RESEARCH ARTICLE

Ethanol Extract of *Spondias pinnata* Leaves Reduce Parasite Number and Increase Macrophage Phagocytosis Capacity of Mice Infected by *Plasmodium berghei*

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Abstract

BACKGROUND: Currently, there is no vaccine against malaria in humans, the development of resistance to anti-malarial drugs, causing the need to find new alternatives to overcome malaria infections. This study aimed to determine effect of *Spondias pinnata* in increasing cellular immunity, especially phagocytosis activity of peritoneal macrophages against *Plasmodium berghei* infection.

METHODS: This was an experimental study with two stages of research, each stage requires 36 Balb/c mice, aged 2 months and weight 20-25 grams. After one week of acclimatization, the mice were put into 6 different groups, each group consisted of 6 mice. The negative control was a group of mice given distilled water for 14 days then infected by *P. berghei* in the 15th day. Meanwhile, T1, T2, T3, T4 and T5 groups were given *S. pinnata* leaves ethanol extract with dose of 25, 50, 100, 200 and 400 mg/kg body weight (BW)/day, respectively, and then infected by *P. berghei* in the 15th day.

RESULTS: The results showed that the lowest parasitemia and the highest capacity of macrophage to phagocytose latex was found in treatment group T3 that received 50 mg/kg BW of *S. pinnata* leaves ethanol extract. Based on analysis of the Pearson correlation test, there was a significant correlation between percent phagocytosis and parasitemia (p<0.05).

CONCLUSION: Ethanol extract of *S. pinnata* leaves lower the parasite number of *P. berghei* in Balb/c mice and increase the capacity of macrophage to phagocytose latex. However, the mechanisms of how *S. pinnata* leaves extract in activating phagocytosis capacity and reducing parasitemia still need further investigation.

KEYWORDS: phagocytosis, *Plasmodium berghei*, parasite number, *Spondias pinnata*

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Introduction

Malaria is the biggest health problem in the world besides HIV/AIDS and tuberculosis. Malaria is the worst infectious disease in the world, especially fatal if caused by the *Plasmodium falciparum* infection.(1,2) Based on World Health Organization (WHO) data in 2018, it was found that around 228 million people were infected by malaria with a death toll of 405,000, 67% of this number were children aged less than 5 years.(3-7)

Problems encountered in malaria include a complex life cycle consisting of various stages of parasite, the unavailability of vaccines, and anti-malaria drug resistance. (8) Currently, the best treatment for uncomplicated malaria worldwide is using artemisinin combination therapies

(ACTs), but since 2017, partial resistance to artemisinins has been reported in several regions. The occurrence of malaria drug resistance threatens the effectiveness of existing malaria therapy, malaria control program and malaria elimination efforts.(9) The threat of parasitic strains that are resistant to currently available drugs, encourages efforts to find new regimens and new strategies for using existing anti-malarial drugs, and the development of new compounds that are ready to be used as anti-malarials.(8)

Plants and herbs are currently being developed to provide an affordable treatment option for various health problems.(10) There were 877 new drugs produced from 1981-2002, 6% came from natural products, 27% derived from natural products, 16% came from synthetic materials developed from natural materials.(11)

Malaria drugs such as quinine, artemisin, atovaquone come from plants. Quinine comes from the plant species Cinchona (Rubiaceae), artemisin, which is currently used as the most effective malaria therapy comes from *Artemisin annua* (Asteraceae), while atovaquone which is the latest malaria treatment comes from the *Talebuia impetiginosa* plant.(12) There are many plants in Indonesia that are used as traditional malaria medicine. *Andrographis paniculata*, *Azadirachta indica*, *Cassia siamea*, *Alstonia scholaris*, *Jasminum quenquenerium*, *Swietenia macrophylla*, *Phylanthus niruri*, *Artocarpus champeden* are examples of plants used traditionally as anti-malarial drugs. Bark extract of *Strychnos ligustrina* was able to inhibit *Plasmodium berghei in vivo*.(12) A local herb that also been used traditionally as anti-malarial drugs *Croton caudatus* Geiseler (CCG).(10)

