

Anti-hemolysis of Aqueous Crude Extract of Siamese Neem Tree (*Azadirachta indica*) during *Plasmodium berghei* Infection in Mice

Voravuth Somsak*, Sukanya Chachiyo, and Ubonwan Jaihan

¹Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi 71170, Thailand

Abstract

Malaria-associated hemolysis is associated with mortality in malaria patients. It has been speculated that oxidative stress and inflammation induced by parasite infection and propagation are involved in its pathophysiology. Hence, this study was aimed to investigate the anti-hemolysis of Siamese neem tree (*Azadirachta indica*) extracts against *Plasmodium berghei* infection in mice. Leaf aqueous crude extract of Siamese neem tree was prepared using hot water method and used for oral treatment in mice. ICR mice were infected with 1×10^7 infected red blood cells of *P. berghei* ANKA by intraperitoneal injection and given the extracts (500, 1,000, and 2,000 mg/kg) for 4-consecutive days. To assess hemolysis, hematocrit levels were then evaluated. Hematocrit level was markedly decreased during malaria parasite propagation in mice. However, anti-hemolytic effect was observed in infected mice treated with the extracts at dose-dependent manners, especially at doses of 1,000 and 2,000 mg/kg. Although hemolysis was observed in pyrimethamine, antimalarial drug, treated group, but it could be protected by combination treatment of pyrimethamine with this extract. In conclusion, aqueous crude extracts of Siamese neem tree exerted anti-hemolysis induced by malaria infection and could be used as combination treatment. This plant may work as potential source in the development of variety of herbal formulations for malarial treatment.

Keywords: Anti-hemolysis; Siamese neem tree; *Azadirachta indica*; *Plasmodium berghei*

Introduction

Malaria, an infectious disease associated with fever, anemia, and other pathologies, is caused by protozoa parasite in genus *Plasmodium*, and transmitted by female *Anopheles* mosquito. Worldwide, clinical cases of malaria were observed in about 270 million people annually resulting in at least 1.5-2.7 million deaths a year [1]. Over 90% of deaths occur within the continent of Africa, mainly among young children [2]. Malaria-associated hemolysis, one of the major life-threatening well-known causes of death in *P. falciparum* and *P. vivax* malaria, occurs between 1-4% of hospitalized adult with a mortality that can reach up to 45% [3]. The pathogenesis of malaria-associated hemolysis is multifactorial and not well characterized, but several hypotheses suggest involvement of cytoadherence of infected red blood cells (iRBC), pro-inflammatory response as well as hemolysis due to oxidative stress [4]. During malaria propagation in RBC, the consumption of hemoglobin by parasites gives rise of considerable amounts of free heme (Fe^{3+}), a molecule that have the ability to induce oxidative stress [5, 6]. The oxidative stress mediated by free heme has been implicated in lipid peroxidation and serious damage in RBC through generation of reactive oxygen intermediates and nitrogen intermediates. Moreover, malaria parasite invasion and subsequent RBC rupture also contributed to pathogenesis of hemolysis [7, 8]. This has prompted research towards the discovery and development of new, safe and affordable anti-hemolysis drugs during malaria infection. In this respect, medicinal plants are potential targets for research.

Recently, interesting in Siamese neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valetton) as promising agents for the prevention or reduction of risk for many human diseases involving oxidative stress such as inflammation, rheumatic, arthritic disorders and treatment of fever and diabetes have increased [9]. It is found throughout Southeast Asia including Laos, Myanmar, Cambodia and Thailand. In Thailand, the young leaves and inflorescences of this plant are commonly eaten as a vegetable. It has been reported that leaf extract of Siamese neem tree showed strong antioxidant and potent anti-inflammation activities. Moreover, anti-diabetes, protection of critical organ damage and hemolysis has also been described in Siamese neem tree extract

[9-13]. However, anti-hemolysis of this extract in malaria has not yet been reported. According to this, the main focus of this study was to investigate anti-hemolytic activity of Siamese neem tree extract either treatment alone or combination with standard antimalarial drug in mice infected with *P. berghei* parasite.

Materials and Methods

Collection of plant material

The leaves of Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valetton) was collected from Suphanburi province, Thailand. The plant samples were compared with the voucher specimen at the Bangkok Herbarium, Botanical Section, Botany and Weed Science Division, Department of Agriculture, Bangkok, and identified by Dr. Sakaewan Ounjaijean, Department of Pharmacy, Faculty of Pharmacy, Payap University. The voucher specimens were deposited at Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi province, Thailand. Samples were cleaned, dried in a hot air oven (55°C) for 6 h, then powdered and passed through a sieve with mesh number 20.

