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Antimalarial activity of Malaysian *Plectranthus amboinicus* against *Plasmodium berghei*

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ABSTRACT

Context: Malaria is a mosquito-borne disease caused by parasitic protozoa from the genus of *Plasmodium*. The protozoans have developed resistance against many of current drugs. It is urgent to find an alternative source of new antimalarial agent. In the effort to discover new antimalarial agents, this research has been conducted on *Plectranthus amboinicus*. **Aims:** This study was conducted to evaluate the toxicity and antiplasmodial properties of *P. amboinicus*. **Materials and Methods:** Acute oral toxicity dose at 5000 mg/kg was conducted to evaluate the safety of this extract. Twenty mice were divided into control and experimental group. All the mice were observed for signs of toxicity, mortality, weight changes and histopathological changes. Antimalarial activity of different extract doses of 50, 200, 400 and 1000 mg/kg were tested *in vivo* against *Plasmodium berghei* infections in mice (five mice for each group) during early, established and residual infections. **Results:** The acute oral toxicity test revealed that no mortality or evidence of adverse effects was seen in the treated mice. The extract significantly reduced the parasitemia by the 50 (P = 0.000), 200 (P = 0.000) and 400 mg/kg doses (P = 0.000) in the *in vivo* prophylactic assay. The percentage chemo-suppression was calculated as 83.33% for 50 mg/kg dose, 75.62% for 200 mg/kg dose and 90.74% for 400 mg/kg dose. Body weight of all treated groups; T1, T2, T3 and T4 also showed enhancement after 7 days posttreatment. Statistically no reduction of parasitemia calculated for curative and suppressive test. **Conclusion:** Thus, this extract may give a promising agent to be used as a prophylactic agent of *P. berghei* infection.

Key words: Antiplasmodial, phytochemicals, *Plectranthus amboinicus*, safety, toxicity

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INTRODUCTION

Malaria is a vector-borne disease that is reported by the Department of Statistics Malaysia, which is increasing each year.[1] It is one of the most serious disease in the world, particularly in Africa and Latin America with high cases.[2] In 2007, an incidence rate of 2.0 was reported with 5456 cases, followed by 2008, 2.7 incidence rate with 7390 cases, in 2009, 2.5 incidence rate with 7010 cases, in 2010, 2.4 incidence rate with 6650 cases and in 2011, 1.8 incidence rate/100,000 Malaysian populations with 5306 cases. It is a malignant disease with 300-515 million cases and that kills 2.7-3 million humans/year worldwide affecting the majority of children below the age of 5 years in the tropical and subtropical regions of the world.[3]

Five species of *Plasmodium*, namely *Plasmodium vivax*, *Plasmodium falciparum, Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*, cause human malaria. The most virulent, *P. falciparum* is responsible for severe clinical malaria and death. In summary, the lifecycle involves humans and female *Anopheles* mosquito as vectors. *Plasmodium berghei* infection in mice undergoes similar lifecycle and has been studied in the laboratory.[4]

Major drugs available for infectious disease and in particular for malaria, have been obtained from plants. Quinine, a successful antimalarial isolated from the bark of *Cinchona succirubra* (*Rubiaceae*) commonly known as "quinas". The other more recent antimalarial is artemisinin, isolated from the traditional Chinese antimalarial *qinghaosu (Artemisia annua)*.[5] *Plectranthus amboinicus* is mentioned to be used as traditional medicinal plant for antimalarial in Comoro.[6]

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The leaves of *P. amboinicus* is bitter, acrid, thermogenic, aromatic and appetizing.[7] It is traditionally used for flatulence, colic, diarrhea and cholera, especially in children, cough, chronic asthma and bronchitis.[8,9] In Malaysia, bruised leaves are applied to burns and their poultice on centipede and scorpion-bites.[8] Although this aromatic herb grows wildly in Malaysia, there is no published report on the use of this plant as antimalarial agent in Malaysia, traditionally or scientifically. Thus, the aim of this study was to evaluate the toxicity contents and determine the potential of this plant to be used as an antimalarial agent.