Spondias pinnata are medium-sized deciduous trees with imparipinnate leaves, polygamous flowers and ovoid greenish-yellow fruits.(13) Genus Spondias plant consists of many species, thus used as traditional medicine to treat various diseases. Plant parts of the genus Spondias from skin, roots, fruit, leaves have various benefits and have been used as traditional medicine in various countries.(14) Ethanol extract of *S. pinnata* contain terpenoid, polyphenols and flavonoids.(13) A study shows *Brassica nigra*, which contains components of alkaloids, flavonoids, tannins, terpenoids such as those of *S. pinnata*, have an effect on *P. berghei* infection.(15)

Other research reported that water extracts from the skin of *S. pinnata* are safe up to 2 g/kg BW of Wistar rats. The administration of the extract for 28 days did not cause changes in general conditions, appetite, body weight, growth, biochemical parameters, hematological values and histopathological abnormalities in body tissue.(15) Research using *S. pinnata* in malaria infections has never been studied.

There has never been a study using *S. pinnata* extract on *P. berghei* infection. *S. pinnata* has never been used traditionally to treat malaria, therefore this study was conducted to determine the immunostimulant effect of *S. pinnata* on *P berghei* infection in mice. Other research showed that administration of *S. pinnata* at a dose of less than 1 g/kg BW was toxicologically safe (13), thus we decided to use range dosage from 1 mg/ BW to 1 g/BW. Based on the background above, we conducted a study to investigate the immunostimulant effect of 25, 50,100, 200 and 400 mg/Kg BW *S. pinnata* extract on parasitemia and the ability of macrophages to phagocytose latex in mice infected with *P. berghei*.

Methods

The treatment of animals models were conducted at the experimental animal of Integrated Biomedic Laboratory, Faculty of Medicine, Universitas Udayana. Macrophage culture was conducted at the Histology Laboratory, Faculty of Medicine, Universitas Udayana. The *P. berghei* used in this study was obtained from Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. The ethical clearance was obtained from the Ethics Committee, Faculty of Medicine, Universitas Udayana (No. 2019.03.1.0985).

Plant Material and Preparation of Extract

S. pinnata leaves used in this study came from the same plantation in Penglipuran Bangli and was determined in the Faculty of Agriculture, Universitas Udayana. The *S. pinnata* extraction process was carried out in accordance to standard procedures at the Food Analysis Laboratory of the Faculty of Agriculture, Universitas Udayana. Procedure for maceration started by approximately 200 grams of *S. pinnata* powder macerated with 1.5 L 80% ethanol at room temperature for 2 days, then filtered. Then the pulp was macerated with 500 mL 80% ethanol at room temperature for 2 days, then the filtrate was collected. The filtrate was concentrated by means of a rotary evaporator at 50°C to obtain an extract that still contains a small volume of solvent. Evaporation of the extraction solvent was continued using an oven at 40°C until a thick extract was obtained.(16)

Animal Experimental

This research was consisted of two stages of research. The first stage investigated the effect of *S. pinnata* on

parasitemia, while the second stage investigated the effect of *S. pinnata* on the ability of macrophages to phagocytose latex. Each stage requires 36 Balb/c mice originated from mice bred in the experimental animal division of the Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Udayana, aged 2 months and weighed 20-25 grams, were divided into 6 groups, each group consisted of 6 rats. Animals were kept in a standard room for animal studies (temperature 22-25°C and 12 h dark-light cycle). All animals got standard feed and *ad libitum* access to tap water. After one week of acclimatization. We divided the mice into six groups, namely negative control (NC), and treatement groups (T1-T5). NC was a group of mice given distilled water for 14 days and then infected by *P. berghei.* The mice were infected by injecting 0.2 mL of inoculum containing $1x10^8$ parasite erythrocytic stage on the 15th day. Parasitemia was observed on the 4th day by dissecting 6 mice from first stage groups, while peritoneal macrophages were isolated from second stage group on the 5th day.(17) T1, T2, T3, T4 and T5 were given leaves ethanol extract of *S. pinnata* dose at 25, 50, 100, 200 and 400 mg/kg body weight (BW)/ day, respectively, then were infected by *P. berghei* in the 15th day. *S. pinnata* leaves extract were administered orally using a 12-gauge sonde in a 24-hour cycle. This study was to determine the immunostimulant effect of *S. pinnata* on *P. berghei* infection in mice, thus *S. pinnata* extract was given for 14 days before mice were infected with *P. berghei*.

