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Antimalarial and Anti-Hemolytic Properties of Aqueous Crude Extract of *Gynostemma pentaphyllum* Leaves against *Plasmodium berghei* Infection in Mice

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Abstract

Continuous emergence of antimalarial drug resistant malaria parasites warrant urgent search for new antimalarials. Traditional medicinal plant extracts have been the main sources for screening antimalarial activity. Accordingly, this study was aimed at investigation the antimalarial and anti-hemolytic properties of aqueous crude extract of *Gynostemma pentaphyllum* leaves against Plasmodium berghei infected mice. Aqueous crude extract of *G. pentaphyllum* leaves have been prepared and tested for acute toxicity and antimalarial efficacy in *P. berghei* ANKA infected mice. At three oral doses of 100, 500 and 1,000 mg/kg of extract were safe, chemosuppressive and thus prevented packed cell volume reduction in a dose-dependent manner compared to the untreated control group. The maximum efficacy was found at the dose of 1,000 mg/kg. This study suggests that the aqueous crude extract of this plant have promising antimalarial activity against *P. berghei* in a dose-dependent manner, which supports the traditional use of this plant for malaria treatment.

Keywords: Antimalarial activity; *Gynostemma pentaphyllum*; Malaria; *Plasmodium berghei*

Introduction

Malaria is one of the most pathogenic diseases in endemic areas of Africa, Latin America, and Asia with more than 350-500 million people in Africa infected by malaria parasite, commonly *Plasmodium falciparum* with 80 million reported clinical cases and more than 2 million deaths annually [1]. The problem is further compounded by the upsurge in the resistance strain of the malaria parasite. This has prompted research towards the discovery and development of new, safe, and affordable antimalarial chemotherapies. 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 [2,3]. Some antimalarials in use today, quinine and artemisinin, were either obtained from plants or developed using their chemical structures as templates [4]. Even though up to 80% of the Thailand population uses traditional medicine especially plant extracts for the management of diseases including malaria, plants are not yet fully explored [5].

Gynostemma pentaphyllum (Cucurbitaceae), known as Jiaogulan in Chinese herbal medicine, is a perennial vine endemic in Southern China, Japan, Korea, and Thailand. This plant is a well-known edible and medicinal plant [6]. Recently, *G. pentaphyllum* has attracted great attention owing to its potent antioxidant, anti-inflammation, anti-cancer, anti-gastric ulcer, immunomodulatory, anti-parasitic, and anti-microbial activities [7-10]. Additionally, it has also been reported to have potential for treating hyperglycemia and hyperlipidemia [11]. Phytochemical studies of this plant had identified the active compounds including gypenosides, and closely related to the ginseng saponin [12,13]. Even though *G. pentaphyllum* is said to be one of the most commonly used medicinal plants in many countries in Asia, especially Thailand, there is still no report of the antimalarial property of this plant. Hence, the aim of this study was to investigate the antimalarial property of *G. pentaphyllum* extract against Plasmodium berghei infected mice.

Materials and Methods

Plant material

The dried leaves of *G. pentaphyllum* was purchased from the Royal Project, Chiang Mai, Thailand. This plant was then identified by Dr. Sakaewan Ounjaijean, Faculty of Pharmacy, Payap University, and a sample with voucher number WTU/PU/GP0910 has been deposited at the Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi, Thailand.

Preparation of crude extract

Aqueous crude extract of *G. pentaphyllum* leaves was prepared as previously described [9]. The dried leaves of *G. pentaphyllum* was ground by using electric blender, and then dissolved with distilled water in a ratio of 1:10 (w:v). Heat with microwave at 360 W for 5 min was then performed, and incubation at room temperature for 3 h was subsequently done with continuously stirring. The extract was filtered through Whatman no. 1 filter paper, and lyophilization was subsequently performed to obtain aqueous crude extract. The extract was kept at -20°C. The lyophilized extract was dissolved in distilled water to obtain appropriate doses for using in mice before experiment.

Experimental mice

The experimental mice used in this study was ICR mice (female, 6-8 weeks old weighting between 30-35 g) purchased from National Laboratory Animal Center, Mahidol University, Thailand. The mice were housed under standard condition at 25-28oC with a 12 h light/ 12 h dark cycle. They were fed with commercial diet pellets and clean water throughout the study ad libitum. All animal experiments were ratified by the Animal Ethic Committee, Western University.

Acute toxicity test

Acute toxicity of aqueous crude extract of *G. pentaphyllum* leaves was carried out as previously described [14]. Groups of naïve mice (5 mice of each) were orally administered 100, 500, 1,000, 1,500, 2,000, 3,000, and 4,000 mg/kg body weight of the extract. 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 first 4 hr and subsequently daily for 30 days.

Rodent malaria parasite

Chloroquine-sensitive Plasmodium berghei strain ANKA (PbANKA) was used. The parasite was maintained in the laboratory by serial passage of 1x107 parasitized erythrocytes in experimental mice. Parasitemia was daily monitored by Wright stained thin blood smear under light microscope with 100x oil immersion lens, and calculation was then performed using formula below.

