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Antimalarial and Anti-hypoglycemic Properties of Siamese Neem Tree (Azadirachta indica) in Plasmodium berghei Infected Mice

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Abstract

The present study has been carried out to investigate antimalarial and anti-hypoglycemic activities of leaf aqueous crude extract of Siamese neem tree (*Azadirachta indica*) against *Plasmodium berghei* infected mice. Groups of ICR mice were treated orally with Siamese neem tree extract (500, 1000, and 2000 mg/kg) after infection with *P. berghei* ANKA. Parasitemia and blood glucose levels were determined. At these doses, Siamese neem tree extract inhibited parasitemia in dose-dependent manner with significance ($P < 0.05$). In addition, anti-hypoglycemic activity has been observed in infected mice treated with Siamese neem tree extracts. In particularly, the highest activities of Siamese neem tree extract were found at dose

2000 mg/kg. These results indicated that leaf aqueous crude extract of Siamese neem tree have antimalarial and anti-hypoglycemic activities against *P. berghei* ANKA infected mice.

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

Background

Malaria is a major health problem in Tropical and Sub-tropical regions. It contributes significantly to the overall malaria burden in Southeast Asia in particularly Thailand. An estimated 3.3 billion of the total world population live in areas with malaria risk and an estimated death of 660,000 [1,2]. This disease is caused by protozoa parasite in genus Plasmodium and transmitted by female Anopheles mosquito. For decades, drug resistance has been one of the main obstacles in the fight against malaria. It is responsible for the spread of malaria to new areas, the recurrence of malaria in areas where the disease had been eradicated and plays an important role in the occurrence and severity of epidemics in some parts of the world [3-6]. Furthermore, the difficulty of creating efficient vaccines and also adverse side effects of the existing antimalarial drugs highlight the urgent need for new antimalarial drugs for treatment of malaria. In addition, malaria-associated hypoglycemia has been reported during malaria parasite infection, and is one of all most causes of death [7-9].

According to several reports, up to 80% of world's populations rely on traditional medicine mainly on herbal remedies as primary source of medicinal agents for the treatment of diseases. Some antimalarial drugs in use today (quinine and artemisinin) were either obtained from plants or developed using their chemical structures as templates [10,11]. In Thailand, it is estimated that about 80% of the populations is still dependent on traditional medicine, which essentially involves the use of plants. Siamese neem tree (*Azadirachta indica* A. Juss var. siamensis Valeton) is one of two varieties of neem of the family Meliaceae, and is found throughout Southeast Asia including Laos, Myanmar, Cambodia, and Thailand [12]. It is used for the treatment of some pathological conditions related to oxidative stress, such as inflammation and skin diseases, rheumatic, arthritic disorders, and treatment of fever and diabetes [13]. However, there are few publication concerning the biological activities of Siamese neem tree against malaria, and it has not yet been reported the activity of this plant against hypoglycemia induced by malaria infection. Hence, Siamese neem tree is an interesting plant for future purpose related to its antioxidant activity including medicinal agents and health supplements. The aim of this study was to investigate the antimalarial and anti-hypoglycemic activities of the leaf aqueous crude extract of Siamese neem tree against P. berghei infection in mice.

Materials and Methods

Plant materials

Leaves of *A. indica* were collected from Kanchanaburi provinces, Thailand. The plant samples were compared with the voucher specimens 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.

Preparation of extracts

Leaf aqueous crude extract of Siamese neem tree was prepared using hot water method as previously described. Dried powder of leaf samples were boiled with distilled water for 6-8h (plant: water = 1:10 w/v), filter was then performed through Whatman no. 1 filter paper. The filtrate was lyophilized to dryness. The dry extract was stored at 4^0C until use. For administration to the animals, the aqueous crude extracts were resuspended in distilled water and vortex to get a clear solution [12,14].

Animals and malaria infection

Female ICR mice (weighting 25-30 g, aged 1 4-6weeks) were kindly provided by Dr. Chairat Uthaipibull at National Center

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for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA). Mice were maintained at a 12:12 light/dark cycle and were given as libitum access to standard pellet diet and water. For infection, mice were inoculated intraperitoneally with a load of 1×107 parasitized erythrocytes of *P. berghei* ANKA (PbANKA). The PbANKA blood stage cryopreservation were also kindly donated by Dr. Chairat Uthaipibull. The parasitemia was daily monitored by microscopy of Giemsa stained thin blood smear. All animal experiments received approval from the Ethical Animal Committee of Faculty of Medical Technology, Western University, and animals were handled according to standard guidelines.

$$\%\text{parasitemia} = \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} \times 100$$

Acute toxicity test

Acute toxicity of leaf aqueous crude extract of Siamese neem tree was carried out [15]. Groups of naïve ICR mice (5 mice of each) were given with the extracts (500, 1000, and 2000 mg/kg) orally by gavage. Signs of toxicity such as death, changes in physical appearance, behavioral change, and organ damage were observed and recorded for 72h.