Preparation of the extracts

The extraction was carried out by boiling dried powdered of leaf samples in distilled water for 6 h (plant: water = 1: 2, w/v). This was followed with vacuum filtration and extract concentration using a

*Corresponding author: Voravuth Somsak, Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi 71170, Thailand, Tel: +66898009939; E-mail: voravuthsomsak@gmail.com

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rotary evaporator at a temperature not exceeding 40°C to yield dried leaf aqueous extracts [13].

Experimental mice

Pathogen free, 4 week old ICR mice were obtained from the National Laboratory Animal Center, Mahidol University, Thailand, and kept for at least one week in animal room at 22-25°C with sterile-filtered tap water and pelleted diet (CP diet 082, Perfect Companion Company, Thailand) *ad libitum*. Experiments were started in 6-week-old mice. Procedures of the animal experiments were ratified by the Ethical Committee on Animal Experimentation, Faculty of Medical Technology, and Western University, Thailand.

Acute toxicity

Acute toxicity test of Siamese neem tree extract was carried out using Lork's method [14]. The mice were randomized into 5 groups of 5 mice each between 20-25 g. The mice were subjected to 24 h fasting (with only water) before administration of extracts. The powdered Siamese neem tree extract was dissolved in 20% Tween-80 and administered in doses of 500, 1,000, 2,000, and 4,000 mg/kg body weight orally. The fifth group served as the control and received only 20% Tween-80. The mice were then observed for signs of toxicity which include but not limited to paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first 4 h and subsequently daily for 7 days.

Rodent malaria parasite

In this study, *Plasmodium berghei* strain ANKA (PbANKA) was used. Mice were inoculated with 1×10^7 iRBC by intraperitoneal (i.p.) injection. Parasite growth was daily monitored by microscopic examination of Giemsa stained thin blood smear under light microscope with 100X oil immersion lens. Percentage of parasitemia (% parasitemia) was determined by counting the number of iRBC out of 200 RBC in random fields of the microscope, and then calculated according to the following formula below.

$$\% \text{parasitemia} = \frac{\text{Total number of iRBC}}{\text{Total number of RBC}} \times 100$$

Total number of RBC

Blood was then collected from the infected mice with a parasitemia between 20-30% by cardiac puncture. Blood was subsequently diluted in normal saline in ratio of 1:10, and 0.3 ml of the diluted blood was then used to infect naïve mice by i.p. injection.

Measurement of hematocrit levels

Percentage of hematocrit (%Hct) was measured by collecting of tail blood into heparinized capillary tube and centrifugation at 10,000 g for 10 min. Proportion of packed RBC and total blood volume was finally calculated.

Antimalarial drug

Standard antimalarial drug, pyrimethamine (PYR) was used in this study. The drug was freshly prepared in dimethyl sulfoxide (DMSO) and administered orally by gavage. Drug dose, expressed as mg/kg body weight, was adjusted at the time of administration according to the weight of each mouse. The dose was based on the ED90 (1 mg/kg) on PbANKA infected mice [15].

Efficacy test *in vivo*

The Peter's 4-day suppressive test against PbANKA infection

in mice was employed [16]. Naïve ICR mice were inoculated by i.p. injection with 1×10^7 iRBC of PbANKA. The mice were then randomly divided into 8 groups of 5 mice each, and treated for 4 consecutive days with 500, 1,000, and 2,000 mg/kg of extracts orally. The control groups were used; normal mice given either 20% Tween-80 or 2,000 mg/kg of extract, untreated groups treated with only 20% Tween-80, and infected mice given either 1 mg/kg of PYR or combination of PYR and 2,000 mg/kg of extract. On day 5 of the experiment, blood was collected for % Hct measurement.

Statistical Analysis

The one way ANOVA test was used to analyze and compared the results at a 95% confidence level. Values of $p < 0.05$ were considered significant. Results were expressed as mean+standard error of mean (SEM).

Results

Acute toxicity test

Behavioral signs of toxicity observed in mice given 4,000 mg/kg of extract include; paw licking, salivation, stretching and reduce activity. There was however no mortality at all dose used.