MATERIALS AND METHODS

Plant material

Leaves of *P. amboinicus* were collected in May 2011 from an orchard in Kuantan, Pahang and identified by a taxonomist in Forest Research Institute Malaysia. The plant specimen (MT11-13) is deposited at the herbarium of Faculty of Pharmacy, International Islamic University Malaysia.

Extraction

Two kg of fresh leaves were ground and extracted with 2.5 L of 95% ethanol, 3 times, (24 h each) by maceration technique at room temperature. The filtrate was then concentrated and evaporated until dry in a vacuum at 60°C using a rotary evaporator (buchi rotavapor R-205). The extractive value (%, w/w) of the dry extract was 1.8%. The dry extract was then stored in a refrigerator at 4°C until use.

Phytochemical screening

Phytochemical screening of the ethanolic extract of the leaves was carried out according to the qualitative phytochemical screening tests to evaluate the presence of its chemical constituents such as alkaloid, flavonoid, saponin, triterpenoid, steroid and phenolic.[10,11]

Animals

The female intelligent character recognition (ICR) mice (mean weight ± standard deviation (SD); 21.65 ± 3.32) and males (25.08 ± 3.46) were obtained from the Animal Center of Universiti Kebangsaan Malaysia. The mice were housed under standard conditions (22°C with 12 h dark and 12 h light) and were maintained on a standard pellet feed and water *ad libitum*. Permission and approval for animal studies were obtained from the Animal Ethics Committee, Kulliyyah of Medicine, International Islamic University Malaysia. During the acclimatization period of 7 days, the animals were observed daily for general condition.

Acute oral toxicity study

The method used in this study was the accepted standard described in the Organization for Economic Cooperation and Development guidelines for the testing of chemical, acute oral toxicity, limit test - cute toxic class method sections 423.[12] A total of 20 mice were divided into one control group and one treated/experimental group, each group consisting of 10 mice (five males and five females). Treated group was dosed orally with 5000 mg/kg extract as a single dose. The animals were fasted for 4 h prior to the administration and subsequent feeding after 1 h. On the day of administration, all the mice observed for signs of toxicity and mortality at 1, 3 and 4 h following the administration and then they were observed daily for 14 days. Observations included changes in the skin and fur, eyes and mucous membrane, behavior pattern, tremors, convulsions, diarrhea, lethargy, salivation and sleep. Individual weights of animals were determined and recorded daily. All test animals (including those that died during 14 days of the test) were sacrificed by ether anesthesia on day 15. All organs such as the heart, lung, kidney, liver and spleen were dissected out, washed in saline solution and weighed.

Parasite inoculation

A chloroquine sensitive strain of *P. berghei* (PZZ1/100) was obtained from the Parasitology Laboratory, School of Bioscience and Biotechnology, University Kebangsaan Malaysia and was maintained by sub-passage in mice. The desired blood volume was drawn from the donor mouse by heart puncture and diluted serially in Alsever's solution. The final suspension would contain about 1×10^6 infected red blood cells (RBCs) in every 0.1 ml suspension. This 0.1 mL suspension was injected into mice intraperitoneally (IP) to initiate infection (modification from Abdulelah *et al.*, 2011).[13] The inoculated animals were then randomized into five mice/cage and maintained in the animal retention room, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, in accordance with the internationally accepted principles for laboratory animal use and care.

In vivo erythrocythic-antiplasmodial assay

A series of experiments were carried out to evaluate the *in vivo* antimalarial activities of the ethanolic leaves extract of *P. amboinicus* (Lour) Spreng. at 50, 200, 400 and 1000 mg/kg doses as compared to control groups treated with distilled water (containing 10% dimethylsulfoxide, the solvent of the test extracts) and reference groups treated with standard drugs (chloroquine 20 mg/kg/day or Fansidar 12 mg/kg/day). Malaria infection was the first established in female mice by the IP administration of donor female ICR mouse blood containing about 1×10^6 parasites. The three different methods of treating malaria infections that is, 4 days suppressive test, curative and prophylactic methods were applied. [14-16] In suppressive test, 2-4 h postinfection, the experimental groups were treated orally. All the treatments were repeated for the next 3 days (D1 to D3). On the 5th day (D4), blood smears were prepared from each mouse and stained with Giemsa's stain.