Antimalarial drug

Chloroquine (CQ) was used to study in vivo drug susceptibility of PbANKA. The drug was freshly prepared in DW and administered orally by gavage. 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 was based on the ED90 (5 mg/kg) of this drug on PbANKA infected mice.

Blood glucose measurement

Tail blood was collected into heparinized microhematocrit tube. The end of tube was sealed with putty and centrifugation was then performed at 10,000 g for 10 min. Plasma was collected into a new 1.5-ml microcentrifuge tube, and used for blood glucose measurement. Blood glucose was measured using a commercial kit (BioSystem S.A. Costa Brava 30, Barcelona, Spain), according to the manufacturer's instruction.

Efficacy test *in vivo*

The standard 4-day suppressive test against PbANKA was employed [16]. Naïve ICR mice were inoculated by intraperitoneal injection with 1x107 parasitized erythrocytes of PbANKA. The mice were randomly divided into 5 groups (5 mice of each) and treated for 4 consecutive days with 500, 1000 and 2000 mg/kg of the extract orally by gavage. Two control groups were used; the positive control was treated daily with 5 mg/kg of CQ while the negative control group was given distilled water. On day 5 of the experiment, blood was collected from tail vein of each mouse and microscopy of Giemsa stained thin blood smear was performed to examine parasitemia, and percentage of inhibition was also calculated as showed below. Moreover, blood glucose levels were also measured as previously described.

$$\%\ \text{inhibition} = ((A-B) \div A) \times 100$$

Where A is the average % parasitemia in negative control group, and B is the average % parasitemia in the test group.

Statistics

Statistical analysis of the data was performed using Graph Pad Prism Software (Graph Pad Software, Inc., USA). The one way ANOVA test was used to analyze and compare the results at a 95% confidence level. Values of $p<0.05$ were considered significant. In addition linear regression of correlate was used to analyze the correlation. Results were expressed as mean+ standard error of mean (SEM).

Results

Acute toxicity test

Acute toxicity studies conducted revealed that the administration of doses of the leaf aqueous crude extract of Siamese neem tree (up to a doses of 2000 mg/kg) did not produce significant changes in behavioral, such as alertness, motor active, breathing, restlessness, diarrhea, convulsions, coma, and appearance of the animals. No death was observed, indicating that the medium lethal dose (LD50) could be greater than 2000 mg/kg. All mice were physically active. These effects were observed during the experimental period.

Malaria-associated hypoglycemia during PbANKA infection

Parasitemia was firstly detectable at day 3 after infection with a parasitemia less than 1% and reached 60% at day 14 after infection (Figure 1A). Additionally, we observed that blood glucose levels were markedly decreased in infected mice (Figure 1A). Strong negative correlation (R2= 0.778) between parasitemia and blood glucose level was also observed (Figure 1B).

Antimalarial activity of Siamese neem tree

The leaf aqueous crude extract of Siamese neem tree exerted antimalarial activity against PbANKA in dose-dependent manner. The extract at doses of 1000 and 2000 mg/kg caused significant ($p<0.05$) suppression with the inhibition of 50% and 82%, respectively when compared to untreated group (negative control) (Figure 2A). The standard drug, CQ caused suppression of 91% inhibition, which was higher than those of the extract treated groups.

Anti-hypoglycemic effect of Siamese neem tree

As showed in Figure 2B, hypoglycemia with significant ($p<0.001$) low levels of blood glucose were observed in untreated group (negative control) and infected mice treated with 500 mg/kg of the extract. Interestingly, the leaf aqueous crude extract of Siamese neem tree exerted anti-hypoglycemia in the extract treated groups, especially at doses of 1000 and 2000 mg/kg.

