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Antimalarial, Anti-hemolytic, Hepatoprotective, and Nephroprotective Activities of *Gynostemma pentaphyllum* Leaf Extract in *Plasmodium berghei* Infection in Mice

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Abstract

Malaria is still a major problem around the world, especially in tropical and sub-tropical zones. Malaria-associated hemolysis and liver and renal injuries have been reported to be causes of death in malaria cases. In this respect, finding plant extracts which have a protective effect against these pathologies induced by malaria are urgently needed. The present study aimed to evaluate the antimalarial, anti-hemolytic, hepatoprotective, and nephroprotective properties of *Gynostemma pentaphyllum* leaf extract (GPE) in *Plasmodium berghei* infected mice. ICR mice were infected intraperitoneally with 1×10^7 parasitized erythrocytes of *P. berghei* ANKA (PbANKA), and given the extract (100, 500, and 1000 mg/kg) orally for 4-consecutive days. Parasitemia, packed cell volume (PCV), alanine aminotransferase (ALT), and creatinine levels were measured. The results showed that during PbANKA infection in mice, parasitemia was increased from day 1 - 14 post-infection with hemolysis, as indicated by the reduction of PCV. Moreover, liver and renal damage during malaria infection were observed, as indicated by the marked increase of ALT and creatinine levels in infected mice. Interestingly, these pathologies induced by PbANKA infection were protected and maintained at normal levels in PbANKA infected mice treated with GPE in a dose-dependent manner. The highest activity was found at a dose of 1000 mg/kg of GPE. No effects on PCV, ALT, or creatinine levels were observed in normal mice treated with 1000 mg/kg of GPE. Moreover, GPE exerted a dose-dependent reduction of parasitemia against PbANKA, with percent inhibitions of 57.6, 78.4, and 89.6 % at doses of 100, 500, and 1000 mg/kg of GPE, respectively. It can be concluded that GPE exerted antimalarial, anti-hemolytic, hepatoprotective, and nephroprotective activities against PbANKA infection in mice.

Keywords: Antimalarial, anti-hemolytic, hepatoprotective, nephroprotective, *Gynostemma pentaphyllum*

Introduction

Malaria is a major parasitic disease with high mortality and morbidity, particularly in Africa, South America, Asia, and Southeast Asia. It has been estimated that about 2 million cases of malaria and 1 million deaths have occurred in the world. This disease is caused by parasites in the genus *Plasmodium*, and transmitted by female *Anopheles* mosquito [1]. Malaria-associated hemolysis, as well as organ damage including liver and renal damage, are well-known causes of death in *P. falciparum* and *P. vivax* severe malaria, occurring in 1 - 4 % of hospitalized patients, with a mortality that can reach up to 60 % [2]. Moreover, due to the lack of effective vaccines to prevent malaria and the emergence of drug resistant malaria parasites, the discovery and development of new, safe, and affordable antimalarial drugs to eliminate the parasites, as well as to protect against liver and renal damage induced by malaria infection, are urgently needed. In this respect, medicinal plant extracts are potential targets; although 80 % of the

Thai population uses traditional medicinal plants for the treatment of several diseases, including malaria, these plant extracts have not yet been fully explored [3]. *Gynostemma pentaphyllum* belongs to the family Cucurbitaceae. It is an edible plant used as a medicine in oriental countries [4]. This plant extract has been investigated for its health benefits in preventing cardiovascular disease, cancer, and microbial infection, and for its lipid and glucose lowering, anti-inflammation, antioxidant, anti-parasite, and multiple organ protection effects [5-8]. However, its antimalarial, anti-hemolysis, and organ protection against malaria effects have not yet been studied. Hence, this study was aimed to evaluate the biological properties, including the antimalarial, anti-hemolysis, and protective effects on liver and renal damage induced by malaria infection, of *G. pentaphyllum* leaf extract in *Plasmodium berghei* infection in mice.