However, 72 h postinfection, the experimental groups were treated and treatments were continued daily until D7 in the curative test. The mean survival time (days) for each group was determined over a period of 30 days post-infection. In the prophylactic test, the mice were administered with the treatment, which was given for 3 consecutive days (D0–D2). On the 4th day (D3), all mice were infected with 1×10^6 *P. berghei* and kept for the next 3 days. On the 7th day, blood smears were prepared from the tail blood of each mouse. The films were then stained with Giemsa's stain to determine parasitized erythrocytes. The percentage of parasitemia was determined by counting the parasitized RBCs out of 9,000 in random fields of the microscope:

% Parasitaemia = (No of parasitized RBC/Total no of RBC counted) ×100

Average percentage chemosuppression was calculated as: 100× (A − B/A) where, A is the mean percentage parasitemia in the negative control group and B is the mean percentage parasitemia in the test group. Body weight had been taken every 2 days and the status of each mouse had been noted daily until 30-days observation for survival analysis.

Statistical analysis

The values are expressed as mean ± SD Results were analyzed statistically using *t*-test, one-way ANOVA and Kaplan–Meier survival Analysis. The significant difference between control and treated groups were considered at $P < 0.05$ level.

RESULTS

Phytochemical screening

Results of the phytochemical screening showed only flavonoid was present in the extract.

Acute oral toxicity study

Mortality rate

All mice (experimental and control groups) survived until the 14th day of observation.

Sign of toxicity

No signs of toxicity were recorded postdosing and during the 14-day observation period in any of the 20 mice (experimental and control group).

Organs and body weight

Results showed no significant differences in weight of organs of both groups [Table 1]. Both the experimental and control group mice gained body weight after 14-day observation period. There were no significant changes ($P = 0.06$) in the body weight throughout the days of observation. Besides, there were also no significant differences ($P = 0.404$) in body weight of the experimental group compared with the control group.

In vivo erythrocythic-antiplasmodial assay

Parasitemia and chemo-suppression

Table 2 shows that by giving doses of 50, 200 and 400 mg/kg before the infection in prophylactic test could reduce the percentage of parasitemia while compared to control mice. Interestingly, results also show that there is not significant reduction of parasitemia if high dose of 1000 mg/kg was given to the mice before infection. Besides, no significant reduction of percentage parasitemia calculated on suppressive and curative test.

Body weight

All mice of treatment groups for prophylactic test showed [Figure 1] enhancement on their body weight after 7 days postinfection except for the control. On the other hands, all treatment groups for suppressive and curative test shows reduction of body weight throughout 30 days observation except for the chloroquine group [Figures 2 and 3].

Survival analysis

Survival analysis on the treatment mice undergo suppressive and curative test [Figures 4 and 5] show that only chloroquine group survived until the end of the study.

Table 1: Effects of oral administration of 5000 mg/kg *P. amboinicus* leaves ethanolic extract on weight of organs of mice

Organ (g)	Group (*n*=10)		*P* value
	Experimental	Control	
Liver	1.69±0.43	1.55±0.36	0.436
Heart	0.15±0.03	0.14±0.03	0.380
Lung	0.38±0.06	0.35±0.05	1.000
Spleen	0.15±0.05	0.15±0.04	0.263
Kidney	0.38±0.09	0.38±0.08	0.979