Discussion

There was a progressive increasing in level of parasitemia as the days progressed from day 3 to 14 in the PbANKA infected mice (Figure 1A). This is in line with the view that parasitemia increases progressively after inoculation or infection until the point of death in the absence of suitable treatment. Interestingly, determination of blood glucose levels showed a progressive decrease in the response to the presence of the parasites, which reached significant valued on 8 day after infection (Figure 1A). Moreover, strong negative correlation (R2= 0.778) between parasitemia and blood glucose was also observed (Figure 1B). Thais could be due in part to the fact that during malaria infection, glucose is rapidly taken up across the parasite plasma membrane through a facilitated hexose transporter and is in turn metabolized through the process of glycolysis [17, 18]. This is accompanied with approximately 100-fold increase in glucose utilization when compared with uninfected erythrocytes thus causing a profound hypoglycemia if untreated [19]. Furthermore, hyperinsulinemia and hypoglycemia during malaria infection has also been described [20].

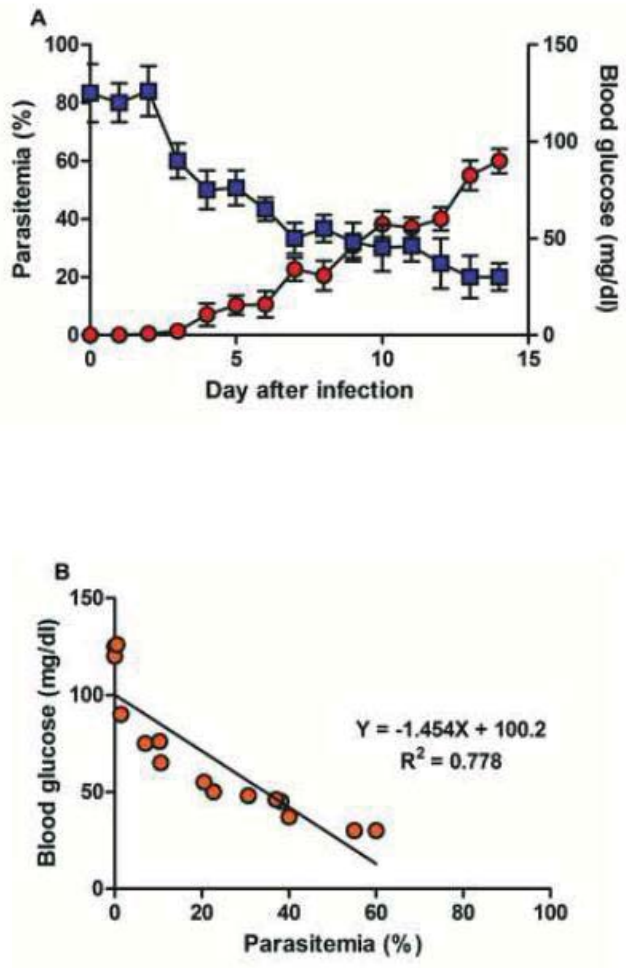

Figure 1: Malaria-associated hypoglycemia induced by *Plasmodium berghei* infection. ICR mice (5 mice of each) were inoculated intraperitoneally with 1x107 parasitized erythrocytes of PbANKA. (A) Parasitemia and blood glucose levels were daily monitored. (B) Correlation of parasitemia and blood glucose was also determined. Results were expressed as mean+SEM. Red circle represented %parasitemia, and blue square represented blood glucose levels.

