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A tablet derived from Andrographis paniculata complements dihydroartemisinin-piperaquine treatment of malaria in pregnant mice

by BastianaBastiana

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A tablet derived from *Andrographis paniculata* complements dihydroartemisinin-piperaquine treatment of malaria in pregnant mice

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Abstract

Objectives: The use of standard antimalarial drugs, such as dihydroartemisinin-piperaquine (DHP) for the treatment of malaria during pregnancy is limited due to the risk of teratogenicity. The alternative is therefore required although few exist. Here we show a phytopharmaceutical drug derived from *Andrographis paniculata* (AS201-01), which is effective as herbal antimalarial both *in vitro* and *in vivo* and may be a suitable alternative when used in complementary treatment with DHP.

Methods: *Plasmodium berghei* infected pregnant BALB/c mice were divided into four groups: G1 (negative control), G2 (AS201-01), G3 (DHP), and G4 (combination of DHP and AS201-01). Pheripheral blood was collected during therapy for counting parasitemia. Placental samples were analyzed for the expression of IFN-γ, TNF- α, IL-10, placental parasite counts and foetal morphology.

Results: Groups G4 and G3 both showed a 100% inhibition of peripheral parasitemia. However, the treatment in G4 was found to be less effective than that in G2 and G3 in preventing placental parasitemia. The G4 treatment was able to reduce the expression of IFN-γ and IL-10, whereas TNF-α was not significantly different from the control group. Foetal morphologic abnormalities were observed in all groups except G2; G4 showed lower percentage of abnormalities compared to G3 and G1.

Conclusions: A combination of *A. paniculata* tablet (AS201-01) with DHP has the potential to reduce the toxicity of DHP in malaria treatment.

Keywords: *Andrographis paniculata*; dihydroartemisinin-piperaquine; foetal morphology; immune system; placental malaria.

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Introduction

Pregnant women are prone to malaria infections due to changes in the immune system during pregnancy [1]. Malaria infections during pregnancy can result in complications for the mother such as anemia, pulmonary edema, renal failure, cerebral malaria, and death. The foetus is also at increased risk of stillbirth, premature delivery, low birth weight neonates and as a result malaria is one of the leading causes of neonatal mortality [2, 3].

Several approaches have been undertaken to reduce malaria in pregnancy. However, both perinatal morbidity and maternal mortality remain high. Difficulties in finding an effective and safe antimalarial drug remains an obstacle to combat malaria infections during pregnancy. The development of antimalarial drugs that are safe with effective therapy targets are therefore needed [4, 5]. For

such cases, artemisinin-based combination therapies (ACTs) are recommended by WHO as the primary choice for the treatment of uncomplicated malaria in the areas where multidrug-resistant malaria is confirmed. The one of ACT regimens used today is dihydroartemisinin-piperaquine (DHP) [6]. DHP is confirmed as safe, effective, and well-tolerated in adult patients. However, their use in pregnant women is still questionable due to limited safety data.

Herbal medicine is currently acceptable worldwide for handling many diseases, including malaria. Several studies have confirmed the effectiveness of some herbal products in treating malaria in both *in vitro* and *in vivo* studies using animals and in clinical trials [7–9]. We recently developed a phytopharmaceutical product (AS201-01); the main ingredient of which is the ethyl acetate fraction of ethanol extract of *Andrographis paniculata*. The marker compound of the tablet, andrographolide, was found to possess therapeutic properties, such as antimalarial and immune stimulant effects [10–12].

Our previous study of the antimalarial activity of AS201-01 showed that they inhibited *Plasmodium berghei* growth with ED_{50} value of 6.75 mg/kg BW [9].

The aim of this study was to determine the complementary effect of a *A. paniculata* derived tablet (AS201-01) combined with DHP on peripheral and placental parasite inhibition, expression of IFN-γ, TNF-α, IL-10 in the placenta and foetal morphology of *P. berghei* infected pregnant mice. To the best of our knowledge, this is the first study to evaluate the complementary effect of a phytopharmaceutical product containing andrographolide combined with antimalarial drug-DHP in the treatment of malaria during pregnancy.

