

HEPATOPROTECTIVE EFFECT OF AQUEOUS CRUDE EXTRACT OF *THUNBERGIA LAURIFOLIA* LEAVES AGAINST *PLASMODIUM BERGHEI* INFECTED MICE

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ABSTRACT:

Background: Hepatotoxicity induced by malaria infection is commonly a significant cause of morbidity and mortality among humans. Hence, identification of medicinal plants that have hepatoprotective activity is urgently needed. Therefore, this study aimed to evaluate the hepatoprotective effect of aqueous crude extract of *Thunbergia laurifolia* leaves in *Plasmodium berghei* infected mice.

Methods: Aqueous crude extract of *T. laurifolia* leaves was prepared and tested for hepatoprotective effect in *P. berghei* infected mice. The standard 4-day suppressive test was undertaken in groups of ICR mice infected with 1×10^7 parasitized erythrocytes, and then orally treated with the extract at doses of 1000, 2000, and 4000 mg/kg for 4 consecutive days. Pyrimethamine (1 mg/kg) was used as positive control, and combination treatment with 4000 mg/kg of extract was also determined. All liver markers were subsequently measured including aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, and albumin.

Results: Hepatotoxicity was induced by malaria infection in mice as indicated by progressive increase of AST and ALT, and marked decrease of cholesterol and albumin, particularly starting on day 4 after infection. Interestingly, hepatoprotective effect was found in infected mice treated with the extract in dose-dependent manner as indicated by the similar levels of all liver markers to normal mice, and the highest activity was found at dose of 4000 mg/kg. Moreover, combination treatment of pyrimethamine and the extract is recommended. Additionally, prolonged survival time of infected mice treated with the extract was also found. No side effect of this extract was observed in normal mice.

Conclusion: Aqueous crude extract of *T. laurifolia* leaves had a hepatoprotective effect during *P. berghei* infection, and can be used as combination treatment with standard antimalarial drugs.

Keywords: Antimalarial, Hepatoprotective, *Thunbergia laurifolia*, *Plasmodium berghei*

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INTRODUCTION

Malaria caused by *Plasmodium* parasites is a major health problem in Africa, South and Central America, and Asia including Thailand, where it is most prevalent, with estimates of more than 70-80 million cases annually [1]. Liver involvement in severe malaria infection is commonly a significant cause of morbidity and mortality among humans [2].

The pathogenesis of liver damage during malaria infection is multifactorial and not well identified, but it is attributed to cytoadherence of parasitized erythrocytes, inflammation and hepatotoxicity due to oxidative stress [3]. Moreover, liver damage and apoptosis induced by hemolysis and hemozoin have also been discussed [2, 4]. Hence, finding of medicinal plants to have hepatoprotective activity are urgently needed.

Thunbergia laurifolia Lindl., commonly known in Thai as Rang Chuet, belong to the family of

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Acanthaceae. This plant has been used as Thai traditional medicine such as antipyretic and detoxifying agent in cases of poisoning [5]. Polyphenols, carotenoid, flavonoids, and apigenin have been reported to be major constituents in *T. laurifolia* leaf extract [6]. The pharmacological properties of *T. laurifolia* leaf extract presented antioxidant, free radical scavenging, anti-inflammation, anti-microbials, anti-cancer, anti-diabetic, and anti-hemolysis [7-10]. It has also been described that this extract has a protective effect on hepatotoxicity [11]. However, protective effect of *T. laurifolia* leaf extract on liver damage induced by malaria infection has not yet been reported. So, this study was aimed to evaluate hepatoprotective effect of aqueous crude extract of *T. laurifolia* leaves against *P. berghei* infected mice.

MATERIALS AND METHODS

Plant materials and preparation of aqueous crude extract

Leaves of *T. laurifolia* used in this study were collected from local areas in Suphanburi province, Thailand, and authenticated by Dr. Sakaewan Ounjaijean, Faculty of Pharmacy, Payap University. Hot water method was used to prepare aqueous crude extract [12]. Leaves were washed, then dried in hot-air oven at 60°C for 6 h, and ground using electric blender in order to obtain fine powder and stored at 4°C. For aqueous crude extract preparation, hot water method with microwave was used. Dried powdered leaves of *T. laurifolia* were dissolved in distilled water with a ratio of 1:10 w/v, and subsequently heat in microwave with 360 W for 5 min. Incubation at room temperature with continuous stirring was performed for overnight to complete extraction. Then, it was filtered using Whatman no. 1 filter paper and drying by freeze dryer to obtain dried aqueous crude extract of *T. laurifolia* leaves at a yield of 32.6% (w/w). Prior to dosing in animals, the dried extract was freshly dissolved in distilled water and adjusted to the desired concentration for experiments.