Materials and methods

Plant material and preparation of crude extract

Dried leaves of *G. pentaphyllum* were purchased from the Royal Project Foundation shop in Suphanburi province, Thailand. This plant material was then ground using an electric blender to obtain a powder sample, and used for the preparation of crude extract by dissolving it in distilled water (1:10). Heating in a microwave at 360 W for 5 min was performed [9], followed by incubation at room temperature for 3 h. Filtering through Whatman no. 1 filter paper was subsequently done in order to collect the filtrate. Freeze-drying was carried out to obtain the aqueous crude extract of *G. pentaphyllum* leaves (GPE), which was stored at –20 °C. Before the experiment, the extract was dissolved in distilled water at appropriate doses.

Experimental mice

Female ICR mice obtained from the National Laboratory Animal Center, Mahidol University, were used. They were kept in an animal room at a temperature between 25 - 28 °C and in a 12 h light/12 h dark cycle. They were freely fed with a standard pellet diet CP082 and drinking water, *ad libitum*. All experiments involving the animal were ratified by the Ethical Animal Committee, Western University.

Acute toxicity test

Groups of ICR mice (5 mice in each) were given GPE orally by gavage, at doses of 100, 500, 1000, 2000, and 4000 mg/kg body weight. These mice were observed for signs of toxicity, which include, but are 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 14 days [10].

Rodent malaria parasite

Plasmodium berghei strain ANKA (PbANKA) was used. The parasite was maintained by a intraperitoneal (IP) injection of 1×10^7 infected red blood cells (iRBC). Parasitemia was monitored daily by microscopy of a Giemsa-stained thin blood smear. Briefly, a thin blood smear from the tail vein of infected mice was placed on a microscopic glass slide. Fixation with absolute methanol for 3s was then performed. The smeared slide was subsequently stained with 10 % Giemsa dye for 10 min. The iRBC was measured under a light microscope with 100× oil immersion lens. Percent of parasitemia was calculated using the formula below;

$$\% \text{ parasitemia} = \frac{\text{Number of iRBC} \times 100}{\text{Number of RBC}} \quad (1)$$

Measurement of packed cell volume (PCV)

Tail blood was collected into a heparinized hematocrit tube and sealed by a crystal seal. Centrifuging was then performed at 3,000 g for 5 min. The percent proportion of PCV and total blood volume was measured and calculated using the formula below;

$$\% \text{ PCV} = \frac{\text{Volume of packed RBC} \times 100}{\text{Total volume of blood}} \quad (2)$$

Measurement of alanine aminotransferase (ALT) and creatinine

Tail blood was collected into a heparinized hematocrit tube and sealed by a crystal seal. Centrifuging was then performed at 3,000 g for 5 min. Plasma was collected as a subject for measurement of ALT and creatinine by commercial kits (BioSystems S.A., Costa Brava, Barcelona, Spain), according to the manufacturer's instructions.

Efficacy test of extract *in vivo*

For the efficacy test of GPE *in vivo*, a standard 4-day suppressive test was carried out [11]. Groups of ICR mice (5 mice in each) were infected with 1×10^7 iRBC of PbANKA by IP injection. They were then administered with 100, 500, and 1000 mg/kg of GPE twice a day for 4 consecutive days (day 0 - 3). At day 4, tail blood was collected to measure parasitemia, PCV, ALT, and creatinine levels. In addition, normal and untreated groups were used as healthy and disease controls.

Statistics

All data was analyzed by GraphPad Prism software. The results were expressed as mean ± standard error of mean (SEM). One-way ANOVA with Tukey post-hoc test was used to compare the mean between tested groups. A significant level was considered to be 95 % confidence, $p < 0.05$.

Results and discussion

For the acute toxicity test of GPE in mice, there were no observed toxic effects at any dose levels throughout the 14 day study period. In addition, the GPE did not produce significant changes in behaviors, such as alertness, motor activity, breathing, restlessness, diarrhea, convulsion, and coma. No death was observed up to the dose of 4000 mg/kg, indicating that the median lethal dose (LD50) could be greater than 4000 mg/kg. The mice were not exposed to doses higher than 4000 mg/kg as, with increasing concentration, the extract precipitated, and the solution became too sticky to handle. In the acute toxicity test, doses higher than 5000 mg/kg are generally not considered to be dose related, and compounds with LD50 lower than 4000 mg/kg are generally considered to be relatively safe [12]. Therefore, GPE can be considered to be non-toxic at acute oral administration.

