Comparison of the provision of champedon trunk shell capsule extract and artesunate on placental histopathologic classification in pregnant mice (*Mus musculus*) malaria model

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ABSTRAK

Tujuan: Membandingkan pengaruh pemberian ekstrak kulit batang cempedak dengan artesunat terhadap klasifikasi histopatologi plasenta pada mencit bunting model malaria.
Bahan dan Metode: Penelitian eksperimental laboratorium pada mencit strain BALBc dengan randomisasi disertai kontrol pembanding. Sebanyak 30 mencit bunting dibagi 3 kelompok. Pada hari-10 tiap kelompok diinfeksikan *P. berghei*. Selanjutnya pada hari-11 tiap kelompok dilakukan pemeriksaan hapusan darah tipis dan bila positif terinfeksi selanjutnya diberikan antimalaria. Kelompok P1 mendapat ekstrak kulit batang cempedak 100 mg/kg BB/hari per sonde dua kali per hari selama 5 hari. Kelompok P2 mendapat artesunat 36,4 mg/kgBB/hari per sonde selama 3 hari dilanjutkan CMC Na selama 2 hari, dan kelompok P3 mendapat plasebo (CMC Na) selama 5 hari. Pada hari-16 mencit dibedah saat kehamilan hari-16 kemudian diambil plasentanya dan dibuat preparat untuk melihat klasifikasi histopatologi plasenta menurut Rogerson.
Hasil: Setelah kelompok yang menerima kapsul kulit batang cempedak, artesunat dan placebo dikelompokkan menurut klasifikasi histopatologi plasenta dari Rogersen, hasil uji Statistik menunjukkan nilai p=0,004 (p<0,05), berarti terdapat dua kelompok yang memiliki perbedaan bermakna, sehingga uji dilanjutkan dengan uji Post Hoc Mann-Whitney. Hasil uji kelompok kapsul kulit batang cempedak dan plasebo diperoleh nilai p=0,007 (p<0,05), menunjukkan perbedaaan bermakna. Pada kelompok artesunat dan plasebo diperoleh nilai p=0,003 (p<0,05), juga menunjukkan perbedaaan bermakna. Hasil uji kelompok ekstrak kulit batang cempedak dan artesunat diperoleh nilai p=0,475 (p<0,05), menunjukkan tidak terdapat perbedaaan yang bermakna.
Simpulan: Klasifikasi histopatologi plasenta pada mencit bunting model malaria yang mendapat antimalaria ekstrak kulit batang cempedak 100 mg/kgBB lebih baik daripada plasebo dan setara dengan artesunat 36,4 mg/kgBB. **(MOG 2017;25:71-76)**

Kata kunci: histopatologi plasenta; cempedak; malaria.

ABSTRACT

Objectives: To compare the effect of chemphedon trunk shell capsule extract with artesunate on placental histopathologic classification in pregnant mice malaria's models.
Materials and Methods: This study was a randomized experimental laboratory study in BALBc strains mice with controls. A total of 30 pregnant mice were divided into 3 groups. On day 10 each group was infected with *P. berghei*. Furthermore, on day 11 each group was subjected to thin blood smear examination and subsequent infection when administered antimalarial positive. Group P1 received Chemphedon trunk shell extract 100 mg/kg BW/day per sonde two times per day for 5 days. Group P2 received 36.4 mg artesunate/kg BW/day for 3 days followed by CMC Na per sonde for 2 days, and group P3 received placebo (CMC Na) for 5 days. On day 16 the mice were dissected on 16 days of pregnancy and the placenta was taken and preparations were made to observe histopathological classification of the placenta according to Rogerson. Grouping was performed according to placental histopathological classification by Rogerson.
Results: Groups receiving cemphedon trunk shell capsule extract, artesunate and placebo revealed p=0.004 (p<0.05), showing that there were two groups with significant difference. To determine which group had significant difference, the test was followed by Mann-Whitney post-hoc test. The results showed chempedon trunk shell capsule group and placebo obtained p=0.007 (p<0.05), indicating significance. Artesunate and placebo groups revealed p=0.003 (p<0.05), also indicating significance. The test results of chemphedon trunk shell capsule extract and artesunate groups showed p=0.475 (p<0.05), indicating no significant differences.
Conclusion: Placental histopathologic classification on pregnant mice malaria's model that received antimalarial chemphedon trunk shell capsule extract of 100 mg/kg BW is better than placebo and equivalent to artesunate of 36.4mg/kg BW. **(MOG 2017;25:71-76)**

Keywords: placental histopathology, cemphedon, malaria.