Experimental mice

Female ICR mice, 4-6 weeks old and weighing between 25-30 g, used in this study were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. They were kept in the animal room maintained at 22-25°C, 12 h light-dark cycle with standard pellet diet 082 and clean water *ad libitum*. All animal procedures and experiments were ratified by the Animal Ethic

Committee, Western University (WTU-EC-1501).

Rodent malaria parasite

Plasmodium berghei strain ANKA (PbANKA), a kind gift of Dr. Chairat Uthaipibull, was used in this study. The parasite was maintained in ICR mice by intraperitoneally injection of 1×10^7 parasitized erythrocytes of PbANKA. Percent parasitemia (% parasitemia) was daily monitored by microscopy of Giemsa stained thin blood smear. Moreover, percent of packed cell volume (% PCV) was also measured according to the following formula:

$$\%PCV = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

Measurement of liver function test

Experiment mice were anesthetized using isoflurane/oxygen gas. Mouse blood was collected by cardiac puncture and transferred into a heparinized vacuum tube. Centrifugation was then performed at 16,000 g for 10 min, and plasma was subsequently collected into a new clean tube. The plasma was used as subject to measure liver function markers including aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, and albumin by Automate analyzer for clinical chemistry test (SYNCHRON LX®20, PCL Holding Co., Ltd).

Standard antimalarial drug

Pyrimethamine (PYR) was used in this study as positive control for antimalarial test *in vivo*. The drug was prepared in DMSO with a drug dose based on the ED90 (1.0 mg/kg) on PbANKA infected mice, and stored at 4°C.

Efficacy test of aqueous crude extract of *T. laurifolia* leaves *in vivo*

For efficacy test *in vivo* of the extract, standard 4-day suppressive test was used [13]. ICR mice were infected with 1×10^7 parasitized erythrocytes of PbANKA by intraperitoneal injection, then infected mice were randomly divided into 4 groups (5 mice of each) and given either with the extracts (1000, 2000, and 4000 mg/kg) or 1 mg/kg of PYR orally by gavage. Control groups were also used including normal mice treated with or without 4000 mg/kg of extract, and untreated control given only distilled water. Moreover, combination treatment of PYR and 4000 mg/kg of extract was also investigated. Treatment was carried out 2 h later after infection and subsequently every 24 h for 4 consecutive days (day0-3). On day 4 of experiment, blood was collected to measure % parasitemia, % PCV, AST, ALT, cholesterol, and albumin levels. In addition, maximum survival time was also determined.

