Leukocyte Profile of Mice Infected with Plasmodium berghei and Treated with Aqueous Extract of Strychnos ligustrina and Its Combination with Dihydroartemisinin Piperaquine Phosphate (DHP)

Profil Leukosit pada Mencit yang Diinfeksi Plasmodium berghei dan Diobati dengan Ekstrak Air Strychnos ligustrina serta Kombinasi dengan Dihydroartemisinin Piperaquine Phosphate (DHP)

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Abstract

Malaria is still one of the most highly pathogenic diseases, which remains a significant problem for public health. Natural compounds are alternative ways as an antimalarial compound that are applied for the artemisinin combination. The aim of this study was to determine the effect of a combination of S. ligustrina extract and DHP on the differential leukocytes percentages. This study used 100 mice which were divided into

Kata kunci: Dihidroartemisinin Piperaquine Phospate; DHP; leukosit; Plasmodium berghei; Strychnos ligustrina

Abstract

Malaria masih merupakan salah satu penyakit yang sangat patogen, dan menjadi masalah signifikan bagi kesehatan masyarakat. Bahan alam merupakan salah satu alternatif antimalaria yang dapat diaplikasikan dengan kombinasi artemisinin. Tujuan dari penelitian ini adalah untuk mengetahui pengaruh penggunaan kombinasi ekstrak S. ligustrina dan DHP terhadap perbedaan persentase leukosit. Penelitian ini menggunakan 100 ekor mencit yang dibagi menjadi lima kelompok. Grup A dan B masing-masing digunakan untuk control negatif dan kelompok control positif. Kelompok C digunakan sebagai obat kontrol obat DHP 222 mg/kg BB. Kelompok D adalah ekstrak air S. ligustrina 300 mg/kg BB. Kelompok E menerima kombinasi antara DHP 111 mg/kg BB dan ekstrak air S. ligustrina 200 mg/kg BB. Hasil menunjukkan bahwa ekstrak DHP dan S. ligustrina memengaruhi pada nilai neutrofil, limfosit, dan penghambatan pertumbuhan serta persentase parasitemia. Hasil studi mengungkapkan bahwa kelompok perlakuan secara signifikan lebih tinggi daripada kelompok A (P<0,05). Persentase limfosit lebih rendah pada kelompok perlakuan, sedangkan persentase eosinofil, monosit, dan basofil tidak berbeda nyata (P>0,05). Studi ini menemukan bahwa kelompok D dan E memiliki efek yang sama pada profil leukosit sebagai kelompok C. Temuan kami menunjukkan bahwa ekstrak air S. ligustrina, serta pengobatan kombinasi dengan DHP, berpotensi mengarah pada penemuan obat antimalaria baru.
Introduction

Malaria is a parasitic disease caused by Plasmodium hemoprotozoa. Malaria can be spread by female mosquitoes of the sporozoite genus Anopheles. Plasmodium ovale, P. vivax, P. malariae, and P. falciparum are among the Plasmodium species that cause malaria in humans (Paisal and Indriyati, 2014). P. berghei is a hemoprotozoa that causes rodent malaria. According to (Raharjo., et al., 2014), the life cycle, morphology, and genetics of P. berghei are comparable to those of P. falciparum, a hemoprotozoa that causes malaria in humans. As a result, P. berghei was used for the malaria model in mice for malaria research.

According to the WHO’s surveillance data from 2018, 40% of the world’s population resides in regions with high rates of malaria. There are around 3 million incidences of severe malaria (malaria complications) and malaria-related fatalities worldwide, as reported from 219 million clinical cases of the disease. P. falciparum has a high death rate and produces most malaria cases (Gultom et al., ., 2019). Based on data from the Ministry of Health of the Republic Indonesia is found to be scattered throughout the islands, especially in eastern Indonesia, such as Papua, West Papua, Maluku, North Maluku and East Nusa Tenggara with a prevalence of 79% of malaria cases in 2011 (Ministry of Health, 2011).

Strychnos ligustrina Blume is one of the Indonesian plants with the potential to be developed as a therapeutic compound. Strychnos ligustrina includes alkaloid chemicals that aid in malaria activity. The stems and roots contain the highest alkaloids of any plant fraction. This plant can be found in West Nusa Tenggara and Bali (Setyawan et al., ., 2014). In malaria-endemic areas with Multi-Drug Resistant Malaria, the World Health Organization (WHO) recommended an artemisinin-based combination treatment (ACT) strategy in 2001. One example of an ACT combination is dihydroartemisinin and piperaquine phosphate (DHP). The development of a combination of S. ligustrina and DHP as a malaria therapy option proposes to reduce the use of DHP to prevent drug resistance. Furthermore, because DHP is produced in another country, it is costly to obtain.

Leukocyte differential is one method for determining the reaction of the body’s defense mechanism in the development of antimalarial drugs. Each form of leukocyte carries out a specific function and is essential in its own way. According to (Saputro et al., ., 2016), the increase and decrease in the number of leukocytes reveal the body’s defense response to diseases and inflammatory agents. The purpose of this study is to examine the differential profile of leukocytes in mice infected with P. berghei and administered a combination of S. ligustrina and DHP extracts. The benefits of this study are intended to provide information on the response of the body’s defense system after being infected with P. berghei and then administered a combination of S. ligustrina and DHP on the value of the body’s defense system.
Materials and Methods

Animal Ethics Commission Research Approval

This research has been approved by the Animal Ethics Commission of the Institute for Research and Community Service (LPPM) IPB under accession numbers 154-2019 IPB.

Experimental Animal Procedure

In this study, 100 DDY mice aged 1.5-2.5 months were employed. Mice were housed in cages that contained 3-5 mice. Ad libitum feeding and bottled water are provided. Mice were acclimatized for seven days before being infected with *P. berghei* and then administered anthelmintic and anti-protozoal for four consecutive days.

Crude extract preparation

A prototype project-scale extraction was conducted at Fits Mandiri Company in Bogor, Indonesia. Thirty-one kilograms of *S. ligustrina* wood shavings, with an 11.8% moisture content, were macerated in distilled water (5% Brix = five grams of solid in one hundred grams of solution).

Treatment of Experiment Animals

The mice were divided into five groups. Each group contained 20 mice. Groups A and B were the untreated and positive control groups that were infected without therapy, respectively. Group C received 222 mg/kg BW of DHP as a treatment. Group D received 300 mg/kg BW of aqueous extract of *S. ligustrina*, whereas Group E received a combination of 200 mg/kg BW *S. ligustrina* water extract and 111 mg/kg BW of DHP. Every two days, the mice’s body weight and temperature were measured.

Propagation of *P. berghei* in Donor Mice

Five mice were prepared for use only as donor animals prior to the start of the experiment. *P. berghei* isolate stored at -80 °C was thawed and 0.2 ml intraperitoneally administered to each animal. Every two days, parasitemia was evaluated, and when it reached 20%, the animals were euthanized with ketamine. Intracardial blood was taken from mice and placed in a 2 ml microtube containing heparin. In addition, the level of parasitemia was measured to prepare the stock of infection doses. Every two days, parasitemia was checked by making blood smears on a microscope slide. After being dried, fixed, and dyed with 10% Giemsa, the slides were observed using a 1,000× magnification. The thin blood smears were observed under a microscope until 1,000 RBCs in each count of the parasites.

*P. berghei* inoculation

Groups B, C, D, and E mice were infected intraperitoneally with $1 \times 10^6$ ml/head of infection. When the parasitemia reaches 5% or six days after infection, the medication is administered orally using gastric tube for four days. For 14 days following infection, blood smear preparations were made every two days.

Leukocyte Differential Quantification

A 1000x magnification microscope was used to observe leukocyte differentiation. Every 100 leukocytes identified were counted and classified as neutrophils, eosinophils, basophils, lymphocytes, or monocytes. To minimize repeated calculations, observations were made in a zig-zag pattern until the leukocyte count reached 100. The percentage of leukocytes discovered was calculated (Kartono, 2015).

Data Analysis

The observed data were processed using the ANOVA (Analysis of Variance) test and then continued with the Duncan multiple range test (DMRT) if it showed a significant difference ($P < 0.05$).

Results and Discussion

Percentage of parasitemia

According to the parasitemia calculations, group B had an increase in parasitemia from day 2 (5.98%) to day 14 (21.49%) after infection (figure 1). All treatment groups saw an increase in parasitemia, however, the peaks of parasitemia in groups C, D, and E were highest on day 6 post-infection (p.i): 15.50%, 20.26%, and 9.97%, respectively. Statistical analysis revealed that the percentage of parasitemia in groups C, and D, was higher than in Group B, while group E was significantly lower than group B from day 8 to
day 14 p.i (P < 0.05). The increase in parasitemia is caused by the asexual phase that occurs in the host’s body, resulting in a large number of merozoites that constantly infect erythrocytes (Widoyono, 2011). Significant differences in parasitemia between groups C, D, and E after day 6 p.i revealed that mouse therapy might reduce the proportion of parasitemia. According to Siswantoro et al. (2011), DHP acts swiftly to eradicate parasites in the body while causing only minor adverse effects. According to Syafii et al. (2016) the alkaloid chemicals found in S. ligustrina extract can prevent parasite growth. S. ligustrina extract and DHP in general are useful for the treatment of malaria by reducing the number of parasites that multiply daily (Lestari, 2020). When DHP enters the body, it is activated by heme from hemoglobin, resulting in the production of numbers of free radicals. These free radicals will kill the parasite by causing damage to the parasite’s feeding vacuole’s lipids and membranes of parasites (Julianto, 2016).

Percentage of Neutrophil

Table 1 reveals that groups B, C, D, and E exhibited an increase in the percentage of neutrophils exceeding the normal limits on day 2 p.i with values of 62.13, 56.63, 46.13, and 52.63%, respectively. The percentage of neutrophils in group B remained above normal from day 2 to day 14 p.i with a final value of 46.5% on day 14 p.i. The maximum percentage of neutrophils in groups C, D, and E occurred on day 6 p.i, or at the height of parasitemia, with values of 59.13; 59.00; and 60.00%, respectively, and gradually decreased to near normal neutrophil values in the following days. Data analysis revealed that group B was significantly different from other treatment groups on each day of observation (P < 0.05). Furthermore, on day 12 p.i there was no significant difference between groups C, D, and E compared to group A, indicating that the percentage of neutrophils in groups C, D, and E mice had reverted to normal (P < 0.05). Neutrophils in mice have a typical range of 20-30% (Provencher et al., 2010). The presence of a neutrophil defensive response against infecting parasites is indicated by a high percentage of neutrophils in mice that exceeds the normal range at the beginning of the observation. According to Rizki et al. (2017), neutrophils contribute as the first line of defense against pathogens that enter the body by killing pathogens through the process of phagocytosis. The reduction in the percentage of neutrophils from the sixth to the fourteenth day after infection was induced by a decrease in the percentage of parasitemia, which was influenced by DHP and S. ligustrina extract in limiting parasite development. According to Komalasari (2019), the average decrease in the percentage of neutrophils corresponded to the level of parasitemia.

Percentage of Lymphocytes

According to Table 2, the average percentage of lymphocytes on day 2 p.i in groups B, C, D, and E were 37.50, 43.00, 53.89, and 36.00%, respectively. The maximum percentage of lymphocytes in group B occurred on day 8 p.i with a final value of 52.63% on day 14 p.i. The maximum percentage of lymphocytes in groups C, D, and E occurred on day 6 p.i, or at the height of parasitemia, with values of 59.13; 59.00; and 60.00%, respectively, and gradually decreased to near normal lymphocyte values in the following days. Data analysis revealed that group B was significantly different from other treatment groups on each day of observation (P < 0.05). Furthermore, on day 12 p.i there was no significant difference between groups C, D, and E compared to group A, indicating that the percentage of lymphocytes in groups C, D, and E mice had reverted to normal (P < 0.05). Lymphocytes in mice have a typical range of 10-40% (Lestari, 2020). The presence of a lymphocyte defensive response against infecting parasites is indicated by a high percentage of lymphocytes in mice that exceeds the normal range at the beginning of the observation. According to Komalasari (2019), the average decrease in the percentage of lymphocytes corresponded to the level of parasitemia.
and 47.13%, respectively, indicating that the percentage of lymphocytes was below normal limits. During the observation period, the percentage of lymphocytes in Group B increased from 37.50% to 49.25% but remained below the normal range. From day 6 with lymphocyte percentage values of 40.50, 40.25, and 40.63% to day 14 p.i with values of 67.75, 66.75, and 66.5%, the percentage of lymphocytes in groups C, D, and E grew towards normal. According to statistical analysis, group B performed statistically significantly more than groups A, C, D, and E from day 8 to day 14 p.i (P < 0.05). On day 10 p.i, Group D differed significantly more than Group C and E (P < 0.05). On the 12th and 14th p.i days, groups C, D, and E were not statistically different from group A (P < 0.05).

The typical range of lymphocyte levels in mice is between 55% and 95%. The low average lymphocyte percentage in groups B, C, D, and E at the start of the observation day was attributable to an increase in neutrophil percentage on the same day as the first line of defense. Because lymphocytes multiply to produce antibodies, the percentage of lymphocytes increases to near normal. Sea S. ligustrina wood powder contains flavonoid chemicals, alkaloids, triterpenoids, steroids, tannins, and hydroquinones, according to (Syafii et al., 2016). Flavonoid chemicals can stimulate cytotoxic T lymphocytes to eliminate parasites and promote lymphocyte proliferation via increasing interleukin-2 activity (Nugroho, 2012; arlinaningrum et al., 2014).

Percentage of Eosinophils

Based on Table 3, the average percentage of eosinophils reveals the value of the percentage of eosinophils that changes but remains within the normal range in all categories. Furthermore, no significant difference was seen in any of the groups (P < 0.05). The usual percentage of eosinophils in mice is 0.4%. A comparable investigation with the administration of the ethyl acetate fraction of afo cloves to the leukocyte response of P. berghei-infected mice found no significant effect or difference in the percentage of eosinophils (Gusdinar, 2019). According to (Kovalszki et al., 2016), hypersensitivity, allergic responses, and parasitic worm infections will increase the percentage of eosinophils.

Percentage of Monocytes

According to Table 4, group B had the highest percentage of monocytes on the 14th day, 3.75%. Monocyte values in groups C, D, and E increased from day 2 to day 10 p.i with values of 0.38, 0.00, and 0.25%, respectively, to 3.25, 0.50, and 1.88%, then decreased until day 14 p.i with values of 0.75, 1.00, and 1.00%, respectively. The data analysis results revealed

Table 2. Average lymphocyte percentage in P. berghei-infected mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>60.3 ± 7.0</td>
</tr>
<tr>
<td>B</td>
<td>37.5 ± 9.5</td>
</tr>
<tr>
<td>C</td>
<td>43.0 ± 11.6</td>
</tr>
<tr>
<td>D</td>
<td>53.8 ± 14.4</td>
</tr>
<tr>
<td>E</td>
<td>47.1 ± 12.5</td>
</tr>
</tbody>
</table>

Note A: negative control group; B: positive control group; C: drug control group; D: extract treatment group; E: combo treatment group. Different superscript letters in the same column indicate significant differences at the level of P <0.05.

Table 3. Average eosinophile percentage in P. berghei-infected mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>B</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>C</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>D</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>E</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Note A: negative control group; B: positive control group; C: drug control group; D: extract treatment group; E: combo treatment group. Different superscript letters in the same column indicate significant differences at the level of P <0.05.
that on day 8 p.i, group C was significantly different from group B, and on day 10 p.i, group C was significantly different from group D (P < 0.05). From day 12 to day 14, there was no significant difference in any of the groups (P < 0.05).

The normal range for mouse monocyte proportion is 0-1.5%. Monocytes can penetrate tissues and develop into macrophages. Monocytes play a crucial role as a second line of defense following neutrophils. Monocytes will consume and eliminate many infections while also acting as antigen presentation cells (APC) (Maryani and Rosdiana, 2020). The high percentage of monocytes in group B 14 days after infection was due to a high percentage of parasitaemia in the blood (Taher, 2019). The difference in the percentage of monocytes in group C vs group D on the 10th day after infection was caused by the effect of treatment as well as the difference in parasitaemia levels between the two groups on that day. The flavonoid chemicals in *S. ligustrina* extract can boost the host reaction in triggering the creation of monocytes, resulting in an increase in the proportion of monocytes (Fatimatuzzahroh et al., 2015). DHP’s active metabolite content helps to eradicate parasites in the body (Siswantoro et al., 2012).

### Percentage of Basophil

Basophils were not found in any of the mouse groups’ blood smears. This indicates that the basophil values in groups A, B, C, D, and E were not significantly different (p< 0.05). Basophils are uncommon in mice and are present in modest numbers (Muhsin, 2017). According to (Soma et al., 2013), basophils play a minor role in parasite infections but are critical in hypersensitivity and allergic reactions.

Our findings suggest that the aqueous extract of *S. ligustrina*, as well as the combination treatment with DHP, have the potential to lead to the discovery of new antimalarial medicines.

### Acknowledgements

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### References


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**Table 4** Average monocyte percentage in *P. berghei*-infected mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Days post infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>B</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>C</td>
<td>0.3 ± 0.7</td>
</tr>
<tr>
<td>D</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>E</td>
<td>0.2 ± 0.4</td>
</tr>
</tbody>
</table>

Note A: negative control group; B: positive control group; C: drug control group; D: extract treatment group; E: combo treatment group. Different superscript letters in the same column indicate significant differences at the level of P <0.05.


Lestari, N. (2020). The effectiveness of the combination of water extract of sea bidara wood (Strychnos ligustrina) and dihydroartemisinin piperakui Phosphate against Plasmodium berghei [skripsi]. Bogor: Institut Pertanian Bogor.


