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Prophylaxic study of Combination Ethyl Acetate Fraction of *Andrographis paniculata* Ness. and Dihidroartemisinin-Piperaquine (DHP) in Malarial Infected Mice

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Abstract

The resistance to some antimalarial drugs has had a major impact on the drug selection for malaria prophylaxis. According to previous research, the combination of *Andrographis paniculata* and Dihydroartemisinin-piperaquine (DHP) can prevent artemisinin resistance, reduce the adverse effects and is highly effective as an antimalarial. This study aims to determine the malaria prophylactic activity and survival time of the combination of *A. paniculata* ethyl acetate fraction with DHP on *P. berghei* infected mice. An in vivo malaria prophylaxis study was conducted based on Peters' prophylactic test method. The combination of *A. paniculata* ethyl acetate fraction at a dose of 100 mg andrographolide/kg BW was administered twice a day per oral for four days and continued for another day after infection, meanwhile DHP at a dose 1,6 mg/kg BW was administered once a day per oral for three days. These results show that the combination of *A. paniculata* ethyl acetate fraction with DHP can inhibit 54,35% of the malaria parasite on day 4 and 44,98% on day 5, which was less active than the monotherapy group of *A. paniculata* ethyl acetate fraction or DHP alone. Also, this combination can increase the survival time of the mice to 6 days.

Keywords

Andrographis paniculata Nees., ethyl acetate fraction, malaria prophylaxis, *Plasmodium berghei* ANKA.

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Introduction

Malaria is an infectious disease caused by Plasmodium parasites and transmitted by the bite of a female Anopheles mosquito. This infection can lead to severe complications and death. A total of 241 million cases of malaria were recorded worldwide in 2020, while in 2019 there were 227 million reported cases. The estimated death rate from malaria has reached 627, 000 (WHO, 2020). Indonesia is one of the countries that has many endemic areas, with 304, 607 reported cases (Kemenkes, 2022). One of the Indonesian government's strategies to control malaria is by utilizing antimalarial drugs both as treatment and as prevention of infection (prophylaxis). One of the prophylactic drugs used in Indonesia up until now is doxycycline. However, doxycycline is acknowledged to have adverse effects on long-term use and is not safe to be taken by pregnant women and children (Savitz and Styka, 2020). Therefore, it is necessary to conduct further research on other drugs to prevent malaria that are safe and effective.

Andrographis paniculata or known as sambiloto, has been used in several Asian countries including Indonesia as a traditional medicine to treat various diseases (Okhuarobo *et al*., 2014). Sambiloto has been proven to have various pharmacological activities such as antioxidant, antibacterial, antimalarial, anti-inflammatory, analgesic, immunostimulant, anticancer, and hepatoprotective (Joselin and Jeeva, 2014). *A. paniculata* has the main content of andrographolide which, as proved by in vitro and in vivo test method, is classified as highly active antimalarial substance, with an ED_{50} of 6.75 mg/kg BW (Widyawaruyanti *et al*., 2017). The highest andrographolide content was found in the ethyl acetate fraction of 96% ethanol extract of *A. paniculata* herb (Hafid *et al*., 2015).

The WHO recommends a combination of antimalarial drugs for the treatment of malaria. This combination therapy aims to increase the effectiveness of therapy, reduce resistance and reduce drug toxicity (WHO, 2001). Based on research by Widyawaruyanti *et al*. (2021), it was concluded that the combination of ethyl acetate fraction of *A. paniculata* and Dihydroartemisinin-piperaquine (DHP) on pregnant female mice infected with *P. berghei*, can inhibited parasites growth at 100% in the peripheral and 50.50% in the placenta. The ethyl acetate fraction of *A. paniculata* also has the potential to reduce the toxicity of the Dihydroartemisinin-piperaquine (DHP) in pregnant mice.

In this study, an in vivo malaria prophylaxis test was conducted from the combination of ethyl acetate fraction of *A. paniculata* herb with Dihydroartemisinin-piperaquine (DHP) on mice infected with *P. berghei*, this study aims to determine the potential of the combination of the two substances as a malaria prophylactic drug.

