ANTIMALARIAL EFFECTS OF METHANOLIC LEAF EXTRACT OF *ANDROGRAPHIS PANICULATA* ON *PLASMODIUM BERGHEI*

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ABSTRACT: Since the emergence of drug resistant strains of malaria parasites, the rate of resistance has been increasing and limiting adequate treatment of malaria. Consequently, there is an urgent global need to isolate new classes of antimalarial compounds from natural sources. The aim of this study was to test *Andrographis paniculata* leaf extract for the ability to treat malaria. Methanolic leaf extract of *A. paniculata* was freshly dissolved in DMSO and diluted with 0.9% NaCl to obtain the doses of 2, 20, and 100 mg/kg. The four-day suppressive, curative effects against established infection and prophylactic models of the extract were carried out by intraperitoneal treatment the extracts in *P. berghei* ANKA infected ICR mice once a day, using chloroquine as a positive control. Parasitemia was then monitored, and percent inhibition was subsequently calculated. It was found that the extracts exerted dose dependent suppressive, prophylactic and curative effects. Interestingly, the extract at a dose of 100 mg/kg showed significantly (P<0.05) the highest activity for inhibition of malaria. Moreover, combination of chloroquine and the extract showed substantial enhancement in their antimalarial activity significantly (P<0.05) when compared to chloroquine treatment alone. It can be concluded that *A. paniculata* leaf extract has potential antimalarial property and in combination with chloroquine could be an effective, alternative source of herbal antimalarial drugs.

Keywords: *Andrographis paniculata* extract, Growth inhibition, *Plasmodium berghei*

INTRODUCTION

Malaria is an infectious disease that is wide spread in tropical and subtropical areas of the world. This disease kills 1 million people annually, especially in Africa and estimated 700,000 of them are children [1]. Malaria is caused by parasite in genus *Plasmodium* and transmitted by female *Anopheles* mosquito. Even though vaccine is the best control option for malaria, however, research on vaccine development is still at preclinical stage and it is predicted that a reliable malaria vaccine is several years away. Therefore, strategy for malaria mainly focuses on antimalarial drugs capable of reducing or eliminating parasites. However, increasing of antimalarial drug resistant *Plasmodium* parasites and emergence of insecticide resistant *Anopheles* mosquitoes are major obstacles to providing effective malaria treatment in endemic areas [2, 3]. This has prompted research towards the discovery and development of new and affordable antimalarial chemotherapies. In this respect, medicinal plants are potential targets for research and development of alternative malarial drugs.

In the present study, *Andrographis paniculata* Burm.f. Nees was selected for evaluation of its antimalarial activity. This plant which is also known as "King of Bitters", is a plant from family *Acanthaceae* that has been used for centuries in

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Asia to treat digestive and respiratory diseases, fever, herpes, throat infection, and many other chronic and infectious diseases, including malaria [4]. The antimalarial activity of *A. paniculata* has been studied in several reports against *P. falciparum in vitro* models either the extract alone or in combination [5-10]. However, there are no evidences of prophylaxis and curative effects of *A. paniculata* against malaria parasites. Hence, this study was aimed to evaluate the antimalarial including prophylaxis, suppressive, and curative effects of *A. paniculata* on *P. berghei* infected mice.

MATERIALS AND METHODS

Plant material and preparation of extract

The leaves of *A. paniculata* were collected from Kanchanaburi province, Thailand, and taxonomic identification was made by Sakaewan Ounjaijean, Ph.D., Faculty of Pharmacy, Payap University, Chiang Mai, Thailand. The voucher specimen was numbered and kept in our research laboratory, Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi, Thailand. For crude extract preparation, the leaves of *A. paniculata* were dried at room temperature, and subsequently powdered mechanically using commercial electrical blender. Powdered plant material (5 g) was extracted twice using methanol (50 ml) for 72 h at room temperature to ensure exhaustive extraction, and then filtered through Whatman No. 1 filter paper. Filtrate was concentrated by evaporation and the residue was stored in aliquots at 4^{o}C until tested. Usually, a 0.4% yield of extract was obtained. Stock solution of extract was prepared in DMSO and diluted with 0.9% NaCl to obtain the doses of 2, 20, and 100 mg/kg.