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INTRODUCTION

Malaria is a tropical and subtropical endemic disease. Based on 2007 data, every year no less than 125 million pregnant women are found in endemic areas. Around the world there are 10,000 women and 200,000 babies die each year from malaria in pregnancy.[1] In addition, malaria in pregnancy is responsible for 400,000 cases of severe anemia and 10,000 maternal deaths each year.[2] Indonesia is a country at risk of malaria. In 2007, there were 1.75 million clinical cases of malaria and the number of malaria positive patients in microscopic examination was 311,000 cases.[3]

An increase in cases of parasitic resistance to antimalarial drugs is the biggest problem in the world of

health. Artesunat is highly effective against multi-drug resistant P. falciparum strains. Therefore, the use of this drug is increasingly widespread to overcome malaria. There are currently problems that of 23 African countries using artesunate as first-line treatment, six countries (Burkina Faso, Democratic Republic of Congo, Erithrea, Gabon, Ghana and Sierra Leone) have reported a treatment failure rate of 10%. In Indonesia, where artesunate-amodiaquine is also a first-line treatment, four out of eight studies showed a treatment failure rate of 10%.[4] Several studies have shown that high doses of artesunate can cause neurotoxicity such as central nervous system damage in rat brains. Several studies have shown an association between growth constraints in the uterus and teratogenesis.[5]

Epidemiological studies show that pregnant women are more susceptible to malaria infections. Malaria infections during pregnancy cause infected red blood cells (iRBCs) to be absorbed into the intervillous space of the placenta and binding of iRBCs to syncytiotrophoblast cells. This is followed by infiltration of inflammatory cells in the intervillous space, resulting in lesions of the placenta, such as necrosis, loss of microvilus and thickening of the trophoblast basement membrane. This phenomenon is referred to as placental malaria. Placental malaria is an important health problem. An estimated 200,000 babies die each year. It is associated with maternal anemia, abortion, prematurity, stillbirth and low birth weight.[5-7]

To overcome those conditions, countermeasures have been done. However, morbidity and mortality rates remain high, requiring new drugs with effective targets, low toxicity and affordability. One method of developed treatments is the use of herbal ingredients, the chempedon (*Artocarpus champeden*) which has been empirically used to treat malarial fever. The use of chempedon as a phytopharmaca product using a multicomponent approach requires a marker compound, instead of employing a strategy to find single substance as that in modern medicine. The marker has been extracted from chempedon trunk shell extract, the morakhalkon A.[9,10]

Eighty percent of standardized ethanol extract of chempedon trunk shell is able to inhibit up to 80% growth of parasite in a dose of 100 mg/kg BW.[11] Artesunate dose of 36.4 mg/kg BW once daily for 3 days is able to inhibit parasitic growth of 83.92%.[12] In phase 2 of this clinical trial, chempedon trunk extract 120 mg per capsule was administered twice daily for five days. On day 4 there were 71.43% of patients who showed negative blood smear, and on day 6 there was 95.26% with negative blood smear. These results were not statistically significant with chempedon trunk shell extract of 120 mg per capsule given twice daily for five days combined with artesunate capsule of 100 mg per capsule for three days. In this treatment, on day 4 as much as 70.59% of the patients showed negative blood smear and on day 6 88.24% showed negative blood smear.[13]

Placental histology is a sensitive method for the diagnosis of malaria in pregnancy. The diagnosis of placental histology is more sensitive (91%) than peripheral blood (47%) or placental blood (63%). Rogerson et al. proposed a modification that divided placental malaria infections based on the significance of parasites, hemozoin pigments and fibrin. Malaria infection of the placenta includes two categories, acute infection (only parasites and minimal hemozoin deposition in macrophages, but not fibrin) and chronic infection (parasitic and hemozoin deposition in fibrin).[6] These histopathologic changes are associated with impaired syncytiotrophoblast and cytotrophoblast.[6,7,14] The presence of accumulated infected erythrocytes, trophoblastic membrane thickening and perivillous fibrin deposits lead to uterine-placental hemodynamic changes and fetal maternal-exchange changes that contribute to fetal growth.[15,16]

It is therefore important to study the effects of chempedon trunk shell extracts that act on the inhibition of intraerythrocytic parasite membrane formation and parasitic food vacuoles. Food vacuole, as a site of hemoglobin metabolism, will inhibit the process of amino acids formation. Similarly, the inhibition of the heme detoxification process results in the accumulation of free heme in toxic food vacuoles and reduced Hz formation.[17] This affects the metabolism that causes parasitic death, which ultimately affects the parasite density and Hz deposition. According to Rogersen, a diagnosis based on placental histology is a sensitive method for the diagnosis of placental malaria. As chempedon trunk shell extract has never been used in pregnant women for malaria treatment and because human involvement is ethically impossible, this study used pregnant BALBc strains mice (Mus muscle) infected with *P. berghei* as models.

