

Antimalarial activity of selected Malaysian medicinal plants

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Abstract

Treatment of malaria infection has becoming extremely challenging due to widespread resistance of the parasite towards available antimalarial drugs. In the present study, we investigated the antimalarial activity of five local Malaysian medicinal plants species including *Eurycoma longifolia*, *Andrographis paniculata*, *Alyxia lucida*, *Ardisia Crispa* and *Orthosiphon Stamineus*. *Plasmodium berghei* ANKA infection in ICR mice was used as model for malaria infection. Malaria was initiated by inoculation of mice with 2×10^7 parasitized red blood cells and treatment with various concentrations of the plant extracts was carried out once daily in a 4-day suppressive test against parasitaemia development. Results showed that *Eurycoma longifolia*, *Andrographis paniculata* and *Ardisia Crispa* exhibited considerable antimalarial activity, whereas *Alyxia lucida* only showed a weak activity and *Orthosiphon stamineus* did not show any antimalarial activity.

Keywords *Eurycoma longifolia*; *Andrographis paniculata*; *Alyxia lucida*; *Ardisia Crispa*; *Orthosiphon stamineus*; Malaria

Introduction

Malaria is still one of the major health problems in many tropical and subtropical countries around the globe. According to the recent estimation by WHO, over 40% of world population is at risk from malaria. The global incidence is estimated at about 300-500 million cases annually, with 1.5-2.7 million deaths every year, 1 million of these among children under 5 years (WHO, 1997). The widespread resistance of malaria parasites against many antimalarial drugs has caused a great problem in controlling the disease. While development of vaccine against the disease has been disappointing, control of malaria infection still very

much depends on the use of effective chemotherapeutic agents. Southeast Asia has been reported to have the greatest problem of drug resistance against malaria. There is evidence of resistance against all anti-malarial drugs including mefloquine, halofantrine and even quinine (Kondrashin and Rooney, 1992). This situation is worsened with the emergence of chloroquine-resistant strains of *Plasmodium falciparum*, the malaria parasite responsible for most of death cases every year. The search for new anti-malarial agents is therefore crucial in the effort of combating the disease.

Plant sources as anti-malarial agents has gain a lot of interests since the discovery of artemisinin, a compound found to be very active against drug resistant malaria parasites, from herb plant *artemisia annua* (Klaymann, 1985). This plant has been used to treat malaria and fever for thousands of years in China. This folklore medicine was the starting point that leads to the isolation of artemisinin, which has now become the frontline therapy for malaria in combination with currently available antimalarial drugs such as piperazine, mefloquine, lumefantrine, naphthoquine etc. (Sinclair et al., 2009; Nsagha et al., 2012). Malaysia has a huge biodiversity and some plants have been identified to possess medicinal values. Screening of plants for their pharmacological and medicinal properties has not been fully explored and therefore creates the need for investigations.

The aim of this study was to evaluate the efficacy of some selected local herbal plants against malaria infection using an *in vivo* animal model of the disease. Five plants have been selected for the study, i.e., *Eurycoma longifolia* (*E. longifolia*), *Ardisia Crispa* (*A. crispa*), *Andrographis Paniculata* (*A. paniculata*), *Alyxia Lucida* (*A. lucida*) and *Orthosiphon Stamineus* (*O. stamineus*). All this plants have been used traditionally for various purposes by the old folks. Some of them have been documented as having scientific and medicinal values. *E. longifolia* for example has been reported to show antihyperglycaemic activity (Husen et al., 2004) and anti-plasmodial activity against *plasmodium yoelii* in mice (Mohd. Ridzuan et al., 2007) and also against *plasmodium falciparum* culture (Sholikhah et al., 2008; Chan et al., 2004).