% parasitemia = Number of parasitized erythrocytes x 100

Number of total erythrocytes

Measurement of packed cell volume

Packed cell volume of mice was carried out by collecting tail blood into heparinized hematocrit tube. Centrifugation was then performed with maximum speed for 10 min using microhematocrit centrifuge. Percentage of packed cell volume (% PCV) was subsequently measured using hematocrit reader.

Antimalarial drug

Standard antimalarial drug, chloroquine diphosphate salt (CQ) was used in this study as positive control. The drug was prepared in distilled water based on the ED90 (5 mg/kg) and administered orally.

Efficacy test *in vivo*

The standard 4-day test was carried out in this study [15]. Groups of naïve ICR mice (5 mice of each) were inoculated by intraperitoneal injection with 1×107 parasitized erythrocytes of PbANKA, and administered orally by gavage twice a day for 4 consecutive days (day 0-3) with 100, 500 and 1,000 mg/kg of *G. pentaphyllum* extracts. Three control groups were used; the normal control was given either with distilled water or the extract (1,000 mg/kg); the untreated control was given distilled water; the drug treatment control was given 5.0 mg/kg of CQ. On day 4, % parasitemia and % PCV were then measured. Moreover, percentage of inhibition was also calculated using formula below.

% inhibition = (Parasitemia of untreated mice – parasitemia of treated mice) x 100

Parasitemia of untreated mice

Statistics

Statistical analysis was performed using GraphPad Prism Software (GraphPad software, Inc., CA, USA). The one way ANOVA with post-hoc Tukey test was used to analyze and compare the results at a 95% confidence level. Values of $p<0.05$ were considered significantly difference. Results were expressed as mean+standard error of mean (SEM).

Results

Acute toxicity test

The signs of toxicity in mice given 2,000, 3,000, and 4,000 mg/kg of aqueous crude extract of *G. pentaphyllum* leaves include; paw licking, salivation, stretching and reduce activity were observed. There was however no mortality at all doses used. This showed that the lethal dose was greater than 1,000 mg/kg. Therefore, 100, 500, and 1,000 mg/kg were suitable doses for using of this study.

Blood stage propagation of PbANKA in mice

ICR mice were infected with PbANKA by intraperitoneal injection, and the course of parasitemia and packed cell volume were determined. Parasitemia was first detectable on day 1 post-infection (< 1%) and reached 65% on day 14 (Figure 1A). Additionally, decreasing of % PCV levels in infected mice from 52% on day 0 to 13% on day 14 post-infection was also observed (Figure 1A). Moreover, with respect to the mortality, a survival rate of 100% was observed until day 12 post-infection (Figure 1B).

Figure 1: Blood stage propagation of PbANKA in mice. ICR mice (5 mice of each) were intraperitoneally infected with 1×107 parasitized erythrocytes of PbANKA. (A) Parasitemia and packed cell volume were daily monitored. (B) Percentage survival of infected mice was also observed. Results were expressed as mean+ SEM.

Antimalarial property of aqueous crude extract of *G. pentaphyllum* leaves against PbANKA infected mice

The aqueous crude extract of *G. pentaphyllum* leaves exerted dose-dependent chemosuppressive effect against PbANKA infected mice (Figure 2). The extract caused a significant ($p<0.05$) suppression with 60%, 65%, and 80% inhibition at the doses of 100, 500, and 1,000 mg/kg, respectively when compared to the untreated control. However, the standard drug, CQ caused chemosuppression of 90%, which was higher than those of the extract treated groups.

Figure 2: Antimalarial activity of aqueous crude extract of G. pentaphyllum leaves against PbANKA. ICR mice (5 mice of each) were intraperitoneally infected with 1×10^7 parasitized erythrocytes of PbANKA, and given orally 100, 500 and 1,000 mg/kg of the extracts twice a day for 4-consecutive days. Parasitemia was subsequently measured. The results were expressed as mean+SEM. UN; untreated, CQ; 5 mg/kg of chloroquine. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to untreated control.

Protective effect of aqueous crude extract of *G. pentaphyllum* leaves on hemolysis induced by PbANKA infection in mice

The aqueous crude extract of *G. pentaphyllum* leaves showed significant ($p<0.05$) protection of hemolysis as indicated by the normal level of % PCV in extract treated mice, while that of untreated group showed significantly ($p<0.05$) decreasing of % PCV, compared to the normal control (Figure 3). The dose-dependent protective effect of this extract was also observed at the doses of 500 and 1,000 mg/kg. However, significant ($p<0.05$) reduction of % PCV was still found in infected mice given 100 mg/kg of the extract. Moreover, no toxic effect on hemolysis was found in normal control treated with the extract.