Animals

Four weeks old male ICR mice weighing 30-35 g used in this study were purchased from National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The mice were kept in an air-conditioned room with 12 h day/12 h night cycle. They were fed with standard pellet diet for mice and had access to clean water *ad libitum*. All experimental laboratory animals were ratified by Ethical Committee of Faculty of Medical Technology, Western University.

Parasite strain

Chloroquine-sensitive strain of *Plasmodium berghei* ANKA (PbANKA) was used in this study. Infection was initiated by intraperitoneal (IP) injection of 1×10^6 PbANKA parasitized erythrocytes into ICR mice. Parasitemia was daily monitored by counting the number of infected erythrocytes per 1,000 erythrocytes under light microscope of Giemsa-stained thin blood smear. Survival rate of infected mice was also calculated. The parasite was kept alive by continuous IP passage in naïve ICR mice every six days.

Antimalarial drug

For *in vivo* drug susceptibility test, chloroquine (CQ; Sigma, St. Louis, Missouri, USA) was used. The drug at chosen dose (5 mg/kg) was freshly prepared in 0.9% NaCl, and kept at 4^{o}C.

Acute toxicity test

Toxicity screening of *A. paniculata* extract was carried out using method as previously described [11]. Three groups of naïve ICR mice (5 mice/group) were given 2, 20, 100, 500, and 100 mg/kg of extract intraperitoneally. Control mice were kept under the same condition without any treatment. The mice were then observed for signs of toxicity which include but not limited to salivation, paw licking, weakness, stretching of the entire body, respiratory distress, coma and death in the first 4 h and subsequently daily 7 days.

Evaluation of prophylactic activity of *A. paniculata* leaf extract

Prophylactic activity of *A. paniculata* leaf extract was carried out according to the method previous described [12]. Groups of naïve ICR mice (5 mice/group) were given 2, 20, and 100 mg/kg/day of the extracts by IP injection for 4 consecutive days. Twenty four hours after final treatment, infection was initiated by IP injection of 1×10^6 PbANKA parasitized erythrocytes. Blood smear was then made at 72 h after infection, and parasitemia was determined by microscopic examination of Giemsa-stained thin blood smear. Two control groups were used; positive control was treated with 5 mg/kg of CQ by IP injection while the negative control was given 0.9% NaCl as untreated group. Percentage of inhibition (% Inhibition) was consequently calculated using formula below.

$$\%\ \text{Inhibition} = \frac{(\text{Parasitemia of negative control} - \text{Parasitemia of treated group})}{\text{Parasitemia of negative control}} \times 100$$

Evaluation of suppressive activity of *A. paniculata* leaf extract

In this respect, the standard 4-day test was employed [13]. Groups of naïve ICR mice (5 mice/group) were inoculated with 1×10^6 PbANKA