A. paniculata has been shown to be efficacious for the relief of rheumatoid arthritis (Burgos et al., 2009) in a placebo-controlled trial. It has also been reported to possess antioxidant, antioedema and analgesic activities (Lin et al., 2009). *A. crispa* was shown to have antiplasmodial activity against *falciparum* cultures (Noor Rain et al., 2007) and other species of this plant which include *Ardisia japonica* and *Ardisia elliptica* has been documented as having anti-HIV and anti-salmonella activities respectively (Dat et al., 2007; Phadungkit & Luanratana, 2006). No scientific documentation on the medicinal property of *A. lucida* has been found so far, apart from the isolation of two new coumarin glycosides from this plant (Lin et al., 1993).

O. stamineus has been shown to exhibit diuretic (Adam et al., 2009), anti-pyretic (Yam et al., 2009) and hypouricaemic (Arafat et al., 2008) activities. Siphonols, which is a chemical constituent of *O. stamineus* extract has been demonstrated as a potent inhibitor of nitric oxide production in lipopolysaccharide-activated macrophages cell line (Awale et al., 2003). With all these scientific evidences, we therefore proposed to further evaluate the scientific and medicinal values of the above said plants in regards to malaria infection.

Materials and methods

Animals

Male ICR mice weighing initially between 18-25g were used throughout this study. The animals were obtained from the Animal Housing Unit, Universiti Sains Malaysia and allowed an unlimited access to food (CRM feeding pellets) and water. Animals were handled as gently as possible and transferred from the animal house to laboratory at least 30-60 min prior to use, in order to minimise the effects of stress.

Rodent malaria parasite

Rodent malaria parasite *Plasmodium berghei* ANKA strain was originally obtained from the Institute of Medical Research, Kuala Lumpur. The parasite has been maintained at the School of Pharmaceutical Sciences, University Sains Malaysia by a combination of passage in male ICR mice and cryoscopic storage.

Chemicals and reagents

Chloroquine, Leishman stain and all of the analytical chemicals and reagents were purchased from Sigma-Aldrich Chemical Company. Leishman's stain used in the staining of *P. berghei* in blood films was prepared from Leishman at 2mg/ml methanol.

Malaria infection

Malaria infection in mice was initiated by intravenous (i.v) inoculation into the tail vein of normal mouse with 0.2 ml blood, diluted to contain 2×10^7 parasitized red blood cells (PRBC) from a donor mouse infected with *P. berghei*. Controls to malaria-infected mice were given an equivalent volume and dilution of normal uninfected red blood cells.

Parasitaemia measurement

Measurement of parasitaemia levels in the animals was carried out by collecting a drop of blood through venesection of the tail from each malarial animal, onto the edge of a microscope slide (single, 76 x 26 mm thickness). The blood was drawn evenly across a second slide to make a thin blood film. The slide was allowed to dry at room temperature before staining with Leishman stain. Slides were viewed under light microscopy with oil immersion (1000x magnification). Leishman positive cells were counted with the aid of a graticule and hand counter. Five fields of approximately 200 cells each were counted and parasitaemia calculated as the percentage of total red blood cells counted containing Leishman positive bodies. The average results from five different fields were then taken as the final percentage of parasitaemia.

Plant samples and extraction

For *E. longifolia*, the root of the plant was used, whereas for *A. Paniculata*, *A. crispa*, *A. Lucida* and *O. Stamineus*, the leaves part were used. The dried powdered roots or leaves of

the plants were extracted using 90% methanol as a solvent in a soxhlet extraction. The solvent was completely removed by vacuum. The methanol extracts were stored in a dessicator until used. A weighed quantity was suspended in 2.5% tween 20 for the experiment.

Treatment of malarial mice with chloroquine

Mice were infected with *P. berghei* as described above and were divided into three groups of six. Chloroquine (7-chloro-4-[4-diethylamino-1-methyl-butylamino]-quinoline, diphosphate salt) was used as a standard and was dissolved in 0.9% sterile pyrogen free saline and a dose of 10mg/kg (i.p) were given in a single daily dose at midday starting from day 1 until day 4 following the inoculation. Controls to chloroquine received an equivalent volume (10ml/kg, i.p) of 0.9% sterile saline. Control uninfected mice group also received an equivalent volume of 0.9% saline (i.p).