Hematological Examination and Parasitemia

Hematological examination and parasitemia were carried out on the 4th day after the mice were infected with *P. berghei*. Hematological examination was done until parasitemia reached 5-10%. Blood thin preparation was done using blood from tail of mice, then placed on the tip of the microscope slide. After the dry smear was fixed with methanol and stained with Giemsa 10% for 15 minutes, then washed with running water, after drying the blood smear was examined to count parasitemia by microscope 1000x magnification with immersion oil. Parasitemia was described the density of blood cells infected by parasites in erythrocytes. Calculation parasitemia was done manually by calculating the percentage of the number of erythrocytes with *P. berghei* in 1000 erythrocytes.(17) The examination was done by a trained analyst, with blinding examination to prevent bias.

Isolation and Culture of Peritoneal Macrophage Cell

Mice were sacrificed by cervical dislocation on day 6th to start the procedure of isolation and culture of peritoneal macrophage cell. Mice were placed in the supine position, the skin of the abdomen was opened and peritoneal sheath was cleaned by 70% alcohol. Then ±3 mL of cold Roswell Park Memorial Institute (RPMI) media was injected into the peritoneal cavity, wait for ±3 minutes to pat the abdomen gently. Peritoneal fluid was removed from the peritoneal cavity by aspirated the fluid with a syringe, especially in a non-fatty part and away from the intestine. The needle containing aspiration material was placed in a beaker filled with ice, then the suspension is inserted into the centrifuge tube. Aspirate is centrifuged at 1,200 rpm, 4°C for 10 minutes. The supernatant is removed, then added 1 mL of complete medium to the obtained pellet. The number of cells was counted with a hemocytometer and the viability determined with trypan blue was then resuspended with complete RPMI medium thus cell suspension with a density of $2.5x10^6$/mL was obtained.(17)

The calculated cell suspension was cultured on 24 microplate wells that had been given a round slips cover, each well was 200 µL ($5x10^5$ cells). Incubated in a 5% CO_2 incubator, 37°C for 60 minutes. Then added a complete medium of 1 mL RPMI per wells and incubated again for 4 hours. Cells were washed with RPMI 2X then added 1 mL complete medium per well and continued incubation for 24 hours. Macrophage cells were ready to be tested for their activity.(17)

Latex Phagocytosis Activity

To determine the activity of *S. pinnata* as an stimulant of cellular immunity in *P. berghei* infection, the phagocytosis assay test was carried out in this study. Latex phagocytosis activity test procedure through the calculation of latex particles using latex beads diameter of 3 µm. Latex beads are resuspended in PBS to obtain a concentration of $2.5x10^7$/mL. Macrophages that were cultured the day before were washed twice with RPMI. The latex suspension was added 200 µL ($5x10^6$)/well or latex density in 1 mL was $2.5x10^7$, and incubated in a 5% CO_2 incubator at 37°C for 60 minutes. The cell was then washed with phosphate buffered saline (PBS) three times to remove particles that were not phagocytosed, dried at room temperature, and fixed with absolute methanol for 30 seconds. Then the methanol was discarded, and wait for it to dry.