Materials and Methods

Materials

The ethyl acetate fraction of 96% ethanol extract of *A. paniculata* herb was obtained from the NPMRD Laboratory, Institute of Tropical Disease (ITD), Universitas Airlangga, with the appearance form of powder, dark green, odorless, and bitter taste, with andrographolide content amount 23.959%.

The dihydroartemisinin-piperaquine (DHP) tablet was purchased from health services in malaria endemic areas and is a natrium-carboxymethylcellulose (CMC-Na).

Animals

Male Balb/C strain mice (*Mus musculus*) were obtained from the NPMRD Animal Laboratory, Institute of Tropical Disease (ITD), Universitas Airlangga. The mice used were aged 2-3 months, body weight (BW) 20 g - 35 g, active, clean white fur, good appetite, and bright eyes. Mice were maintained on standard animal pellets and water ad libitum at NPMRD Animal Laboratory, ITD, Universitas Airlangga.

Malaria Parasite

Plasmodium berghei ANKA strain parasites were obtained and maintained at the NPMRD Malaria Laboratory, ITD, Universitas Airlangga.

Methods

In Vivo Malaria Prophylaxis Activity Set

The prophylactic test method was conducted based on Peter's prophylactic test. All *A. paniculata* ethyl acetate fraction suspension was given orally for four days, on the fifth day the mice were infected by injecting 200 µL of erythrocytes containing 1 x 10^6 *P. berghei* parasites intraperitoneally. Administration of the *A. paniculata* suspension was continued for four days after infection (D_0-D_3). Thin blood smear samples were taken daily to examine and calculate the parasitemia percentage for six days (D_0-D_6) using a microscope. Malaria prophylaxis activity was determined by the difference between the mean value of parasitemia of negative control and experimental groups that were expressed as inhibition percentage. Inhibition percentage was calculated with the following formula:

$$100\% - \left(\frac{Xe}{Xk} x100\%\right)$$

Xe = % Parasitemia growth of experimental group

Xk = % Parasitemia growth of negative control

Various therapy model groups were conducted in this research as follows: Negative control group (K(-)): Given CMC-Na 0,5% twice a day for four days before and four days after infection; Positive control group (K(+)): Given CMC-Na 0.5% twice a day for four days before infection followed by DHP at a dose of 1.6 mg/kg BW for three days after infection; Treatment group (P1): Given the ethyl acetate fraction of *A. paniculata* that equivalent to 100 mg andrographolide/kg BW twice a day for four days before infection, followed by the combination of DHP 1.6 mg/kg BW once a day for three days and ethyl acetate fraction of *A. paniculata* that equivalent to 100 mg andrographolide/kg BW twice a day for four days after infection; Treatment group (P2): Given ethyl acetate fraction of *A. paniculata* that equivalent to 100 mg andrographolide/kg BW twice a day for four days before infection and followed by another four days after infection.

Determination of Mean Survival Time (MST)

Determination of mean survival time was performed based on Somsak *et al*. (2016); the survival time of *P. berghei* infected mice was observed for 14 days after infection (D0-D13). Mean survival time was calculated with the following formula:

$$\text{MST} = \frac{\textit{Sum of survival time (days) of all mice in a group}}{\textit{Total number of mice in that group}}$$

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical analysis of data was performed using GraphPad Prism version 8, the one-way ANOVA was used for analysis and to compare the results at 95% confidence level. Value $p<0.05$ was considered significant.

Results and Discussion

Malaria Prophylaxis Acticity

Based This study aims to determine the malaria prophylactic activity and survival time of the combination of sambiloto (*A. paniculata*) ethyl acetate fraction with dihydroartemisinin-piperaquine (DHP) of *P. berghei* infected BALB/c strain mice. Based on the results of previous studies, the combination of *A. paniculata* with the antimalarial drug dihydroartemisinin-piperaquine (DHP) on pregnant female mice obtained the results of inhibition percentage of *P. berghei* by 50.50% in the placenta and 100% in the peripheral. This study was conducted to determine the activity of this combination as a prophylaxis with the DHP dose lowered to 1/100 of the normal human dose. This was also done to prevent overuse, especially of first line antimalarial drugs according to the WHO recommendation, as well as to reduce side effects due to the use of DHP, which can increase heart QT prolongation, risk of miscarriage in first trimester pregnant women, and can cause GI tract disturbances which are the main factors of non-compliance in the treatment of malaria patients (Chatio *et al*., 2016).

