Schizonticidal effect of a combination of *Amaranthus spinosus* L. and *Andrographis paniculata* Burm. f./Nees extracts in *Plasmodium berghei*-infected mice

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Abstrak

Latar belakang: *Bayamduri (Amaranthus spinosus L.) dan sambiloto (Andrographis paniculata* Burm F*) adalah herbal tradisional yang digunakan untuk pengobatan malaria. Tujuan penelitian ini adalah untuk menentukan aktivitas anti malaria skizontisidal kombinasi kedua ekstrak terhadap mencit yang diinfeksi Plasmodium berghei.*

Metode: *Mencit jantan (galur* Balb/c*) dengan berat 28-30 g, 7-8 minggu, dibagi menjadi 5 kelompok secara acak, tiap kelompok terdiri atas 4 ekor mencit. Kelompok A: kontrol negatif, kelompok perlakuan dengan ekstrak tanaman (kelompok B, C, D) diberikan 1 kali per hari selama 7 hari. Kelompok B: Amaranthus 10 mg/kgBB, kelompok C: Andrographis 2 mg/kgBB, kelompok D: kombinasi ekstrak Amaranthus + Andrographis 10 mg + 2 mg/kgBB, dan kelompok E: diberi klorokuin 10 mg/kgBB sekali sehari selama 3 hari.*

Hasil: *Terjadi peningkatan berat badan hanya pada kelompok D, peningkatan kadar hemoglobin pada semua kelompok perlakuan (C, D dan E secara bermakna dibandingkan dengan kontrol, p < 0,05). Aktivitas skizontisidal darah terlihat pada semua kelompok perlakuan, aktivitas tertinggi hampir 90% terlihat pada kelompok D dan E. Tingkat* survival *adalah 100% pada semua kelompok.*

Kesimpulan: *Kombinasi ekstrak Amaranthus dan Andrographis (10 mg + 2 mg/kgBB) menunjukkan aktivitas skizontisidal darah sama baiknya dengan pemberian klorokuin 10 mg/kgBB.* ***(Med J Indones. 2012;21:66-70)***

Abstract

Background: *Amaranthus spinosus* and *Andrographis paniculata* are traditionally used as antimalarial herbs, but the combination of both has not yet been tested. The aim of this study was to determine the schizonticidal anti-malaria effect of a combination in *Plasmodium berghei*-infected mice.

Methods: Male mice (Balb/c strain) weighing 28-30 g, 7-8 weeks old, were randomly devided into 5 groups of 4 animals each. Group A: controls (nil) and 4 treatment groups (B, C, D, and E). Group B: Amarathus 10 mg/kgBW, group C: Andrographis 2 mg/kgBW, group D: combination of Amaranthus + Andrographis 10 mg + 2 mg/kgBW. All treatment with plant extracts was administered orally, once per day for 7 days. Group E was given chloroquine 10 mg/kgBW, once a day orally, for 3 days.

Results: The body weigh increased only in group D, hemoglobin concentration increased significantly vs controls ($p < 0.05$) in treatment groups C, D, and E, and blood schizonticidal activity was seen in all treatment groups, highest at almost 90% in groups D and E. Survival rate was 100% in all groups.

Conclusion: The combination of Amaranthus and Andrographis (10 mg + 2 mg/kgBW) exerts the same blood schizonticidal activity as chloroquine 10 mg/kgBW. ***(Med J Indones. 2012;21:66-70)***

Keywords: *Amaranthus spinosus, Andrographis paniculata, Balb/c mice, Plasmodium berghei, schizonticidal effect*

Malaria affects 300-500 million people and causes 1-2 million(s) of deaths every year.[1] Drug resistance, limited ability to store and distribute medicines and poverty of the target population are some of the major obstacles to providing effective malaria treatment in endemic areas.[2] In Indonesia, various attempts were made to control malaria. Although impregnated bed nets are successful means for vector control,[3] it is difficult to eradicate malaria in total, because of the complex interrelation between environmental factors, mosquito vectors, chemicals and drugs used.[4] Biological barriers towards toxic and otherwise problematic chemicals applied, but also infrastructure, administrative and educational limitations together with low income in the endemic areas reduce the efficacy of eradication programs.[5]