After drying, coverslips were daubed with Giemsa 10% for 15 minutes. The percentage of cells that phagocytose latex particles and the number of latex particles that were phagocytosed were counted from 100 cells examined under a light microscope with a magnification of 400x. From each subject examined, 3 slides were made.(18)

Statistical Analysis

Data processing was performed using IBM Statistical Package for Social Sciences for Windows (SPSS) version 23.0 (IBM Coorporation, Armonk, NY, USA), with $p<0.05$ was significance. Parasitemia (P) and Percentage of Phagocytosis (PP) were compared using analysis of variance followed by Tukey Honest Significant Difference (HSD) test.

Results

Six groups of mice with an average weight of 20-25 g were used in this study. The six groups were compared in terms of age, and body weight, parasitemia, percentage of phagocytosis. Data were expressed as mean±SD of six replications. The distribution of body weight between the six groups at baseline was similar.

In the T1-T5 treatment group there were a significant decrease of parasitemia, *i.e*., the number of erythrocytes infected with *P. berghei* and also a significant increase of the percentage of peritoneal macrophages that phagocytosis of latex particles when compared with the control group (NC) $p<0.05$ (Table 1).

Furthermore, from the One Way Anova analysis and the Tukey Honest Significant Difference test, we found significant differences in parasitemia and the percentage of phagocytosis between the treatment and control groups ($p<0.05$) (Table 1). Based on the analysis of the Pearson correlation test, it was found that there was a significant correlation between percent phagocytosis and parasitemia ($p<0.05$).

The ethanol extract of *S. pinnata* leaves with the highest efficacy in reducing parasitemia was found in the T2 group who received 50 mg/kg/day for 14 days (Figure 1). The phagocytic ability of macrophages appears to be improved by the administration of *S.pinnata* ethanol extract, especially in the T1 and T2 groups who received *S.pinnata* ethanol extract at doses of 25 and 50 mg/kg/day for 14 days. This study was found that the higher the percentage of latex phagocytosis macrophages, the lower the parasitemia (Figure 1).

Erythrocyte infected with *P. berghei* with Giemsa staining and 400x magnification were indicated by ring form inside the erythrocyte cytoplasm (Figure 2). The highest erythrocytes infected with *P. berghei* was found in the control group without administration of ethanol extract of *S. pinnata* leaves (Figure 2).

Macrophage phagocytosis test with the calculation of latex particles using cultured macrophage cells. Macrophages were the main phagocytic cells that fight invasion of pathogens such as bacteria, parasites and viruses through the mechanism of phagocytosis. The ability of macrophage phagocytosis can be measured by its ability to phagocytose latex particles *in vitro*. Macrophages that phagocytose latex were characterized by latex particles attached to the surface of the macrophage or inside the macrophage (Figure 3). The ability of these macrophages appeared to be increased in the group receiving ethanol extract of *S. pinnata* leaves compared to the control group (Table 1).

Discussion

The results of this study indicate that the ethanol extract of *S.pinnata* has the effect of reducing parasitemia. In this study, the effect of weight on parasitemia was controlled because the weight of the mice did not differ significantly. The results of this study indicate that the ethanol extract of *S.pinnata* has the effect of increasing the phagocytosis activity of macrophages as measured by its ability to phagocytose latex particles. Factors that affect the phagocytic activity of macrophages are cytokines, chemokines, plasmodium species, strains and serotypes.

Table 1. Mean values and standard deviation obtained within the computer-assisted analysis of mice infected by *P. berghei* after treated with *S. pinnata* leaves extract.

Characteristics	Group (n=6)						*p* -value
	NC	T1	T2	T3	T4	T5	
W0	23.3±1.9	24.7±0.5	23.2±2.2	23.8±2	22.5±2.7	23.7±2.3	0.604
W1	27.2±1	26.3±3.3	26.2±4.7	23.8±5.5	24.7±4.1	29.3±5.6	0.33
Phagocytosis (%)	1.2±0.4	14.3±1	34.3±1	8.7±0.8	10.5±5	12.5±1	0
Parasitemia	10.9±2	2.9±1.5	2.5±0.7	4.7±0.6	4.2±1	5.30±4	0

W0: weight before administration of ethanol leaves extract of *S. pinnata*; W1: weight after administration of ethanol leaves extract of *S. pinnata*. *p*-value was tested with One Way Anova, $p\leq0.05$ is statistically significant difference.