Materials and methods

Samples

AS201-01 containing ethyl acetate fraction of *A. paniculata* (equivalent to 35 mg of andrographolide each tablet) was produced at The Faculty of Pharmacy, Universitas Airlangga. The DHP tablets containing fixed-dose of 40 mg dihydroartemisinin and 320 mg piperaquine for each tablet (D-ARTEPP™) was purchased from Guilin Pharmaceutical Co., Ltd., China. Healthy female BALB/c mice (8–12 weeks old, 25–30 g in weight) were obtained from Universitas Gadjah Mada and kept at the animal facilities of Institute of Tropical Disease, Universitas Airlangga.

Experimental animals

BALB/c female mice, aged 8–12 weeks were obtained from LPPT-Gadjah Mada University, Yogyakarta. Mice were subsequently adapted to the dark and light cycle for 12/12 h and fed and drinking ad libitum at the Institute of Tropical Disease (ITD) experimental animal laboratory, Universitas Airlangga. This experimental study has received ethical approval from the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga No: 2.KE.185.10.2019.

Parasitic infection

P. berghei ANKA was obtained from the Eijkman Institute (Eijkman Institute for Molecular Biology), Jakarta and the subsequent treatment was carried out at ITD, Universitas Airlangga by doing a combination of passage on female mice and cryoscopic storage. Malaria was induced by injecting 1×10^6 infected erythrocytes in 200 µL of physiological saline solution intra peritoneally. Parasitemia was monitored starting on day two after infection through microscopic examination of peripheral blood smears from the tails of mice with 10% Giemsa staining. The percentage of parasitemia was calculated under a light microscope with a 100× objective lens [13, 14].

Timing and pregnancy monitoring

Female mice were injected with pregnant mare serum gonadotropin (PMSG, foligon) at a dose of 5 IU and then injected with human chorionic gonadotropin (hCG, Chorulon) 48 h after PMSG injection. After injection, female mice were collected and monomated with male mice (one female:one male) and the vaginal plug was examined after 17 h. The presence of a vaginal plug defined day 0 of pregnancy.

Research design

In vivo antimalarial activity was carried out based on the Peter Test [15]. Infection is carried out intraperitoneally by injecting 1×10^6 infected erythrocytes in 200 µL of physiological saline solution on day nine of pregnancy and therapeutic treatment started on day 11–14 of pregnancy. In total, 20 female mice were divided into four study groups (n=5): Group 1 (G1): The placebo control group, infected pregnant mice given a placebo: CMC-Na two times a day for four days. Group 2 (G2): The AS201-01 group, infected pregnant mice given AS201-01 tablets (tablets equivalent to andrographolide 25 mg/kg bw) two times daily for four days. Group 3 (G3): The DHP group, infected pregnant mice given DHP tablets (tablets equivalent to 1.25 mg of dihydroartemisinin and 9.98 mg piperaquine phosphate/kg bw) once daily for three days. Group 4 (G4): The combination of AS201-01 tablets and DHP group, pregnant mice were given AS201-01 tablets (tablets equivalent to 25 mg andrographolide/kg bw given two times a day for four days) and DHP tablets (tablets equivalent to 1.25 mg dihydroartemisinin and 9.98 mg piperaquine phosphate/kg bw), once a day on the first day of treatment for one day.

Placental collection

On day 15 of pregnancy, placental tissue was removed. Mice in all groups were euthanised by anesthesia using chloroform and dissected by opening the abdominal wall. The required sample was taken. Placental tissue was collected and then fixed in 10% formalin for at least 24 h.

Peripheral parasitemia evaluation

Parasitemia was monitored until day 5, which was one day after treatment was stopped, by observing the peripheral blood smear taken from the tails of mice through staining with Giemsa 10% [13]. The peripheral blood smear was examined under a light microscope. The percentage of parasitemia and the percentage of inhibition parasitic growth are calculated as follows:

The percentage of parasitemia=[(growth of parasitemia observed in the treatment group)/(growth of parasitemia observed in the untreated group)] × 100%.