Treatment of malarial mice with plant extracts

Methanol extracts of the five plant species were assessed for *in vivo* activity in a four-day suppressive test against *P. berghei* infection in mice. Mice were inoculated with 0.2ml of 2×10^7 PRBC intravenously as described above. All extracts were dissolved in 2.5% tween 20 and diluted with water (for injection) to provide doses of 10, 50 and 100 mg/kg body weight. The extracts were administered in a single daily dose intraperitoneally according to their body weight from day 1 until day 4 post infection. Control to the extract-treated malarial group received equivalent volume (i.v) and dilution of 2.5% tween 20 in water for injection. Parasitaemia development in the infected mice was monitored daily throughout the treatment.

Statistical analysis

Statistical analysis of the data obtained in this study was performed by one-way ANOVA followed by Tukey as a single post hoc test. $P < 0.05$ was taken as statistically significant.

Results

No significant difference was observed between the percentage parasitaemia in saline-treated (10ml/kg, i.p) malarial mice ($3.0 \pm 0.2\%$) and chloroquine-treated (10mg/kg, i.p) malarial mice ($3.0 \pm 0.3\%$) on day 1 following inoculation with the parasite (Table 1). On day 2, chloroquine treatment in malarial mice significantly inhibited the development of parasitaemia ($3.4 \pm 0.3\%$) as compared to saline-treated malarial mice ($12.0 \pm 2.5\%$, $P < 0.0001$).

Table 1. The effects of chloroquine treatment on percentage parasitaemia in malaria-infected mice.

Group/Treatment	Percentage parasitaemia (%)			
	Day 1	Day 2	Day 3	Day 4
Control uninfected	2.00 ± 0.75	2.50 ± 0.20	1.50 ± 0.10	1.00 ± 0.10
Malaria + Saline	3.00 ± 0.20	12.00 ± 2.50	27.00 ± 8.20	54.00 ± 6.00
Malaria + Chloroquine	3.00 ± 0.30	$3.40 \pm 0.30^{***}$	$2.50 \pm 0.10^{***}$	$2.00 \pm 0.10^{***}$

Table 2. The effects of different doses of plant extracts on percentage parasitaemia in malarial mice. Number in parenthesis indicates the percentage inhibition on parasitaemia development in malarial mice treated with the various doses of plant extracts as compared to malarial mice treated with tween 20.

Plant	Days post infection	Group/dose of plant extracts				
		Control uninfected	Malaria + Tween 20	Malaria + 10mg/kg	Malaria + 50mg/kg	Malaria + 100mg/kg
<i>Eurycoma Longifolia</i>	1	2.00 ± 0.20	3.00 ± 0.98	1.63 ± 0.26	2.50 ± 0.29	2.50 ± 0.29
	2	1.50 ± 0.10	6.33 ± 0.83	2.75 ± 0.37* (57%)	2.75 ± 0.48* (57%)	Died
	3	1.50 ± 0.10	17.11 ± 1.46	4.75 ± 1.35* (72%)	Died	Died
	4	2.00 ± 0.10	32.33 ± 4.06	7.00 ± 3.08* (78%)	Died	Died
<i>Andrographis Paniculata</i>	1	2.00 ± 0.10	2.10 ± 0.98	1.89 ± 1.67	1.67 ± 0.24	1.50 ± 0.22
	2	2.00 ± 0.20	6.30 ± 0.81	4.15 ± 0.63* (34%)	3.90 ± 0.66* (38%)	3.60 ± 0.56* (43%)
	3	1.50 ± 0.10	17.00 ± 1.39	9.53 ± 2.10* (44%)	8.10 ± 2.35* (52%)	6.50 ± 2.08* (62%)
	4	1.00 ± 0.10	31.90 ± 4.00	15.10 ± 5.27* (53%)	13.00 ± 4.00* (59%)	11.50 ± 3.78* (64%)
<i>Alyxia Lucida</i>	1	2.00 ± 0.10	1.99 ± 0.95	1.88 ± 0.35	2.00 ± 0.33	2.38 ± 0.46
	2	1.00 ± 0.10	6.29 ± 0.85	4.50 ± 0.49* (28%)	4.70 ± 0.44* (25%)	4.68 ± 0.23* (26%)
	3	1.50 ± 0.10	17.20 ± 1.49	14.63 ± 2.92	13.90 ± 2.36	12.00 ± 3.50
	4	2.00 ± 0.10	32.50 ± 4.07	23.88 ± 7.70	22.10 ± 6.57	27.61 ± 3.22
<i>Ardisia crispa</i>	1	2.00 ± 0.10	3.50 ± 0.70	2.60 ± 0.30	2.60 ± 0.16	3.00 ± 0.29
	2	2.00 ± 0.20	10.00 ± 0.85	5.00 ± 0.61* (50%)	4.70 ± 0.39* (53%)	4.52 ± 0.85* (55%)
	3	1.50 ± 0.10	25.83 ± 2.35	12.60 ± 1.69* (51%)	15.00 ± 1.37* (42%)	16.11 ± 2.06* (38%)
	4	1.00 ± 0.10	45.81 ± 4.78	26.70 ± 3.97* (42%)	28.50 ± 3.04* (38%)	29.68 ± 3.53* (35%)
<i>Orthosiphon Stamineus</i>	1	2.30 ± 0.10	2.16 ± 0.17	3.83 ± 0.90	3.17 ± 0.31	3.00 ± 0.37
	2	2.00 ± 0.10	4.67 ± 0.33	5.17 ± 0.48	5.00 ± 0.37	5.00 ± 0.26
	3	1.00 ± 0.10	20.00 ± 1.20	25.00 ± 4.68	22.00 ± 1.95	22.30 ± 1.65
	4	1.50 ± 0.10	45.00 ± 2.92	48.33 ± 5.66	46.17 ± 2.68	46.70 ± 4.67

