Effect of Aqueous Crude Extract of Tinospora Crispa on Plasmodium Berghei Induced Liver Damage in Mice

Voravuth Somsak*, Jutapit Kittitorn1, Sukanya Chachiyo1, Somdet Srichairatanakool2, and Chairat Uthaipibul3
1Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi 71170, Thailand
2Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
3Protein-Ligand Engineering and Molecular Biology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand

Abstract
Malaria is still a serious problem with increasing of mortality in children annually. One of major causes of death in malaria, organ damage especially liver, has been observed. Hence, we aimed to investigate hepatoprotective effect of traditional plant, Tinospora crispa during malaria infection using Plasmodium berghei infected mice as in vivo model. Aqueous crude extract of T. crispa was freshly prepared. For in vivo test, groups of ICR mice were intraperitoneally injected with 0–106 parasitized erythrocytes of P. berghei ANKA, and given with the extract at doses 10, 50, and 500 mg/kg twice a day for 4-consecutive days. Aspartate aminotransferase and alanine aminotransferase are measured for liver damage while albumin measurement is for liver function. The results showed that liver damage was observed during malaria infection as indicating by significantly (p<0.05) increase of AST and ALT, and markedly decrease of albumin. Interestingly, T. crispa extract exerted hepatoprotective effect during malaria infection. The highest hepatoprotective activity of the extract was shown at dose of 500 mg/kg. Additionally, there were no any toxics to liver function in normal mice treated with this extract. It can be concluded that aqueous crude extract of T. crispa exerts hepatoprotective effect during P. berghei infection.

Keywords: Tinospora crispa; Plasmodium berghei; Liver damage

Introduction
Malaria is an endemic infectious disease that is wide spread in tropical and sub-tropical regions of the world. This disease kills 1 million people annually, and an approximated 700,000 of them are children. Malaria is caused by parasitic protozoa in the genus Plasmodium, and transmitted by female Anopheles mosquito [1]. The manifestations of severe malaria include cerebral malaria, severe anemia, pulmonary edema, acute kidney injury, hypoglycemia, acidosis, and liver involvement [2]. Liver is an important organ involved during malaria infection and development. It has been reported that malaria parasite causes liver damage, clinical jaundice, and liver dysfunction in 2.5-5.3% of cases in endemic countries [3]. This has prompted research towards the development and discovery of new, safe and affordable compounds to protect liver damage during malaria infection. In this respect, medicinal plants are potential targets for research. Thailand is malaria endemic area with an abundance of diverse plant life widely used as traditional medicines to treat tropical diseases including malaria [4]. However, these plants are not fully explored.

Tinospora crispa is an indigenous climber plant that commonly grows wild in Southeast Asia including Thailand. Its stem has been used for various therapeutic purposes such as diabetes, hypertension, diarrhea, and anti-parasites [5-7]. In vitro and in vivo studies revealed that T. crispa produced considerable antimalarial effect [6,8]. It has also been described that crude extract of T. crispa had an in vivo antimalarial effect against P. yoelii [9]. Moreover, liver damage induced by oxidative condition could be protected and treated with T. crispa extract [7,10,11]. During liver damage development, liver enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are markedly increased while albumin level is decreased, and can be used as biological markers [12]. This has led our interest to explore the biological properties of T. crispa in P. berghei-induced liver damage using the biological markers including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin levels in plasma.

Materials and Methods

Plant materials
Fresh stems of Tinospora crispa were collected from its natural environment in Kanchanaburi province, Thailand, during February to March 2014. The samples were identified by Dr. Sakaewen Ounjaijain, Faculty of Pharmacy, Payap University, Chiang Mai, Thailand. Stems were minced into small pieces and dried in ventilated oven at 60°C for 72 h, after which the dried plants were grounded and extracted with distilled water at the proportion of 1:10 (w/v) [6]. Briefly, 250 g of dried powdered plant materials were extracted with distilled water at 45°C for 3 h with frequent agitation. The extract was filtered through Whatman no.1 filter paper, and evaporation was then performed to remove solvent. Crude extract was kept at -20°C. Before experiment, powdered extract was dissolved in distilled water to obtain appropriate doses for use in mice.

Experimental mice
Specific-pathogen-free, female ICR mice Mus musculus, aged 6-8 weeks old and weighted 30-35 g were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. They were kept at 22-25°C with 12 h light/dark cycle, and given standard mouse pellet and water ad libitum. Procedures of the
animal experiments were ratified by the Ethical Committee on Animal Experimentation, Faculty of Medical Technology, Western University.