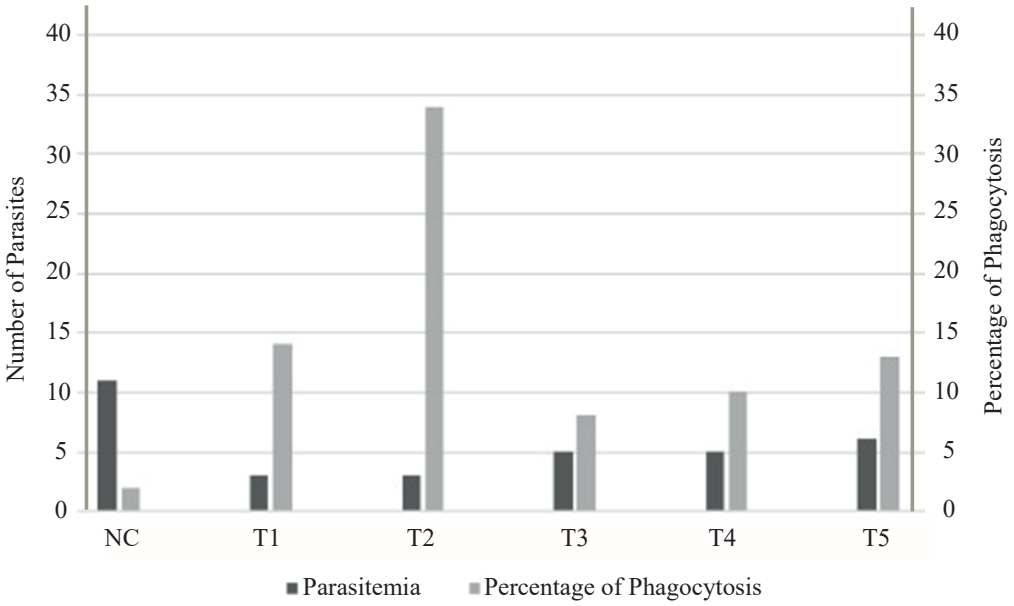

Figure 1. Relation between parasitemia and phagocytosis activity of peritoneal macrophage of mice infected by *P. berghei* after 14 days of *S. pinnata* leaves extract administration.