Malaria-associated hemolysis development during PbANKA infection

As showed in Figure 1A, parasitemia was firstly detectable at day 2 after infection with a parasitemia of $< 1\%$, and reached 65% at day 12 after infection. Next, we observed that % Hct was markedly decreased in infected mice (Figure 1B), and strong negative correlation ($R^2 = 0.8173$) between % parasitemia and % Hct was also observed (Figure 1C). Additionally, PbANKA infected mice survived within 2 weeks (Figure 1D).

Anti-hemolytic activity of Siamese neem tree extract against PbANKA infected mice

The results showed that aqueous crude extracts of Siamese neem tree exerted dose-dependent anti-hemolytic effects against PbANKA infection in mice (Figure 2). Untreated control and 500 mg/kg of extract treated mice presented significant ($p < 0.01$) decrease in % Hct, compared to normal mice. The highest anti-hemolytic activity was found in infected mice treated with 2,000 mg/kg of extract. Moreover, significant ($p < 0.01$) hemolysis was also found in PYR treated group. However, normal % Hct level was observed in combination treatment between PYR and the extract. Additionally, there were no any effects on % Hct in normal ICR mice treated with the extract at a maximum dose of 2,000 mg/kg.

Discussion

Study of the anti-hemolytic effect of aqueous crude extract of Siamese neem tree was carried out on ICR mice experimentally infected with PbANKA. The choice of this plant extract was based on previous reports of its antioxidant and free radical scavenging properties [13]. The present results showed that during blood stage propagation and development of PbANKA, % Hct level was markedly decreased and infected mice would die from severe hemolysis and anemia. The hemolysis induced by malaria infection is proposed to be a consequence of parasite development in RBC and exacerbated RBC membrane against products of oxidative stress releasing during infection [2,3]. Moreover, RBC destruction during blood stage of infection accumulates high levels of toxic free heme in circulation that, in turn, has the ability

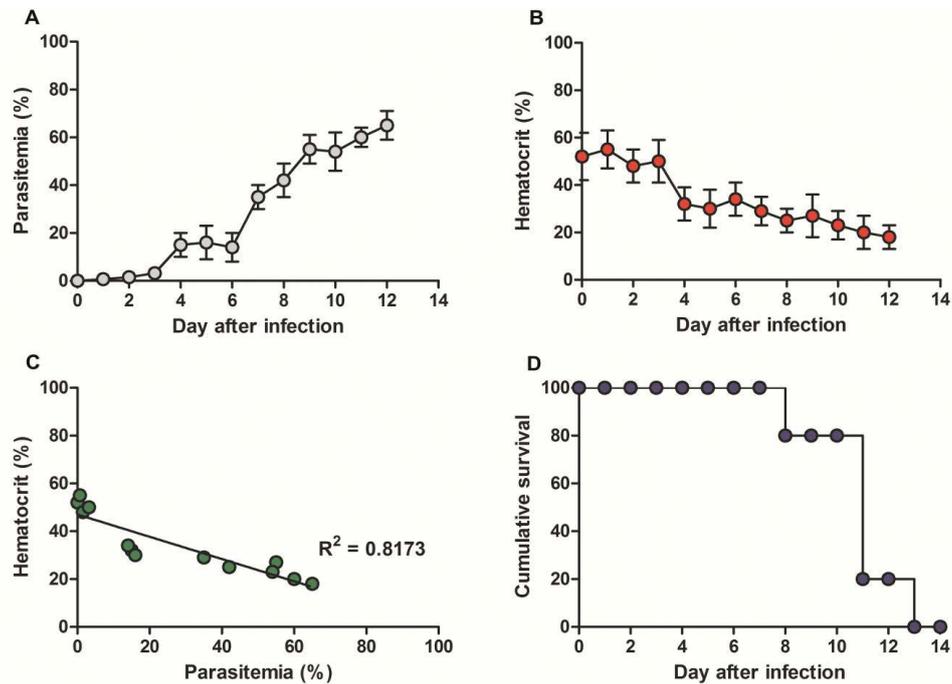


Figure 1: Malaria-associated hemolysis development during *Plasmodium berghei* ANKA infection. ICR mice were infected with 1×10^7 iRBC of PbANKA by i.p. injection. (A) Parasitemia and (B) hematocrit were daily monitored as previously described in Materials and Methods section. (C) Correlation between parasitemia and hematocrit was also determined. (D) Cumulative survival of PbANKA infected mice. Results were expressed as mean \pm SEM.