Since the first malaria vaccine against *Plasmodium falciparum* is still in phase 3 trial,[6,7] prevention and treatment of malaria relies strongly on small molecule inhibitors of plasmodial growth,[2] but chemotherapy has been greatly hampered by the emergence of parasite resistance towards antimalarials, especially in *Plasmodium falciparum*, the species responsible for most deaths from malaria.[8]

Chloroquine is used as a standard antimalarial drug and is still effective, although parasite resistance already exists

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in some areas in Indonesia.[9] Subsequent generations of chemical antimalarials widely developed similar plasmodial drug resitance, which makes it necessary to search for new solutions including the application of anti-malarials derived from plants and herbs. This approach appears appropriate, because Indonesia is rich in medicinal plants, many of which have been used traditionally by our ancestors in the prevention and treatment of malaria.

Thus, our research compares the anti-malarial potential of two herbal extracts, spiny Amaranth (*Amaranthus spinosus* L.) and Andrographis (*Andrographis paniculata* Burm. f./Nees) with the combination of both herbs in a malaria mouse model. The aim of this study is to determine the schizonticidal effect of this combination vs chloroquine by growth inhibition of *Plasmodium berghei in vivo*, which to our knowledge has not been investigated before.

METHODS

Plant material and preparation of extracts

Samples of spiny Amaranth (*Amaranthus spinosus* L.*,* Indonesian name *bayam duri*) and Andrographis, also known as King of Bitters or as Kalmegh (*Andrographis paniculata* Burm. f./Nees*,* Indonesian name *sambiloto)* were extracted with ethanol (70%) and dried in the Laboratory of National Institute of Health Research and Development - Ministry of Health (NIHRD- MoH) Indonesia.

Air-dried powdered plant materials (500 g) were extracted twice using 70% ethanol (1 L) for 72 hours at room temperature to ensure exhaustive extraction. The filtrates were concentrated under reduced pressure (*in vacuo*) in a Rotavapor Yamato (Ogawa Seiki Ltd., Tokyo), followed by removal of residual water at 40°C in a Dry Oven Seima (Ogawa Seiki Ltd., Tokyo). Extract yields of 70 g Amaranth and 66.5 g Andrographis were obtained. The extracts were stored at 40°C until needed for testing. For treatment, the dry extracts were dissolved in water at concentrations, which allowed the administration of a single daily dose in 0.3 mL; Amaranth, 1 mg/mL; Andrographis, 0.2 mg/mL and for the combination: Amaranth, 2 mg/mL together with Andrographis, 0.4 mg/mL. Chloroquine was dissolved at 1 mg/mL.

Parasite strain

We used a chloroquine-sensitive *Plasmodium berghei* (Pb) strain to assess the *in vivo* intrinsic antimalarial activity. The parasite strain was kept alive by continuous re-infestation into Balb/c mice.

Animals

Male mice (Balb/c strain) weighing 28-30 g, 7-8 weeks from Animal Laboratory of NIHRD-MoH Indonesia breeding, were used for this study. The animals were kept in an air-conditioned room, were fed standard mouse diet and had access to clean water *ad libitum*. The animals were caged in groups of four and housed in the Centre of Animal Laboratory of NIHRD-MoH, Jakarta, where the assays were performed between July 2010 and April 2011, after approval by the Ethical Committee of Faculty of Medicine, Universitas Indonesia.

Inoculum

Parasitized erythrocytes were obtained from a donor-infected mouse by cardiac puncture in heparin and diluted with sterile blood from male mice at the same age. Animals were inoculated intraperitoneally with infected blood suspension (0.2 mL) containing 10^6 parasitized erythrocytes lethal inoculums on day zero (D0). Infected mice with parasitemia of 1-5% were allocated to 5 groups of four mice each.

Treatment

Animal group A. Control (nil); group B. Amaranth treatment (10 mg/kgBW; once per day for 7 days); group C. Andrographis treatment (2 mg/kgBW; once per day for 7 days); group D. Combination treatment (10 mg + 2 mg/kgBW; once per day for 7 days); group E. Chloroquine treatment (10 mg/kgBW; once per day for 3 days). All treatment was administered *per os*.

Evaluation of blood schizonticidal activity in early infection (4-days test)

The blood schizonticidal activities of the plant extracts and chloroquine (diphosphate salt, SIGMA) as standard drug were assessed by classical 4-day suppressive test.[10] From day 0 (D0) to day 3 (D3), the aqueous extract solutions were administered once a day to *Plasmodium berghei*-infected mice. Subsequently, tail blood smear was taken and stained with Giemsa 3%. To determine parasitemia, the parasitized red blood cells per 1000 red blood cells were counted blindedly.