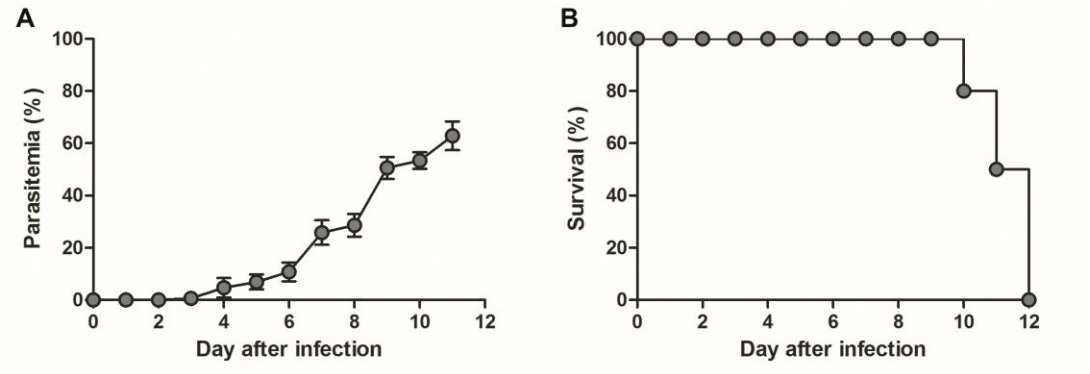

Figure 1 Blood stage propagation of *Plasmodium berghei* ANKA infection in mice. Five ICR mice were intraperitoneally inoculated with $1x10^6$ PbANKA parasitized erythrocytes. (A) Parasitemia was then monitored by Giemsa stained thin blood smear and (B) survival of infected hosts were also observed. Results represent the mean ± SE. * $p<0.05$ compared with day 0.

parasitized erythrocytes by IP injection, and 2 h later, the extracts at doses of 2, 20, and 100 mg/kg/day were treated by IP injection for 4 consecutive days (Day 0-3). On day 4, parasitemia was then determined by microscopic examination of Giemsa-stained thin blood smear. Positive control group was treated 5 mg/kg of CQ by IP injection and untreated group was given 0.9% NaCl. Percentage of inhibition (% Inhibition) was consequently calculated as previous described above.

Evaluation of curative activity of *A. paniculata* leaf extract

The curative test of the extract was carried out according to the previously method [14]. Groups of naïve ICR mice (5 mice/group) were infected with 1×10^6 PbANKA parasitized erythrocytes by IP injection. Seventy two hours later, the infected mice were treated with the extracts at the doses of 2, 20, and 100 mg/kg/day by IP injection for 4 consecutive days. CQ (5 mg/kg/day) was given to the positive control and 0.9% NaCl was given to the untreated groups. Twenty four hours after final treatment, parasitemia was consequently determined by microscopic examination of Giemsa-stained thin blood smear. Percentage of inhibition (% Inhibition) was consequently calculated as previous described above.

Combination treatment of *A. paniculata* leaf extract and chloroquine

Groups of naïve ICR mice (5 mice/group) were inoculated with 1×10^6 PbANKA parasitized erythrocytes by IP injection. Two hours after infection, the extract (100 mg/kg) and CQ (5 mg/kg) were treated by IP injection once a day for 4 consecutive days. CQ (5 mg/kg) and 0.9% NaCl were used as positive and untreated groups, respectively. Twenty four hours after final treatment, parasitemia was consequently determined by microscopic examination of Giemsa-stained thin blood smear. Percentage of inhibition (% Inhibition) was consequently calculated as previous described above. By the end of the experiment, survival of infected mice was also observed.

Statistical analysis

Results were expressed as mean ± SE. Statistical significant of the differences was analyzed using one-way ANOVA. A value of $p<0.05$ was considered significant.

RESULTS

Blood stage propagation of *Plasmodium berghei* ANKA *in vivo*

In order to determine blood stage propagation of PbANKA in mice, parasitemia was daily monitored by microscopy of Giemsa-stained thin blood smear. Parasitemia was firstly detectable on day 2 after infection with a parasitemia of 0.6%, and increased significantly on day 4 after infection with a parasitemia of 4.7% (Figure 1a). Infected mice survived with 100% until day 9, began to die from day 10 and died completely on day 12 (Figure 1b).

Acute toxicity test

Behavioral signs of toxicity were not found in mice given 2, 20, and 100 mg/kg of the extract. Moreover, there was no mortality at all the dose levels used. However, the extract at doses more than 100 mg/kg showed some toxicity signs such as changes in physical appearance, behavioral change, organ damage, and death.

