

ANTIMALARIAL ACTIVITY OF STEM EXTRACT OF *TINOSPORA CRISPA* AGAINST *PLASMODIUM BERGHEI* INFECTION IN MICE

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ABSTRACT: Malaria is caused by protozoa parasite *Plasmodium* and transmitted by female *Anopheles* mosquito. It causes suffering and death to millions of people each year in tropical and sub-tropical areas including Thailand. However, it has been reported that emergence and drug-resistant parasites were increasing. Hence, novel and effective antimalarial drugs are urgently needed. Medicinal plant extracts have been interested to use for treatment in several disease including malaria. In this study, antimalarial activity of *Tinospora crispa* extract against *P. berghei* infection in mice was investigated. Methanol extraction of dried stem powder of *T. crispa* was prepared and total polyphenolic content was then measured using Folin-Ciocalteu method. For *in vivo* test, ICR mice were inoculated with *P. berghei* ANKA and treated intraperitoneally with crude extract at doses of 20, 100, and 200 mg/kg. It was found that crude extract of *T. crispa* has antimalarial activity in dose-dependent manner, especially at doses of 100 and 200 mg/kg with percent inhibition of 35% and 50%, respectively. However, the extract at dose of 20 mg/kg had no any antimalarial effect. Interestingly, percent inhibition was increased in combination treatment of pyrimethamine with the extract in dose-dependent manner. Complete inhibition and parasitemia was clear by combination treatment with 200 mg/kg of extract and pyrimethamine. In conclusion, *T. crispa* extract has an antimalarial activity in dose-dependent manner and strong inhibitory effect when using as combination treatment with pyrimethamine.

Keywords: Antimalarial, *Plasmodium berghei*, *Tinospora crispa*

INTRODUCTION

Malaria is still a serious health problem for people around the world, especially children under five-year of age in Sub-Sahara Africa and estimated 1 million deaths each year [1]. This disease is caused by the protozoa parasite *Plasmodium* and transmitted by the female *Anopheles* mosquito [2]. Because of the lack of effective malarial vaccine, antimalarial drug still is the most importance. Since the emergence of drug resistant strains of *Plasmodium* parasites, the rate of resistance has been increasing [3]. Hence, novel effective antimalarial drugs are urgently

needed. In this respect, medicinal plants are potential targets for research and development of alternative antimalarial drugs with novel modes of action. During last decade, several reports have been conducted to explore antimalarial activities of many plants, including *Carcica papaya* [4], and *Tinospora corpa* [5]. Moreover, total polyphenolic content in these plant extracts has been shown to strongly correlate with its properties including antioxidant, anti-inflammation, anti-cancer, and antimalarial [6-8]. In the present study, Thai medicinal plant *Tinospora crispa* was interesting for evaluation of its antimalarial activity *in vivo*. This plant has been used regularly by people in Southeast Asia as hepato-protective, fever-reducing

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drugs, and antihyperglycemic properties since ancient time [9-11]. Moreover, some studies have also reported its antimalarial property against *P. yoelii* [12].

This study was aimed to evaluate the antimalarial activity of *T. crispera* extract on *P. berghei* infection in mice.

MATERIALS AND METHODS

Preparation of plant crude extract

Thai medicinal plant *Tinospora crispera* was collected from its natural environment in Kanchanaburi Province, Thailand. A voucher specimen has been deposited in the Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai Province, Thailand. The stems of this plant were washed and cut into small pieces and subsequently dried in oven at 40°C for 3 days. Then, they were ground into fine powder for extraction. Powder samples (20g) were extracted with 200 ml of methanol and placed in room temperature shaker for 3 days. Subsequently, it was filtered and the filtrate was dried at 40°C using rotatory vacuum evaporator. The dried crude extracts were prepared in DMSO and diluted with 0.9% NaCl to obtain final doses for using in the animals including 20, 100, and 200 mg/kg with a final less than 10% of DMSO concentration. The extracts were kept at -20°C until used [13].