This study had the result that on the fourth day after infection, the combination of *A. paniculata* ethyl acetate fraction with dihydroartemisinin-piperaquine (DHP) was able to inhibit parasite growth (D4) by 54.35%. The group that was able to inhibit the highest parasite growth was the P2 group that was given monotherapy of *A. paniculata* ethyl acetate fraction, which was 75.09%. This was suspected to be an interaction between the substances contained in the *A. paniculata* ethyl acetate fraction with DHP. Drug interactions could be divided into three groups. Pharmaceutical interactions are physicochemical interactions between drugs that change their pharmacological activity. Pharmacokinetic interactions occur when one drug affects the absorption, distribution (protein binding), metabolism and excretion of another drug. Pharmacodynamic interaction occurs when the activity of a drug changes due to the influence of another drug on the site of action (Suryawati, 1995). Pharmaceutic interaction is unlikely to occur in this study because the administration was not done by combining DHP and *A. paniculata* ethyl acetate fraction in one dosage suspension. The administration of the suspension was given orally in sequence, the suspension of *A. paniculata* ethyl acetate fraction was given first, then followed by DHP suspension. Therefore, possible interactions are pharmacokinetics or pharmacodynamics. Andrographolides are known to significantly increase the production of the hepatic enzyme cytochrome CYP4501A in the body (Jarukamjorna and Nemotob, 2008). With the increase of the enzymes, it can also increase the elimination rate of dihydroartemisinin which affects the pharmacology of dihydroartemisinin, reducing bioavailability, the volume of distribution and half-life of dihydroartemisinin (Erhirhie *et al*., 2021). This interaction resulted in dihydroartemisinin being ineffective. In addition, based on the mechanism of action of each compound described, it is known that andrographolide and piperaquine have the same mechanism of action, they inhibit heme detoxification which causes parasite death. So, it is likely that the two compounds compete to bind to the same receptor, which could cause a delay in activity or reduce activity in one or both compounds (Palleria *et al*., 2013). Some of these factors are suspected to be the cause of the combination of *A. paniculata* ethyl acetate

fraction with DHP having lower inhibition activity. Whereas on the fifth day after infection, the highest percent inhibition, of 62.66%, was the positive control. This might be because andrographolides are eliminated in the body in a period of 6- 10 hours (Panossian *et al*., 2000), while DHP contains piperaquine which has a half-life up to 20 days in the human body (Chotsiri *et al*., 2019). Therefore, piperaquine in DHP can stay longer in the blood and inhibit parasite growth. The results of malaria prophylaxis activity are summarized in Table 1. ANOVA analysis showed that there wasn't a significant difference between models of therapy. The results obtained sig. = 0.074 (sig. > 0.05).

Table 1. Malaria Prophylaxis Activity on *P. berghei* Infected Mice

Group	% Parasitemia						% Parasite Inhibition	
	D_0	D_1	D_2	D_3	D_4	D_5	D_4	D_5
K (-)	0	0.03^{a}±0.08	0.63^{a}±0.43	3.72^{a}±2.70	9.15^{a}±1.68	16.71^{a}±1.90	-	-
K (+)	0	0.03^{a}±0.08	0.24^{b}±0.20	1.03^{b}±0.66	2.66^{b}±1.58	6.26^{b}±2.69	70.96^{b}±17.30	62.66^{b}±16.15
P1	0	0.06^{b}±0.09	0.42^{a}±0.13	1.18^{b}±0.37	4.18^{b}±0.82	9.24^{b}±1.41	54.35^{a}±8.95	44.98^{a}±8.49
P2	0	0.02^{a}±0.04	0.18^{b}±0.15^{a}	0.53^{a}±0.39	2.28^{a}±1.17	6.98^{b}±2.76	75.09^{b}±12.75	58.24^{b}±16.49