*P<0.05 significant difference between extract-treated malarial mice and tween 20-treated malarial mice.

Percentage parasitaemia in chloroquine-treated malarial mice remained around 2-3% throughout the infection, whereas parasitaemia development in saline-treated malarial mice progressed as that normally seen in untreated malarial mice. Control animals treated with saline showed percentage parasitaemia of around 1 - 3%, which served as the baseline level.

During treatment with *E. longifolia*, all malarial mice receiving 100mg/kg of the extracts died on day 2 following treatment (Table 2). In malarial group receiving 50mg/kg *E. longifolia* extract, 50% mortality was observed on day 2 post inoculation, followed by 100% mortality on day 3. Malarial mice treated with 10mg/kg *E. longifolia* extract however, survived throughout the experiment. Physical signs of toxicity such as writhing, palpitation and gasping were obvious in malarial mice treated with 50 and 100mg/kg *E. longifolia* extracts following the intraperitoneal administration. Malarial mice treated with 10 mg/kg *E. longifolia* extract showed significant inhibition of parasitaemia development as compared to malarial mice treated with the vehicle tween 20. Day 2 observation on parasitaemia development in malarial group receiving 10mg/kg *E. longifolia* extract showed a percentage inhibition of 57% followed by 72% inhibition on day 3 and 78% inhibition on day 4. The 50mg/kg extract of *E. longifolia* also inhibited 57% of parasitaemia development on day 2 following infection even though only 50% of the mice in this group survived on that day. All malarial mice treated with tween 20 showed normal progression of parasitaemia development throughout the infection (Table 2).

A. paniculata extracts also showed considerable antimalarial activities (Table 2). Treatment with different concentrations of the extracts (10, 50 and 100mg/kg body weight) was also carried out on the first day following inoculation with *P.berghei*. On day 2 post infection, treatment with 10mg/kg dose of *A. paniculata* extract was found to cause 34% inhibition of parasite growth as compared to malarial mice treated with tween 20. Thirty eight percent inhibitions were observed with 50mg/kg dose of the extract whereas 100mg/kg dose caused 43% inhibition on the same day. On day 3, percentage inhibitions on parasitaemia were 44%, 52% and 62% for the 10, 50 and 100mg/kg dose of *A. paniculata* extract respectively. This was followed by 53%, 59% and 64% inhibitions on day 4 following infection for 10, 50 and 100mg/kg dose respectively. The inhibitory properties of the different concentrations of *A. paniculata* extracts on parasite development were found to be dose dependent. The higher the concentration of extracts, the higher the percentage inhibitions on parasite development. No mortality was observed in this group. The methanol extracts of *A. paniculata* did not cause any obvious physical signs of toxicity during the treatment even at a high dose of 100mg/kg.