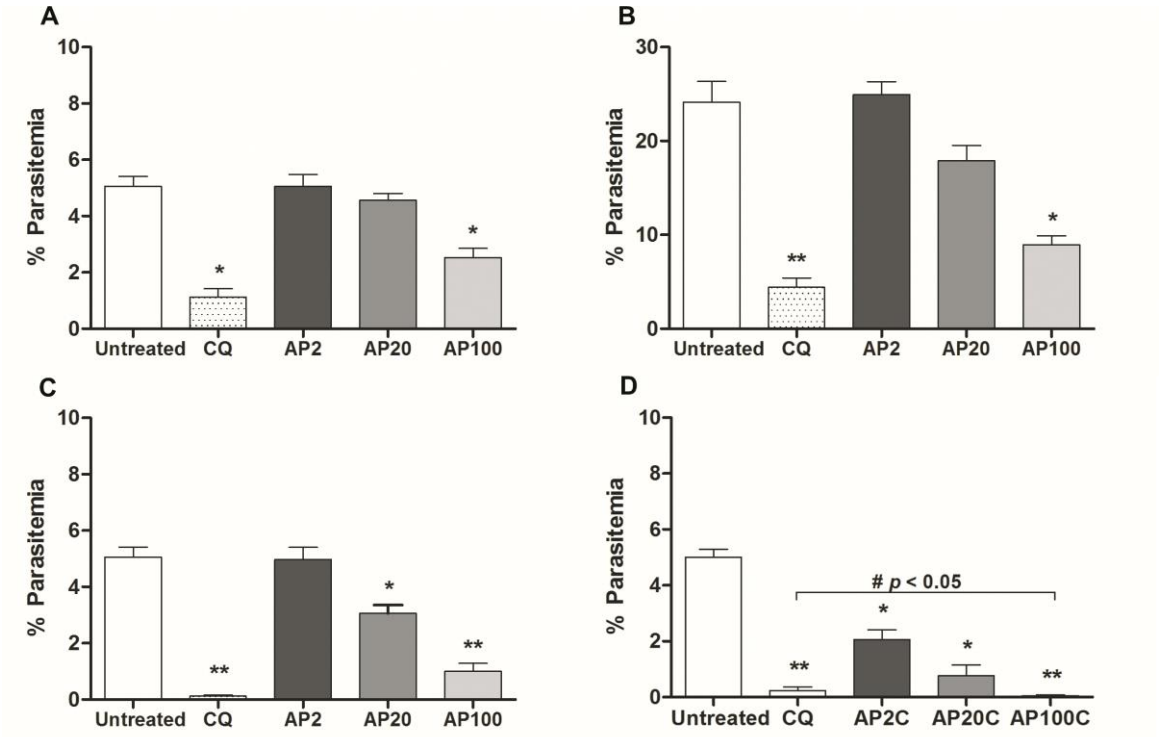

Figure 2 Antimalarial effects of *Andrographis paniculata* methanolic leaf extract on *Plasmodium berghei* ANKA infected mice. (A) Prophylactic effect, (B) curative effect, (C) suppressive effect, and (D) combination effect with chloroquine. Results represent the mean ± SE. * $p<0.05$ compared with Untreated group. ** $p<0.01$ compared with Untreated group. # $p<0.05$ compared with CQ group.

Prophylactic property of *Andrographis paniculata* leaf extract

Protective effect of *A. paniculata* leaf extract against PbANKA was carried out. As shown in Figure 2a, the extract exerted dose dependent inhibition, especially 100 mg/kg of the extract showed significant ($p<0.05$) protective effect with 65% inhibition, compared to untreated group. In addition, CQ had strong protective effect with an inhibition of 90%.

Curative effect of *Andrographis paniculata* leaf extract

In order to use this extract for treatment malaria, curative effect of *A. paniculata* leaf extract was performed. It was observed that the methanolic leaf extract of *A. paniculata* produced dose dependent reduction in parasite levels in the extract treated groups, with a similar reduction in the CQ treated group. Interestingly, 100 mg/kg of the extract showed significant ($p<0.05$) reduction with an inhibition of 75%, compared to untreated group. While that of the CQ treated group was 90% inhibition (Figure 2b).

Suppressive test of *Andrographis paniculata* leaf extract

From standard 4-day suppressive test, it was found that the methanolic leaf extract of *A. paniculata* exerted dose dependent suppressive effect against PbANKA. As shown in Figure 2c, the extracts at doses 20 and 100 mg/kg resulted 54% and 91% inhibition, respectively. However, 5 mg/kg of CQ was the highest antimalarial activity with 95% inhibition. It caused a significant ($p<0.05$) suppression when compared to the untreated control.