Total polyphenolic content

Total polyphenolic content in the extract was measured using Folin-Ciocalteu method [14]. Gallic acid was used as standard at concentration ranging 0.01-0.05 mg/ml, and prepared by diluting the stock solution with distilled water. The extract was prepared at concentration of 1 mg/ml. Briefly, 0.1 ml of extract previously diluted 10-fold with distilled water was transferred into a test tube containing 0.75 ml of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min, and then 0.75 ml of 6% (v/v) of sodium carbonate was added and mixed gently. After incubating at room temperature without light for 120 min, the absorbance was read at 765 nm. Calculation of total polyphenolic content in the extract was then performed using linear equation of gallic acid standard curve. Total polyphenolic content was expressed as mg gallic acid equivalents (GAE)/g of samples.

Experimental mice

Pathogen-free female ICR mice weighting 30-35 g, 6-8 weeks old from National Laboratory Animal Center, Mahidol University, Thailand were

used in this study. They were kept at an animal room with temperature of 22-25°C and given standard mouse pellet diet and clean water *ad libitum*. All experiments involving animals were ratified by Animal Ethical Committee of Faculty of Medical Technology, Western University, Kanchanaburi, Thailand.

Rodent malaria parasite

Rodent malaria parasite used in this study was a chloroquine-sensitive *Plasmodium berghei* ANKA (PbANKA) strain. This strain was maintained by continuous re-infestation into ICR mice. Tail blood was collected from mice for parasitemia monitoring by microscopic examination of Giemsa stained thin blood smear. Parasitemia was calculated using formula below.

$$\% \text{ Parasitemia} = \frac{\text{Number of infected erythrocytes}}{\text{Number of total erythrocytes}} \times 100$$

Standard antimalarial drug

Pyrimethamine, antifolate drug, was used as a standard antimalarial control and for study drug susceptibility of PbANKA *in vivo*. The drug was freshly prepared in DMSO and administered orally by gavage [15]. Drug dose, expressed in mg/kg of body weight, was based on the ED90 of this drug, 1.0 mg/kg, on PbANKA infected ICR mice.

Efficacy test of extract *in vivo*

In order to evaluate antimalarial activity of *T. crispera* extract *in vivo*, standard 4-day suppressive test was used [16]. Groups of 3 ICR mice were inoculated with 1×10^6 infected erythrocytes of PbANKA by intraperitoneal injection. The extracts in the doses of 20, 100, and 200 mg/kg were treated intraperitoneally 2 h after inoculation and once a day for 4 consecutive days either the extract alone or in combination with 1.0 mg/kg of pyrimethamine. Parasitemia was consequently measured on day 4 after infection by standard microscopy of Giemsa-stained thin blood smear, and percent inhibition was then calculated. Moreover, 2 controls including untreated and pyrimethamine treated groups were also performed. Survival time of all groups was also observed.

- Group 1 : 10% DMSO
- Group 2 : 1.0 mg/kg of pyrimethamine
- Group 3 : 20 mg/kg of extract
- Group 4 : 100 mg/kg of extract
- Group 5 : 200 mg/kg of extract
- Group 6 : 20 mg/kg of extract + 1.0 mg/kg of pyrimethamine
- Group 7 : 100 mg/kg of extract + 1.0 mg/kg of pyrimethamine
- Group 8 : 200 mg/kg of extract + 1.0 mg/kg of pyrimethamine