Percentage of growth inhibition of the parasites was calculated by the following formula

$$\text{Growth inhibition (\%)} = \frac{\text{Parasitemia in control} - \text{parasitemia with treatment}}{\text{Parasitemia in control}} \times 100\%$$

Survival rate

By the end of the experiment, after 7 days (D6), all mice still alive were counted and expressed as percentage of the total number of four in each group.

Data analysis

The results were analyzed statistically using one-way ANOVA to identify the differences between treated groups and controls. Differences were considered significant at $p < 0.05$.

RESULTS

Table 1. Average of mouse body weight before and after treatment

	A	B	C	D	E
Average BW (D0)	32.4	32.4	31.2	30.4	31.1
Average BW (D6)	30.7	31.4	29.4	30.9	31.1
%ofchange	-5.3	-3.1	-5.8	+1.6	0

Loss of body weight (BW) after inoculation with Pb was 5.3% in controls (A) and 5.8% in group C. The difference between both group was not significant. In group B, the loss of BW was only 3.1% , i.e., loss of BW after inoculation was reduced by more than 40% in group B relative to controls. No loss in BW was observed in group E, whereas in group D (combination A+B) BW slightly increased by 1.6%. The latter difference was highly significant ($p < 0.01$) vs controls (group A). No change in BW before and after treatment was seen with chloroquine (group E, Table 1).

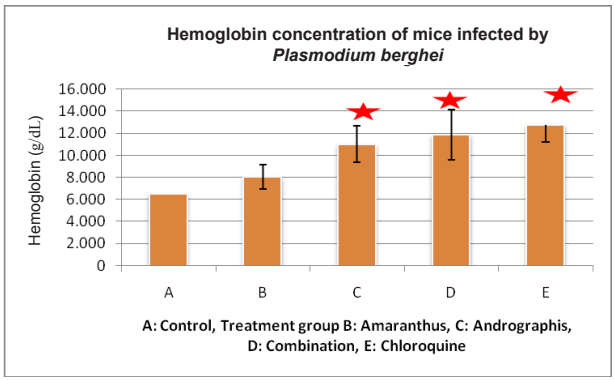

Figure 1. Hemoglobin (Hb) concentration [g/dL] on day 6; values of groups C, D, and E (marked with stars) are significantly different from control group A ($p < 0.05$)

Mean hemoglobin values [g/dL] on day 6 (D6) were 6.6 (0.544) in the control group A, 8.1 (1.090) in group B, 11.3 (1.625) in group C, 11.9 (2.296) in group D, and 12.8 (1.567) in group E. The last three values (marked with stars in figure 1) were significantly different from controls ($p < 0.05$).

Mean blood schizonticidal activity of treatment, i.e., inhibition of parasitemia on day 3 (D3) was 39.42% (20.047) in group B, 48.19% (14.900) in group C, 88.17% (4.494) in group D, and 89.11% (1.100) in group E (Figure 2).

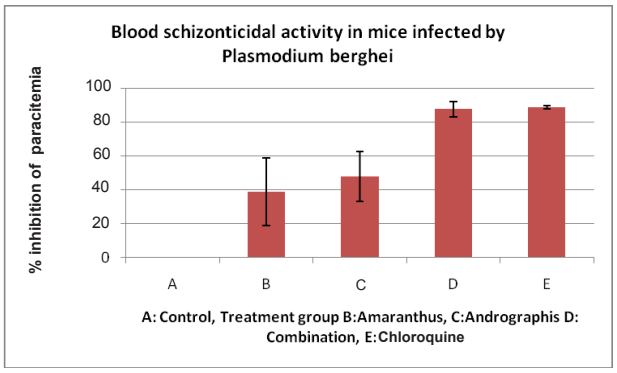

Figure 2. Blood schizonticidal activity in mice infected by Plasmodium berghei

Survival rate: at the end of the experiment, four mice in each group were still alive, i.e., the survival rate was 100% in each group (data not shown).