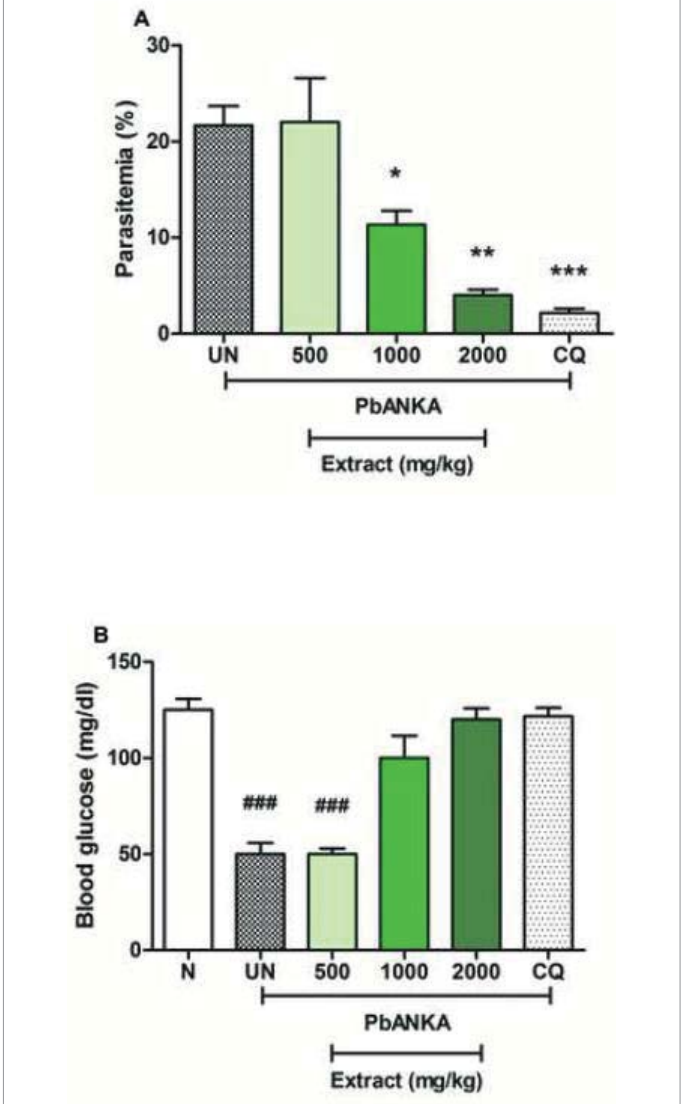

Figure 2: Anti-hypoglycemic effect of Siamese neem tree extract against *Plasmodium berghei* infection. Groups of ICR mice (5 mice of each) were inoculated with 1x107 parasitized erythrocytes of PbANKA by intraperitoneal injection, and subsequently given 500, 1000, and 2000 mg/kg of the extracts orally for 4-consecutive days. On day 5 of experiment, (A) parasitemia and (B) blood glucose were measured. Results were expressed as mean+SEM.

During malaria infection, the leaf aqueous crude extract of Siamese neem tree produced a dose-dependent antimalarial activity against PbANKA. The extract caused a significant ($p<0.05$) antimalarial when compared to the untreated control, especially at dose of 2000 mg/kg showed the highest activity (Figure 2A). The standard drug, CQ caused chemosuppression, which was higher than those of the extract treated groups. It has been reported that the antioxidant potential was related to antimalarial activity in several plant extracts [21-24]. Hence, flavonoids and polyphenolic compounds in Siamese neem tree, and its potent antioxidant activity might play a role to inhibit PbANKA growth in vivo. In addition, it has been described that azadirachtin and nimbin, most active compounds in Siamese neem tree might also play a role in antimalarial activity [25-27]. Interestingly, oxidative damage in order to inhibit malaria parasite of artemisinin has been reported, and might related to antimalarial property of Siamese neem tree extract. Moreover, it has been reported antimalarial activity was found in either A. indica leaf extract alone or in combination with artesunate in dose-dependent manners [28]. Azadirachtin and nimbin were considered to be active compounds in this extract [29,30]. However, mode of action and other mechanisms should be searched for. As showed in Figure B, hypoglycemia with significant ($p<0.001$) low level of blood glucose was found in untreated group. Interestingly, the leaf aqueous crude extract of Siamese neem tree presented anti-hypoglycemia in the extract treated groups, especially at dose of 2000 mg/kg showed the highest activity. Several studies have been reported the activity of many plant extracts that have antioxidant activity could control blood glucose levels. Knowledge of properties and constituents of Siamese neem tree such as flavonoids and polyphenolic compounds suggests that biological activity of Siamese neem tree to maintain and control blood glucose level might be similar to other plant extracts. Inhibition of glycolysis and hexose transporter of infected erythrocytes

might be properties of Siamese neem tree on blood glucose levels. In addition, beneficial effect of Siamese neem tree on insulin may be due to the antioxidant capacity of this extract. It has been also described that leaf aqueous crude extract of Siamese neem tree had significant antioxidant potential [12,14,31,32]. However, no antimalarial and anti-hypoglycemic activities were observed in PbANKA infected mice treated with 500 mg/kg of the extract, might be due to the fact that low levels of active compounds and antioxidant activity. It is interesting to note that leaf aqueous crude extract of Siamese neem tree was found the antimalarial and anti-hypoglycemia against *P. berghei* infection in mice.

Although the bioactive compounds and mechanism are yet to be identified, the results of this study provided the basis for further studies. For all results, the leaf aqueous crude extract of Siamese neem tree produced a reduction in parasitemia level in the extract treated group; there was also a similar reduction in the chloroquine treated group. In addition, this extract showed anti-hypoglycemic activity against *P. berghei*-induced hypoglycemia. This finding is sufficient to say that Siamese neem tree extract has antimalarial and anti-hypoglycemic activities against malaria parasites. Acute toxicity of extract of Siamese neem tree observation that no death with up to an oral dose of 2000 mg/kg could indicate that the extract is very safe.

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