^{a,b} Different superscripts in the same column showed significant differences ($p<0.05$)

Mean Survival Time (MST)

The results of the mean survival time observation showed that all mice in the treatment group could not survive for 14 days. This might be due to several possibilities, including the *P. berghei* parasite provided by the laboratory that infected mice was too infectious due to genetic mutations during the culturing process. In addition, it could also be caused by host factors (mice) that have a poor immune system. The results of mean survival time are summarized in Table 2. In P1 group, which was given a combination of *A. paniculata* ethyl acetate fraction equivalent to 100 mg andrographolide/kg BW with 1.6 mg/kg BW DHP, all mice died on the eighth day after infection with an average survival time of six days. Whereas in the positive control group and monotherapy treatment of *A. paniculata* ethyl acetate fraction equivalent to 100 mg andrografolida/kg BW mice (P2) had an average survival time longer than the combination group, the P2 group also had an average survival time longer than K(+), which was eight days. This is aligned with the percent inhibition value obtained in group P2 which is slightly greater than the K (+) group so that the parasite growth can be suppressed, and the severity of infection is slow to occur. It can also be caused because the *A. paniculata* ethyl acetate fraction also contains andrographolide, 14-deoxy-11,12-didehydroan-drographide and 14-deoxyandrographolide, which is proven to have activity as an immunostimulant agent in mice, so the immune system in group P2 mice is stronger than group K (+) and can survive longer.

Table 2. Mean Survival Time (MST) for each Treatment Group.

Group	% Inhibition		*Mean Survival Time* (MST)
	D_4	D_5	
K (-)	-	-	5.4[a]±0.55
K (+)	70.96[b]±17.30	62.66[b]±16.15	7.0[b]±1.22
P1	54.35[a]±8.95	44.98[a]±8.49	6.2[b]±0.45
P2	75.09[b]±12.75	58.24[b]±16.49	7.8[b]±1.64

[a,b] Different superscripts in the same column showed significant differences ($p<0.05$)

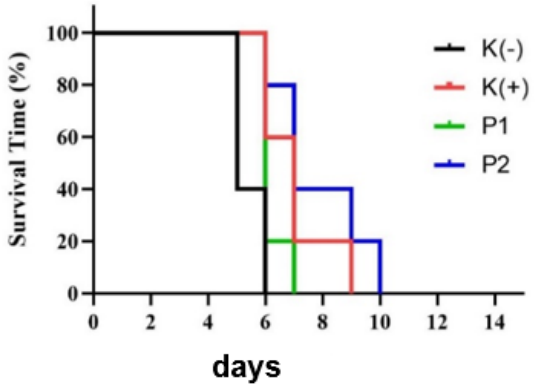

Figure 1. Survival Time Profile. K (-) dead on the fifth day and survived until the sixth day; K (+) dead on the sixth day and survived until the ninth day; P1 dead on the sixth day and survived until the seventh day; P2 dead on the sixth day and survived until the first day.

Conclusion

The combination of *A. paniculata* ethyl acetate fraction with DHP showed prophylactic activity with inhibition percentage of 54.35% on day 4 and 44.98% on day 5 post-infection. The prophylactic activity in this combination was lower than the monotherapy of *A. paniculata* ethyl acetate fraction, which was 75.09% on day 4 and 58.24% on day 5 post-infection, as well as DHP monotherapy, which was 70.96% on day 4 and 62.66% on day 5 after infection. In addition, the combination of *A. paniculata* ethyl acetate fraction with DHP increased the survival time value for six days, while the DHP monotherapy group had a survival time of seven days and the *A. paniculata* ethyl acetate fraction monotherapy group had a survival time of eight days.

Approval of Ethical Commission

Ethical clearance received an approval from Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia with the protocol no: 1.KEH.040.03.2023.

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Author's Contribution

HKN: Contributed to the conceptualization, methodology, data collection, data analysis, and writing of the original draft, as well as project administration. AS: Supervised the study, provided methodological input, validated the findings, and contributed to the review and editing of the manuscript. HP: Involved in supervision, methodology, validation, and manuscript review and editing. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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