The percentage of inhibition=100% – (percentage of parasitemia)

Placental parasitemia evaluation

Placental tissue was fixed in 10% formalin, then histopathologically processed through the stages of dehydration, rehydration, blocking and cutting of placental tissue. Tissue was then stained with hematoxylin-eosin (HE) and then evaluated under a light microscope using a 100× objective lens [16, 17].

Expression of IFN-γ cytokines, IL-10 and TNF-α placenta

IFN-γ, TNF-α and IL-10 expressions were examined in the entire field of view of the mice placentas (syncytiotrophoblast cells, decidua, macrophage monocyte and lymphocyte cells in the intervillus space, blood vessel endothelium) after immunohistochemical coatings using each of IFN-γ, TNF-α and IL-10 primary antibodies. IFN-γ, TNF-α and IL-10 expressions in each sample were evaluated semiquantitatively as stated by the modified Remmele method, where the Remmele scale index (Immuno Reactive Score/IRS) is the result of multiplying the percentage score of immunoreactive cells with the colour intensity score of immunoreactive cells [18].

Morphological examination of foetal bones

Foetal morphology was assessed qualitatively by looking at the morphology of the *cranium*, *costae*, vertebrae, and anterior and posterior extremities after Alizarin staining of the mice foetuses.

Statistical analysis

Data was analyzed using one-way Anova test carried out with SPSS software for Windows program, followed by the Tukey HSD multiple comparison test to find out which groups were significantly different. If the data were not normally distributed, the nonparametric test Kruskal-Wallis test was used. The level of significance (α) was 0.05.

Results

Pheripheral and placental parasite inhibition of AS201-01 combined with DHP tablets

The groups receiving a combination of AS201-01 with DHP (G4) and DHP alone (G3) had a peripheral parasite inhibition rate of 100%; the AS201-01 group (G2) was 22.87% (Figure 1A). The placental parasite growth inhibition rate for groups G2, G3 and G4 was 75.73, 90.29 and 50.50% respectively (Figure 1B).

Effects of a combination of AS201-01 tablet and DHP on IFN-γ, IL-10, and TNF-α cytokine expression in the placenta

The effect of the administration of a combination of AS201-01 and DHP on cytokines was measured using an immunohistochemical method with a modified Remmelle scale. The mean result of cytokine expression as shown in Table 1 and Figures 2–4. For the all treatment groups, IFN-γ and IL-10 expression decreased significantly compared to the placebo control group (G1), whereas there was no significant change in TNF-α expression.

Effects on foetal morphology

Alizarin staining was carried out on mice foetuses to examine foetal morphology. The stained foetus was then subjected to morphological observations of the *cranium*, *costae*, vertebrae and extremities (Table 2). Abnormal morphology of foetal bones is expressed as a percentage of the amount (%) in terms of the condition of the bones of *cranium*, rib (*costae*), spine (vertebrae) and extremities. Only in the AS201-01 group (G2) was foetal bone morphology normal. Morphological abnormalities in the other groups showed *cranium* bones were partially formed

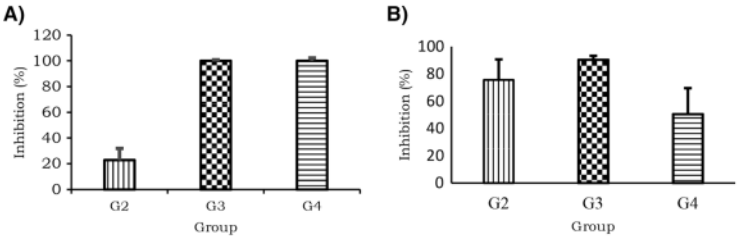

Figure 1: Parasite inhibition,
(A) inhibition of peripheral parasitemia,
(B) inhibition of placental parasitemia.