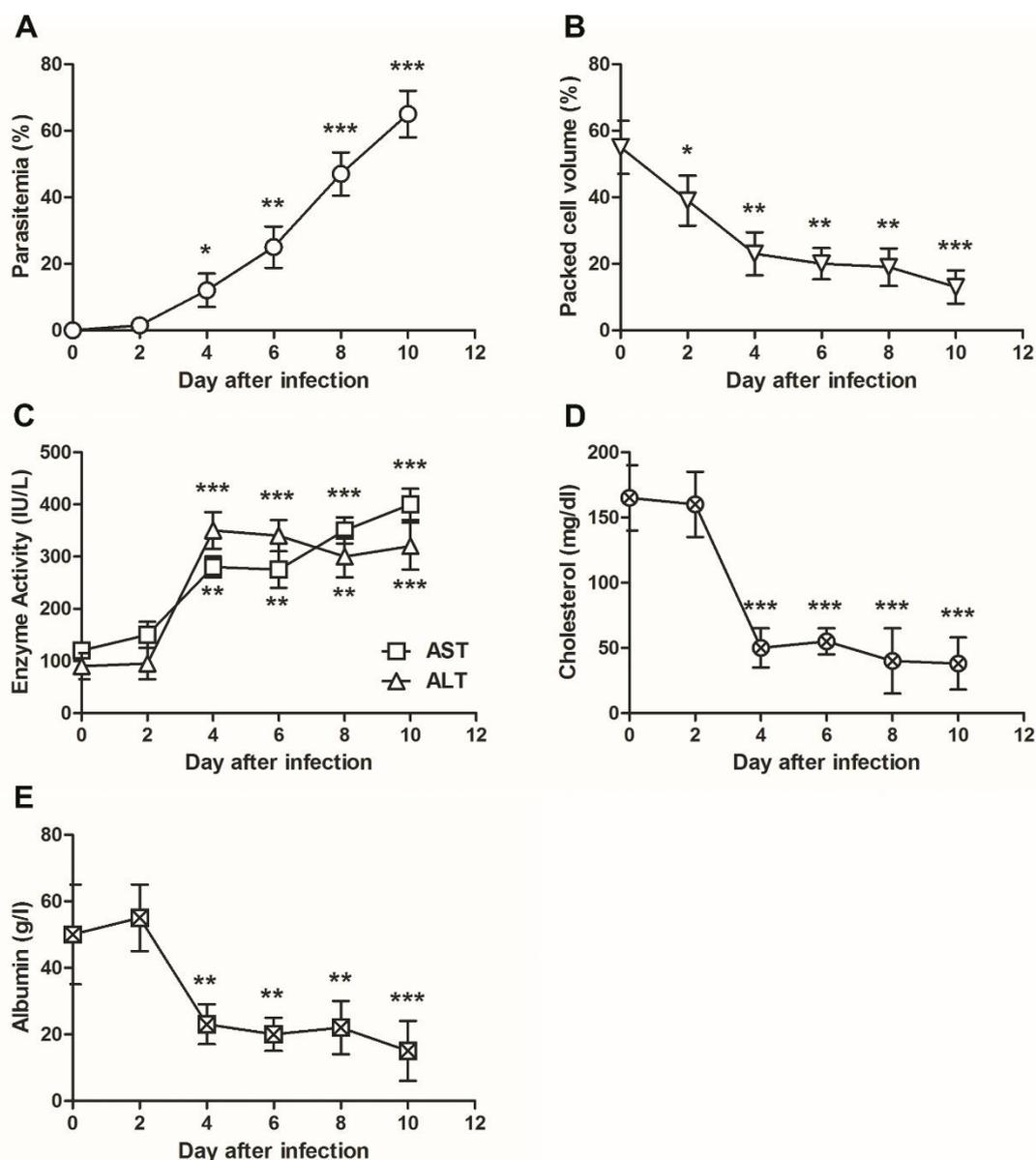


Figure 1 Hepatotoxicity induced by *Plasmodium berghei* ANKA infection in ICR mice. ICR mice (5 mice of each) were infected with 1×10^7 parasitized erythrocytes of PbANKA by intraperitoneal injection. (A) Parasitemia, (B) packed cell volume, (C) aspartate and alanine aminotransferase, (D) cholesterol, and (E) albumin levels were monitored. (F) Survival time of infected mice was also observed. Results were expressed as mean \pm SE. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to day 0 after infection

Statistics

Statistical analysis was performed using GraphPad Prism Software (GraphPad software, Inc., CA, USA). All results were expressed as mean \pm standard error of mean (SE). In addition, significance was considered at 95% confidence with $p < 0.05$ using the one way ANOVA.

RESULTS

Hepatotoxicity during PbANKA infection in mice

During PbANKA infection in ICR mice, it was found that parasitemia was markedly increased and

firstly observed on day 2 after infection (1.5% parasitemia) until infected mice died on day 10 with a parasitemia of 65% (Figure 1A). A decrease in %PCV with a correlation of parasite growth was also observed (Figure 1B). Interestingly, hepatotoxicity in PbANKA infected ICR mice was found as indicated by markedly increasing of AST and ALT, and a decrease in cholesterol and albumin. Particularly, the significant ($p < 0.05$) levels were firstly observed on day 4 after infection (Figure 1C-E). Survival time of PbANKA infected ICR mice was 10 days (Figure 1F).

