Asian Journal of Pharmacognosy (2018) 2(1): 5-12

Asian Journal of Pharmacognosy

Research Article

Antimalarial activity of *Morus alba* leaf extract against *Plasmodium berghei* infection in mice

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Abstract

Malaria is one of the most important parasitic diseases in the World. The choice for the treatment is highly limited due to antimalarial drug resistance. The use of medicinal plant in the treatment of malaria is a common practice in many countries where the malaria is mostly endemic. The present study is attempted to investigate the antimalarial activity of *Morus alba* leaf extract against

Plasmodium berghei infected mice. In this study, aqueous crude extract of *M. alba* leaves was prepared, and acute toxicity test was also conducted. The standard 4-days test was used to determine parasite inhibition. Male ICR mice were randomly grouped into five groups of five mice each. They were then given orally with the extracts (100, 200, and 500 mg/kg) once a day for 4-consecutive day. The untreated and positive controls were given distilled water and chloroquine (10 mg/kg), respectively. The level of parasitemia, packed cell volume, variation in body weight, and survival time of mice were used to determine the antimalarial activity of the extract. It was found that the aqueous crude extract of *M. alba* leaves significantly ($p < 0.05$) inhibited parasitemia in a dose-dependent manner, prevented body weight loss, and packed cell volume reduction at doses of 100, 200, and 500 mg/kg. Additionally, the extract prolonged the mean survival time of *P. berghei* infected mice, compared to the untreated control. It can be concluded that aqueous crude extract of *M. alba* leaves possess acceptable antimalarial activity with non-toxicity. However, further investigation should be pursued on the bioactive compounds responsible for the observed antimalarial action of this plant.

Keywords: medicinal plants, antiprotozoal, pharmacology

Introduction

Malaria is one of the major parasitic diseases responsible for the high rate of mortality and morbidity in malaria endemic areas such as sub-Saharan Africa, Central and Latin America, and Asia. It was estimated 300–500 million people are affected by malaria throughout the world annually. The World Health Organization (WHO) indicates that 95% of malaria-related deaths occur in sub-Saharan Africa, with children younger than 5 years of age and pregnant women being the most severely affected (WHO 2016). Malaria is caused by Apicomplexan protozoan parasites of the genus *Plasmodium*. Malaria control has relied on two ways namely the control of the *Anopheles* mosquito vector and the effective case management (White, Pukrittayakamee et al. 2014). Unfortunately, drug resistance has been one of the main obstacles in the malaria control. It is responsible for the spread of malaria to new areas, the recurrence of malaria in the areas where the disease had been eradicated and plays an important role in the severity of epidemics in some parts of the World (White 2004). This highlight the urgent need for novel, well-tolerated, and affordable anti-malarial chemotherapies.According to several researches, up to 80% of world's populations rely on traditional medicine mainly on herbal extracts as primary source of medicinal agents for the treatment of diseases. Moreover, the use of medicinal plant extracts in malaria treatment is a common practice in many countries where the disease is mostly endemic (Tariq, Adnan et al. 2016). Some standard antimalarial drugs in use today, quinine and artemisinin, were obtained from plants. This explains why a lot of current research focuses on plant-derived products as they can be sourced easily, locally available, and can be selected on the basis of their ethnomedicinal use (Ashley and White 2005). Although up to 80% of the Thai population uses traditional medicinal plants for the management of diseases including malaria, these plants are not yet fully explored (Khuankaew, Srithi et al. 2014).*Morus alba* L. (Mulberry) leaf belongs to the Moraceae family, distributed mainly in the temperate and subtropical regions. It has been traditionally used in Japan, Korea, China, and other Asian countries including Thailand as herbal tea and herbal medicine (Gryn-Rynko, Bazylak et al. 2016). *M. alba* leaf extract contains abundant varieties of polyphenols, including chlorogenic acid, rutin, isoquercitrin, quercetin, astragalin,

kaempferol, and other flavonoids which are considered strong antioxidant, anti-inflammation, and anti-proliferative activities (Liu and Willison 2013). Recent researches have reported that it shows anti-atherosclerosis, anti-hypertension, anti-obesity, anti-diabetic, hepatoprotective, neuroprotective, antiviral, and antimicrobial activities (Chan, Lye et al. 2016). However, the antimalarial activity of *M. alba* leaf extract has not yet been described. Hence, the present study was aimed to investigate antimalarial activity of the aqueous crude extract of *M. alba* leaves against *P. berghei* infected mice.