Table 1: IFN-γ, IL-10 and TNF-α cytokine expressions in the control and treatment groups.

Cytokine	Group, Mean ± SD			
	G1	G2	G3	G4
IFN-γ	6.80 ± 1.30	3.64 ± 1.25*	3.08 ± 0.70*	1.16 ± 0.26*
IL-10	5.84 ± 1.31	2.92 ± 0.94*	2.32 ± 0.50*	1.80 ± 0.87*
TNF-α	7.72 ± 2.31	3.40 ± 0.81	3.12 ± 1.22	5.08 ± 1.50

*Showed a significant difference compared to the placebo control group (G1).

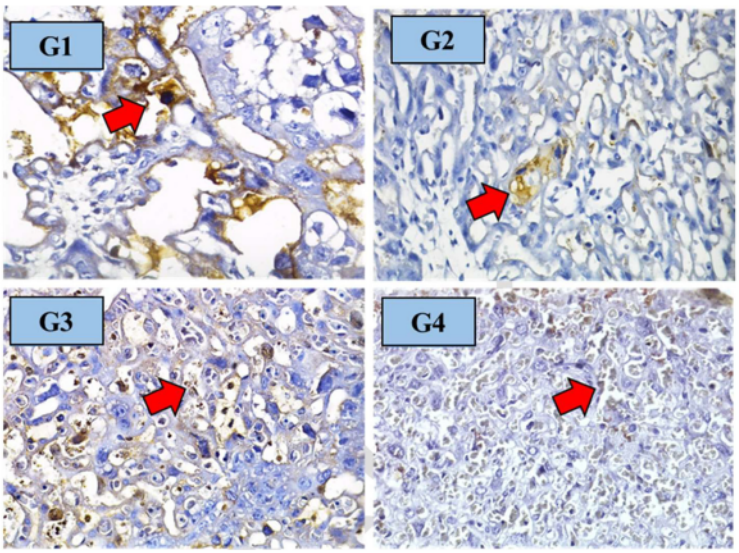

Figure 2: Expression of IFN-γ in placenta between treatment groups. The expression of IFN-γ are characterized by a yellowish to chromogenous brown color (arrow); G2, G3, and G4 appears to decrease significantly than the control group G1 (immunohistochemical staining, 400 times magnification; Nikon H600L microscope; 300 MP camera DS Fi2).

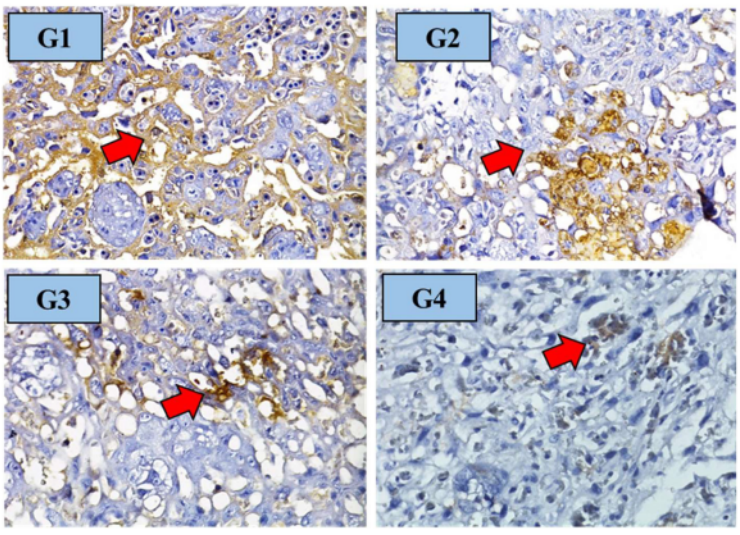

Figure 3: Expression of IL-10 in placenta between treatment groups. The expression of IL-10 are characterized by yellow to chromogen brown (arrows); G2, G3, and G4 appears to decrease significantly than the control group G1 (immunohistochemical staining, 400 times enlargement; Nikon H600L microscope DS Fi2 camera 300 megapixels).

