ORIGINAL ARTICLE

Antimalarial Activity of Mahagony Seed Ethanolic Extract in BALB/c Mice Infected with *Plasmodium berghei* ANKA and The Correlation of Parasitemia and Plasma Level of IFN-γ

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ABSTRACT

Introduction: The genus of *Plasmodium* is a parasite that can cause malaria. Interferon gamma (IFN-γ) is a inflammatory sitokin, that can control *Plasmodium* in the blood. World Health Organization (WHO) recommends Artemisinin-based Combination Therapies (ACTs) to treat malaria. However, some ethnic communities in Indonesia still consume herbal, one of which is mahogany seeds. Its effect on the immune system has not been much explained. **Materials and Methods:** Twenty-five male BALB/c mice were infected with *Plasmodium berghei* ANKA and divided into 5 groups. Groups I-III were treated with mahagony seeds ethanolic extract (MSEE) at the concentrations of 100%, 50% and 25% respectively. Group IV was a negative control (NC) which was treated with 0.5% sodium carboxymethyl cellulose (CMCNa). Group V was a positive control (PC) which was treated with dihydroartemisinin-piperaquine (DHP) at 187.2 mg/kgBW. Treatments were given for four consecutive days. Parasitemia was observed every day from the first day to the fourth day post infection. Plasma level of IFN-γ were measured by Sandwich ELISA. **Results:** Two way ANOVA analysis showed a significant difference in parasitemia based on the treatment day (p=0.000) and the treatment group (p=0.000) in infected mice. Treatment of MSEE showed a significant effect on plasma level of IFN-γ (p=0.032) in infected mice. Spearmen's rho bivariate correlation test showed a significant positive correlation between parasitemia on the fourth day post treatment and plasma level of IFN-γ (p=0.006; r=0.589). **Conclusion:** Antimalarial activity of MSEE was shown by the decreased of parasitemia along with the decreased of IFN-γ level on the day 4 post infection in a concentration dependent manner.

Keywords: Malaria, mahagony seed, *Plasmodium berghei* ANKA, parasitemia, IFN-γ

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INTRODUCTION

Malaria is a serious disease and remains the leading cause of death worldwide. Malaria is caused by a parasite of the genus *Plasmodium*, which is transmitted to humans through the bite of an infected female *Anopheles* mosquito (1). It was estimated that there were 241 million cases of malaria worldwide in 2020 in 85 malaria endemic countries, with 627.000 deaths (2). *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi* are five *Plasmodium* species possess the ability to infect humans (3).

Plasmodium infection induces the production of IFN-γ from various innate and adaptive cells at various stages of the life cycle. IFN-γ produced by CD4+ T cells optimally activates CD8+ T cells, B cells and macrophages. IFN-γ affects isotype transfer in B cells which leads to the production of cytophylic antibodies capable of binding free parasites and blocking red blood cell invasion, mediating parasite clearance through opsonization, and binding to the surface of infected red blood cells by promoting antibody dependent phagocytosis. IFN-γ production from CD4+ T cells also optimally activates

macrophages to phagocytose infected and parasite-free red blood cells (4).

The first-line treatment for malaria, currently recommended by WHO, is Artemisinin-based Combination Therapies (ACTs) (3). However, some ethnic communities in Indonesia still consume herbal plants that already exist and are obtained from generation to generation, one of which is mahogany seeds (*Swietenia mahagoni* (L.) Jacq.) (5). These plants have been reported to have antimalarial activity both in vitro and in vivo (6,7), but its effect on the immune system has not been much explained. This study aims to analyze the antimalarial activity of mahogany seeds ethanolic extract (MSEE) in BALB/c mice infected with *Plasmodium berghei* ANKA and the correlation between parasitemia and plasma level of IFN-γ.