MATERIALS AND METHODS

This was a laboratory experimental study on BALBc strain mice (*Mus musculus*) with randomization with comparative control. This study was conducted at ITD Airlangga University and Embryology Laboratory, Faculty of Veterinary Medicine, Airlangga University from November 2013 to January 2014.

The sample of this study was 30 pregnant BALBc strain mice of 25-30 g divided into 3 groups, ie P1, P2 and P3. On day 10 each group was inoculated by *P. berghei*.

Furthermore, on day 11 thin blood smear examination was performed on each group, and, if positively infected, antimalarial was then administered. Group P1 received chempedon trunk shell capsule bark of 100 mg/kg/day per sonde twice daily for 5 days. P2 group received artesunate 36.4 mg/kgBW/day per sonde for 3 days, followed by CMC Na for 2 days, and P3 group received placebo (CMC Na) for 5 days. On day 16 post-treatment samples were taken.

The inclusion criteria in this study were BALBc mice aged 2 - 3 months, never pregnant, weight 25-30 grams and healthy. The exclusion criteria were having been used as experimental animals, disabled, and not pregnant after mating. The dropout criteria were uninfected after the third day of inoculation (12th day of pregnancy), reabsorbed pregnancy, and died after treatment. The classification of placental histopathology according to Rogerson suggests that the greater the classification, the better the histopathology because the accumulation of parasites and Hz pigments, which have a direct effect on fetomaternal blood flow, is less.[6,18]

Table 1. Classification of malaria infections in the placenta.[2]

Pathological classification	Description
1	Parasite is present No pigment (Hz) found
2	Parasite and pigment (Hz) found in monocytes
3	Parasite and pigment (Hz) found in fibrin
4	No parasite is presenet Only pigment
5	No parasite and pigment

RESULTS AND DISCUSSION

Of 30 mice that were dissected and met the criteria, placental tissue samples were taken and then histologic preparations were made. The slides were stained with Hematoxilin-Eosin as the basic staining for histologic preparations. This histopathological examination was intended to determine the degree of plasmodium infection in the placenta according to Rogerson's method which has been modified by the classification method.

Table 2 shows that after the data were classified by classification, the results in placebo group showed that plasmodium was present in all mice models. The artesunate group had 8 mice with positive plasmodium and 2 mice with negative plasmodium. The group receiving chempedon trunk shell extract capsule showed 6 mice had positive plasmodium and 4 negative plasmadium.

Table 2. Rogersen placental histopathology classification in pregnant mice (*Mus musculus*) malaria model with various treatments

Histopathological classification	Placebo	Artesunate	Chempedon
1	1		
2	5		1
3	4	8	5
4		2	2
5			2

The placental histopathologic profile in P3 treatment group (*P. berghei* without therapy) in Table 2 shows all placentas had *P. berghei*, pigment hz in monocytes (classifications 1, 2 and 3), increased rosetting, severe erythrocyte damage and attached to endothelial wall (Figure 1 slide A, slide B and C). It implies necrosis of syncytiotrophoblasts, microvilal loss and thickening of trophoblast basal membrane, causing reduced blood flow to the placenta and the fetus, resulting in abortion, premature birth, stillbirth, and low birth weight.[6]

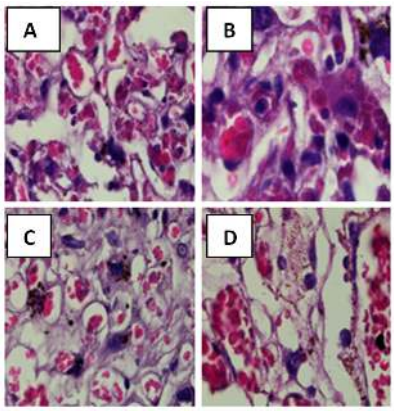

Figure 1. Placental histopathology A: Classification 1, B: Classification 2, C: Classification 3 and D: Classification 4 (HE staining, 1000x enlargement, microscope Nikon H600L, DS Fi2 300 megapixel camera).