P. amboinensis: Pomacentrus amboinensis

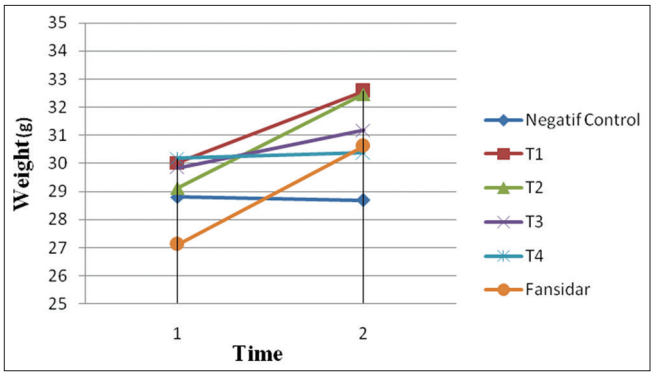

Figure 1: Graph of body weight of mice in a different group of treatments in prophylactic test. Time 1 = 1st day of treatment (day 0), time 2 = 7 days posttreatment (D7) and 4 days postinfection

DISCUSSION

In this study, phytochemical analysis, acute oral toxicity and antiplasmodial study of ethanolic extract of *P. amboinicus* leaves were carried out. Phytochemical analysis revealed flavonoids as the only chemical constituents in the extract tested. Resulting from experimental evidence that flavonoids may modulate allergens, viruses and carcinogens, it can be said that the extract has a potential to be biological response modifiers such as antiallergic, antiinflammatory, antimicrobial, anticarcinogens and cardioprotective agents.[17,18] Based on the above-mentioned results, the acute oral LD_{50} in experimental mice were found to be in excess of 5000 mg/kg. This value showed that the extract is considered not to present a significant acute toxic risk if swallowed.[19] This supports the popularity and wide usage of this plant in folk medicine practices.

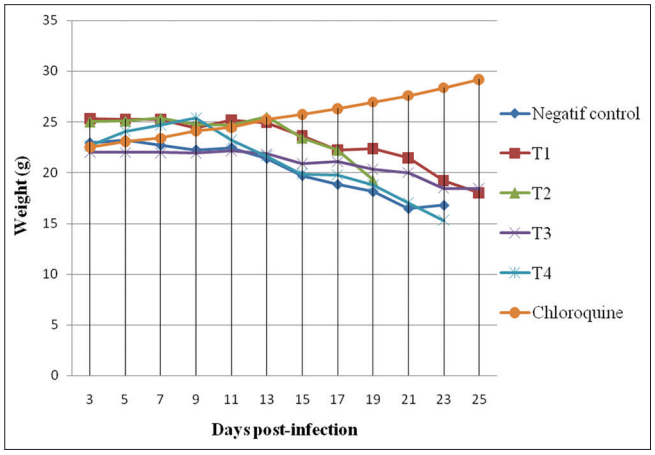

Figure 2: Graph of body weight of mice in a different group of treatment throughout 30 days of suppressive test

Previous study proved that the aqueous extract of the leaves of *P. amboinicus* is able to reduce the parasitemia caused by *P. berghei yoelli* in suppressive test in mice, thus support the use of this leaves as folkloric medicinal plant to treat malaria fever in India.[20] In this study, Malaysian *P. amboinicus* also showed some potential to be used as antimalarial. No reduction of parasitemia found in suppressive and curative tests, but the extract showed some potential to be used as a prophylactic agent in malaria. This condition may be due to accumulation of phytochemical constituents that may exert antimalarial action either by causing elevation of RBC oxidation or by inhibiting protein synthesis.[21]

CONCLUSION

The ethanolic extract of *P. amboinicus* leaves (Malaysian isolated) contained only flavonoid and was proven to be scientifically as safe for human consumption. No reduction of parasitemia found in suppressive and curative tests,

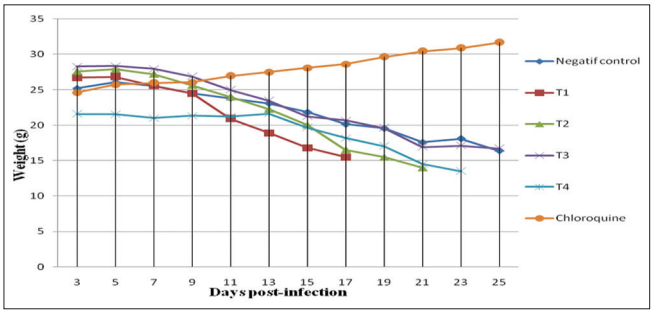

Figure 3: Graph of body weight of mice in a different group of treatment throughout 30 days of curative test