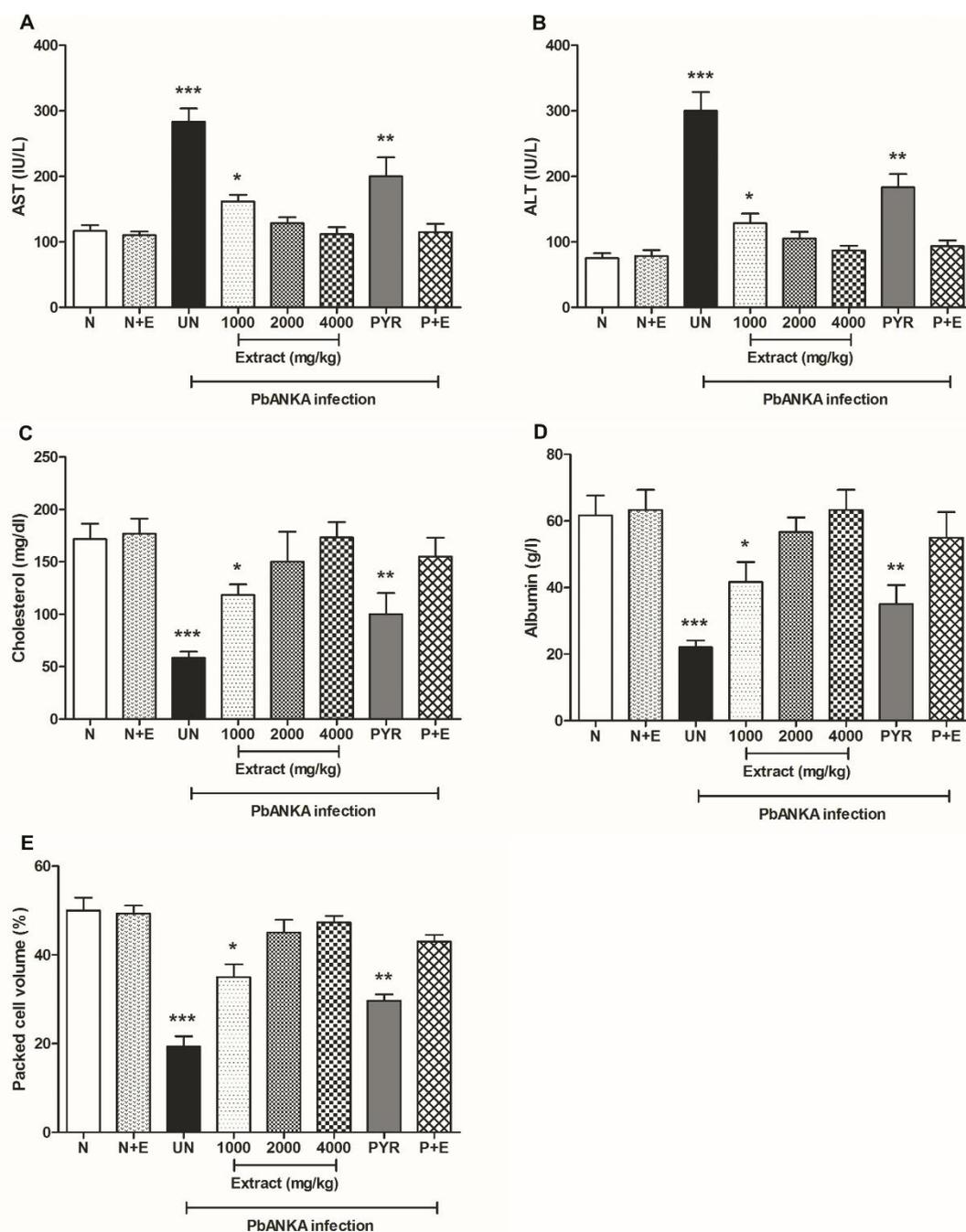


Figure 2 Hepatoprotective effect of aqueous crude extract of *Thunbergia laurifolia* leaves against *Plasmodium berghei* ANKA infected ICR mice. Groups of ICR mice (5 mice of each) were infected with 1×10^7 parasitized erythrocytes of *PbANKA* by intraperitoneal injection and orally treated with 1000, 2000, and 4000 mg/kg of extract for 4 consecutive days. (A) Aspartate aminotransferase, (B) alanine aminotransferase, (C) cholesterol, (D) albumin, and (E) packed cell volume were subsequently measured. Results were expressed as mean \pm SE. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to normal mice. N; normal mice, N+E; normal mice treated with 4000 mg/kg of extract, UN; untreated mice, PYR; 1 mg/kg of pyrimethamine, and P+E; combination treatment with 1 mg/kg of pyrimethamine and 4000 mg/kg of extract.

Protective effect of aqueous crude extract of *T. laurifolia* leaves on liver damage induced by *PbANKA* infection

During *PbANKA* infection in ICR mice, hepatotoxicity was found in untreated control group as indicated by significantly ($p < 0.01$) increasing of

AST and ALT (Figure 2A-B, UN), and decreasing of cholesterol and albumin levels (Figure 2C-D, UN). Moreover, a significant ($p < 0.01$) decrease in % PCV was also observed in untreated control group (Figure 2E, UN). Interestingly, hepatoprotective effect was found in *PbANKA* infected mice treated

Table 1 Survival time of *Plasmodium berghei* ANKA infected ICR mice and treatment with aqueous crude extract of *Thunbergia laurifolia* leaves