Materials and methods

Plant material and preparation: Commercial dried leaves of *Morus alba* was purchased from the Royal Project shop at Chiang Mai, Thailand. The plant material was identified by Dr. Sakaewan Ounjaijean from the Faculty of Pharmacy, Payap University, Chiang Mai, Thailand. The microwave-assisted water extraction method was used to prepare the aqueous crude extract of *M. alba* leaves (Li and Jiang 2010). The powdered dried leaves of this plant was prepared using electric blender. For extraction, 10 g of the plant material was dissolved in 100 ml of distilled water, microwave at 360 W for 5 min was subsequently performed. After incubation at room temperature for 3 h, filtration through Whatman no. 1 filter paper was done in order to obtain filtrate. Lyophilization was then carried out to remove the solvent, and the aqueous crude extract of *M. alba* leaves (MAE) was obtained. The extract was stored at -20^{o}C.

Experimental mice: Pathogen-free, male ICR mice, 6-8 weeks old, weighting 25–30 g obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand, were used for this study. All the experimental mice were sustained under standard condition, temperature of 22 ± 3°C, 50–70% relative humidity and 12 h light/dark cycle, with standard pellet diet and water *ad libitum*. All the experimental protocols were carried out in compliance with the Principles of Laboratory Animal Care (NIH) based on the approval of the Animal Ethical Committee of Western University.

Acute toxicity test: The acute toxicity of the MAE was tested as previously described (Lorke 1983). ICR mice were used by randomly dividing into 6 groups of 5 mice per group. They were then administered orally by gavage with 0.2 ml of the extracts (100, 200, 500, 1,000, and 2,000 mg/kg) in a single dose. The mice were observed continuously for 1 h, followed by 4 h observations for 24 h and thereafter daily for 14 days, for any manifestation of toxicity.

Rodent malaria parasite: Chloroquine sensitive *Plasmodium berghei* ANKA strain (PbANKA) provided by MR4 (Malaria Research and Reference Reagent Resource Center) was used. This parasite was sustained by mechanical serial passage of blood from infected mice to non-infected ones on weekly basis. On each day of experiment, blood from the tail vein of each mouse was smeared on a microscopic slide to make thin film, stained with 10% Wright-Giemsa in phosphate buffet, pH 7.2 for 15 min and examined under light microscope at 100x in order to monitor the parasitemia level. Percent parasitemia was determined and calculated using the following formula:

$$\text{Percent parasitemia (\% parasitemia)} = \frac{\text{Number of parasitized erythrocytes}}{\text{Number of Erythrocytes}} \times 100$$

Antimalarial drug: Chloroquine diphosphate salt (CQ; Sigma, Saint Louis, Missouri, USA) was used to determine the drug susceptibility of PbANKA. The drug at chosen dose of 10 mg/kg (sub-curative dose) was prepared in distilled water (DW) and administered orally by gavage.

Packed cell volume determination: Packed cell volume (PCV) was measured in order to predict the effectiveness of the extract in preventing hemolysis resulting from increased parasitemia. Blood from tail of each mouse was collected and filled up to $3/4^{th}$ of their volume in heparinized capillary tubes, then sealed at the dry end with sealing clay. Centrifugation was subsequently done for 5 min at 12,000 rpm, and PCV was determined using a standard micro-hematocrit reader. PCV is a measure of the proportion of erythrocytes to plasma and measured before inoculating the parasite and after treatment using the following formula:

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

Monitoring of body weight (BW): In the suppressive test, body weight of each infected mice was measured before infection on day 0 and after treatment on day 4. The body weight of each mouse was measured using a sensitive digital weighing balance.