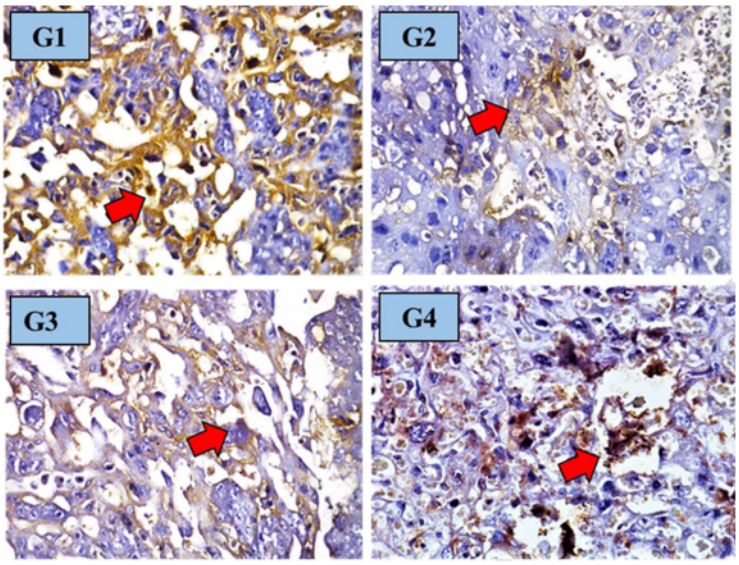

Figure 4: Expression of TNF-α in placenta between treatment groups. The expression of TNF-α in the G2, G3, and G4 showed no significantly difference compared to the control group G1. The expression of TNF-α are characterized by a yellowish to chromogen brown color (arrows). Immunohistochemical staining, 400 times magnification; Nikon H600L microscope; camera DS Fi2 300 megapixels.

Table 2: Qualitative foetal morphological data.

Abnormality fetal bone	Group				p-Value
	G1, %	G2, %	G3, %	G4, %	
Cranium (not formed, malformations)	100	0	100	80	0.000
Costae (shortening of *costae*, malformations)	40	0	40	40	0.139
Abnormal vertebrae extremities	100	0	100	40	0.000
Shortening of the extremities	100	0	60	80	0.005

as well as malformations and shortening of *cranium* size; rib bones in some cases were not formed, malformed or shortened; spines were shortened or the lumbar sacrum segment was not formed; anterior and posterior extremities bones were also shortened. Figures 5–8 showed selected foetal bone morphology between treatment groups.

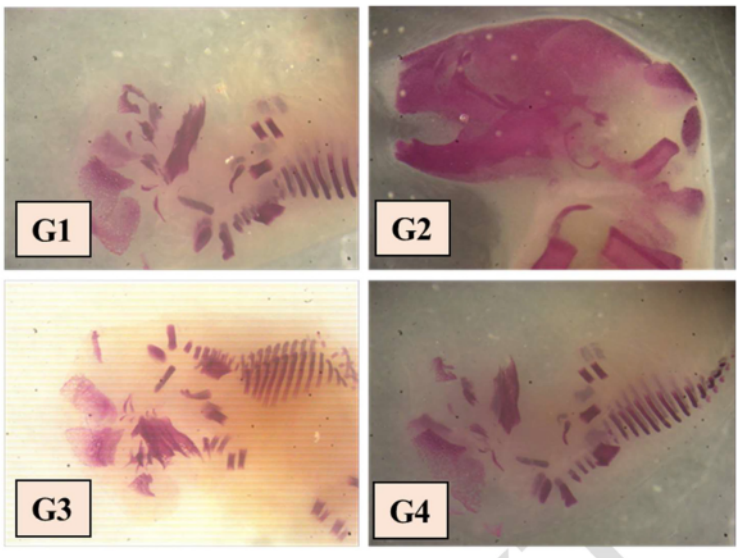

Figure 5: Alizarin staining of *cranium* bones. G1, G3, and G4 showed abnormal *cranium* bone formation in the form of malformations and shortening of cranium size. G2 showed normal cranium morphology.

