Hepato-protective effect of *Azadirachta indica* leaf aqueous extract against *Plasmodium berghei* infected mice

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ABSTRACT

Malaria is caused by protozoa parasite in genus *Plasmodium* and transmitted by *Anopheles* mosquito. It is estimated 1 million deaths annually, most of them are children less than 5 years of age in sub-Saharan Africa. Causes of death in malaria are variable including severe anemia, cerebral malaria, and liver damage and failure. Especially in liver damage during malaria infection is a major focus of this study. The objective of this study was to determine protective effect of *Azadirachta indica* leaf extract against *Plasmodium berghei*-induced liver damage by using aspartate and alanine aminotransferase (AST and ALT, respectively) as biological markers. Aqueous leaf extract of *A. indica* was prepared. For *in vivo* test, ICR mice were inoculated with 1x10⁷ infected red blood cells of *P. berghei* ANKA. The extract (1,000 mg/kg) was orally given twice a day for 6 consecutive days, and AST and ALT were then measured using commercial kits. It was found that AST and ALT levels in plasma were significantly (*p*<0.05) increased on day 6 post infection resulting to parasite development *in vivo*. Interestingly, infected mice treated with this extract, AST and ALT levels were normalized significantly and no difference to normal mice. It can be concluded that *A. indica* leaf extract exerted protective effect on liver damage during malaria infection. However, active components and mechanism of action should be studied in more detail for validating this extract as alternative malaria treatment.

Keywords: Hepato-protective effect, *Azadirachta indica*, *Plasmodium berghei*
1. INTRODUCTION

Malaria is an infectious disease with ravaging effects in the world. It is estimated that half the world’s population is at risk of malaria, and that 1-2 million annual deaths can be attributed to malaria alone [1, 2]. This disease is caused by protozoa parasite *Plasmodium* and transmitted by female *Anopheles* mosquito. The causes of death in malaria involve severe anemia, cerebral malaria, and organ damage and failure [3-5]. In organ damage, liver is a critical organ and one of most targets for malaria. Malaria-associated liver damage and failure occurs between 2-5% of hospitalized patients with a mortality that can reach up to 45% [6-8]. This has prompted research towards the discovery and development of compounds to treatment and protects liver damage during malaria infection. In this respect, plant resources are potential targets for research and development of alternative drugs.

*Azadirachta indica*, commonly known as Neem, is found throughout Southeast Asia including Laos, Myanmar, Cambodia and Thailand. This plant is used for treatment of some pathological conditions related to oxidative disorders such as inflammation and skin diseases, rheumatic disorders and treatment of fever and diabetes [9, 10]. Moreover, extract of *A. indica* has been used traditionally to treat malaria in several endemic countries. For *in vitro* study, *A. indica* leaf extract possess inhibitory activity on *P. falciparum* asexual stage [11-13]. However, there is no publication concerning the biological activities of *A. indica* in protection of liver damage during malaria infection. The aim of this study was to investigate protective effect of aqueous leaf extract of *A. indica* against *P. berghei*-induced liver damage.

2. MATERIALS AND METHODS

Plant material and preparation of extract

Fresh leaves of *Azadirachta indica* were collected in Kanchanaburi, Thailand. The sample was identified by Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand. The Voucher specimen was deposited at Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Thailand. Sample was cleaned, dried in a hot air oven (55°C) for 6 h then powdered. Dried powder of leaf sample was separately boiled with distilled water (DW) for overnight at room temperature (plant: water = 1: 20, w/v). The suspension was centrifuged at 5,000 g for 20 min and filtered through Whatman no. 1 filter paper [14]. The filtrate was then lyophilized to yield 11.9% (w/w) and stored at -20°C. The extract was dissolved in DW at a suitable concentration prior to experiment.

Experimental animal

Free-pathogen 4-week female ICR mice with 25-30 g purchased from National Laboratory Animal Center, Mahidol University, Thailand were used. They were kept at 25°C with 12 h day/night cycle, and fed with standard pellet diet and clean water *ad libitum*. All experimental animals were ratified by the Animal Ethical Committee from Western University, Thailand.