In P2 group, consisting of mice infected with *P. berghei* and received artesunate therapy (Table 2), placental histopathologic classification showed 8 mice placenta with positive plasmodium, Hz pigment in fibrin, increased rosetting erythrocyte and mild erythrocyte defects. In P1 group, consisting of mice infected with *P. berghei* and treated with chempedon trunk shell extract (Table 2), placental histopathologic classification showed 6 placentas of mice with positive plasmodium, Hz pigment in fibrin, increased rosetting erythrocyte and mild erythrocyte defects. There were 2 placentas without plasmodium.

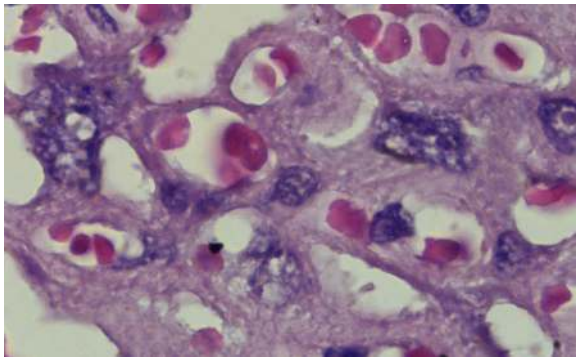

Figure 2. Placental presentation without plasmodium infection (classification 5) (HE staining, 1000 x magnification, microscope Nikon H 600L, DS Fi2 300 megapixel camera).

Table 3. Classification of placental histopathology of pregnant mice malaria model with various treatments.

Histopathological classification	Placebo	Artesunate	Chempedon	p
Medium	2	3	3	0.004
Minimum	1	3	2	
Maximum	3	4	5	

Kruskal Wallis test yields p=0.004, meaning there were at least two groups that had significant differences. To find out which groups that had significant differences, the test was continued with Mann-Whitney Post-Hoc test.

Table 4. Classification of placental histopathology of pregnant malaria model compared with chempedon trunk shell extract with placebo

Histopathological classification	Placebo	Chempedon	p
Medium	2	3	0.007
Minimum	1	2	
Maximum	3	5	

Table 5. Classification of placental histopathology of pregnant mice malaria model versus artesunate with placebo

Histopathological classification	Placebo	Artesunate	p
Medium	2	3	0.003
Minimum	1	3	
Maximum	3	4	

Statistical analysis showed that placental histopathology classification between antimalarial-receiving groups in the form of chempedon trunk shell extract, artesunate and placebo using Kruskal-Wallis test had p=0.004. The results of this analysis showed significant differences ($p < 0.05$), which means that at least two groups had significant differences. To determine which groups had different inhibition of placental histopathological changes, a Mann-Whitney Post Hoc test was performed.

Table 6. Classification of placental histopathology of pregnant bunting malaria model versus artesunate with chempedon trunk shell extract

Histopathological classification	Artesunate	Chempedon	p
Medium	3	3	0.475
Minimum	3	2	
Maximum	4	4	

Post Hoc Mann-Whitney test of chempedon trunk shell extract and placebo group resulted in p=0.007 with significant value of $p \leq 0.05$, indicating a significant difference in placental histopathology classification in pregnant mice malaria model between those receiving chempedon trunk shell extract and placebo. Inhibition of *P. berghei* growth was due to chempedon trunk shell extract that contained prenylated flavonoid compound and was given in a dose of 100 mg/kg BW mice (twice daily for 5 days). The study by Widyawaruyanti et al (2007-2008), which gave the extract from the first day after the mice were infected, reported that malaria parasite growth could be inhibited up to 80% in a dose of 100 mg/kg BW. The prenylated flavonoid compound of *A. champeden* may cause delayed growth of malaria parasite beginning in the first 24 hours as an early stage of trophozoid formation, thereby inhibiting the ring-stage formation.[12]

Chempedon trunk shell extract as a phytopharmaca material containing flavonoid compounds can serve as antimalarial by inhibiting the development of malarial parasites from ring stages to tropozoites and causing schizont to grow with abnormal morphology, inhibiting hemoglobin degeneration, detoxificating of parasitic heme, and inhibiting New Permeation Pathway (NPP) on erythrocyte membranes induced by parasites.[19]