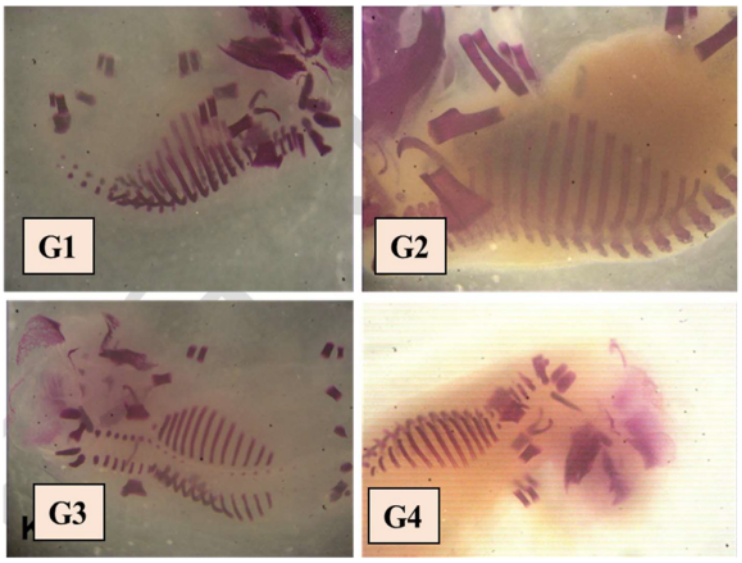

Figure 6: Alizarin staining of *costae* bone. G1, G3, and G4 showed abnormal *costae* of bone formation in the form of shortening of the size of the costae. G2 showed normal *costae* morphology.

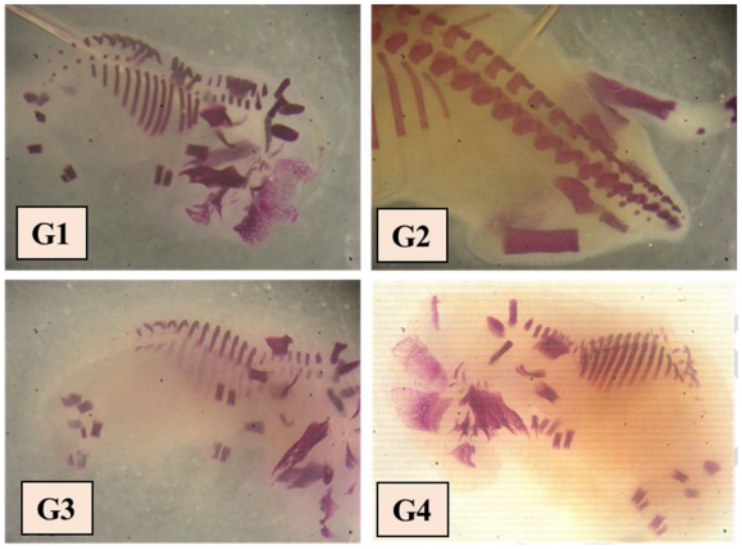

Figure 7: Alizarin staining of vertebrae bone. G1, G3, and G4 showed abnormal costae of bone formation in the form of shortening of the size of the vertebrae. G2 showed normal vertebrae morphology.

Discussion

This study has demonstrated that pregnant mice receiving a DHP and AS201-01 combination treatment displayed complete inhibition of peripheral and 50.50% placental growth of *P. berghei*.