During PbANKA infection in mice, parasitemia was first detected on day 1 post-infection, with a parasitemia lower than 1 %, and reached to 65 % at day 12 until the infected mice died. Moreover, the onset of hemolysis and liver and renal injuries in PbANKA infected mice was at day 4 post-infection, and the incidence of these pathologies were confirmed through manifestations including a decrease in PCV levels, as well as increases in ALT and creatinine levels, in infected mice (**Figures 1(a) - 1(d)**). Hemolysis, as indicated by PCV reduction, is considered a hallmark of both human and rodent malaria; infected mice may suffer from severe anemia because of the rapid destruction of erythrocytes, by parasitemia and/or spleen reticuloendothelial cells. Moreover, *P. berghei* increased erythrocyte fragility and led to subsequent reduction of PCV in infected mice [13-15]. The pathogenesis of malaria-associated liver and renal injuries is not well characterized, but several hypotheses suggest the involvement of cytoadherence of parasitized erythrocytes, proinflammatory response, and hepato- and nephrotoxicity due to oxidative stress [16-18]. It is well-established that the pathogenesis of severe malaria is associated with an upregulation of proinflammatory cytokines [19]. During the intraerythrocytic stage, hemoglobin consumption by malaria parasites gives rise of considerable amounts of free heme, a molecule that has the ability to induce oxidative stress [20]. The oxidative stress mediated by toxic free heme has been implicated in lipoprotein oxidation and damage in different organs, such as the liver and kidney, through the generation of reactive oxygen and nitrogen species (ROS and NOs) by host cells [21]. Moreover, ox-LDL upregulates the expression of adhesion molecules, facilitating the cytoadherence of infected erythrocytes [22]. The sequestration and adhesion of parasitized erythrocytes to endothelial cells

compromises the vascular permeability of vital organs. The changes in the endothelial permeability contribute to alterations of microvascular patterns and proinflammatory cytokine release [23,24].