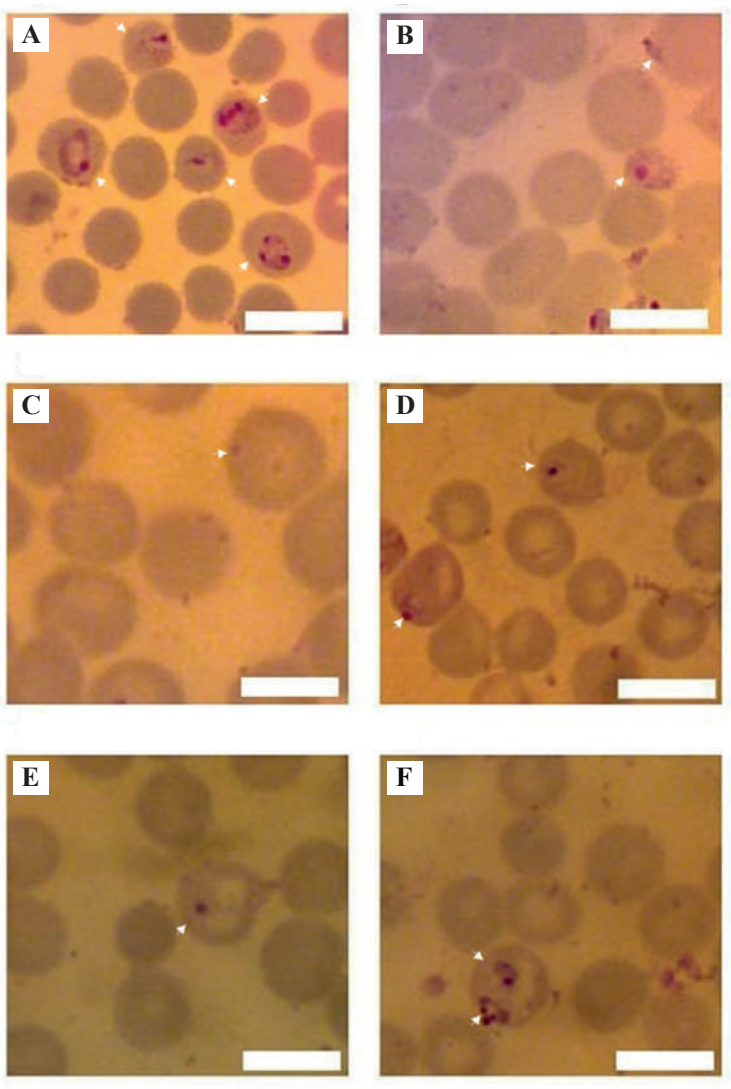

Figure 2. Morphology of erythrocyte infected by *P. berghei*. A: NC; B: T1; C: T2; D: T3; E: T4; F: T5. Cell in passage were documented under an upright light microscope using Giemsa Staining. White bar: 10 µm. White arrow: ring formation inside the erythrocyte cytoplasm.

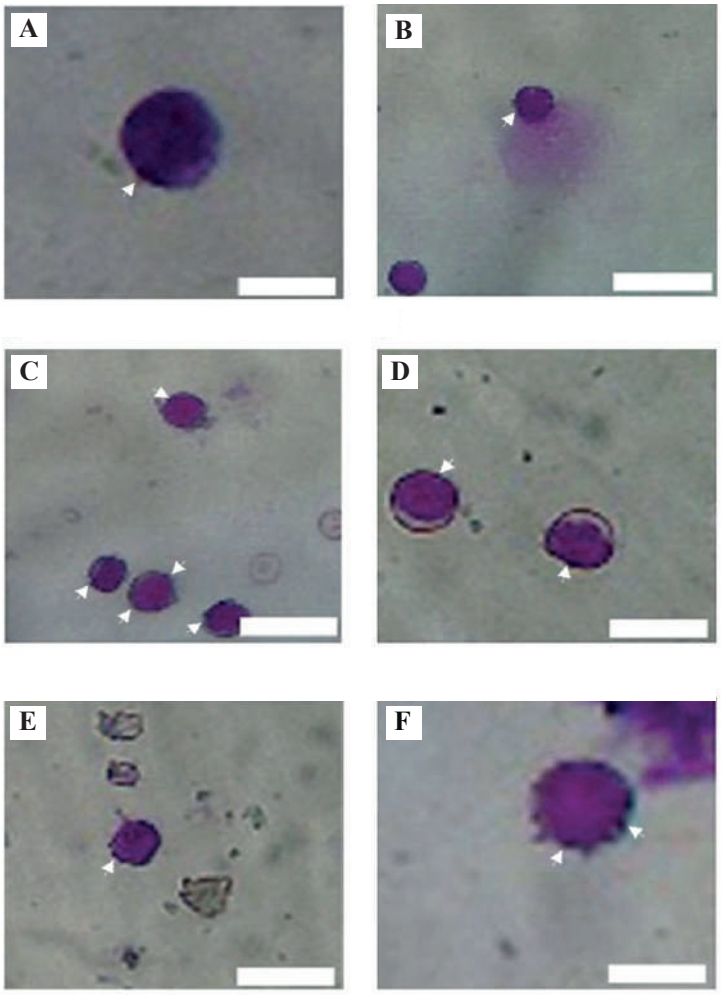

Figure 3. Morphology of Macrophage derived from peritoneal cavity of mice A: NC; B: T1; C: T2; D: T3; E: T4; F: T5. Cell in passage were documented under an inverted light microscope using Giemsa Staining. White bar: 10 µm. White arrow: latex particles attached to the surface of the macrophage.