Extracts of *A. lucida* (10, 50 and 100mg/kg body weight) only exhibited a small potency of its antimalarial activity (Table 2). Significant inhibition on parasitaemia was only observed on day 2 post infection with 28%, 25% and 26% inhibition for the 10, 50 and 100mg/kg dose respectively. No significant results were observed for all concentrations of the extract during treatment on day 3 and day 4 post infection. No mortality or physical signs of toxicity were observed in any of the animals during treatment with the extracts.

Methanol extracts of *A. crispa* also showed considerable antimalarial activity. On day 2 post infection, 50%, 53% and 55% inhibition of parasitaemia were observed for all the doses (10, 50 and 100mg/kg respectively). On day 3, percentage inhibitions of parasitaemia were

51% for 10mg/kg does, 42% for 50mg/kg dose and 38% for 100mg/kg dose, followed by 42%, 38% and 35% inhibition on day 4 for 10, 50 and 100mg/kg *A. crispera* extracts respectively. Mortality was not recorded during treatment with *A. crispera* extracts and neither the physical signs of toxicity were observed throughout the experiment indicating that methanol extracts of this plant is also safe to be used in mice even up to 100mg/kg concentration.

For *O. stamineus*, all of the animals in malarial groups treated with various concentrations of the extracts showed a normal progressive parasitaemia development as in malarial group treated with tween 20. No significant inhibition on parasitaemia development was observed during treatment with *O. stamineus* extracts.

Discussion

Data in this study indicated that chloroquine treatment (10mg/kg, i.p) during the infection completely abolished the parasites. Physical signs of illness (diarrhea, lethargy, piloerection, reduced locomotor activity etc.) normally seen in malaria-infected mice were absent in chloroquine-treated malarial mice and they appeared healthy throughout the infection and treatment. Results with chloroquine indicate that the malarial model used in this study is sensitive towards antimalarial agent and therefore justify its use in screening of antimalarial properties from other sources.

Among the five plant extracts tested against malaria infection in this study, *E. longifolia* exhibited the most potent antimalarial activity. Inhibition on parasitaemia reached almost 80% even at the lowest dose of 10mg/kg. However, at the higher doses (50 and 100mg/kg) of the extract, increase mortality was observed among the treated malarial mice. This event could be due to the presence of toxic substance(s) in *E. longifolia* extracts which is expected to be highly concentrated in the higher doses. The high mortality rate could not be due to the infection as mortality are not normally observed during day 2 and day 3 following infection as already established before. *E. longifolia* extract has thus far being tested *in vitro* against *P. falciparum* strains and not much information is available on its possible antimalarial activity *in vivo*. A few chemical constituents isolated from *E. longifolia* were thought to be responsible for its antimalarial properties. Chan *et al.* (1986) reported that 10-hydroxycanthin-6-one, eurycomalactone, eurycomanone and eurycomanol isolated from *E. longifolia* exhibited potential antimalarial activity *in vitro* against the chloroquine resistant *P. falciparum*. 7-methoxy-P-carboline-1-propionic is another compound isolated from *E. longifolia* that possessed antimalarial activity against *P. falciparum* strains (Kardono *et al.*, 1991). We have demonstrated here in our study, the *in vivo* antimalarial property of *E. longifolia* extract and also in the meantime its potential toxicity in living animals. Further studies are therefore necessary to be carried out to determine whether the compounds responsible for the toxicity shown by *E. longifolia* extract in the living animals are the same compounds responsible for its antimalarial activity.