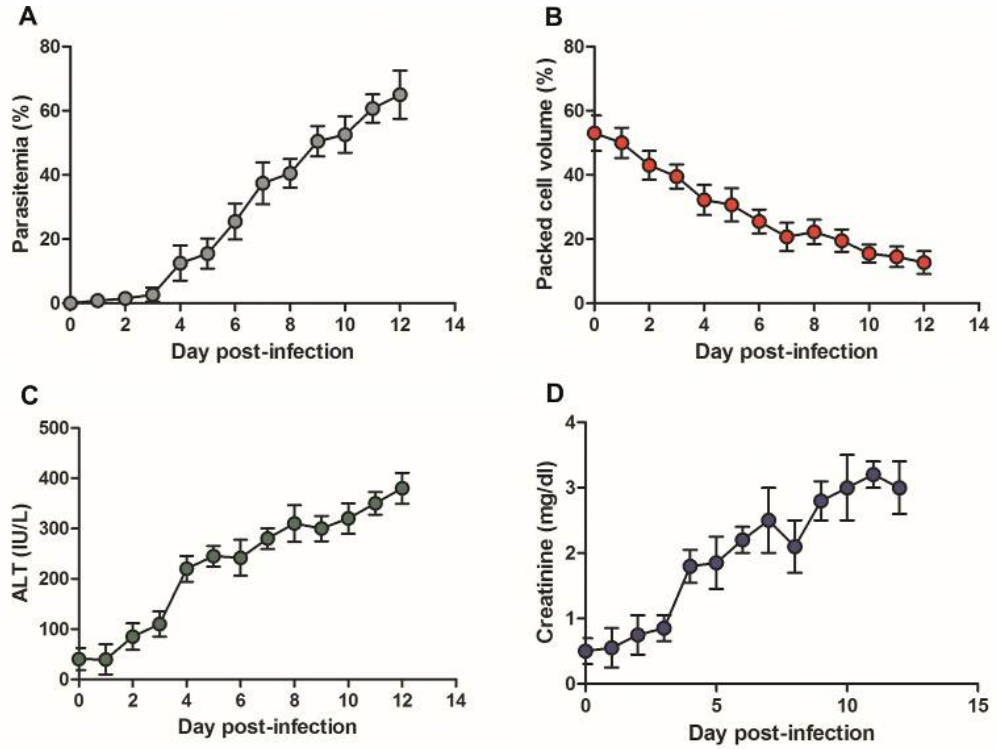

Figure 1 Malaria-associated hemolysis and liver and renal injuries. ICR mice (5 mice) were infected intraperitoneally with 1×10^7 parasitized erythrocytes of PbANKA. (A) % parasitemia, (B) % PCV, (C) ALT, and (D) creatinine levels were monitored daily. The results are expressed as mean ± SEM.

For the efficacy test *in vivo*, GPE exerted protective effects on hemolysis and liver and renal injuries induced by PbANKA infection in mice (**Figure 2**). During PbANKA infection, hemolysis with liver and renal injuries were observed, as indicated by significant ($p < 0.001$) reduction of PCV, and marked increases in ALT and creatinine levels, compared to the normal group (**Figures 2(a) - 2(c)**, UN). Interestingly, significant ($p < 0.01$) increases of PCV levels, and decreases of ALT and creatinine levels, with a dose-dependent manner were observed in infected mice treated with GPE, compared to the untreated group (**Figures 2(a) - 2(c)**, GPE 100, 500, and 1000 mg/kg). The highest activity was shown at a dose of 1000 mg/kg. Moreover, there were no effects on PCV, ALT, or creatinine levels in normal mice treated with 1000 mg/kg of GPE. It can be suggested that the antioxidant activity of this extract might play a central role in protecting erythrocyte destruction, as well as protecting against organ damage, during malaria infection [25]. The rapid growth of malaria parasites with concomitant elevation in free radical production leads to an imbalance of plasma oxidants and the antioxidant system, resulting in oxidative stress [26]. Consequently, the oxidative stress leads to the oxidation of membrane proteins and lipids, hemolysis, endothelial cells, and other vital organ damage [27-30]. Polyphenols and flavonoids, active compounds in GPE, have been revealed to have potent antioxidant properties, and protected erythrocytes and protected against damage to several organs induced by oxidative stress [31]. The active compounds, including polyphenols and flavonoids, in GPE have been described previously [6]. It has been revealed that polyphenols and flavonoids inhibit the production of free radicals and oxidants by

interfering with or inhibiting the metabolic pathway of malaria parasites [32]. Additionally, these compounds promoted an increase in the total antioxidant capacity, and minimized the oxidative damage to the host. Hence, the antioxidant activity of GPE was considered to have protective effects on these pathologies induced by PbANKA infection in mice.

Surprisingly, GPE exerted a dose-dependent inhibitory effect against PbANKA infected mice. The extract caused a significant ($p < 0.01$) inhibition, compared to untreated mice, with percent inhibitions of 57.6, 78.4, and 89.6 % at the doses of 100, 500, and 1000 mg/kg of GPE, respectively. The antimalarial activity of this extract might be attributed to the presence of active compounds such as alkaloids, flavonoids, polyphenols, terpenoids, quinine, and gypenoside. These active compounds, which have antioxidant activity, may also contribute to antimalarial activity [33-36]. Antioxidant effects can inhibit heme polymerization, and unpolymerized heme is very toxic for malaria parasites in erythrocytes [37]. However, the active compounds responsible for the observed activity need to be identified in future studies.