MATERIALS AND METHODS

Ethical clearance and research design

The proposal of this research has been reviewed by Ethical Committee of Universitas Airlangga Faculty of Dental Medicine as described on certificate number 779/HRECC.FODM/XII/2019. This research is a true experimental research with a post test only control group design. The treatment was given using the standard test method for curative activity (8).

Experimental animals

The test animals in this study was BALB/c strain male mice aged 2-3 months, with a weight of ±20-30g, wich were maintained and developed in the Animal Laboratory of the Department of Biochemistry, Airlangga University, Surabaya. The test animals were acclimatized for 7 days at a temperature of 25 °C with an exchange of dark and light every 12 hours and were given standard feed ad libitum. The mice were sacrificed after anasthesized by intraperitoneal injection of 87 mg/kg bodyweight ketamine. The dead animals were then burned in the incenerator.

Plant material and MSEE preparation

The mahogany seeds used in this study were seeds from the mahogany plant species *Swietenia mahagoni* (L.) Jacq. They were obtained, determined and tested for phytochemical content at the Integrated Service Unit [Unit Pelayanan Terpadu, UPT] of the Herbal Materia Medika Laboratory, Batu City, East Java Province. *EEMS* was obtained from the extraction of mahogany seed powder by maceration technique using 96% ethanol. MSEE was made into three concentrations, 100%, 50% and 25% respectively. The 100% MSEE concentration was originally from maceration without is the result of maceration without dilution (crude extract). The extract was diluted using dilution using 0.5% CMCNa solution. MSEE concentration of 50% (v/v) was prepared in a ratio of 1:1 (3 mL crude extract : 3 mL CMCNa 0.5%) and MSEE concentration of 25% (v/v) was prepared in a ratio of 1:3 (1.5 mL crude extract : 4.5mL CMCNa 0.5%). The results of the MSEE phytochemical test showed the presence of the flavonoids, tannins and triterpenoids.

Infection of mice

Frozen isolates of *P. berghei* ANKA were thawed at 37 °C, then inoculated in 0.2 mL blood from donor mice intraperitoneally. Parasiemia was observed daily on Giemsa-stained thin smear. When parasitemia reached 10-20%, mice were sacrificed and blood were collected by cardiac punctured to infect the test mice. Twenty-five male BALB/c mice were infected with 0.2 mL of blood containing $1x10^6$ of infected red blood cells from donor mice. Test mice were then divided into 5 groups. Group I was treated with 100% MSEE, Group II was with 50% MSEE, Group III was with 25% MSEE, Group IV was negative control (NC) given 0.5% CMCNa and Group V was positive control (PC) treated with antimalarial drug of dihydroartemisinin-piperaquin (DHP) at 187.2 mg/kgBW. The treatments were given when parasitemia reached ±1%. The treatment was given for four consecutive days. Parasitemia was observed as described above. On the fourth day post treatment, the test mice were sacrificed to collect blood by cardiac punctured prior to the measurement of IFN-γ level (10).

Measurement of IFN-γ level

Plasma level of IFN-γ were measured on using the ELISA (Enzyme-Linked Immunosorbent Assay) Kit (The BioLegend, Singapore).

Statistical analyses

The differences in mean of parasitemia were analyzed using two way analysis of variance (ANOVA), followed by Post Hoc (Tukey) analysis. The difference in mean of IFN-γ levels were analyzed using Kruskal Wallis, followed by Mann-Whitney U analysis. The correlation between parasitemia and IFN-γ level were analyzed using the Spearman correlation analysis. Values were statistically significant at $p < 0.05$.

RESULTS

Antimalarial activity of MSEE

The mean parasitemia of each treatment group is shown in Figure. The mean parasitemia in NC group has increased normally since the first day to the fourth day post treatment. Parasitemia in the PC group increased on the first day then continued to decrease until the fourth day post treatment. Parasitemia in the all group treated with MSEE on day four post treatment were lower than NC group, but higher than the PC group. This result indicated the antimalarial activity of MSEE.