Figure 3: Anti-hemolysis of aqueous crude extract of G. pentaphyllum leaves during PbANKA infection in mice. ICR mice (5 mice of each) were intraperitoneally infected with 1×10^7 parasitized erythrocytes of PbANKA, and given orally 100, 500 and 1,000 mg/kg of the extracts twice a day for 4-consecutive days. Percentage of packed cell volume was then investigated. The results were expressed as mean+SEM. N; normal, N+E; normal mice treated with 1,000 mg/kg of extract, UN; untreated, CQ; 5 mg/kg of chloroquine. **$p<0.01$ compared to normal control.

Discussion

Medicinal plant extracts are frequently considered to be less toxicity than synthetic ones. Therefore, a growing number of peoples are turning to alternative therapy, including medicinal plant extracts. Although the plant extracts have been used in clinical treatment, however the active compounds and their modes of action remain to be investigated. In the present study, the antimalarial and anti-hemolytic properties of aqueous crude extract of *G. pentaphyllum* leaves in PbANKA infected mice were tested. For acute toxicity test of aqueous crude extract of *G. pentaphyllum* leaves, oral administration did not show changes in general appearance of the experimental mice until the end of 30 days. Furthermore, no signs of toxicity and death were found in the mice receiving the extract up to a dose of 1,000 mg/kg, which is about 10 times the minimum effective dose (100 mg/kg). If a test substance has a lethal dose higher than 3 times the minimum effective dose, it can be a good candidate for further studies [16]. Hence, absence of toxicity and mortality up to an oral dose of 1,000 mg/kg could indicate that the test extracts were safe and this could explain the routine use of the plant by the local people for traditional treatment of malaria.

During PbANKA infection in ICR mice, increasing of % parasitemia and decreasing of % PCV were observed, and infected mice would die from severe anemia. Malaria-associated hemolysis is proposed to be a consequence of parasite development in erythrocytes as well as exacerbated erythrocyte membrane against products of oxidative stress releasing during infection [17]. Moreover, the destruction of erythrocytes during blood stage of infection accumulates high levels of toxic free heme in circulation that, in turn, has the ability to induce oxidative stress from production of hydroxyl radicals via the Fenton/Harber-Weiss reaction [18]. Moreover, lipid peroxidation of erythrocyte membrane followed by hemolysis has also been suggested [19]. Additionally, recruitment of inflammation during pathogenesis of malaria-associated hemolysis also contributes to increase the occurrence of hemolytic events [20,21].

The standard 4-day is a test commonly used for in vivo antimalarial phytochemical screening in which >30% inhibition following treatment makes a product to be considered active [16,22]. Accordingly, the aqueous crude extract of *G. pentaphyllum* leaves which showed 80% inhibition at 100, 500, and 1,000 mg/kg, respectively can be classified as active. The dose-dependent manner in chemosuppression could be attributed to the low dose of the compounds in natural products and as such their activity may be undetectable in lower doses. This increased percent inhibition of parasitemia with increased dose was observed by other studies on different plant species [23-26]. Alkaloids, polyphenolic compounds, terpenoids, flavonoids and gypenoside in this extract could be responsible for its antimalarial activity [27-30]. Moreover, it has been reported that gypenoside and terpenoids showed strong antimalarial, anti-microbials and anti-cancer activities [31]. Particularly, the extract at maximum dose of 1,000 mg/kg was highly antimalarial activity indicating the dominant presence of antimalarial bioactive compounds in this extract.

Severe anemia is the general features of PbANKA infected mice, and the ideal antimalarial compounds from medicinal plant extracts are expected to prevent anemia. The absence of significant % PCV reduction among extract treated mice at the doses of 500 and 1,000 mg/kg of the aqueous crude extract of *G. pentaphyllum* leaves may indicate the protective activity of this extract. Moreover, observing a significantly lower % PCV reduction among the same groups of mice at the highest dose (1,000 mg/kg) shows the presence of antimalarial compounds in the dose administered. It can be suggested that polyphenols and flavonoid contents in this extract might play a central role to protect erythrocytes from oxidative stress and inflammation induced by malaria infection [32]. Moreover, malaria can cause metabolic acidosis via erythrocyte destruction followed by severe anemia [33]. *G. pentaphyllum* leaf extract has been reported to maintain blood pH as well as protect erythrocytes from acidosis [34]. However, it appears that the activity of the extract at dose of 100 mg/kg was not strong enough to significantly prevent % PCV reduction among PbANKA infected mice.

It can be concluded that when oral administered, no adverse effects were noted for the plant extract ranging from 100-1,000 mg/kg doses signifying the safety of the extract in mice via the oral route. Interestingly, antimalarial and anti-hemolytic activities were observed in PbANKA infected mice administered with the aqueous crude extract of *G. pentaphyllum* leaves. Moreover, 1,000 mg/kg of the

extract was showed to have the strongest activity. Hence, the antimalarial activity and no toxicity of this extract may confirm the use of the extract against malaria. However, the finding is only preliminary and thus confirmatory studies followed by isolation and characterization of the active antimalarial compounds of the extract that are responsible for the observed malaria inhibition thereby resulting in prevention of % PCV reduction in the PbANKA infected mice are recommended.

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