DISCUSSION

In our mouse model, the two extracts, Amaranth and Andrographis, exert different effects on parameters measured. Loss of BW after infection with *Plasmodium berghei* was counteracted by Amaranth extract (loss reduced from 5.3% to 3.1%) but not by Andrographis (loss 5.8%). With chloroquine, there was no change in BW development over 7 days, whereas with the combination of Amaranth and Andrographis, we observed a slight increase in BW by 1.6%. The difference between control group and the combination of plant extracts was highly significant ($p < 0.001$).

Hemoglobin concentration was higher in all treatment groups than in controls: Andrographis, the combination of the two plant extracts and chloroquine increased the hemoglobin concentration significantly vs controls, whereas the slight increase by Amaranth was not significantly different from control.

From these results, we can conclude that – at the dosage applied - Amaranth exerts a stronger beneficial effect on BW development than Andrographis, whereas the latter increases hemoglobin concentration more effectively.

The specific parameter measured, blood schizonticidal activity i.e., inhibition of parasitemia in the infected mice is similar for both extracts, almost 40% in the Amaranth group B and almost 50% in the Andrographis group C, whereas in group D with the combination of both extracts and in the chloroquine group E we observe almost 90% inhibition of parasitemia.

In general, the dosage of Amaranth (10 mg/kgBW) is about 5 times higher than Andrographis (2 mg/kgBW). The dosage was chosen from our own preliminary

experiments (not shown). Other authors had used higher dosages: in a dose range from 100 to 900 mg/kgBW, Hilou et al[11] had found ED50 of 789 mg/kgBW and extrapolated LD50 of 1473 mg/kgBW for their Amaranth stem bark extracts.[11] We applied the lowest dose in our range of 10 mg/kgBW and observed a stronger inhibition of parasitemia (39.42%) than they (15.38%); at 300 mg/kgBW these authors reported a value of 30.94%, further increasing at higher dosages.[11] It was our intention to test the combination of lowest dosages of the two extracts. Although concentrations of the active principles of our own extracts have not been determined, from our results it can be assumed that our extracts are more effective than those used by other authors, e.g. Hilou et al.[11]

According to Stintzing et al,[12] *Amaranthus spinosus* contains 23.87 mg of amaranthine equivalents per 100 g of extract. Hilou et al[11] discussed possible ways of action of betanin and amaranthine as active principles of the antimalarial effect. The chelation of Ca, Mg and Fe ions by these compounds may remove essential cofactors from enzymes of malaria parasites. Chelated cofactors are no longer available for the plasmodial metabolism.[11] Especially the betain structure of these compounds provides the opportunity for variation of pH-dependent membrane permeability and impermeability, which is an important pharmakokinetic aspect in the efficacy of antimalarial drugs.[13]

Betanin and amaranthine contain quarternary nitrogens, which block the parasite's intracellular choline transport and inhibit its phosphatidylcholine biosynthesis.[14] Moreover, the antioxidant capacity of these compounds[15] may be involved in the inhibition of heme polymerisation; unpolymerised heme is toxic to intraerythrocytic plasmodia.[16] The increase of hemoglobin in our study supports this aspect as one possible mechanism of action, especially for Andrographis and the combination of both extracts.

The active principle of Andrographis is andrographolide. On day 12 of their experimental procedure, Mishra et al[17] found 50% inhibition of parasitemia vs control at a dosis of 7 mg/kgBW, intraperitoneally. The antimalarial activity of Andrographis was ascribed to (re)activation of antioxidant enzymes, mainly SOD.[18]

Unwanted side effects or toxicity to the host organism has not been reported from Andrographis malaria studies with the mouse model. Adverse effects reported from human trials are considered mild, infrequent and self-limiting.[19]

For the combination of the extracts from Amaranth and Andrographis, various mechanisms of action are suggested, i) chelation of metal ions necessary for the parasite's metabolism, ii) increase of hemoglobin and inhibition of heme polymerisation and iii) activation of SOD. After we could show that there are additive or perhaps even synergistic pharmacodynamic effects of this combination, more detailed studies should follow.

Our study was primarily designed to see the schizonticidal effect of two extracts and their combination. In future studies, we should modify the conditions in the survival test, especially the number of animals should be increased in order to detect differences between the treatment groups in survival rate. In our combination with 10 mg/kgBW of Amaranth extract and 2 mg/kgBW of Andrographis, we reach the same blood schizonticidal antimalarial effect as with 10 mg/kgBW of chloroquine. This is a very satisfying first result, which has to be confirmed and can possibly still be improved by variation of the doses in the combination, before it can be considered for application in human patients.

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