Two Way ANOVA analysis to find out the the differences of parasitemia between day post treatment and between treatment groups resulted in significant differences, then was continued with the Post Hoc (Tukey) test. A summary of the results of the Post Hoc (Tukey) test is

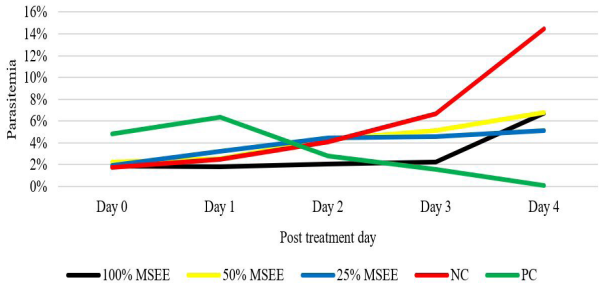

Fig. 1: The mean of parasitemia from day 0 to day 4 post treatment in BALB/c mice infected with *P. berghei* ANKA which were given MSEE and the control group.

presented in Table I and II.

Based on the category of the treatment day, Post Hoc (Tukey) test showed a significance difference mean of parasitemia at day 1 and 4, day 2 and 4, day 3 and 4 post treatment and between treatment groups 100% MSEE, 50% MSEE and 25% MSEE with NC. Significant differences also occurred between the 50% MSEE treatment group with PC and between the NC and PC group, but not between the 100%, 50% and 25% MSEE concentrations.

Table I: Result of Post Hoc (Tukey) analysis of parasitemia based on treatment day category in BALB/c mice infected with *P. berghei* ANKA which were given MSEE and the control group

Treatment day	p-value	Information
Day 1 and day 2 post treatment	0.975	Not significant
Day 1 and day 3 post treatment	0.579	Not significant
Day 1 and day 4 post treatment	0.000*	Significant
Day 2 and day 3 post treatment	0.825	Not significant
Day 2 and day 4 post treatment	0.000*	Significant
Day 3 and day 4 post treatment	0.000*	Significant

* p-value less than 0.05 was considered significant.

Table II: Result of Post Hoc (Tukey) analysis of parasitemia based on treatment group category in BALB/c mice infected with *P. berghei* ANKA which were given MSEE and the control group

Groups	p-value	Information
NC and PC	0.000*	Significant
NC and 100% MSEE	0.000*	Significant
NC and 50% MSEE	0.010*	Significant
NC and 25% MSEE	0.002*	Significant
PC and 100% MSEE	0.938	Not significant
PC and 50% MSEE	0.030*	Significant
PC and 25% MSEE	0.103	Not significant
100% MSEE and 50% MSEE	0.178	Not significant
100% MSEE and 25% MSEE	0.420	Not significant
50% MSEE and 25% MSEE	0.986	Not significant

* p-value less than 0.05 was considered significant.

Antimalarial activity of MSEE was also shown by the percentage of parasites' growth and its inhibition, where both percentages rising in a concentration-dependent manner (Table III). They were lower than those of PC group. The inhibition of parasites' growth by DHP in PC group was very high (137%) as parasitemia reached 0% at the end of experiment (Figure 1). Probit analysis based on the percentage of inhibition of MSEE resulted in an ED50 was 69.67%.

Table III: Percent growth and inhibition of parasites in BALB/c mice infected with *P. berghei* ANKA which were given MSEE and the control group

Groups	% of Growth	% of Inhibition
100% MSEE	1.21	62
50% MSEE	1.13	65
25% MSEE	0.18	75
NC	3.19	0
PC	-1.18	137

Plasma level of IFN-γ

The *P. berghei* ANKA-infected group which treated with 100% MSEE showed the highest mean of plasma level of IFN-γ than other groups, while the lowest plasma level of IFN-γ was shown by PC group. The plasma level of IFN-γ in MSEE-treated groups increased along with the increased of MSEE concentration. The Kruskal Wallis analysis showed the differences in the mean of plasma levels of IFN-γ among groups of mice treated with 100%, 50%, 25% MSEE, NC and PC treatment groups (p= 0.032). Mann-Whitney U analysis resulted in a significant difference in plasma level of IFN- γ in all groups when compared with those in PC group (p = 0.021) (Table IV). However, the comparison among MSEE-treated groups and between MSEE-treated groups with NC were insignificantly different (p> 0.05). A summary of the mean of plasma levels and results of the Mann-Whitney U analysis is presented in Table IV. Bivariate correlation analysis was performed to determine the relationship between parasitemia on the fourth day post treatment with IFN-γ levels. The results of the Spearman's rho correlation test showed a significant positive relationship between parasitemia on the fourth day post treatment and IFN-γ levels (correlation

Table IV: A summary of the mean of plasma levels of IFN-γ and their significant differences between MSEE-treated groups and PC group analyzed by Mann Whitney U test

Groups	Mean SD	p*
100% MSEE	473.53±550.82	0.021
50% MSEE	195.41±42.27	0.021
25% MSEE	158.24±89.08	0.021
NC	293.88±154.51	0.021
PC	26.76±15.27	

*Significantly different when compared with PC.

coefficient (r) = 0.589 and p= 0.006).

DISCUSSION

This current study proved the antimalarial activity of MSEE as shown by the decreased of parasitemia and IFN-γ level at the end of the experiment when compared with those in mice without any MSEE treatment (NC group), although the percentages inhibition of the parasites' growth were lower compared with that of PC group.

The antimalarial activity of MSEE in this study was probably caused by the bioactive compounds contained in MSEE. Based on the phytochemical test, the MSEE used in this study was positive for flavonoids, tannins and triterpenoids. Flavonoids inhibit the biosynthesis of fatty acids in the biochemistry of parasites and inhibit the entry of myoinositol into infected erythrocytes during the erythrocytic phase. These compound is required by *Plasmodium* for the development of the parasite stage during the erythrocytic phase (11,12). Flavonoids and their derivatives play a role in blocking the formation of haemozoin by forming free heme complexes which can be toxic to parasites so that parasites die (13).

Tannins can inhibit protease activity (14). Proteases are a group of enzymes that play an important role in the growth and invasion of *Plasmodium* parasites (15). In the erythrocytic stage, *Plasmodium* uses host hemoglobin as a food source for the development of the parasite stage (16). Aspartate and cysteine protease are used by *Plasmodium* to degrade hemoglobin into amino acids as a source of nutrition (17). The tannin content in MSEE may play a role in the inhibition of the *Plasmodium* protease enzyme, so that its growth and development are inhibited and prevent invasion of new erythrocytes. Oleanolic acid which is one of the triterpenoid compounds has anti*plasmodium* activity caused by its incorporation into the erythrocyte membrane and causes erythrocyte changes to form stomatocytes, thus interfering with the growth of *P. falciparum* strain 3D7 in vitro (18). The invasion and growth of *Plasmodium* in erythrocytes depends on the integrity and normal function of the erythrocyte membrane. Changes in the erythrocyte membrane will interfere with the growth of *Plasmodium* (19). The triterpenoid content in the MSEE in this study probably caused changes in the erythrocyte membrane, so that their growth was disturbed and the merozoites were unable to invade new erythrocytes.

The level of IFN-γ in the NC group (293.88 ± 154.51) was higher than that in PC group (26.76 ± 15.27). The high parasitemia in NC group was responded by the host by secreting IFN-γ. The higher parasitemia, the higher molecules which act as pathogen associated molecular pattern (PAMP) to activate more macrophage and dendritic cells to induce the IFN-γ secretion by immune system tries to eliminate parasites by secreting IFN-γ cytokines. IFN-γ is a primary cytokine used to activate macrophages and promote the killing of intracellular pathogens (20). IFN-γ is a Th1-derived pro-inflammatory cytokine that is important for the elimination of intracellular parasites (21).

