

Antiplasmodial properties of some Malaysian medicinal plants

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Abstract. Seven Malaysian medicinal plants were screened for their antiplasmodial activities *in vitro*. These plants were selected based on their traditional claims for treatment or to relieve fever. The plant extracts were obtained from Forest Research Institute Malaysia (FRIM). The antiplasmodial activities were carried out using the pLDH assay to *Plasmodium falciparum* D10 strain (sensitive strain) while the cytotoxic activities were carried out towards Madin-Darby bovine kidney (MDBK) cells using MTT assay. The concentration of extracts used for both screening assays were from the highest concentration 64 µg/ml, two fold dilution to the lowest concentration 0.03 µg/ml. *Goniothalamus macrophyllus* (stem extract) showed more than 60% growth inhibition while *Goniothalamus scortechinii* root and stem extract showed a 90% and more than 80% growth inhibition at the last concentration tested, 0.03 µg/ml. The *G. scortechinii* (leaves extract) showed an IC₅₀ (50 % growth inhibition) at 8.53 µg/ml, *Ardisia crispa* (leaves extract) demonstrated an IC₅₀ at 5.90 ± 0.14 µg/ml while *Croton argyratus* (leaves extract) showed a percentage inhibition of more than 60 % at the tested concentration. *Blumea balsamifera* root and stem showed an IC₅₀ at 26.25 ± 2.47 µg/ml and 7.75 ± 0.35 µg/ml respectively. *Agathis borneensis* (leaves extract) demonstrated a 50 % growth inhibition at 11.00 ± 1.41 µg/ml. The study gives preliminary scientific evidence of these plant extracts in line with their traditional claims.

INTRODUCTION

Natural resources from tropical rain forest are a promising source of biologically active compounds. Drug discovery program in the tropics often focuses on this readily accessible resources. Malaysia is rich in natural resources, of the more than 20,000 species of angiosperms and 600 species of ferns in Malaysia, 1,082 species (15%) and 76 species (13%), respectively, are reported to have medicinal properties (Mansor Puteh, 2005). While some species are more popular than others, many form the basis of herbal remedies to treat ailments. The importance

of the region's diverse medicinal plants lies not only in their chemotherapeutic value in traditional health care but also in their potential as source of new chemical entities for drug discovery (Mansor Puteh, 2005). In Malaysia, most malaria cases are caused by *Plasmodium falciparum*. The disease remains a public health problem where, clinical signs of fever, headache, and shivering are often suggestive of malaria fever.

Present drugs have become ineffective because of the occurrence of resistant *P. falciparum*, particularly to chloroquine. Chloroquine resistance has been reported in Malaysia in 1963 (Montgomery & Eyles,

1963). Subsequently, several reports on chloroquine resistance have been reported in Sabah, West Malaysia (Clyde *et al.*, 1973). Resistance of *P. falciparum* to combination of chloroquine and pyrimethamine (Dondero *et al.*, 1976), sulfadoxine/pyrimethamine (SDX/PYR) resistant (Black *et al.*, 1982; Ponnampalam, 1982) have been reported including widespread resistance to both chloroquine and SDX/PYR in endemic areas of Peninsular Malaysia (Lokman Hakim *et al.*, 1996).

Historically the majority of anti-malarial drugs has been derived from medicinal plants or from structures modelled on plant lead compounds. In this study, some Malaysian medicinal plants were screened for their potential as antiplasmodial activities and cytotoxic activities. The selection of plants were based on their traditional claims.

MATERIALS AND METHODS

Chemicals and reagents

Chloroquine (Sigma, C-6628), dimethyl sulfoxide (DMSO, Sigma D-5876), MALSTAT (Flow Inc. Portlan USA), RPMI medium 1640 (Gibco, Invitrogen Corporation, USA), HEPES (Gibco, Life Technologies), nitro blue tetrazolium (NBT, Sigma, N-6876), phenazine ethosulphate (PES, Sigma P-4544) and MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, M-2128) were used.

Plant materials

Origin of collection and identification

Plants used in the study are listed with botanical names, families, voucher specimen number, local names, and the traditional claims as stated in Table 1. The pictures of the plants are as shown in Fig. 1. The specimens were collected and authenticated by Forest Research Institute Malaysia (FRIM) and they are deposited at the FRIM herbarium.

Preparation of extracts

Briefly the plant materials were prepared

by air drying and grounded to mesh size 40-60 using grinding machine, Wiley mill. They were then subjected to exhaustive soxhlet extraction with methanol (Analytical grade, BDH Laboratory). The resulting extracts were filtered through Whatman paper No.1 and concentrated using a rotary evaporator and kept at -20°C until required.

Preparation of test samples

Stock solutions of the crude extracts were prepared by dissolving 20 mg in 1 ml of DMSO, to make an initial concentration of 20 mg/ml. The initial concentration was then serially diluted half fold in DMSO in a 96-well flat bottomed microtiter plate over 12 concentrations (this plate is referred as DMSO plate). All of these concentrations were then diluted to 16-folds in millipore water by transferring 15 µl of each of the concentration in DMSO plate correspondingly to wells of a new microtiter plate each containing 225 µl of water (this plate referred as watery plate). This plate was prepared one day prior to test and kept at -20°C. On the day of test, the watery plate was taken out from the -20°C freezer and thawed at 37°C. Ten microliter from each concentration (from each well) of the watery plate was transferred correspondingly to a new microtiter plate in duplicates (referred as test plate). The test plate was then ready to use for pLDH assay and MTT assay. The watery plate for positive and the negative control were prepared by adding 15 µl of DMSO to 225 µl of water into each wells of a new 96-wells microtiter plate. Then 10 µl of this dilution was seeded into the control wells in the test plate. Chloroquine was used as a substance control and it was diluted similarly as the plant extract dilution.

In vitro culture of *P. falciparum*

Plasmodium falciparum chloroquine sensitive strain, D10, was used. It was obtained from the Malaria Research and Reference Reagent Resource Center (MR4) with MR4 number 201. The strain was grown using the standard *in vitro* culture

Table 1. List of plants used for the antiplasmodial activity with their traditional claims

Plant species (Family), (Voucher specimens number)	Local name	Plant part and traditional claims
<i>Agathis borneensis</i> Warb Synonym, <i>Agathis alba</i> Foxw, <i>Agathis loranthifolia</i> Salisb, <i>Agathis dammara</i> (Lambert) L.G Richard (Araucariaceae), (FRI45957)	Raja kayu, Damar laut, Kayu Damar Bukit	The powdered wood is used to treat headache and myalgia
<i>Aralidium pinnatifidum</i> (Cornaceae), (FRI45577)	Hempedu buaya, Tampong tulang, Tentulang, and Tampong tulang.	The leaves are placed on abdomen of children having fever. They are mixed with coconut oil and heated and placed on the skin to break the fever by causing perspiration.
<i>Ardisia crispa</i> Thunb. Synonym, <i>Ardisia littoralis</i> Andr., <i>Ardisia humilis</i> Vahl (Myrsinaceae), (FRI45481)	Mata itik, Mata ayam, Mata pelanduk, Daun bisa hati.	The leaves or the roots are used to treat fever, diarrhoea and liver poisoning.
<i>Blumea balsamifera</i> , Synonym, <i>Conyza balsamifera</i> L. (Asteraceae), (FRI45482)	Sembong, Sembuh, Sembang, telinga kerbau, