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SEQUESTRATION OF ERYTHROCYTE INFECTED BY *Plasmodium berghei* ANKA IN MICE LIVER TREATED WITH ETHANOL EXTRACT OF PEARL GRASS *(Hedyotis corymbosa (L.) Lamk)*

SEQUESTRASI ERITROSIT TERINFEKSI Plasmodium berghei ANKA *PADA LIVER MENCIT YANG DIBERI EKSTRAK ETANOL RUMPUT MUTIARA (Hedyotis corymbosa (L.) Lamk)*

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Original Research Article
Penelitian

A B S T R A C T

Background: *Malaria is a parasitic infection caused by the Plasmodium genus. Certain Plasmodium species can evade the immune system by sequestering internal organs, including the liver. The ethanolic extract of pearl grass (Hedyotis corymbosa (L.) Lamk) (EEPG) has been reported to have an antimalarial activity in reducing parasitemia and hepatomegaly in Plasmodium berghei ANKA-infected mice.* ***Purpose:*** *To analyze the effect of EEPG administration on the sequestration of P. berghei ANKA-infected erythrocytes in the livers of BALB/c mice.* ***Method****: P. berghei ANKA-infected mice were treated with EEPG at doses of 250, 300, and 350 mg/kg BW. The positive and negative control groups received dihydroartemisinin-piperaquine (DHP) 187.2 mg/kgBW and 1% CMCNa, respectively. The treatments were administered for four consecutive days, followed by observation of parasitemia on Giemsa-stained tail blood smears. On day five, mice were sacrificed for liver removal. Sequestrations were observed on HE-stained slides of mouse livers. The differences in sequestration between treatment groups were analyzed using One-way ANOVA and Games-howell post-hoc analysis. The correlation between parasitemia and sequestration was analyzed using the Pearson correlation test.* ***Result:*** *The percentage reduction of the number of infected erythrocyte sequestration in EEPG-treated groups was 81.74%, 77.72%, and 77.70%, respectively, while the positive control group was 91.14%. Parasitemia was correlated with the number of erythrocytes sequestration (p-value < 0.05).* ***Conclusion:*** *EEPG was able to decrease parasitemia along with the decrease in the number of infected erythrocytes sequestration in the liver. These results indicated that EEPG is a potential source of natural antimalarial drugs, especially for reducing sequestration in the liver.*

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A B S T R A K

Latar belakang: Malaria merupakan penyakit yang disebabkan oleh infeksi protozoa genus *Plasmodium*. Sekuestrasi mengacu pada fenomena eritrosit yang terinfeksi menghindari sistem imun inang dengan melekat pada pembuluh darah mikro di otak dan organ visceral. Beberapa spesies dapat menghindar dari sistem imun dengan bersequestrasi di beberapa organ dalam, termasuk liver. Rumput mutiara atau *Hedyotis corymbose* (*L.*) *Lamk* memiliki aktivitas menurunkan parasitemia dan hepatomegali pada mencit terinfeksi *Plasmodium berghei* ANKA. **Tujuan:** Menganalisis pengaruh pemberian Ekstrak Etanol Rumput Mutiara (EERM) terhadap parasitemia dan sequestrasi eritrosit terinfeksi *P.berghei* ANKA pada liver mencit BALB/c. **Metode:** Penelitian ini menggunakan mencit BALB/c diinfeksi *P.berghei* ANKA dan diberi EERM dengan dosis 250, 300, dan 350 mg/kgBB. Kelompok kontrol positif dan negatif diberikan dihidroartemisinin piperaquine (DHP) 187,2 mg/kgBB dan CMCNa 1% berturut-turut. Parasitemia diamati selama 4 hari. Sequestrasi diamati pada preparat liver mencit yang diwarnai dengan HE. Perbedaan antar kelompok dianalisis dengan *One-way* ANOVA dan *post-hoc Games-howell*. Korelasi antara parasitemia dan sequestrasi dianalisis dengan uji *Pearson*. **Hasil:** Persentase penurunan jumlah sekuestrasi eritrosit terinfeksi pada kelompok perlakuan EERM dosis 250, 300, dan 350 mg/kgBB masing-masing 81,74%, 77,72%, dan 77,70%, sedangkan kelompok kontrol positif 91,14%. Terdapat korelasi antara parasitemia dan jumlah sequestrasi eritrosit terinfeksi *P.berghei* ANKA (p-value < 0,05). **Kesimpulan:** EERM mampu menurunkan parasitemia dan jumlah sekuestrasi eritrosit yang terinfeksi pada liver mencit. Hasil ini menunjukkan EERM merupakan sumber obat antimalaria alami yang potensial, terutama untuk mengurangi sekuestrasi pada liver.

Kata kunci:
Antimalaria, Rumput Mutiara, *Plasmodum berghei* ANKA, Sequestrasi

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INTRODUCTION

Malaria is a disease transmitted through the bite of the female *Anopheles* mosquito and is caused by protozoa of the genus *Plasmodium* (WHO, 2024). In humans, malaria is commonly caused by five species of *Plasmodium*, namely (1) *Plasmodium falciparum* and (2) *Plasmodium vivax* (Loy *et al*., 2017), (3) *Plasmodium malariae* (Plenderleith *et al*., 2022), (4) *Plasmodium ovale* (Okafor and Finnigan, 2025), and (5) *Plasmodium knowlesi* (Bin Said *et al*., 2022). Species of *P. falciparum* account for the highest number of malaria-related deaths (99%) compared to other species of *Plasmodium*. One of the reasons is the ability of this species to evade the immune system by sequestrating and rosetting. Sequestration refers to a phenomenon where infected erythrocytes accumulate in the brain, liver, and lungs (Belachew, 2018; Viriyavejakul *et al*., 2014). The process of sequestration in *P. falciparum* infection is mediated by the binding of *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) and its receptors on the surface of endothelial cells of microvasculatures in visceral organs, mainly CD36 (Fonager *et al*., 2012) ICAM-1 (Cunningham *et al*., 2017) VCAM-1, E-selectin, and chondroitin sulfate (Mahamar *et al*., 2017). Inflammatory responses triggered by ruptured infected erythrocytes further support this process by inducing cytokines such as TNF-α, which increase cytoadhesive endothelium surface protein levels like ICAM-1 (Gallego-Delgado *et al*., 2014; Storm and Craig, 2014). The phenomenon has been reported to occur in various mouse strains infected with various strains of *Plasmodium berghei* (Franke-Fayard *et al*., 2010; Wardani *et al*., 2020). Furthermore, Schizont Membrane-Associated Cytoadherence (SMAC) has been reported as a protein that is involved in the sequestration of *P. berghei*-infected erythrocytes in mice (Fonager *et al*., 2012).

Pearl grass (*Hedyotis corymbosa* (*L.*) *Lamk*) or also known as *Oldenlandia corymbosa* is an herbaceous plant of the *Rubiaceae* family. Phytochemically analysis has shown that it contains phytochemical proteins, polysaccharides, polyphenols, tannins, flavonoids, saponins, steroids, triterpenes, and glycosides (Das *et al*., 2019). Traditionally, this plant has been used as an antipyretic, anti-inflammatory, antibacterial, diuretic, anti-stomach ulcer, anti-dysentery, postpartum medicine, and for digestive disorders (Soemardji *et al*., 2015). The antimalarial properties of pearl grass have demonstrated its ability to inhibit the growth of *P. falciparum* in an in vitro culture by methanolic extract of pearl grass (Mishra *et al*., 2009) as well as *P. berghei* in BALB/c mice by Ethanolic Extract of Pearl Grass (EEPG) that has been reported previously by our research group (Putri *et al*., 2023). However, the effect of pearl grass extract on the sequestration of malaria-infected erythrocytes has not been reported either in human or in mouse malaria. This study aimed to analyze the effect of EEPG on the parasitemia and sequestration of erythrocytes infected with *P. berghei* ANKA in the livers of BALB/c mice.

MATERIAL AND METHOD

Preparation of the *Ethanolic Extract of Pearl Grass* (EEPG)

The EEPG was prepared by maceration of simplicia in 70% ethanol solvent in the Laboratory of Herbal Materia Medika, Batu City, East Java Province, Indonesia. The doses of EEPG used were 250 mg/kgBW, 300 mg/kgBW, and 350 mg/kgBW in 1% CMCNa (Putri *et al*, 2023).

Research design

This study was an experimental laboratory research employing a post-test only control group design. The experiment was conducted between March 2022 and May 2022 across three different: Departments Biochemistry, Anatomical Pathology and Medical Parasitology, Faculty of Medicine, Universitas Airlangga. Twenty-five mice were injected intraperitoneally with 200 µL of *P. berghei*-infected blood obtained from donor mice.

The mice were then randomly divided into five groups. Group 1 - 3 received 250 mg/kgBW, 300 mg/kgBW, and 350 mg/kgBW of EEPG, respectively. Group 4 served as the positive control group and was treated with 187.2 mg/kgBW of dihydroartemisinin-piperaquine (DHP). Group 5 acted as the negative control group and received 1% CMCNa. All treatments were administered orally once a day for four consecutive days.

On day five, a drop of blood was collected from each mouse's tail to prepare thin blood smears, which were then stained with Giemsa-stain for parasitemia determination. Subsequently, all mice were sacrificed, and their livers were processed into Hematoxylin Eosin (HE)-stained slides for anatomical pathology examination. The number of infected erythrocyte sequestrations was observed within 5 fields of view per microscopy slide (Wardani *et al*., 2020). The number of infected erythrocytes sequestration reduction due to EEPG administrations was calculated using the following Formula (1). In which NS: number of *P. berghei*-infected erythrocytes sequestration, TG: EEPG treated group and NG: negative control group

$$\% \text{ Reduction} = \frac{\text{Mean of NS in NG - Mean of NS in TG}}{\text{Mean of NS in NG}} \times 100\% \quad .....(1)$$

Data analysis

The sequestration data were statistically analyzed using a One-way ANOVA followed by Games-howell post-hoc tests. Correlations between variables were assessed using the Pearson correlation test.

RESULT

Sequestration of *P. berghei* ANKA-infected erythrocytes in mice the liver

Microscopic observation of the HE-stained mice liver tissues revealed a purple color of the nuclei of the hepatic cells, the clear red of healthy erythrocytes, the purple color of infected erythrocytes containing parasites, and the purple color of various leukocytes. An overview of the HE-stained mice livers is presented in Figure 1. Based on Figure 1, sequestrations of *P. berghei* ANKA-infected erythrocytes were observed in the sinusoids of the liver. Infected erythrocytes can be distinguished from healthy erythrocytes by the presence of the parasite and malarial pigment inside erythrocytes. Sequestration of infected erythrocytes could be observed in all treatment groups albeit at varying levels. In the positive control group, the presence of malarial pigment (hemozoin) was relatively low compared to both the negative control group and the EEPG-treated groups. The comparative mean sequestration level in the positive control group was lowest compared to that in the negative control group and the EEPG-treated groups. The mean number of *P. berghei* ANKA-infected erythrocytes sequestration between the EEPG-treated group is presented in Figure 2.

Based on Figure 2, sequestrations of *P. berghei* ANKA-infected erythrocytes were observed in the sinusoids of the liver. Infected erythrocytes can be distinguished from healthy erythrocytes by the presence of the parasite and malarial pigment inside erythrocytes. Sequestration of infected erythrocytes was present in all groups, with varied numbers. In the positive control group, malarial pigment (hemozoin) was observed in relatively small amounts compared to that in the negative control and the EEPG-treated groups. The mean sequestration in the positive control group was lower than that in the negative control and the EEPG-treated groups. The comparative mean number of *P. berghei* ANKA-infected erythrocytes sequestration from the EEPG-treated group compared to the control group is presented in Figure 3.

Correlation between parasitemia and infected erythrocytes sequestration

The percentage of parasitemia on the fourth day post-treatment is presented in Figure 3. The Positive Control Group (POS) exhibited the lowest parasitemia among the groups (0.36%). The Negative Control Group (NEG) showed the highest parasitemia (23.06%). Statistical analysis using ANOVA followed by Games-howell post-hoc tests revealed insignificant differences of parasitemia among three groups of mice treated with EEPG (p-value > 0.05). However, parasitemia levels of EEPG250 (p-value = 0.021), EEPG300 (p-value = 0.021), and EEPG350 (p-value = 0.035) were significantly different from those in the POS group (p-value = 0.001), as well as with the NEG group (p-value = 0.001). The correlation between parasitemia and sequestration of erythrocytes infected with *P. berghei* ANKA was analyzed using the Pearson correlation test revealed a significant correlation (p-value = 0.002), with a moderate strength of 0.041 - 0.600 (Schober *et al.*, 2018).

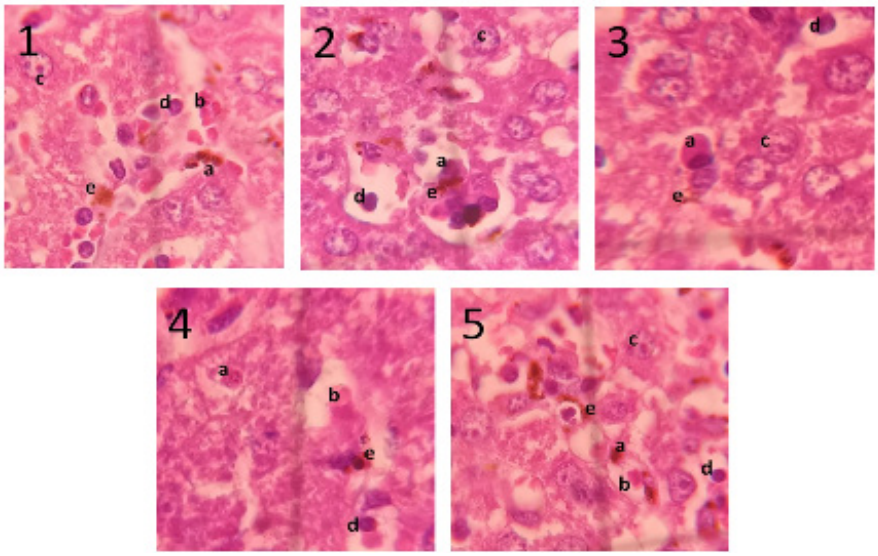

Figure 1. Histopathology images of HE-stained BALB/c tested mice livers. Microscopic slide observation 1) EEPG250, 2) EEPG300, 3) EEPG350, 4) POS, and 5) NEG, observed with 1000x magnification. Note: a) infected erythrocytes b) healthy erythrocytes c) hepatocyte's nuclei, d) leukocytes, e) hemozoin. (EEPG = Ethanolic Extract of Pearl Grass)

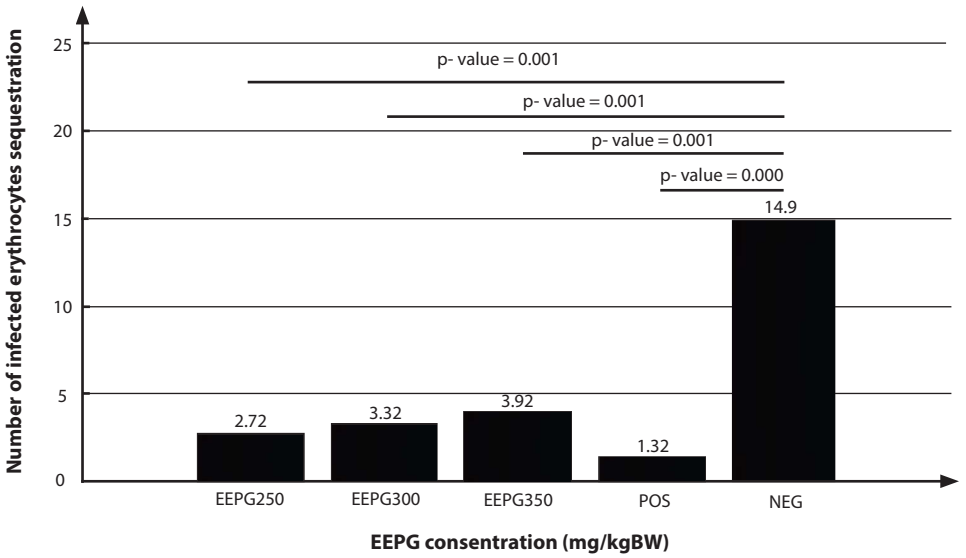

Figure 2. Mean and standard deviation (SD) of the number of *P. berghei* ANKA-infected erythrocytes sequestration in control and treatment groups. (EEPG = Ethanolic Extract of Pearl Grass)

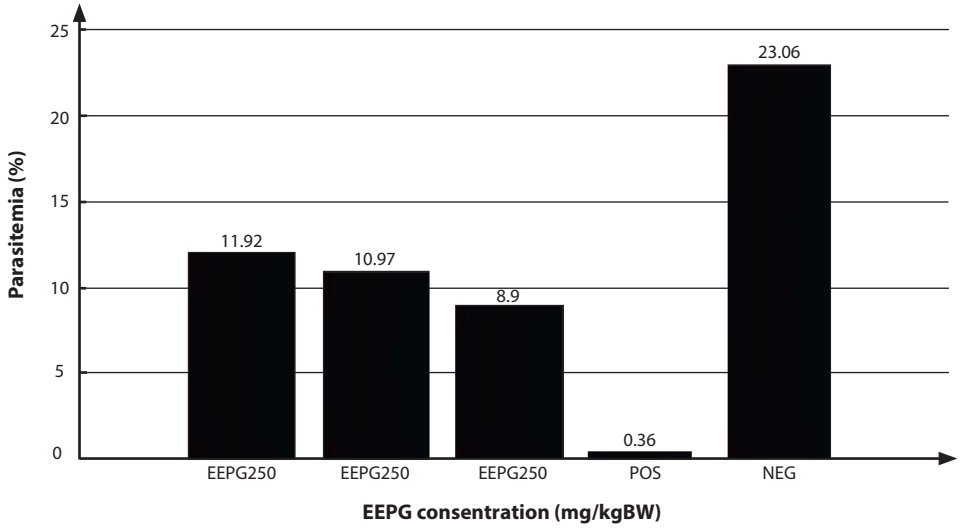

Figure 3. Percentage of parasitemia on the fourth day post-treatment using EEPG in mice infected with *P. berghei* ANKA. (EEPG = Ethanolic Extract of Pearl Grass)

DISCUSSION

The *P. falciparum* parasite is responsible for 99% of malaria-related deaths due to its ability to evade the host immune system through sequestration of infected erythrocytes in visceral organs (Belachew, 2018). Sequestration is a hallmark of *P. falciparum* infection where infected erythrocytes accumulate in vital organs such as the liver, brain, lungs, kidneys, subcutaneous tissue, and placenta. This process occurs via a receptor-ligand attachment mechanism, involving *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) binds to host receptors such as CD36 and ICAM-1 expressed by the endothelial cells of venules in those vital organs (Wassmer and Grau, 2017). The sequestrations of erythrocytes infected with schizonts of *P. berghei* have also been observed in the lungs, liver, spleen, and adipose tissue organs known to express CD36 (Franke-Fayard *et al*., 2010; Wardani *et al*., 2020). The receptors of CD36 and ICAM-1 have a major role in interacting with parasite-infected erythrocytes in small blood vessels and mediate the sequestration phenomenon, both in humans and mice. The liver and spleen ICAM-1 receptor is a crucial receptor for the attachment of infected erythrocytes. Test mice lacking ICAM-1 exhibited significant parasitemia followed by reduced parasite accumulation in the liver and spleen. ICAM-1 expression is raised as a result of the production of cytokines at sequestration sites, which causes anemia and weight loss (Cunningham *et al*., 2017). Sequestration of *P. falciparum*-infected erythrocytes in humans causes cerebral malaria (Bruneel, 2019) as well as *P. berghei*-infected erythrocytes in experimental cerebral malaria in C67BL mice (Ghazanfari *et al*., 2018). However,

experimental cerebral malaria in is strain dependent in mice. Although sequestrations of *P. berghei*-infected erythrocytes were found in BALB/c mice, these mice do not typically develop cerebral malaria (Rai *et al*., 2023).

In the present study, treatment with EEPG was able to reduce the number of sequestrations of *P. berghei*-infected erythrocytes in the liver significantly compared to the untreated group (NEG group). Previous studies have shown that pearl grass can enhance Nitric Oxide (NO) production by intraperitoneal macrophages in mice infected with *Salmonella typhimurium* (Sahat, 2006). NO is a well-known anti-adhesive molecule that inhibits platelet aggregation and leukocyte adhesion to endothelial cells (Gao *et al*., 2018). In falciparum malaria, NO has been postulated to have pathogenic and protective roles. NO is broadly recognized as an anti-inflammatory mediator that contributes to host survival in many animal models of infection, including a number of rodent models of malaria (Weinberg *et al*., 2008). The protective action of NO against *P. falciparum* by either inhibiting parasite growth (Hempel *et al*., 2014) or killing parasites intracellularly (Vasquez *et al*., 2021). NO protects against falciparum malaria by controlling infected erythrocytes' adherence to vascular endothelium, similar to how it reduces leukocyte recruitment during inflammation (Serirom *et al*., 2003). The increase of NO production leads to the reduction of adhesion molecules on endothelial cells and therefore decreases the number of infected erythrocytes sequestration (Wardani *et al*., 2020). Similarly, the increase in NO production due to the EEPG treatment in mice resulted in a decrease in adhesion molecules, leading to the decrease of infected erythrocytes sequestration.

Inflammatory processes play an important role in malaria pathology, including the sequestration process in falciparum malaria which is a consequence of the release of pro-inflammatory cytokines. In contrast with NO, the pro-inflammatory cytokines, especially TNF-α, increase the expression of molecules on the endothelial surface where erythrocytes attach during the sequestration process (Clark *et al*., 2006; Zuniga *et al*., 2022), such as ICAM-1 (Lennartz *et al*., 2019). Quercetin, a flavonoid compound found in pearl grass can reduce TNF-α levels in mice infected with *P. berghei* (Ali *et al*., 2021), that leads to the reduction of sequestration.

The EEPG was shown to reduce parasitemia in BALB/c mice infected with *P. berghei* compared to the negative control group, which indicated that EEPG possessed antimalarial activity (Putri *et al*., 2023). Therefore, the EEPG was tested against the sequestrations of erythrocytes infected with *P. bergei* ANKA in infected mice livers. Pearson test analysis revealed a significant correlation between parasitemia and the number of sequestrations of *P. berghei* ANKA-infected erythrocytes (p-value = 0.002) with moderate correlation (Pearson correlation = 0.41 – 0.60) (Schober *et al*., 2018). This result suggests that an increase in parasitemia is followed by an increase in the number of sequestrations of infected erythrocytes. This correlation indicates that sequestration of infected erythrocytes is directly affected by the level of parasitemia because *P. berghei* ANKA-infected erythrocytes in the peripheral blood will sequester inside visceral organs through cytoadherence on the endothelial cells of blood vessels. In this study, mice with higher parasitemia, tended to exhibit a greater number of infected erythrocytes sequestration. However, the correlation of sequestration and parasitemia differs between humans and mice. Sequestration of *P. falciparum*-infected erythrocytes with mature stages in humans relates to the vascular endothelial cell activation and mechanical blockage of blood flow in small blood vessels (Oelschlegel *et al*., 2024) causing these stages to disappear from the peripheral blood (Franke-Fayard *et al*., 2010), therefore, resulting in low levels of parasitemia (Antwi-Baffour *et al*., 2023). However, this is not the case with *P. berghei*-infected erythrocytes. Immature and mature parasite-infected erythrocytes remain in circulation shortly post-infection (Franke-Fayard *et al*., 2010), parasitemia remains high and increases progressively until death. In this study, EEPG possessed antimalarial property as well as reduced sequestration is a promising plant-derived treatment for malaria.

CONCLUSION

Ethanolic Extract of Pearl Grass (EEPG) was able to decrease parasitemia along with the decreased in the number of infected erythrocytes sequestration in the liver. This effect is likely attributed to the quercetin-content of EEPG, which increases NO production and suppresses TNF-α leading to the reduction of adhesion molecules on endothelial cells. Consequently, the reduced number of infected erythrocytes sequestration. EEPG is a potential antimalarial therapy that may develop into an antimalarial drug.

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AUTHOR CONTRIBUTION

N. F. C. performed *P. berghei* ANKA infection, mouse treatments, prepared thin blood smears, counted the parasitemia, sacrificed the mice, and drafted the manucricipt. J. A. P. col¬lected blood from mice. N. F. C. and J. A. P. organized and analyzed the data. NFC, JAP and H. A. observed the sequestration on the HE-stained liver slides. H. A. designed and led the research project, and finalizing the manuscript. All authors read and approved the final manuscript.

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Not applicable

DATA AVAILABILITY

The dataset generated and/or analyzed during the current study are not publicly available due to all the data have been summarized and presented in the manuscript, but the raw data are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors state there is no conflict of interest with the parties involved in this study.

ETHICAL APPROVAL

The ethical approval was obtained from the Ethics Committee of the Faculty of Medicine, Universitas Airlangga as stated on the certificate number 88/EC/KEPK/FKUA/2022 issued on 19th May 2022.

INFORMED CONSENT

Not applicable

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