In this study, the PC group had the lowest IFN-γ levels compared to other groups. This is probably due to parasitemia in PC group is low, so it does not stimulate the secretion of IFN-γ cytokines. The treatment of dihydroartemisinin therapy to BALB/c mice infected with *P. berghei* ANKA showed a decrease in IFN-γ levels compared to untreated infected mice (22). In this study, the same thing happened, the levels of IFN-γ in PC group that given with DHP were lower than those in NC. This results indicated that DHP is a potent antimalarial drug which was able to suppress the production of proinflamation cytokine such as IFN-γ.

The mean of IFN-γ level was increased along with the increased of MSEE concentration. However, this was not statistically significant. IFN-γ levels in the 100% MSEE group had a standard deviation greater than the mean in that group. This happened because one sample of the study had a much higher IFN-γ levels than the other sample. Although the mice as samples in this study have attempted to have small variations with uniformity of sex, age and body weight of mice, each mice may have different abilities in responding to a given infection. The immune system between individuals varies widely (23). Every individual has a specific immune system and is different from each other (24), that cause each individual responds differently to a given infection.

When compared to the PC group, the mean IFN-γ levels in the MSEE group (100%, 50%, and 25%) were greater and showed a statistically significant difference. This is most likely relating to parasitemia as shown by the Spearman's correlation analysis. A potent antimalarial drug used as PC showed a sharp decrease in parasitemia, and reached 0% on day 4 post treatment along with the decreased of IFN-γ level. Likewise, the decreased of IFN-γ levels in MSEE-treated group of mice were followed by the decreases of parasitemia in a concentration dependent manner. This proved the role of IFN-Z in parasite elimination. There was no statistically significant difference of the IFN-γ levels between NC and all MSEE treatment groups. This is probably because parasitemia in NC can stimulate cell activation to secrete IFN-γ in response to infection, whereas in the MSEE group besides parasitemia, the compounds contained can also activate cells to secrete IFN-γ, so that in all these groups there are conditions that can trigger cell activation in secreting IFN-γ.

The significant correlation between parasitemia on the fourth day post treatment with IFN-γ levels showed that the immune cells in BALB/c mice tried to eliminate *P. berghei* ANKA infection by secreting IFN-γ. In this study,

there was a tendency for IFN-γ levels to be higher in the group with higher parasitemia. The same was true of studies assessing cytokine and antibody profiles in *P. falciparum* infected individuals in Southwest Nigeria. The results showed that IFN-γ levels were higher in the group with symptomatic malaria, which had a higher percentage of parasitemia compared to asymptomatic malaria patients with lower parasitemia (25). An association between IFN-γ levels and parasitemia were reported in *P. falciparum* infection which showed an increase in IFN-γ levels at an increase in parasitemia levels (26). Another study showed that there was no relationship between IFN-γ levels and parasite density, but descriptively, IFN-γ levels were higher in the group with higher parasite density (27).

IFN-γ provides important protection during the erythrocytic stage of *Plasmodium* infection (28). Erythrocytic stage triggers a strong IFN-γ response during acute infection in *P. berghei*, *P. yoelii* and *P. chabaudi* infections in mice as well as in *P. falciparum* infections in humans (29). However, high IFN-γ secretion during erythrocytic infection is associated with a predisposition to severe malaria, such as cerebral malaria. Proinflammatory cytokines such as IFN-γ are illustrated as double swords. Under normal conditions, these cytokines are essential for controlling parasite growth and sustained protection against disease pathologies, but excessive and uncontrolled secretion can lead to multiple immunopathologies

CONCLUSION

This study proved the antimalarial activity of MSEE in BALB/c mice infected with P. bergei ANKA. Treatment of MSEE caused a significant difference in the decreased of parasitemia and mean of IFN-γ levels in a concentration dependent manner. The decreased of parasitemia correlated with the decreased of IFN-γ levels at the day four post treatment .

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