Mean survival time: For *in vivo* antimalarial model, monitoring of mortality and the number of days from the time of inoculation of parasite until death was recorded in each mouse for 30 days. The mean survival time (MST) for each group was calculated as follows:

$$\text{MST} = \frac{\text{Sum of survival time of all mice in a group}}{\text{Total number of mice in the group}}$$

Suppressive test: For investigation of chemosuppression of the MAE, standard 4-day test was carried out (Peters 1975). Five groups of ICR mice (5 mice of each) were inoculated with $1x10^7$ parasitized erythrocytes of PbANKA by intraperitoneal injection. The treatment was started 2 h post-infection by administer orally with 100, 200, and 500 mg/kg of the extract once a day for 4-consecutive days. Untreated and positive groups were given DW and 10 mg/kg of CQ, respectively. On the fifth day, parasitemia, PCV, and body weight were measured. Calculation of percentage of inhibition was performed using the formula below. Moreover, MST was also monitored.

$$\%\ \text{inhibition} = \frac{(\%\ \text{parasitemia of untreated} - \%\ \text{parasitemia of extract treated}) \times 100}{\%\ \text{parasitemia of untreated}}$$

Statistics: Data was analyzed using GraphPad Prism v. 5.01 (GraphPad Software, Inc., CA, USA). The results were expressed as mean + standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to compare among positive, negative, and treatment groups. Statistically significant value was considered at 95% confidence and $p < 0.05$.

Results

The MAE did not cause mortality or any sign of behavioral change that is indicative of acute toxicity in the mice after oral administration of extract (100 to 2,000 mg/kg). Therefore, the plant can be considered safe according to the Organization for Economic Cooperation and Development (OECD) guideline which recommends a maximum dose of 2,000 mg/kg for acute toxicity (OECD 2001). Therefore, this could justify the common use of the MAE in Thailand traditional medicine to treat malaria and other ailments. In the standard 4-day test, the MAE showed significant ($p < 0.05$) inhibition in all the doses against PbANKA infected mice in a dose dependent manner with 40.3, 58.6 and 75.4% inhibition at 100, 200 and 500 mg/kg of the extract, respectively (Table 1). However, the MAE did not clear the parasite completely on D4. According to the previous finding, a compound is considered active when the % inhibition is > 30% (Krettli, Adebayo et al. 2009). Hence, a remarkable suppression suggests the potency of the MAE against malaria. The antimalarial activity of the MAE might be attributed to the presence of bioactive secondary metabolites in the crude extract. Basically, different secondary metabolites, such as polyphenols, flavonoids, quercetin, and kaempferol have been reported from the extract of *M. alba* leaves (Chan, Lye et al. 2016). These metabolites have antimalarial activities. However, the active compounds responsible for this observation need to be identified. Hence, the antimalarial activity observed in the MAE could have resulted from single or in combined action of the above metabolites (Rasoanaivo, Wright et al. 2011).

The possible mechanisms might be through antioxidant, immunomodulatory, inhibition of protein synthesis, interference with the parasites invasion of new erythrocytes, or any other unknown mechanisms (Rasoanaivo, Wright et al. 2011; Builders, Uguru et al. 2012).The result showed a significant ($p < 0.05$) reduction in PCV between D0 and D4 in untreated group (Table 2). The PbANKA infected mice may suffer from severe anemia because of rapid erythrocyte destruction, either by parasitemia or spleen RE system (Taylor and Hurd 2001). Further, PbANKA increased erythrocyte fragility and led to subsequent reduction of PCV in infected mice (HOT. and AO. 2009). Interestingly, mice treated with MAE demonstrated a dose dependent effect on the PCV value of PbANKA infected mice at all the three dose levels. Body weight loss is one manifestation of PbANKA infected mice. There was significant ($p < 0.05$) change between D0 and D4 in BW of untreated mice. It is due to the depressant action on the appetite of the mice and the consequences of disturbed metabolic function and hypoglycemic effect of the parasite (Basir, Rahiman et al. 2012).