Mann-Whitney Post Hoc test of artesunate and placebo groups yielded p=0.003 with a significant value of $p \leq 0.05$, indicating significant difference in placental histopathology of the pregnant mice malaria model between those receiving artesunate and placebo. Artesunate was given from the first day after the mice were infected and given for three days. Artesunate is known to work specifically during the schizontiside blood stage. Characteristics of artesunate is that it rapidly eliminates parasites in the body for about 48 hours. After oral administration in humans, artesunate is hydrolyzed by

stomach acid, then absorbed rapidly but not perfectly with an average absorption time of 0.78 hours. Peak concentration in plasma is achieved after oral administration 1 - 2 hours and eliminated from the body after 3 hours.[20]

Artesunate is metabolized in the liver and spleen, but short half-life (t1/2=1-2 hours) increases its potential to persist or re-emerging of asexual parasites in peripheral blood if artesunate is given as monotherapy in a short period of time. Artesunate action mechanism that has been disclosed revealed that artesunate acts by inhibiting the calcium-dependent ATP-ase enzyme (PfA TP6). Free radicals produced by artemycin bind and inhibit PfATP6 irreversibly and specifically. The AT Pase function of the Na+/K + ion pump complex system is to regulate ion content in the cell. Failure of PfATP6 function results in a drastic reduction of potassium ions in the cells that is highly lethal for the parasite.[20,24]

In placebo group that acted as a control (CMC-Na), decrease in the number of parasitemia was not visible, while, instead, its number did increase. This is supported by the results of other studies that mice infected with *P. berghei* without treatment and maintained at room temperature rapidly increased the rate of parasitemia.[21]

Mann-Whitney Post Hoc test of chempedon shell trunk and artesunat groups yielded p=0.475 with significance value p<0,05, so statistically there was no significant difference. It shows that chempedon trunk shell extract of 100 mg/kg BW can act as antimalarial because there was no significant difference in placenta histopathology classification between 100 mg/kg BW chempedon trunk shell and artesunate. However, in histopathologic classification examination (Table 2) in P1 group (chempedon trunk shell extract) there were 4 mice without parasites in the placenta (classifications 4 and 5) in which 2 did not show hz (classification 5), while in P2 group (artesunate) 2 mice were found without parasites (classification 4), although it was not statistically significant.

In group P1 there were 2 mice without plasmodium and hz. This suggests that chempedon trunk shell extract acts on a food vacuole that acts as a hemoglobin degeneration process inhibitor and parasitic heme detoxification that results in parasitic death. Khalkan, a flavonoid compound from chempedon trunk shell extract, has activity of inhibiting the growth of parasites through the mechanism of cysteine protease enzymes inhibition.[9,10] Hemoglobin provides most of the parasite amino acid requirements for the synthesis of proteins. Hemoglobin degradation is a central metabolic process in the growth and maturation of malarial parasites. Detoxification of heme to Hz, as a process of biocrystallization, is a major mechanism occurring in the malaria parasite. Hz has been used as a biomarker of malarial infection acquired in large quantities in reticulo-circulation during infection.[22] Since the tropical phase, the parasite RNA content has increased, and since that process DNA replication has been 8-32 times. This rapid growth of parasites also increases the need for amino acids to be used in the process of protein synthesis.[9,17]

In P2 group, artesunate was administered for three days with the aim of knowing its effectiveness as antimalarial when compared with the extract of chempedon trunk shell for five days. This was due to increased parasitemia in the placenta in P2 group, which after the third day artesunate was replaced by CMC-Na. It is possible that the artesunate is eliminated from the body after the third day relating to short half-life, where the peak concentration in plasma is achieved after oral administration 1-2 hours and is eliminated from the body after 4 hours.[24] After day 3 artesunate function as antimalarial will be reduced and unable to inhibit parasite growth compared to chempedon trunk shell given for five days. The administration of artesunate in a dose of 36.4 mg/kgBW once a day for 3 days was able to inhibit the growth of parasite as much as 83.92%.[12] The parasite decline was still more than 3%. This amount was considered as to still be able to increase the number of red blood cells infected with *P. berghei* because, according to Dewi et al., (1996), the number of parasitemia is considered positive if at least 2-3%. Therefore, with the number of red blood cells infected by *P. berghei* was higher than 2-3%, it would facilitate *P. berghei* to develop again after the administration was discontinued on the third day.[23]

CONCLUSION

The provision of chempedon trunk shell extract as an alternative for antimalarial therapy in pregnant mice malaria model results in a placental histopathology classification similar to that of artesunate.

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