Rodent malaria parasite

Chloroquine sensitive strain of *Plasmodium berghei* ANKA (PbANKA) gifted from Dr. Chris Janse at Leiden University was used in this study. Parasite was grown in animal by intraperitoneal (IP) injection of 1x10^7 infected red blood cells (iRBC). Parasitemia was monitored daily by Giemsa stained thin blood smear under light microscope with x100 oil immersion lens. When parasitemia reached to 15-20%, mechanical passage was performed into new ICR mice. Moreover, assessment of liver function during malaria infection was also determined.

Assessment of liver function

Enzyme markers, aspartate and alanine aminotransferase (AST and ALT) were measured using commercial kit (BioSystems, Spain), according to manufacturer’s instruction. Blood was collected by cardiac puncture and centrifugation was then performed to collect serum. The serum was used as subject for measurement.

Efficacy test *in vivo*

The *in vivo* test was based on standard Peters’ test [15]. Groups of ICR mice (5 mice of each) were inoculated with 1x10^7 iRBC of PbANKA by IP injection. The extract (1,000 mg/kg) was given orally twice a day for 6 consecutive days. Blood was collected then AST and ALT were measured. Normal and untreated mice were used as controls.

Statistics

Statistical analysis was performed using GraphPad Prism software. Data was expressed as mean ± standard error of mean (SEM). One-way ANOVA was used to compare the data, and significance was considered at 95% confident, *p* < 0.05.
3. RESULTS

In order to examine blood stage propagation of PbANKA infection in mice, parasitemia was daily monitored. Figure 1A showed that parasitemia was first detectable on day 2 post infection with a parasitemia of <1%. Parasite was growing and parasitemia reached to 60% on day 10 post infection. Infected mice died on day 11 post infection. During *Plasmodium* propagation in RBC, ring form is developed into trophozoite and schizont containing thousand merozoites. Then, merozoites are released into bloodstream and re-invade new RBC. At this phenomenon, RBC was destroyed and hemolysis is occurred resulting to severe anemia [16, 17]. Therefore, infected mice could die from severe anemia. In addition, levels of AST and ALT in plasma were increased during parasite development, and significance ($p < 0.05$) was firstly observed on day 6 post infection (Figure 1B). It was suggested that during hemolysis, free radical is increased followed by oxidative stress and inflammation is developed. Hemolysis is the important factor in the onset and progress of severe anemia mainly by producing oxidative stress [18, 19]. It has been reported that oxidative stress could damage several organs, especially liver [20, 21]. The mechanism suggested involvement of cytoadherence of iRBC, pro-inflammatory response as well as hepatitis due to oxidative stress [6]. So, biological markers for liver function, AST and ALT were then increased.

For efficacy test *in vivo*, aqueous leaf extract of *A. indica* was given orally twice a day for 6 consecutive days in PbANKA infected mice. The results showed that AST and ALT were decreased and normalized into normal levels in infected mice treated with this extract, and there were no significant difference when compared to normal mice (Figure 2A and B). Moreover, no toxicity was observed in normal mice treated with the extract as indicated by levels of AST and ALT were normal (data not shown). The extract with a dose of 1,000 mg/kg has been described to have no any toxicity to mice [22]. Moreover, it has been reported that aqueous leaf extract of *A. indica* exhibited antioxidant activity as well as anti-inflammation with potent free radical scavenging [14, 23]. Hence, these properties of the extract might exert protective effect on liver damage during malaria infection in order to inhibit free radical and oxidative stress followed by protection of liver. Moreover, it has been reported that the efficacy of this extract is attributed to limonoid, a class of highly oxygenated terpenoids, endowed with a range of biological properties including insecticidal, anti-microbial, anti-inflammation, and immune-modulatory activities [24].
Figure 2. Efficacy of aqueous leaf extract of *Azadirachta indica* on liver damage during *Plasmodium berghei* infection. Groups of ICR mice (5 mice of each) were inoculated with 1x10^7 iRBC of PbANKA by IP injection. The extract (1,000 mg/kg) was given orally twice a day for 6 consecutive days, and (A) AST and (B) ALT were consequently measured as previously described. Results were expressed as mean ± SEM. * p < 0.05, compared to normal group. # p < 0.05, compared to untreated group.

4. CONCLUSIONS

The aqueous leaf extract of *A. indica* showed the protective effect on liver damage during *P. berghei* infection in mice as indicated by normalizing of AST and ALT levels. Further work should include the separation and identification of active components and mechanism of action.

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REFERENCES