Group of <i>P. berghei</i> ANKA infected ICR mice	Maximal survival time (Days)
Untreated mice (distilled water)	10
1000 mg/kg of extract	25
2000 mg/kg of extract	28
4000 mg/kg of extract	30
Pyrimethamine (1 mg/kg)	28

with the extract at doses of 2000 and 4000 mg/kg indicated by the levels of AST, ALT, cholesterol, and albumin were all similar levels to normal mice (Figure 2A-D, Extract). Moreover, anti-hemolysis during malaria infection of the extract was also presented (Figure 2E, Extract). Surprisingly, significant ($p < 0.05$) hepatotoxicity and hemolysis were found in infected mice treated with PYR (Figure 2A-E, PYR). However, normal levels of all liver function markers and PCV were observed in combination treatment of PYR and 4000 mg/kg of extract (Figure 2A-E, P+E). In addition, no any toxic effects on liver function and hemolysis were found in normal mice treated with 4000 mg/kg of extract (Figure 2A-E, N+E). Prolong survival time (30 days) was also observed in PbANKA infected mice treated with the extract (Table 1).

DISCUSSION

In the present study, the hepatoprotective effect of aqueous crude extract of *T. laurifolia* leaves against PbANKA infection in ICR mice was investigated. It has been reported that liver damage occurred during malaria infection in blood stage, especially in chronic infection as indicated by increasing of AST, ALT, alkaline phosphatase, total bilirubin and decreasing of albumin [2]. In our finding, liver damage was also induced by PbANKA infection as indicated by significant increasing levels of AST and ALT, and decreasing of cholesterol and albumin with firstly observed on day 4 after infection. AST and ALT are liver enzyme markers that are increased during the liver damages. In addition, cholesterol and albumin are biomolecules that are generated by liver, and can be used as markers for abnormal function of liver when decreasing of these markers are found. From our finding, it can be suggested that during malaria infection, oxidative stress and inflammation were progressively increased and caused organ damage and failure, especially liver and renal [14-16]. Moreover, death of malaria patients by liver damage and failure has also been reported [17]. Therefore, oxidative stress and inflammation during malaria infection might play a critical role in liver damage and death of infected mice within 2 weeks in our

study. Additionally, activation of NF- κ B p65 and apoptosis of hepatocytes in malaria infection has been described [2]. It has also been suggested that hemozoin induced liver inflammation and damage during rodent malaria parasite infection in mice [4]. Furthermore, during malaria infection in blood stage, PCV was markedly decreased in response to parasite growth. It can be discussed that the decrease in %PCV and hemolysis always occurred when malaria parasites fully developed into mature schizonts containing thousands merozoites and burst out into the blood circulation. During hemolysis of infected erythrocytes, oxidative stress and inflammation are also developed and can make other erythrocytes in both normal and infected ones hemolysis [8]. So, malaria patients might die from acute hemolysis and severe anemia.

For efficacy test *in vivo* of aqueous crude extract of *T. laurifolia* leaves, the extract showed hepatoprotective effect with anti-hemolysis activities during PbANKA infection in ICR mice. It has been described that *T. laurifolia* leaf extract contained high polyphenolic content and flavonoids, and presented potent antioxidant, free radical scavenging and anti-inflammation [6, 9]. Moreover, it has been reported previously that this extract exerted hepatoprotective and anti-hemolytic effects [8, 9, 11]. Hence, antioxidant, anti-inflammation, and anti-hemolytic activities of this extract containing polyphenolic content and flavonoids might likely protect liver damage and hemolysis induced by PbANKA infection in mice in our study. Furthermore, apigenin, major component of *T. laurifolia* leaf extract, has been reported to have strong antioxidant, anti-microbials, and hepatoprotective effect in oxidative stress condition [18-21]. This compound might also be mentioned in this study. Interestingly, hepatotoxicity was found in infected mice treated with PYR. It has been described that PYR could induce oxidative damage to vital organs such as liver, renal and heart, and caused hemolysis [22]. So, oxidative stress induced by PYR treatment should play a role in liver damage and decreasing of PCV. However, hepatotoxicity and hemolysis induced by PYR could be protected by combination treatment with *T. laurifolia* leaf

extract. Antioxidant, anti-inflammation and hepatoprotective effects of this extract might likely protect liver damage and erythrocytes from oxidative stress and inflammation induced by both malaria infection and PYR [9]. For hepatoprotective and anti-hemolytic effects of this extract during malaria infection, prolong survival time of mice was found up to 1 month. It can be concluded that aqueous crude extract of *T. laurifolia* leaves protects liver damage and hemolysis during malaria infection, and prolong survival time of malaria infected mice. Hence, this justifies the traditional usage of this extract as malaria treatment.

In conclusion, the study reveals that aqueous crude extract of *T. laurifolia* leaves presented hepatoprotective effect during *P. berghei* infection as indicated by normal levels of AST, ALT, cholesterol, and albumin with prolong survival time of infected mice. Moreover, this extract can be used as combination treatment with standard antimalarial drugs.

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