The ability of *A. paniculata* to inhibit peripheral parasites both *in vitro* and *in vivo* has been demonstrated in previous studies [7–9, 19]. However, a single *A. paniculata* tablet (AS201-01) was not optimal against parasites in the peripheral in this study. We believe this is due to the use of pregnant mice in this study that are immunocompromised compared to non-pregrant mice. Nevertheless, the AS201-01 showed a higher parasitic inhibitory ability in the placenta than in the peripheral (Figure 2). This result is in line with research conducted by Wahdi et al. [20], that demonstrated the administration of AS201-01 to pregnant mice infected with *P. berghei* can reduce the expression of chondroitin sulfate (CSA). CSA is a special receptor that binds to parasitic infected erythrocytes (PfEMP-1) in the placenta [6]. Decreased CSA expression will cause the binding of PfEMP-1 to decrease, resulting in decreased

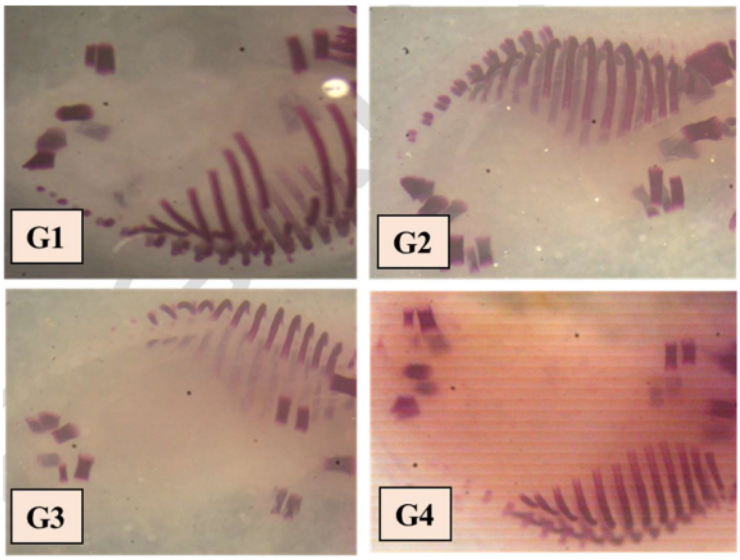

Figure 8: Alizarin staining of extremities bones. G1, G3, and G4 showed abnormal extremities of bone formation in the form of shortening of the size of the extremities. G2 showed normal extremities morphology.

adhesion of infected erythrocytes in the placenta so that ultimately lowers parasitic abundance [20]. Optimal drug combination doses should be found to eliminate parasitic growth in the peripheral and placenta.

Combination treatment is the simultaneous use of two or more drugs, each of which has a different mechanism and targeting action in treating malaria. The selected ideal combination drug should be able to work safely, quickly and effectively, and be tolerated by all age groups [21]. The complementary advantages of AS201-01 in combination with DHP is in line with research conducted by Zein et al., which reported that A. paniculata had *in vitro* anti-malarial activity in combination with chloroquine, artemisinin, against *Plasmodium falciparum*; both *A. paniculata* extracts or treatment combined with artemisinin was reported to have significant antimalarial effects. Increasing *A. paniculata* dose yielded higher antimalarial activity [22]. The antimalarial ability of *A. paniculata* was also described by Widyawaruyanti et al., which found that *A. paniculata* was able to reduce the density of placental parasites in pregnant mice infected with *P. berghei* [23]. The active ingredient in AS201-01 is andrographolide, the main active component of *A. paniculata*. Based on previous research, the anti-malarial activity of andrographolide is due to the effects on the ring stage of the parasite [7]. In addition, andrographolide has been shown to have schizonticidal activity and works on parasitic food vacuoles by inhibiting the detoxification process of heme [24, 25]. AS201-01 can also increase TGF-β expression. Increased TGF-β expression in the placenta can decrease the expression of adhesion molecules that reduces parasite abundance [23]. DHP is a standard antimalarial drug that contains dihydroartemisinin and piperaquine phosphate. Dihydroartemisinin is an active ingredient of artemisinin that binds to iron ions and produces free radicals or Reactive oxygen species (ROS) which will inhibit and modify various molecules that cause the death of parasites [26, 27], whereas piperaquine works on a late trophozoite stage of parasite [28]. The combination of AS201-01 and DHP works on their respective target action in inhibiting the growth of parasites. The right combination of dosage and duration of use of these two treatments will be able to produce the most effective combination in reducing peripheral and placental malaria and requires further study.