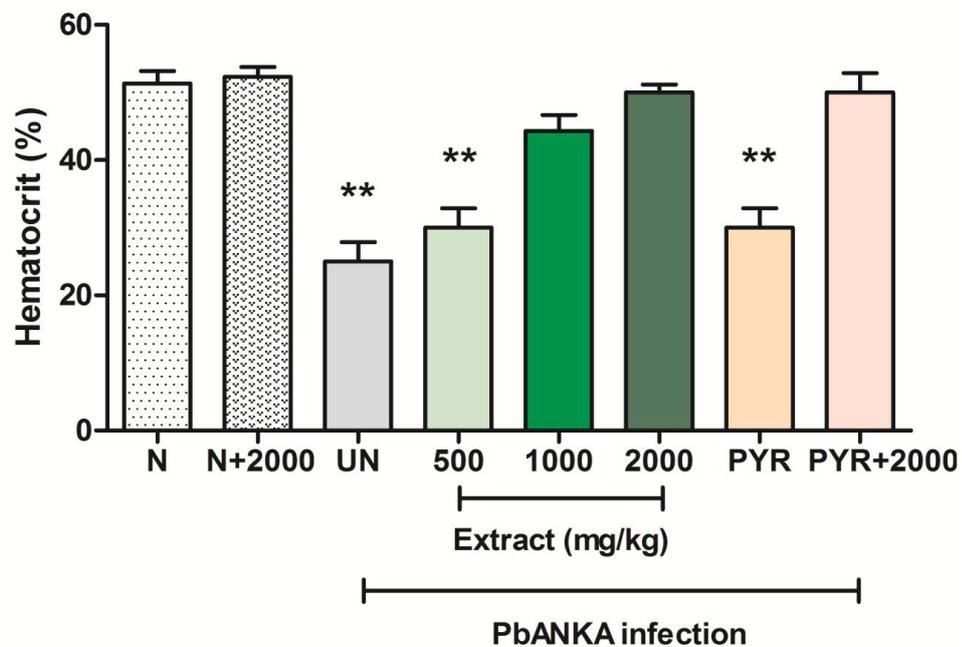


Figure 2: Anti-hemolytic activity of Siamese neem tree extract against *Plasmodium berghei* ANKA infected mice. Groups of ICR mice were randomly infected with 1×10^7 iRBC of PbANKA by i.p. injection. They were then given the extracts at doses of 500, 1,000, and 2,000 mg/kg for 4 consecutive days. On day 5 of experiment, hematocrit levels were measured. Results were expressed as mean \pm SEM. ** $p < 0.01$ compared to normal. N; normal mice, UN; untreated mice, PYR; 1 mg/kg of pyrimethamine, 500, 1,000, and 2,000; extracts at doses of 500, 1,000, and 2,000 mg/kg, respectively, N+2000; normal mice treated with 2,000 mg/kg of extract, PYR+2000; combination treatment of 1 mg/kg of pyrimethamine and 2,000 mg/kg of extract.

to induce oxidative stress from production of hydroxyl radicals via the Fenton/Harber-Weiss reaction [8,17]. Additionally, lipid peroxidation of RBC membrane followed by hemolysis has also been described [4]. Moreover, recruitment of inflammation during pathogenesis of malaria-associated hemolysis also contributes to increase the occurrence of hemolytic events [17].

For the efficacy test *in vivo* of aqueous crude extract of Siamese neem tree against PbANKA induced hemolysis presented the same level of % Hct compared with normal control, especially the extract at the doses of 1,000 and 2,000 mg/kg. It can be suggested that polyphenols and flavonoid contents in these extracts as well as alkaloids, terpenes, and saponins might play an important role to protect RBC from oxidative stress and inflammation induced by malaria infection [9,12,13]. It has been also reported that polyphenolic contents strongly positive correlated to antioxidant activity in the extract. Furthermore, metabolic acidosis induced by malaria infection, development, and RBC destruction followed by severe anemia has also been described [18]. Antioxidant property of Siamese neem tree extract has been reported to maintain blood pH as well as protect RBC from acidosis [19-21]. Interestingly, hemolysis was also observed in PYR treated group. However, normal level of % Hct was found in combination treatment between PYR and Siamese neem tree extract. Hemolysis in PYR treatment might be due to the oxidative stress and inflammation induced by this drug [22].

It is interesting to note that aqueous crude extract of Siamese neem tree was found to have anti-hemolytic activity against *P. berghei* infected mice. In addition, combination treatment with standard antimalarial pyrimethamine is recommended. Although the bioactive components and mechanism are yet to be identified, the results of this study provide the basis for further studies. Other mechanisms of action should also be considered.

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