Combination treatment of *Andrographis paniculata* leaf extract and chloroquine

The efficacy of the extract in combination with CQ studied in PbANKA infected mice was shown in Figure 2d. Its performance was compared with that of CQ. It was found that combination of CQ with the extracts, 2, 20, and 100 mg/kg showed dose dependent inhibition of 73%, 96%, and 99%, respectively. Surprisingly, percent inhibition of the extract at 100 mg/kg with CQ treatment was increased significantly ($p<0.05$), compared with CQ treatment group. Moreover, the decrease of parasitemia level in response to test drug combination extended period of life in dose-dependent manner (Table 1).

DISCUSSION

The rodent model of malaria has been employed in this research for evaluation of

Table 1 Combination effect of *Andrographis paniculata* methanolic leaf extract and chloroquine on survival time of *Plasmodium berghei* ANKA infected mice

Treatment	Survival time (day)
Untreated mice	11
5 mg/kg of CQ	25
2 mg/kg of extract	11
20 mg/kg of extract	15
100 mg/kg of extract	20
5 mg/kg of CQ + 2 mg/kg of extract	13
5 mg/kg of CQ + 20 mg/kg of extract	20
5 mg/kg of CQ + 100 mg/kg of extract	30

antimalarial property of *A. paniculata* leaf extract. *Plasmodium berghei* ANKA is normally used in the evaluation of treatment outcomes; hence it was an appropriate parasite for this study. Moreover, since this parasite is sensitive to CQ, this drug was used as the standard drug in this study. In addition, the choice of 4 weeks old mice for the study was done to avoid the effect of anemia in the old mice and the effect of possible physiological changes associated with aging may induce on the treatment outcomes [15]. *Andrographis paniculata* has been reported to have diverse pharmacological potential including antiviral, anti-inflammatory, and anticancer activities [16-18]. Moreover, antimalarial of *A. paniculata* either crude extract or andrographolide have been reported against *P. falciparum* and *P. berghei* [5, 19]. However, protective and curative effects of this plant extract have not been reported. Hence, this investigation is the first report an application of *A. paniculata* extract on the treatment of *P. berghei* in both prophylactic, suppressive, and curative activities. In addition, combination treatment of the extract and CQ were also investigated. The results obtained from our study showed significant decrease in parasitemia of PbANKA infected mice treated with the methanolic leaf extract of *A. paniculata*. This significant inhibition of parasitemia observed was dose dependent. In particularly, the extract at dose of 100 mg/kg showed the highest inhibition in prophylactic, suppressive, and curative tests. This effect was however lower in group that received low dose. This effect may be attributed to the presence of andrographolide, major active compound in this extract [20]. It was found evidently on the ring stage inhibition of the parasite [19]. The anti-inflammation of *A. paniculata* extract and andrographolide includes inhibition of the nuclear transcription factor-kappa B (NF-κB), making it a therapeutic target for the treatment of cancer [21, 22]. The role of NF-κB is also important in malaria as mentioned in a recent study [23]. During malaria infection, NF-κB is activated and translocate into nucleus, where it binds to the DNA regulatory site to regulate specific gene expression, especially cell signaling for parasite growth and development. Therefore, the inhibition of transcription factor of *A. paniculata* extract against PbANKA might be a critical process to inhibit blood stage propagation of parasites in vivo. There are several reports on drug combinations showing their in vitro and in vivo inhibitions on malaria parasites [8, 24, 25]. In this study, combination treatment between *A. paniculata* leaf extract and CQ in interesting in the fight against PbANKA. The extract (100 mg/kg) exhibited high level of inhibition activity in PbANKA infected mice when administered with CQ by IP route without any toxicity. The decreasing of parasitemia in response of combination treatment and extended periods of life, observed with the mice is the main finding of the present study. It can be concluded that methanolic leaf extract of *A. paniculata* evidently has the effect of antimalarial against PbANKA in vivo with a maximum effect at a dose of 100 mg/kg. Moreover, the effect of this extract as antimalarial has been revealed both in a monotherapy and in a combination.

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