Anti-hypoglycemic effect of aqueous leaf extract of Siamese neem tree 
(Azadirachta indica) in Plasmodium berghei infected mice

Somsak, V., Srichairatanakool, S. and Uthaipibull, C.

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
3National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand

Abstract
Hypoglycemia has been reported during malaria infection in blood stage, and can cause of death in malaria endemic countries. Hence, in this study was aimed to evaluate anti-hypoglycemic effect of Siamese neem tree extract in P. berghei infected mice. The aqueous leaf extract of Siamese neem tree was freshly prepared and used for orally treatment. Groups of ICR mice were infected intraperitoneally with 6x10⁶ infected erythrocytes of P. berghei ANKA, and subsequently given with the extract twice a day for 4 consecutive days. Blood glucose levels were then measured. Normal and untreated mice were used as healthy and disease controls, respectively. The results showed that hypoglycemia was developed during P. berghei ANKA infection in mice as indicated by decreasing of blood glucose level. However, blood glucose level in the extract treated mice was similar to normal group. It can be summarized that aqueous leaf extract of Siamese neem tree exhibited anti-hypoglycemic effect against P. berghei ANKA infected mice.

Introduction
Malaria caused by Plasmodium parasite is a major public health problem in tropical and subtropical countries with estimates of 700,000 cases annually (Kar et al., 2014). During malaria infection, hypoglycemia is recognized as serious complication malaria and occurred between 1-4% of hospitalized patients with a mortality that can reach up to 45% (Onyiriuka et al., 2012). The glucose metabolism in malaria infection is affected by several factors including drug treatment, fever, parasite metabolism, hormonal changes, cytokines, fasting and gastrointestinal disturbances (Elased et al., 1996). This has prompted research towards the discovery and development of new, safe and affordable drugs to protect the hypoglycemia during malaria infection. In this respect, medical plant resources are potential targets for this research.

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, Mynmar, Cambodia and Thailand. This plant is used for the treatment of some pathological conditions related to oxidative disorders such as inflammation and skin diseases, rheumatic, arthritis, fever, and diabetes (Kitdamrongtham et al., 2014; Sithisarn et al., 2005; Sithisarn et al., 2006). However, publications concerning the activity of Siamese neem tree in hypoglycemic condition have not yet been reported. Hence, the aim of this study was to evaluate the anti-hypoglycemic activity of aqueous leaf extract of Siamese neem tree on P. berghei infection in mice.

Materials and Methods
Plant material and preparation of crude extract
Leaves of Siamese neem tree (Azadirachta indica) were obtained from Kanchanaburi province, and specimen was then verified by Dr. Sakaewan Ounjaijene, Department of Pharmacology, Faculty of Pharmacy, Payap University, Chiang Mai, Thailand. Dried powder leaves (50°C in hot air oven for 30 min) were extracted using hot distilled water (plant:water = 1:20 w/v) for 6-8 h, then filtered. The filtrate was evaporated to dryness on boiling water bath to obtain dried extract (Sithisarn et al., 2006).

Experiment mice
Naïve ICR mice weighting 25-30 g, aged 4-6
weeks were purchased from National Laboratory Animal Center (NLAC), Mahidol University. They were kept in animal room with temperature 22-25°C, 12 h day/night cycle, and fed with standard pellet and clean water ad libitum. All animal experiments were ratified by Animal Ethical Committee, Western University.

Rodent malaria parasite

Plasmodium berghei strain ANKA (PbANKA) was used in this study. The parasite was maintained in naïve ICR mice by intraperitoneal (IP) injection of $6 \times 10^6$ infected erythrocytes. Parasite propagation was daily monitored as percent parasitemia by Giemsa stained blood smear.

Measurement of blood glucose

Tail blood was collected into heparinized hematocrit tubes. Centrifugation was then performed at 10,000 g for 10 min. Plasma was collected into new 1.5-ml microcentrifuge tube and blood glucose measurement was subsequently done using commercial kit (BioSystems S.A. Costa Brava, Barcelona, Spain).

Efficacy test in vivo

The standard 4-day suppressive test was used to evaluate efficacy of the extract (Peters, 1975). Groups of ICR mice (5 mice of each) were randomly divided, and infected intraperitoneally with $6 \times 10^6$ infected erythrocytes of PbANKA. They were given with 500, 1500, and 3000 mg/kg of the extract twice a day for 4 consecutive days. Blood glucose levels were then measured as previously described. Normal and untreated mice were used as healthy and disease controls, respectively.

Statistical analysis

All data was analyzed using GraphPad Prism (GraphPad Software, Inc., USA). The results were expressed as mean±standard error of mean (SEM). The one-way ANOVA was used to test and compare the results at a 95% confidence. Values of $p<0.05$ was considered significance.

Results and Discussion

Malaria-associated hypoglycemia development during PbANKA infection

In order to determine hypoglycemia during malaria infection, naïve ICR mice were infected intraperitoneally with $6 \times 10^6$ infected erythrocytes of PbANKA. Hypoglycemia was subsequently investigated by measuring blood glucose level. As shown in Figure 1A, there was a progressive increase in line of parasitemia as the days progressed from day 2 to 14 in the infected mice. Parasitemia increased progressively after inoculation until the point of death in the absence of suitable treatment. Next, we observed that blood glucose level was markedly decreased starting at day 6 after infection (Figure 1B), and all infected mice died within 2 weeks (Figure 1C). This could be due in part to the fact that during malaria infection, glucose is rapidly taken up across the parasite membrane through a facilitated hexose transporter and is in turn metabolized through
the process of glycolysis (Tjhin et al., 2013). This is accompanied with approximately 100-fold increase in glucose utilization when compared with uninfected erythrocytes thus causing a profound hypoglycemia (Woodrow et al., 2000). Furthermore, impairment of glucose production caused by the inhibition of gluconeogenesis during severe malaria infection has previously been discussed and also led to hypoglycemia (Eltahir et al., 2010; Geoffrion et al., 1985; van Thien et al., 2004).

Anti-hypoglycemia of aqueous leaf extract of Siamese neem tree during PbANKA infection

In order to investigate anti-hypoglycemic effect of the extract, PbANKA infected ICR mice were given the extract orally by gavage twice a day for 4 consecutive days. Blood glucose levels were measured and compared to normal and untreated groups. It was found that glucose level was significantly lower (p<0.05) in the untreated group compared to the normal control (Figure 2). Interestingly, aqueous leaf extract of Siamese neem tree exerted dose dependent anti-hypoglycemic effect against PbANKA infected mice, especially at the doses of 1,500 and 3,000 mg/kg. This could be due to a fall in glycolysis activity within the cells of mice in these groups (Elased & Playfair, 1994). Moreover, antioxidant properties of Siamese neem tree extract and its active compound have been reported to protect the cell from oxidative damage and control glucose homeostasis (Sithisarn et al., 2005). The result also showed that the extract might have no significant side effects on glucose level in normal mice.

Conclusion

The results of this study showed that aqueous leaf extract of Siamese neem tree exhibited anti-hypoglycemia during PbANKA infection in mice.

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