group 1 (P1) at 13.4% and the negative control group (K-) was 24.9%, so it can be concluded that treatment group 2 (P2) had the highest level of effectiveness among the other groups. The results of the post hoc test can be seen in table 5.

Table 5.Post hoc test results on mean degree of parasitemia in male mice of the balb/c strain

Group	**Mean difference**	**IC 95%**		***p-value***
		Min	**Max**	
K- vs P1	11,5	6,56	16,43	0,000
K- vs P2	14,5	9,56	19,43	0,000
K- vs P3	12,5	7,56	17,43	0,000
P1 vs P2	3,00	-1,93	7,93	0,350
P1 vs P3	1,00	-3,93	5,93	0,941
P2 vs P3	-2,00	-6.93	2,93	0,674

DISCUSSION

Based on the results of research that has been carried out, it was found that administration of johar flower extract was proven to affect and inhibit the growth of Plasmodium berghei which was infected in male mice of the Balb/c strain. The results of this study showed that the mean percentage degree of parasitemia in the negative group (K-) had a higher average than the other treatment groups, namely 24.9%, while in the treatment group 1 (P1) it was 13.4%, treatment 2 (P2) was 10 .3%, and treatment 3 (P3) was 12.2%.So it can be concluded that administering the extract to treatment group 2 proved effective in reducing the degree of parasitemia. This proves that johar flower extract has an inhibitory effect on the growth of Plasmodium berghei.Johar flower extract (Cassia siamea) given to Balb/c mice infected with Plasmodium berghei contains chemical compounds that affect inhibiting the degree of parasitemia of mice infected with Plasmodium berghei. One of the chemical compounds contained in johar flower extract is flavonoids which have been studied to be involved in the antiplasmodial activity process.[17] This is in line with research conducted by Mamadou Kamagate and his friends in 2014 at the University of Felix Houphouet Boigny-Abidjan where they researched to prove that the compounds contained in johar plant extracts consisting of leaves, stems, flowers, and roots can be used as antimalarials.[14]

Specific research regarding the effect of johar flower extract on the degree of parasitemia of mice infected with Plasmodium berghei has not been carried out in Indonesia. However, there has been research discussing the effectiveness of johar leaves as an antimalarial, such as research conducted by Wiwied Ekasari and friends in 2003 where they made an extract. Johar leaf ethanol was then given orally to mice infected with *Plasmodium berghei* and the ED50 result from *Cassia siamea* leaves was 8.43 kg/BW. with a dose of 200mg/kgbw, the highest percentage of inhibition was 56.90%. 8 The flavonoid chemical compounds contained in johar leaves and flowers were identified as effective antimalarials. This flavonoid works as an antimalarial by acting as an antioxidant that will transport free radicals thereby preventing oxidative damage caused by *Plasmodium berghei* infection in male Balb/c mice where this infection will produce inflammatory cytokines in the form of interferon-gamma which will activate macrophages in synthesizing nitric oxide. and other ROS compounds that can cause malaria complications.[18]

Flavonoids work as antiplasmodium by inhibiting the NPP (New Permetion Pathway) system which is a selective channel for anions and is permeable to chemicals needed by parasites to grow so that if NPP is inhibited then the parasites will not get nutrients to be able to grow, flavonoids inhibit parasite growth through malaria parasite food vacuoles by inhibiting the hemoglobin degradation

process.[19] Other research has been conducted on the effectiveness of flavonoid compounds from herbal plant extracts as antiplasmodium, including *merkubung (Macaranga Gigantea)* leaf extract.[20,26] *Securidaca longepedunculata Fresen* leaf extract.[21] Breadfruit *(Artocarpus altilis)* leaf extract.[22] Ethanol extract of pineapple leaves *(Ananas comosus).*[23] Extract of sugarcane leaves *(Sugarcane).*[24] Based on several studies that have been presented, it can be proven that many herbal plants have an antimalarial effect on *Plasmodium berghei*.

Based on research conducted by Ezrani Tasiam and friends, administering a dose of 1000mg/kgBW johar flower extract *(Cassia siamea)* does not have a better effect on reducing the degree of parasitemia than administering a 100mg/kg BW dose of johar flower *(Cassia siamea)* extract because It is possible that this dose has exceeded the maximum effective dose (ED50) or may have reached the lethal dose (LD50) of Johar flower extract, but this cannot be confirmed directly by researchers because the research was carried out on Johar flower leaves, not Johar flowers. so further research is still needed regarding the toxic dose of Johar flower extract.[25]

CONCLUSIONS AND SUGGESTIONS

Administration of johar flower extract *(Cassia siamea)* at a dose of 10 mg/kgbw, 100 mg/kgbw, and 1000 mg/kgbw was proven to affect antimalarial activity with an emphasis on the percentage of parasitemia degrees of mice infected with *Plasmodium berghei* by obtaining significantly different results compared to controls. negative. Based on the results of this study, it was found that a dose of 100 mg/kgBB johar flower extract *(Cassia siamea)* in treatment group 2 had the highest antimalarial effect with an average degree of parasitemia of 10.34%. In this research, there are still shortcomings so it is necessary to carry out further research regarding testing the effectiveness of johar flower extract *(Cassia siamea)* on mice infected with *Plasmodium berghei*. Then further research was carried out regarding the toxicity test of johar flower extract *(Cassia siamea)* to determine a safe dose as an alternative antimalarial. It is also necessary to carry out further research regarding phytochemical testing of active compounds contained in johar flower extract *(Cassia siamea)* so that we can know for certain which compounds play an active role as antimalarials in johar flower extract *(Cassia siamea).*

THANK-YOU NOTE

On this occasion, the author would like to express his deepest gratitude to all parties involved in this research, namely, Dr. Dewa Ayu Agus Sri Laksemi, S.Ked., M.Sc. as the first supervisor, Dr. Ni Luh Putu Eka Diartini, S.Ked. M.Si as the second supervisor and Dr. Dr. I Made Sudarmaja, M.Kes as examining lecturer.

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