Table 2: Erythrocythic-antiplasmodial assay of the ethanolic leaves extract of *P. amboinicus* during early, established and residual *P. berghei* infection

Test	Drug/ extract	Dose (mg/kg)	Percentage of parasitemia (mean±SD)	Percentage of chemo-suppression	*P* value
Prophylactic test	Control	0.2 mL	6.48±0.58	00.00	
	Extracts	50	1.08±0.11	83.33	0.000***
		200	1.58±0.40	75.62	0.000***
		400	0.60±0.25	90.74	0.000***
		1000	7.33±1.04	Nil	0.176
	Fansidar	1.2	1.22±0.40	81.17	0.000***
Suppressive test	Control	0.2 mL	5.02±0.52	00.00	
	Extracts	50	4.25±0.35	15.33	0.175
		200	5.99±0.31	Nil	0.059
		400	7.22±0.92	Nil	0.000***
		1000	8.24±0.63	Nil	0.000***
	Chloroquine	20	0	100	0.000***
Curative test	Control	0.2 mL	6.51±0.24	00.00	
	Extracts	50	10.30±0.35	Nil	0.000***
		200	17.76±0.70	Nil	0.000***
		400	21.62±0.64	Nil	0.000***
		1000	11.4±1.00	Nil	0.000***
	Chloroquine	20	0	100	0.000***

***P<0.001 as compared with control. n=5 for each group of treatments. *P. amboinensis: Pomacentrus amboinensis, P. berghei: Plasmodium berghei*, SD: Standard deviation

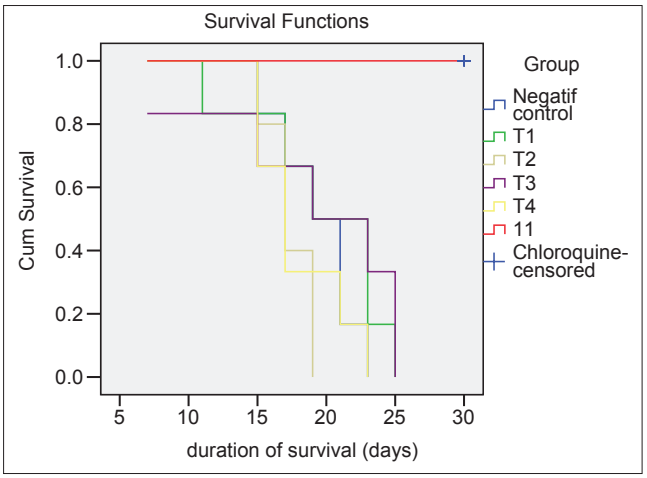

Figure 4: Graph of Kaplan–Meier survival function of different group of treatment throughout 30 days of suppressive

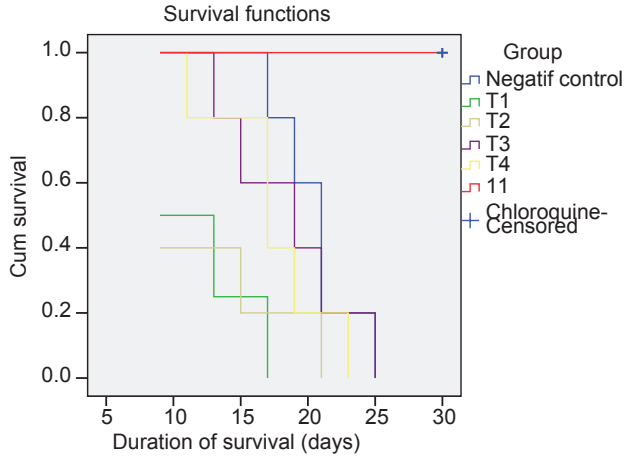

Figure 5: Graph of Kaplan–Meier survival function of different group of treatment throughout 30 days of curative test

but the extract showed some potential to be used as a prophylactic agent in malaria. The isolation of active compound of this extract may give a promising drug molecule to be served as a prophylactic agent of malaria in mice.

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