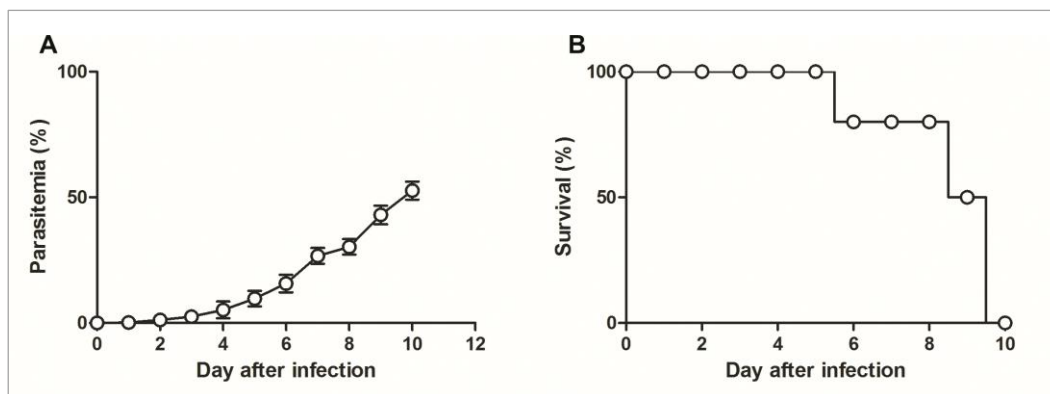


Figure 1 Propagation of blood stage *P. berghei* ANKA infection in ICR mice. Three naïve ICR mice were inoculated with 1×10^6 infected erythrocyte of PbANKA by IP injection, and parasitemia (A) and survival of infected mice (B) were daily monitored. Line graphs were expressed as mean \pm standard error of mean (SEM).

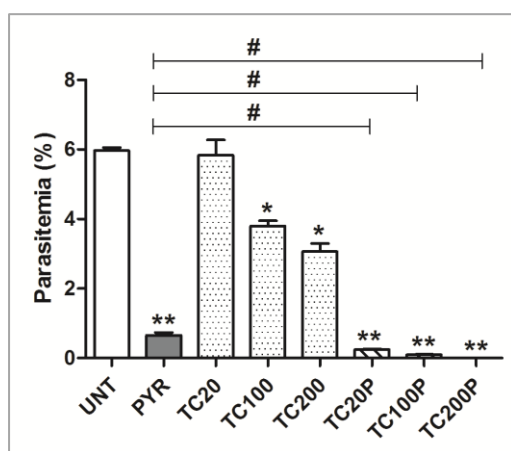


Figure 2 Effect of *T. crispa* stem extract on *P. berghei* ANKA infection in mice. Naïve ICR mice were inoculated with 1×10^6 infected erythrocytes of PbANKA by IP injection. The extracts at doses of 20, 100, and 200 mg/kg (TC20, TC100, and TC200 respectively) were treated intraperitoneally once a day for 4-consecutive days. Pyrimethamine (1.0 mg/kg) was used as positive control (PYR) and untreated group was given 10% DMSO (UNT). Moreover, combination treatment of pyrimethamine and the extracts (TC20P, TC100P, and TC200P) was also carried out. Bar graphs were expressed as mean \pm standard error of mean (SEM). * $p < 0.05$ compared with UNT. ** $p < 0.01$ compared with UNT. # $p < 0.05$ compared with PYR.

In addition, percent inhibition was then calculated using formula below.

$$\% \text{ Inhibition} = \frac{\text{Mean parasitemia of Untreated group} - \text{Mean parasitemia of Treated group}}{\text{Mean parasitemia of Untreated group}} \times 100$$

Statistics

Statistical analysis of the data was performed using GraphPad Prism Software (GraphPad software, Inc., US). The one way ANOVA test was used to analyze and compare the results at a 99% confidence level. Values of $p < 0.01$ were considered significant. Results were expressed as mean \pm standard error of mean (SEM).

RESULTS

Total polyphenolic content of *Tinospora crispa* stem extract

The contents of total polyphenols were measured by Folin-Ciocalteu method in term of gallic acid equivalent with standard curve equation $Y = 0.001X - 0.0006$, $R^2 = 0.9999$. It was found that total polyphenolic content of *T. crispa* stem extract with methanol was 29.13 ± 3.31 mg GAE/g dry weight.

Propagation of blood stage *Plasmodium berghei* ANKA infection in mice

ICR mice were inoculated with 1×10^6 infected erythrocytes of PbANKA, and parasitemia was then daily monitored by microscopic examination of Giemsa-stained thin blood smear. It was found that parasitemia was first detectable on day 2 after infection, and reached to 50% on day 10 with the death of all infected mice on day 10 (Figure 1a-b).

Antimalarial activities of *Tinospora crispa* stem extract

To investigate antimalarial activity of *T. crispa* stem extract, standard 4-day suppressive test was performed using the doses of this extract at 20, 100, and 200 mg/kg. As showed in Figure 2, *T. crispa* stem extract showed inhibitory effect on the parasite in the dose-dependent manner, especially at the doses of 100 and 200 mg/kg with percent inhibition of 35% and 50% respectively.