Interestingly, the MAE treated mice had shown no significant deviation in BW between D4 and D0 in all three dose levels (Table 2). The MAE might lead to increased weight gain including appetite stimulant activity, and it is nutritionally endowed with vitamin B such as vitamin B1, which maintains appetite and growth; vitamin B2, which prevents weight loss; and vitamin B3, which maintains the normal function of the gastrointestinal tract (Muluye, Melese et al. 2015). The MST

is another parameter evaluates the antimalarial activity of plant extracts. Accordingly, the plant extract that can prolong the MST of PbANKA infected mice compared to the untreated controls are considered as active agents against malaria (Oliveira, Dolabela et al. 2009). The results showed that the MST of MAE treated mice was significantly ($p < 0.05$) higher compared to the untreated group. Moreover, it was prolonged in a dose dependent manner (Table 3). This might be due to the antimalarial activity of the extract. However, the MST of mice treated with the MAE were shorter as compared to positive control; this might be the fast elimination phase of the extract.

Table 1 Chemosuppressive activity of aqueous crude extract of *M. alba* leaves against *P. berghei* ANKA infection in mice

Description	Dose (mg/kg/day)	% parasitemia	% inhibition
DW	0.2 ml	15.7 + 4.04	0
MAE	100	8.7 + 1.66	40.3*
	200	6.5 + 1.70	58.6**
	500	3.7 + 0.25	75.4***
CQ	10	0.73 + 0.25	94.9***

The results were presented as mean + SEM, n = 5, DW; distilled water (untreated group), MAE; aqueous crude extract of *M. alba* leaves, CQ; chloroquine (positive group), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to untreated group

Table 2 Effect of the aqueous crude extract of *M. alba* leaves on packed cell volume and body weight in *P. berghei* ANKA infected mice

Description	Dose (mg/kg/day)	PCV		BW (g)	
		D0	D4	D0	D4
DW	0.2 ml	52.8 + 0.48	40.6 + 0.64*	28.4 + 0.33	22.7 + 0.25*
MAE	100	51.7 + 0.74	49.6 + 0.87	27.6 + 0.24	26.2 + 0.37
	200	52.1 + 1.01	50.5 + 0.52	26.9 + 0.15	27.2 + 0.55
	500	51.8 + 0.99	50.4 + 0.52	27.0 + 0.31	28.6 + 0.40
CQ	10	54.7 + 0.27	53.4 + 0.21	27.8 + 0.46	28.2 + 0.27

The results were presented as mean + SEM, n = 5, PCV; packed cell volume, BW; body weight, DW; distilled water (untreated group), MAE; aqueous crude extract of *M. alba* leaves, CQ; chloroquine (positive group), * $p < 0.05$ compared between D0 and D4

Table 3 Effect of the aqueous crude extract of *M. alba* leaves on mean survival time of *P. berghei* ANKA infected mice

Description	Dose (mg/kg/day)	MST (day)
DW	0.2 ml	7.2 + 0.37
MAE	100	12.2 + 0.25*
	200	19.8 + 0.28**
	500	23.7 + 0.45**
CQ	10	29.6 + 0.42***

The results were presented as mean + SEM, n = 5, MST; mean survival time, DW; distilled water (untreated group), MAE; aqueous crude extract of *M. alba* leaves, CQ; chloroquine (positive group), * $p < 0.05$ compared to untreated group, ** $p < 0.01$ compared to untreated group, *** $p < 0.001$ compared to untreated group

Conclusion

From all finding of this study, it can be concluded that the aqueous crude extract of *M. alba* leaves exhibited a reasonable antimalarial activity against *P. berghei* ANKA infection in mice as well as lack of toxicity. However, this finding is only preliminary; and thus confirmatory studies followed by isolation and characterization of the active antimalarial compounds that are responsible for the observed parasite suppression thereby resulting in increased MST, BW loss prevention and PCV reduction in the P. berghei infected mice are recommended.

Acknowledgement

We would like to thank students of Department of Clinical Chemistry, Faculty of Medical Technology, Western University for help in animal experiments and plant extraction. The authors also thank Dr. Sakaewan Ounjaijean for her help in the excellent discussion about the plant extraction.

Declaration of Conflict of Interest

No conflict of interest associated with this work.

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