The effect of the combination of AS201-01 and DHP as well as DHP on their own resulted in foetal morphological abnormalities although lower abnormalities were found in the combination group. AS201-01 treatment only resulted in no morphological abnormality which shows that DHP is likely the dominant factor in treatment related foetal morphological abnormalities. A study by Longo et al. on *in vitro* mouse embryos found that dihydroartemisinin on influenced primitive red blood cells during the hematopoiesis process in the yolk sac. High dihydroartemisinin concentration and prolonged exposure inhibited angiogenesis, causing tissue damage and defects in embryonic morphology [29]. Studies conducted in rats, rabbits or mice shows a general effect that an increase in the dose of artemisinin and its derivatives (artesunate, arteether, dihydroartemisisnin, and arthemeters), either singly or in combination with other drugs during pregnancy is associated with an increase in post-implantation loss with

total resorption at higher dose levels. Some studies also show instant morphological abnormalities at dose levels that cause foetal resorption, without maternal toxicity [29–31].

When AS201-01 was used as the only treatment, there was no morphological abnormality, which shows that there is no toxic effect of AS201-01 on the foetus. This is in line with previous studies reported by Mishra et al. that there was no toxicity effect of *A. paniculata* extract in the organ systems of foetuses from treated mothers [32]. *A. paniculata* extract tested using the Brine shrimp Lethality assay method found no toxic effect on *Artemia salina* larvae [33]. Furthermore, Bardi et al. found a hepatoprotective effect of a *A. paniculata* ethanol extract in thioacetam-induced mice [34].

Combination therapy of AS201-01 with DHP caused a significant reduction in IFN-γ pro-inflammatory cytokines ($p<0.05$) and anti-inflammatory cytokines IL-10 in the placenta ($p<0.05$). However, expression of TNF-α pro-inflammatory cytokines were not significantly affected compared to the placebo control ($p>0.05$).

The balance between the pro- and anti-inflammatory responses contributes substantially to the infection outcome. When malaria infection starts, the host will produce pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-1, and IL-6 which play a critical role in controlling parasitic abundance. Increased anti-inflammatory cytokines such as IL-4, IL-5, IL-6 and IL-10 will occur later in the infection process as a mechanism to reduce the expression of pro-inflammatory cytokines. The excessive pro-inflammatory mediators be fatal when excessive, and cannot be regulated. Hence, the balance between pro-inflammatory and anti-inflammatory responses is needed and play an important role in the regulation of an effective immune response to malaria [35–37]. In this study, there was no significant difference in TNF-α expression of the combination group (G4) and DHP group (G3) compared to the placebo control group (G1). TNF-α levels in the combination group and DHP group were likely to cause morphological abnormalities in the foetus as high and excessive levels of TNF-α can lead to the extensive and severe tissue damage [36, 38–40]. However, in the AS 201-01 group (G2), TNF-α expression was also not significantly decreased compared to the placebo group, yet there was found to be normal foetal morphology. For that reason, AS 201-01 may be able to provide a protection to the foetus through an antioxidant effect that may inhibit the production of ROS resulted from the binding of PfEMP-1 with CSA [20]. *A. paniculata* is reported to have an antioxidant activity that can prevent oxidative stress from damaged cells. Andrographolide in *A. paniculata* can significantly increase the activity of antioxidant enzymes such as Catalase, Superoxide dismutase, and gluthation S-transferase [41]. However, when used in combination with DHP in this study, the protective effect of AS201-01 was not observed. Increasing AS201-01 dose may provide protection to the foetus although this would require further research.

Conclusions

The AS201-01 tablet containing the ethyl acetate fraction of *A. paniculata* exhibits the potential to reduce the dihydroartemisinin-piperaquine (DHP) toxicity of malaria treatment in pregnant mice.

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