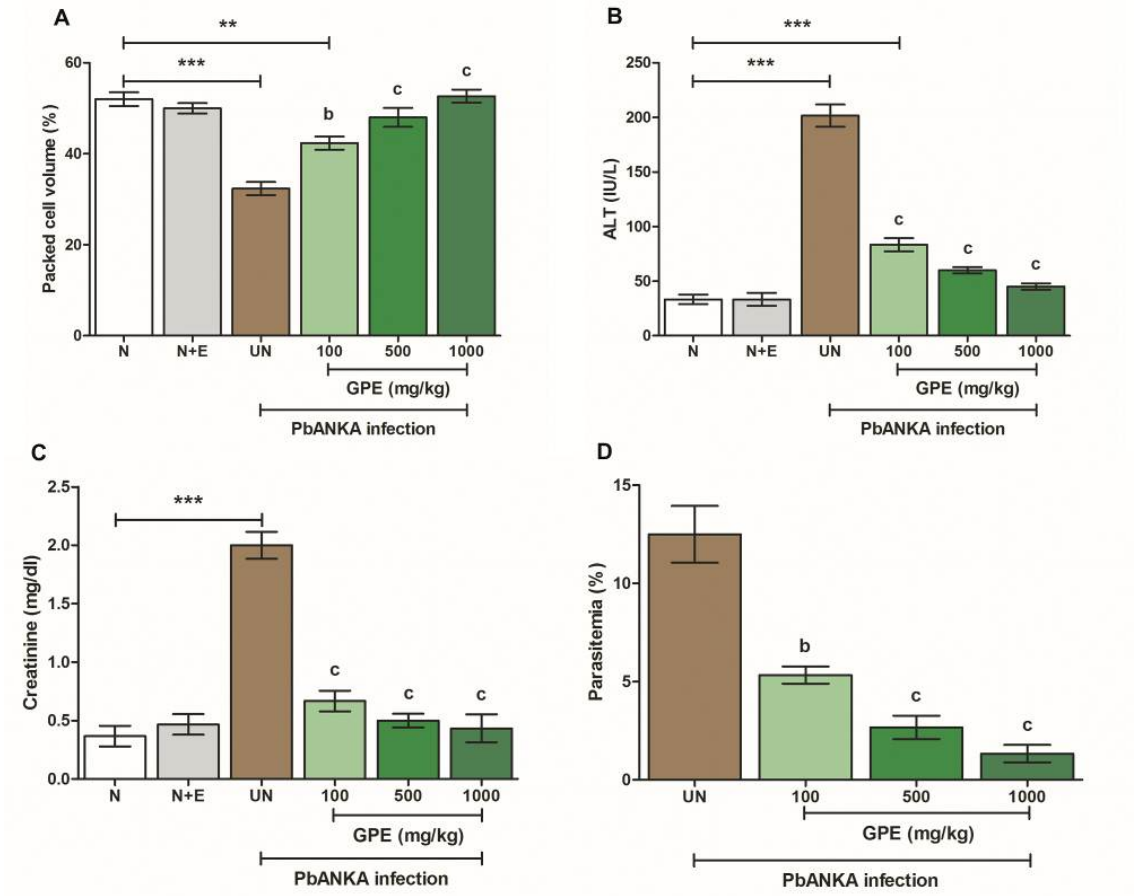

Figure 2 Efficacy of GPE in PbANKA infection in mice. Groups of mice (5 mice in each) were infected intraperitoneally with 1×10^7 parasitized erythrocytes of PbANKA, and then given GPE at doses of 100, 500, and 1000 mg/kg orally for 4 consecutive days. (A) % PCV, (B) ALT, and (C) creatinine levels were measured. Additionally, (D) % parasitemia was also determined. The results are expressed as mean $\pm$ SEM. ** $p < 0.01$ and *** $p < 0.001$, compared to normal mice, b $p < 0.01$ and c $p < 0.001$, compared to untreated mice. N; normal mice, N+E; normal mice treated with 1000 mg/kg of GPE, and UN; untreated mice.

Conclusions

The results obtained in the present study showed that the aqueous crude extract of *G. pentaphyllum* leaves was safe and possessed a significant protective effect against hemolysis and liver and renal damage induced by *P. berghei* infection in mice. Additionally, this extract also exerted antimalarial activity, as seen in its ability to suppress *P. berghei* infection in mice. Hence, *G. pentaphyllum* leaf extract represents a promising source of alternative antimalarial compound for downstream clinical development.

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