The Effect of Ethanol Extract of Pearl Grass (Hedyotis corymbosa (L.) Lamk) on TNF-α and IL-10 Levels in Mice Infected with Plasmodium berghei

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ABSTRACT

Pearl grass (Hedyotis corymbose, (L.) Lamk) has been reported to have antimalarial activity against Plasmodium falciparum and Plasmodium berghei because of its chemical contents as well as anti-inflammatory activity. This current study aimed to analyse the effect of ethanolic extract of pearl grass (EEHC) on the serum level of TNF-α and IL-10 in mice infected with P. berghei. Fifty male BALB/c mice were infected with 1x10⁶ P. berghei-infected erythrocytes from donor mice, and then were randomly divided into five groups. Each group contained 10 mice. Group 1 was a positive control group, and treated with 187.2 mg/Kg BW antimalaria drug of dihydroartemisinin piperaquine (DHP). Group 2 was a negative control group without any additional drugs. Group 3, 4, and 5 were treated with 250, 300, and 350 mg/Kg BW of EEHC, respectively. A curative test of EEHC was started on day three post-infection, followed by daily treatments of two hundred microliters of EEHC for four consecutive days. Parasitemia was observed on day five post-treatment on Giemsa-stained tail blood smears followed by cardiac puncture to obtain sera prior to TNF-α and IL-10 measurement by ELISA. The findings showed that higher doses of EEHC reduced parasitemia, which increases their therapeutic impact. Serum level of TNF-α was lower in mice treated with higher doses of EEHC, while IL-10 was vice versa. Serum level of TNF-α was correlated with percentage parasitemia, whereas IL-10 was not. These findings demonstrated EEHC’s anti-inflammatory effect against malaria in mice, suggesting that it may be developed as an antimalarial drug in the future.

Keywords: Ethanolic extract of Hedyotis corymbose; mouse malaria; anti-inflammatory; parasitemia.

INTRODUCTION

Since ancient times, medicinal plants have been identified and employed in conventional medical procedures. For a multitude of purposes, including defense and protection against insects, fungi, disease, and herbivorous mammals, plants manufacture hundreds of chemical compounds (1). Herbal medicine has a reputation for treating parasite infections and boosting immunity. One of the plants that has now been used as herbal medicine and is widely consumed by some people of Indonesia is pearl grass (Hedyotis corymbose (L.) Lamk) or Oldenlandia corymbose Linn. Chemical investigation of this plants reported that H. corymbose (L.) Lamk contained triterpenes, anthraquinones, coumarins, phenolics and their derivatives, flavonoids, carboxylic acids, iridoids glycosides (2)(3), oleanolic acid and ursolic acid (4)(5)(6)(7).

Biological studies on H. corymbose (L.) Lamk have been reported to have antioxidant and anticarcinogenic properties (8)(9)(10)(11), anti-inflammatory and hepatoprotective (12)(13), antibacterial (14), and antimalaria activities against Plasmodium falciparum (15) and Plasmodium berghei (15)(16).

Malaria is an infectious disease caused by protozoa from the genera of Plasmodium and transmitted by female Anopheles mosquito (17). Recently, malaria cases in Indonesia have decreased along with the increase in the number of districts and cities that have been certified as malaria-free areas, however, some areas remain endemic for malaria, such as Papua, West Papua, Maluku, and East Nusa Tenggara Provinces (18).
Many plant extracts have been tested for antimalaria activities against *P. berghei* infection in mice before being developed into antimalaria drugs (19)(20)(21).

Antimalaria activities of *H. corymbose* (L) Lamk has been reported in previous studies (15)(16), however, the effect of the extract on the inflammatory response in mice infected *P. berghei* has not been documented. This study aimed to find out the effect of ethanolic extract of *H. corymbosa* (L) Lamk (EEHC) on the serum level of pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and the anti-inflammatory cytokine interleukin-10 (IL-10) in mice infected with rodent malaria parasite, *P. berghei*.

**MATERIALS AND METHODS**

**Ethical approval**
The ethical clearance of this research was approved by the Research Ethical Committee of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia as stated on certificate number 161/HRECC.FODM/IV/2021.

**Preparation of EEHC**
The EEHC was prepared by maceration of simplicial in 70% ethanol solvent in the Laboratory of Herbal Materia Medika, Batu City, East Java Province, Indonesia. The EEHC was then used to prepare doses of 250 mg/kgBW, 300 mg/kgBW, and 350 mg/kgBW in 1% CMCNa (16).

**Experimental protocol**
The protocol for the in vivo experiment was a four-day test (22)(16). After a week of acclimatization, fifty male BALB/c mice were infected with 1x10⁶ *P. berghei* ANKA-infected erythrocytes from donor mice and then were randomly divided into five groups. Each group contained 10 mice. Group 1 was a positive control group, and treated with 187.2 mg/Kg BW antimalaria drug of dihydroartemisinin piperaquine (DHP). Group 2 was a negative control group without any additional drug. Group 3, 4 and 5 were treated with 250, 300, and 350 mg/Kg BW of EEHC, respectively. A curative test was started on day three post infection, followed by daily treatments of two hundred microliters of EEHC for four consecutive days.

**Parasitaemia determination**
Parasitaemia was observed on day five post-treatment on Giemsa-stained tail blood smears (23). Briefly, a tiny incision was made using surgical scissors on the tip of the tail, and a single drop of blood was applied to an object of glass to make a thin smear. Once the smear had dried, it was fixed with methanol and stained for ten minutes with a 10% Giemsa solution (24). Using oil immersion and a 1,000 magnification of light microscopy, parasitaemia was observed. The following formula was used to determine the number of infected erythrocytes per 1,000 erythrocytes.

\[
\text{Parasitemia} = \frac{\text{Number of infected erythrocytes}}{\text{Total number of infected erythrocytes counts}} \times 100
\]

**Serum collection**
Sera were collected on day five post-treatment. After ketamine anaesthetised mice were sacrificed and blood was collected by cardiac puncture. Blood from each mouse was put into a gel clot tube to separate serum from blood. Sera were used for cytokines measurements by enzyme-linked immunosorbent assay (ELISA).

**Measurement of TNF-α and IL-10 by ELISA**
The procedure of ELISA was done by following the attached protocol on the manufacturers of ELISA kits for mouse TNF-α and IL-10 (Elabscience, Maryland, USA). Absorbances were read using an ELISA reader at a wavelength of 450 nm (Humareader, Faridabad, India). The concentrations of cytokines were obtained automatically from the computer connected to the ELISA reader.

**Data analysis**
The data was normally distributed. One-way analysis of variance (ANOVA) was used to evaluate the data followed by Post Hoc Bonferroni analysis. The Pearson test was used to assess the correlation between parasitemia and serum levels of TNF-α and IL-10. The outcome was deemed statistically significant when p<0.05 and the 95% confidence level were met. Analyses were performed using SPSS 25 for Windows (IBM Corp., New York, USA).

**RESULTS**
**Parasitaemia and curative effect**
Parasitaemia on day four post-treatment is presented in Table 1 together with its curative effect. In this current study, parasitemia percentage is needed to correlate with the serum level of TNF-α and IL-10. The effect of EEHC against parasitemia in mice infected with *P. berghei* showed a decrease in parasitemia in doses dose-dependent manner. In contrast, the higher the dose of EEHC the higher the curative effect (Table 1).

**TABLE 1: Parasitemia and curative effect of EEHC on day four post-EEHC treatment.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parasitaemia (%)</th>
<th>Curative Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>39.93</td>
<td></td>
</tr>
<tr>
<td>POS</td>
<td>0</td>
<td>100.00</td>
</tr>
<tr>
<td>EEHC250</td>
<td>31.11</td>
<td>20.90</td>
</tr>
<tr>
<td>EEHC300</td>
<td>18.95</td>
<td>51.32</td>
</tr>
<tr>
<td>EEHC350</td>
<td>8.20</td>
<td>77.86</td>
</tr>
</tbody>
</table>

**Effect of EEHC on the level of TNF-α and IL-10**
The data of serum level of TNF-α and IL-10 are presented in Figure 1. The serum level of TNF-α was decreased along with the increased of doses of EEHC. The highest concentration of TNF-α was found in NEG group, while the lowest was in the POS group. Analysis statistic using ANOVA followed by Post Hoc Bonferroni test showed that the serum level of TNF-α in NEG group was significantly different with those of EEHC300 (p=0.002), and EEHC350 (p=0.000), but was different insignificantly with EEHC250. The POS group was different significantly from all groups (p=0.000).
 While, EEHC250 was different insignificantly with EEHC300 (p= 1.000), as well as in EEHC300 with EEHC350 (p= 0.321). However, EEHC250 was different significantly from EEHC350 (p= 0.012).

On the other hand, the serum level of IL-10 was increased along with the increased doses of EEHC as shown in Figure 1. The serum level of IL-10 in the NEG group was different significantly from those in the POS (p=0.000) and EEHC250 group (p=0.007) as well as in EEHC300 and EEHC350 (p=0.000) groups. In the POS group, the IL-10 level was completely different significantly from those in EEHC250, EEHC300, and EEHC350 (p= 0.000). However, EEHC250 was different insignificantly from EEHC300 (p= 0.158), but different significantly from EEHC350 (p= 0.032).

**Correlation between parasitemia and TNF-α, IL-10 levels, and the doses of EEHC**

Decreasing of proinflammatory response was followed by the increase of anti-inflammatory response. Only parasitemia on day four was used to correlate with TNF-α and IL-10 levels and doses of EEHC. The correlation between parasitemia and the levels of TNF-α, IL-10, and doses of EEHC are presented in Figure 2. Serum level of TNF-α was correlated with percentage parasitemia (p= 0.000) whereas IL-10 was not. The level of TNF-α showed a positive strong correlation with parasitemia (p= 0.000; Pearson correlation= 0.887). The higher the parasitemia the higher the TNF-α level. The correlation of TNF-α level to the doses of EEHC was correlated insignificantly (p=0.231) but showed a negative strong correlation (-0.248). The higher the dose of EEHC the lower TNF-α levels. On the other hand, the serum level of IL-10 showed an insignificant correlation with parasitemia (p= 0.417; Pearson correlation= -0.170) nor with the dose of EEHC (p= 0.490; Pearson correlation= 0.145). When parasitemia decreased, the level of IL-10 increased. Parasitaemia was correlated significantly with the doses of given EEHC (p= 0.009). The higher the dose of EEHC caused decreasing parasitemia as shown in Figure 1.

**DISCUSSION**

Malaria parasitemia indicates the severity of infection as well as the antimalarial activity of herb extract administration. The increased parasitemia in the negative control group (NEG) indicated that the infection developed tends to be severe. In contrast, the decrease of parasitemia to 0% in mice treated with DHP showed the recovery of mice from malaria infection and proved the effective antimalaria drug of DHP. The results are similar to the reported studies (15)(16). It has been proven that the parasitemia in mice treated with EEHC fell between those two groups, indicating the antimalarial activity of EEHC in reducing parasitemia and the degree of infection. However, the two studies used different methods, resulting in different outcomes. This current research followed the method as described in (16).

The percentage of parasitemia can be also used as a reference to determine host immunity to malaria infection. The host’s immune system releases proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8, IL-12 (p70), and IFN-γ, in reaction to the parasite during the blood infection stage, and affect the growth and removal of the parasite (25). In this study, proinflammatory immune response against malaria infection was measured based on the serum level of TNF-α. The highest proinflammatory immune response was seen in the NEG group, where the infection was developed normally without any antimalaria drug interference, where parasitemia was the highest percentage among other groups. Elevated TNF-α in the blood stages of infection suppresses parasite growth and boosts immunity by activating the cellular immune system. Meanwhile, a high level of TNF-α production during the late stage of infection can cause tissue damage and exacerbate malaria (26). In the POS group, as long as the parasitemia dropped after DHP treatment, the proinflammatory response also significantly decreased, followed by the increased anti-inflammatory response.

EEHC gave effect to the decrease of TNF-α response as the increased doses. EEHC contains flavonoids. Flavonoids have been shown to have antimalarial activity and can also increase the chemosuppression of malaria parasites when combined with antimalarial drugs (15)(27). Flavonoids have phenolic OH groups which are responsible for their acidic properties. Although the antimalarial mechanism of action of flavonoids is not completely understood, it is believed that flavonoids act by inhibiting the biosynthesis of fatty acids that play an important role in the synthesis of parasite cell membranes or by targeting certain functional biomolecules such as enzymes, proteins or DNA that are important for the survival of malaria parasites. Furthermore, the phenolic OH groups of polyphenolic flavonoids can be converted into stable phenoxy anions under cellular oxidative stress in vivo which in turn results in oxidative damage to cellular components or tissue damage in parasites (28).

**FIGURE 1**: Serum level of TNF-α and IL-10 in mice infected with *P. berghei* on day four post EEHC treatment.
Furthermore, a significant correlation between parasitemia and TNF-α levels indicates a possible mechanism of parasite elimination in this study was through the production of TNF-α.

Anti-inflammatory immune response indicates the host defends against infection. In this study, where this response was measured based on the serum level of IL-10, the highest response was showed by EEHC350 followed by EEHC300 and EEHC250. The levels of IL-10 among EEHC-treated groups were different significantly (p<0.05), but when EEHC300 and EEHC350 were compared with those in the POS group were different insignificantly (p>0.05). This result indicated that EEHC was able to increase the anti-inflammatory response against P. berghei ANKA infection. Pearl grass contains flavonoids which have the capacity to trigger the release of anti-inflammatory cytokines, thereby increasing the serum level of anti-inflammatory cytokines such as IL-10 and inhibiting pro-inflammatory cytokines like TNF-α (29)(30). Pearl grass has been reported to have anti-inflammatory activity against rat paw edema in combination with two other herbal extracts (31). The decreased TNF-α concentration due to the immunostimulatory of polysaccharides from H. corymbose (L.) Lamk in an in vitro culture of RAW264.7 cells decreased the concentration of TNF-α along with NO and IL-1β (32).

Limitations of the study
The small volume of mice’s sera only allows us to analyze the limited type of cytokines. This study did not involve other parameters related to malaria inflammation.

CONCLUSION
The extract of pearl grass currently has been widely used either in experiments or in daily practice as it is available as a food supplement. This research may be the first report on the anti-inflammatory effect of EEHC against mouse malaria, potentially allowing for its future development as an antimalarial drug.

ACKNOWLEDGEMENT
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REFERENCES