(17-19) Dysregulation cytokine and chemokine affect the function of macrophages as an effector on the natural immune system.(17,20) However, in this study the mechanism that causes phagocytic activity of macrophages increased and parasitemia decreased due to the administration of *S. pinnata* leaves ethanol extract had not established.

P. berghei is species that caused lethal malaria in mice. Phagocytosis by macrophages was varied depend on species and serotype of Plasmodium. Differences in plasmodium species giving rise to different capacities to induce the production of O_2 metabolites by macrophages, thus affecting the final course of the disease.(21) Leaves of *S. pinnata* contain flavonoids, tannins, gums, alkaloids, saponins and terpenoids. It has mentioned in previous research that phenolic and flavonoid content is equivalent to gallic acid and quercetin.(22)

Various studies have shown that in the physiopathogenesis of malaria, free radicals are formed through oxidative stress.(21,23,24) The discovery of high concentrations of plasma malondialdehyde which is a byproduct of lipid peroxidase is evidence that the level of oxidative stress in malaria increases. Plasmodium infection will trigger gamma interferon-producing Th1 cells, which causes activated macrophages to produce nitric oxide (NO) and reactive oxygen species (ROS) which can kill parasites.(25) However, ROS and oxidative stress cause pathologies of malaria infection, also contribute to complication of malaria.(21,23-25) Antioxidants have been shown to reduce the negative effects of oxidative stress, including ROS, which occurs in malaria infection. (24,26,27)

The increase in cellular immune response found in this study hypothetically could be due to the content of terpenoids and flavonoids found in the ethanol extract of *S. pinnata* leaves. Previous research mentioned *S. pinnata* leaves contain high phenol and flavonoid components. Other parts of the plant such as fruit are also a large source of energy. *S. pinnata* has an antioxidant effect.(22) In this study, there was a slight decrease in cellular immune response in mice that received ethanol extract *S. pinnata* at doses of 100, 200 and 400 mg/kg BW/day compared to those who received 25 and 50 mg/kg BW/day. However, the

cellular immune response was still higher than the group that only received distilled water (NC).

There has never been a study that tested *S. pinnata* in plasmodium infections. However, there have been studies using bark extract of *S. pinnata* in helminths namely *Pheretima postuma*, an Indian earth worm.(28) The results of this study are in line with other studies that found antituberculosis extract activity at a concentration of 50 mg/ mL. That research showed that methanol extract of *S. pinnata* leaves contained triterpenoids and flavonoids.(29)

Flavonoids has immunostimulatory effect, may be due to cell mediated and humoral antibody mediated immune response.(30) There was an review regarding biological role of flavonoids and terpenoids in the treatment of parasite namely leishmaniasis. Flavonoids and terpenoids are major secondary metabolite used in the treatment of leishmaniasis by producing therapeutic efficacy against visceral leishmaniasis. Their potential is equivalent to the leishmaniasis drugs on the market.(30) Our study were not excluding the impact of the used solvent in extraction process. Some factors that might influence the increase of phagocytic activity of peritoneal macrophages thus reducing the density of *P. bergei* parasites due to administration of *S. pinnata* leaves ethanol extract also has not been determined so far.

Conclusion

Ethanol extract of *S. pinnata* leaves lower the parasite number of *P. berghei* in Balb/c mice and increase the capacity of macrophage to phagocytose latex. The ethanol extract of *S. pinnata* leaves with the highest efficacy in reducing parasitemia and phagocytic ability was found in the T2 group which received 50 mg/kg/day for 14 days. Based on the analysis of the Pearson correlation test it was found that there was a significant correlation between percent phagocytosis and parasitemia.

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Authors Contribution

SL involved in concepting and planning the research, KA, AD and ED performed the data acquisition/collection and result interpretation, KT and MS calculated the experimental data and performed the analysis, DW drafted the manuscript and designed the figures, LA and KS aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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