Table 1 Survival time of *P. berghei* ANKA infected ICR mice and treatment with either *T. crispa* stem extract alone or combination with pyrimethamine

Group of <i>P. berghei</i> ANKA infected ICR mice	Survival time (Day)
Untreated mice (10% DMSO)	9
1.0 mg/kg of pyrimethamine	21
20 mg/kg of extract	9
100 mg/kg of extract	15
200 mg/kg of extract	19
20 mg/kg of extract + 1.0 mg/kg of pyrimethamine	23
100 mg/kg of extract + 1.0 mg/kg of pyrimethamine	27
200 mg/kg of extract + 1.0 mg/kg of pyrimethamine	29

The extract caused a significant ($p < 0.01$) suppression when compared to the untreated group. However, the extract at doses of 20 mg/kg had no any antimalarial effect. For pyrimethamine treatment, it was found that 1.0 mg/kg of pyrimethamine showed inhibition effect on parasite growth with 90% inhibition. Interestingly, combination treatment of *T. crispa* stem extract with 1.0 mg/kg of pyrimethamine showed significantly higher antimalarial activity than pyrimethamine treated alone as dose-dependent manner of the extract. Surprisingly, the combination of pyrimethamine and 200 mg/kg of the extract presented completely inhibit parasite growth. Moreover, all treatment groups survived significantly compared to untreated group, except infected mice treated with 20 mg/kg of extract (Table 1).

DISCUSSION

The rodent malaria *P. berghei* ANKA was used in this study for prediction of efficacy of antimalarial activity of *T. crispa* stem extract. Stem extract of *T. crispa* has been reported to have several effects including antioxidant, anti-inflammation, anti-proliferation, and reduce blood sugar in diabetic mice [17-20]. In addition, it has also been showed that stem extract of this plant had antimalarial property in *P. yoelii* infected mice [12]. The previous study was found that methanol extract of *T. crispa* could extend the life of PbANKA infected mice when treated intraperitoneally at a dose of 5 mg/kg [21]. Another report showed a suppressive property of *T. crispa* aqueous extract given orally in *P. yoelii* infected mice and found 53.8% inhibition at a dose of 110 mg/kg [5]. Hence, to our knowledge this study is the first report showing the antimalarial activity of stem extract of *T. crispa* in the treatment alone and in combination with pyrimethamine against PbANKA in ICR mice. The result of total polyphenolic content in *T. crispa* stem extract showed 29.13 ± 3.31 mgGAE/g dry weight. It has

been reported that total polyphenolic content contributed to the antioxidant and anti-inflammation activities [22, 23]. Moreover, it also has been shown polyphenolic content might also contribute to antimalarial activity in *T. crispa* [24-27]. Our results showed that *T. crispa* stem extract suppressed the growth of PbANKA when it was used once a day by IP injection for 4 days as a dose-dependent manner. Moreover, the extract at dose of 200 mg/kg had the highest suppression activity. However, at dose of 20 mg/kg of this extract could not inhibit parasite growth *in vivo*. This may be because the dose of 20 mg/kg was too low to inhibit parasite propagation. The major components in this extract, diterpenoids (borapetoside A, B, and C) might play an important role for antimalarial activity. Borapetosides have been reported to inhibit cancer cell growth *in vitro* and strong activities to protect cells and tissues from oxidative stress and inflammation induced by reactive oxygen species in mice [9, 19]. In addition, previous report showed the blockage of protein synthesis in *P. falciparum* is the main target for antimalarial drugs [21, 28]. Therefore, we believe that this major component in *T. crispa* extract might have effect to inhibit parasite growth. For combination treatment of stem extract of *T. crispa* and pyrimethamine showed a more significant inhibition of parasite growth compared to pyrimethamine treated alone in a dose-dependent manner. The combination of pyrimethamine and 200 mg/kg of stem extract of *T. crispa* showed completely inhibition in parasite growth, and also prolonged survival times of infected mice. Thus, this extract enhanced the antimalarial activity of pyrimethamine via a pharmacodynamics interaction and could be used for combination therapy.

CONCLUSION

The findings of this study show that *Tinospora crispa* stem extract has antimalarial activity in both extract treatment alone and in combination with

standard antimalarial pyrimethamine. Moreover, the treatment prolonged the survival time and increased the cure rate. It can be further explored to study and develop this extract as supplement for using malaria treatment and combination therapy.

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