**Rodent malaria parasite**

Chloroquine-sensitive *Plasmodium berghei* strain ANKA (PbANKA) was used. They were kept alive by intraperitoneal (IP) passage in mice. Parasitemia was daily monitored by microscopic examination of Giemsa stained thin blood smear.

**Acute toxicity test**

The acute toxicity test of aqueous crude extract of *T. crispa* was carried out as previously described [13]. Groups of naïve ICR mice (5 mice of each) were given orally by gavage with 10, 50, 500, 1000, and 5000 mg/kg. The mice were observed for signs of toxicity which include but not limited to paw licking, salivation, stretching of the entire body, weight loss, weakness, respiratory distress and death in the first 4 h and subsequently daily for 7 days [14].

**Antimalarial drug**

Standard antimalarial drug, chloroquine diphosphate salt was used to study in vivo drug susceptibility of PbANKA. The drug was freshly prepared in distilled water and administered orally by gavage [15]. Drug dose, expressed in mg/kg of body weight, was adjusted at the time of administration according to the weight of each mouse. The dose of 8 mg/kg was based on the ED90 of this drug on PbANKA infected mice.

**Assessment of liver function tests**

AST, ALT, and albumin were used as indicators of liver function. Tail blood was collected into heparinized hematocrit tubes and centrifuged at 10,000 g for 10 min. Plasma was then collected and used as subjects for measurement of AST, ALT, and albumin using a commercial kit (BioSystems) according to manufacturer’s instruction.

**Efficacy test in vivo**

Hepatoprotective effect of aqueous crude extract of *T. crispa* was done based on standard Peter’s test [16]. Groups of ICR mice (5 mice of each) were randomly divided, and infection was then performed with 6x10^6 parasitized erythrocytes of PbANKA by IP injection. Four hours after infection, they were given orally with 10, 50, 500, 1000, and 5000 mg/kg of the extracts, and every 24 h twice a day for 4-consecutive days. Three control groups were used; normal controls were given either distilled water or the extract while untreated control was given only distilled water. On day 8 of experiment, tail blood was collected and plasma was then used to measure AST, ALT, and albumin for antimalarial activity, 8 mg/kg of chloroquine was used as positive control to treat infected mice once a day for 4-consecutive days. Parasitemia was then measured on day 8 of experiment.

**Statistics**

Statistical analysis was done using GraphPad Prism software. The results were presented as mean ± standard error of mean (SEM). One-way ANOVA was used to compare several treatment groups. Significant differences were considered at 95% confidence, p<0.05.

**Results**

**Acute toxicity test**

Behavioral signs of toxicity observed in all mice treated with 1000 and 5000 mg/kg of extracts include paw licking, salivation, stretching, weight loss, and reduced activity. These signs of toxicity were firstly observed within 4 h and until 7 days. There was however no mortality at all the doses used. Moreover, no any signs of toxicity could be observed in all mice treated with 500 mg/kg of extracts and lower doses.

**Impairment of liver function during *Plasmodium berghei* ANKA infection**

Parasitemia was first detectable on day 2 post-infection with a parasitemia of 0.5%, and reached 65% on day 12 (Figure 1A). Next, we observed that AST and ALT activities were markedly increased in infected mice, and first significant (p<0.05) increases were found on day 8 post-infection (266 and 127 IU/l, respectively) (Figure 1B). Additionally, we also observed decreases of albumin levels in infected mice, and firstly significant (p<0.05) decreases were also observed on day 8 post-infection (23 g/dl) (Figure 1C).

**Hepatoprotection of aqueous crude extracts of *Tinospora crispa* during *Plasmodium berghei* ANKA infection**

It was observed that aqueous crude extract of *T. crispa* at dose of 500 mg/kg produced hepatoprotective effect by reducing of AST (57 IU/l) and ALT (24 IU/l), and increasing of albumin levels (41 g/dl) in extract treated groups, with similar levels in normal control (70 and 34 IU/l for AST and ALT, and 44 g/dl for albumin) (Figure 2A-C). However, there were significantly (p<0.01) increase in AST and ALT, and decrease in albumin levels in untreated groups and infected mice treated with 10 mg/kg of the extract. Although all biological markers were significantly (p<0.05) different in 50 mg/kg of extract treated mice when compared to untreated control, but significant (p<0.05) differences of all markers were still detectable when compared to normal. Moreover, parasitemia was significantly (p<0.01) decreased in infected mice treated with 500 mg/kg of extract, compared to untreated group. However, no significantly difference were observed in infected mice treated with 10 and 50 mg/kg of extracts, compared to untreated group (Figure 2D). In addition, prolong survival time (27 days) was also observed in infected mice treated with 500 mg/kg of extract (Table 1).