A. paniculata extracts also exhibited considerably significant antimalarial activity in this study but with inhibitory potency on parasitaemia less than that observed with *E. longifolia*. A previous study by Dua *et al.* (2004) revealed that *A. paniculata* root extract contained xanthenes which were thought to be responsible for its antimalarial property. This xanthone was reported to show substantial anti-plasmodial activity against *P. falciparum* and

also against *P. berghei* infection in Swiss Albino mice (Dua *et al.*, 2004). In our study, the leaves part of the plant was used which was reported to contain compounds like andrographolide, diterpene glucoside and flavonoids (Akbar, 2011). There is no report documenting the presence of xanthenes in the leaves extract of *A. Paniculata* so far, so the active principle(s) responsible for antimalarial activity in the leaves extract of this plant remains elusive. Andrographolide and diterpene glucoside have not been linked to antimalarial activity so far, but flavonoids from other plants extracts for examples, flavonoids from *Artemisia indica* (Chanphen *et al.*, 1998), *Siparuna andina* (Jenett-Siems *et al.*, 2000) and *Abrus precatorius* (Limmatvapirat *et al.*, 2004) have been reported to exhibit antiplasmodial activity. From these previous findings, it may be possible that the flavonoids contents of *A. Paniculata* leaves extract is responsible for the observed *in vivo* antimalarial activity in this study.

A. crispa extract also showed potential antimalarial activity with more than 50% inhibition on parasite growth recorded in this study. *A. crispa* extract has been shown in a previous study to be effective in inhibiting the growth of *P. falciparum* culture (Noor Rain *et al.*, 2007). So far, no investigation on *in vivo* malarial model using *P. berghei* has been carried out using *A. crispa* extract. Reports from previous studies showed that two active compounds have been isolated from *A. crispa* extract so far, i.e., benzoquinonoid which exhibited antimetastatic and antitumor effects (Kang *et al.*, 2001) and saponins which was reported to have uterine contracting property (Jansakul *et al.*, 1987). The possible active compounds responsible for *A. crispa* antimalarial activity in our study is not known at this stage since thus far, no previous study associating benzoquinonoid and saponins with antimalarial activity.

For *A. lucida*, the extracts only exhibited weak antimalarial activity and the low potency of the extract may not be able to curb parasites growth during the late stages (day 3 and 4) of infection which is normally associated with critically high percentage of parasitaemia. Little is known about this plant property, apart from isolation of coumarin glycosides from the inner bark of this plant in a previous study (Lin *et al.*, 1993). Antiplasmodial activity of coumarin compounds has been reported in a few studies (Oketch-Rabah *et al.*, 1997; Yenjai *et al.*, 2000; Kohler *et al.*, 2001; Argotte-Ramos *et al.*, 2006). Whether coumarin compound also exist in the leaves extract of *A. lucida* used in our study and is responsible for the weak antimalarial activity cannot be deduced at this stage as not many informations can be gathered regarding this plant from the previous study. *O. stamineus* extracts showed no significant inhibition on the parasites growth at all doses tested throughout the treatment suggesting that the extracts of this plant do not possess any antimalarial activity.

In conclusion, three of the five plant extracts used in this study showed considerable antimalarial activities that are worth to be investigated further. This includes *E. longifolia*, *A. paniculata* and *A. crispa*. Further studies are suggested to elucidate their antimalarial properties and also to isolate the active substance(s) responsible for their antimalarial activity. *E. longifolia* extract was shown to be the most potent among the three plants but was found to be highly toxic in the animals. Elimination of toxic substances in the extracts is therefore necessary for a better understanding of its antimalarial property. Even though *A. lucida* extracts showed some antimalarial activity but its potency is too low as compared with other extracts used in this study. From the data observed, increasing *A. lucida* extract concentrations will not results in any increase in its potency since the inhibitory effects on parasites

growth was found to be dose independent. Therefore, further investigation on *A. lucida* for its antimalarial properties may not be beneficial. *O. Stamineus* extracts did not show any antimalarial activity at all and therefore no further work should be considered for this extracts in antimalarial study.

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Conflicts of interest

The authors declare that there is no conflict of interest in the writing of the manuscript and there is also no financial competing interest in regards to the funding source.

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