**Discussion**

In the present study, we aimed to investigate the effect of aqueous crude extract of *T. crispa* on *P. berghei* ANKA induced liver damage. For acute toxicity test of *T. crispa*, toxicity signs were observed in mice given 1000 and 5000 mg/kg of extracts. It has been reported to use *T. crispa* extract at a dose of 110 mg/kg for treatment orally in mice and no mortality has been observed [8]. However, the ethanol extract was different from our water extract, thus using a dose of 500 mg/kg in this study was possible. Impairment of liver function during malaria infection has been previously reported with increasing of AST and ALT activities. Liver change and damage in severe malaria infection often include hyperplastic Kupfer cells, fatty change, portal tract inflammation, cholestasis, sequestration of parasitized erythrocytes, and the deposition of hemozoin pigment [17]. Moreover, apoptosis in the hepatocytes has been reported in animal models, linked to activation of mitochondrial pathway, release of reactive oxygen species and induction by glycosylphosphatidylinositol, a major membrane-associated protein of malaria parasites [18-21]. From our finding liver damage induced by PbANKA infection in mice was protected by treatment of aqueous crude extract of *T. crispa*. However, the doses of 10 and 50 mg/kg were too low to protect liver damage. Additionally, no any effects on liver function were found in normal mice treated with 500 mg/kg of the extract. Interestingly, the extract at dose of 500 mg/kg exerted antimalarial activity and prolong survival in infected mice. It has been reported that total polyphenolic content might contribute
Figure 1: Impairment of liver function during Plasmodium berghei ANKA infection. (A) Parasitemia of ICR mice infected with 6x10^6 parasitized erythrocytes of PbANKA. Liver function was assessed by enzyme activities of (B) AST and ALT, and (C) level of albumin. Results represented the mean ± SEM. *p<0.05, **p<0.01, compared to day 0.

Figure 2: Hepatoprotective effect of aqueous crude extract of Tinospora crispa during Plasmodium berghei infection. ICR mice were IP injection of 6x10^6 parasitized erythrocytes of PbANKA, and given orally with the extract 10, 50, and 500 mg/kg twice a day for 4-consecutive days. On day 8 of experiment, liver function was assessed by enzyme activities of (A) AST, (B) ALT, and (C) albumin in plasma. Moreover, (D) parasitemia was also determined. Results were expressed as mean ± SEM. *p<0.05, **p<0.01, compared to normal control. #p<0.05, ##p<0.01, compared to untreated control. N; normal, U; untreated, CQ; chloroquine.
to the antioxidant activity in *T. crispa* extract, and then protect liver damage from oxidative stress induced by malaria infection [5,21]. In addition, borapetoside, apigenin and magnoflorine presented in stem extract of *T. crispa* might play this activity [7,22]. In addition, it has been reported antimalarial activity of *T. crispa* extract against *P. yoelii* infected mice [9]. Moreover, catechin, a potent antioxidant, was also found in this extract, and might show the hepatoprotection during malaria infection [23]. All together, we observed that aqueous crude extract found in this extract, and might show the hepatoprotection during *P. berghei* infection in mice. The results suggested that *T. crispa* is a valuable source of natural hepatoprotective compound and can be potentially developed as alternative drugs in combination with standard antimalarials.

**Table 1:** Survival time of *Plasmodium berghei* ANKA infected mice and treatment with aqueous crude extract of *Tinospora crispa*.

<table>
<thead>
<tr>
<th>Group of <em>P. berghei</em> ANKA infected ICR mice</th>
<th>Survival time (Day)</th>
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<tbody>
<tr>
<td>Untreated mice</td>
<td>12</td>
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<tr>
<td>10 mg/kg of extract</td>
<td>12</td>
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<tr>
<td>50 mg/kg of extract</td>
<td>18</td>
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<tr>
<td>500 mg/kg of extract</td>
<td>27</td>
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<tr>
<td>8 mg/kg of chloroquine</td>
<td>30</td>
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**References**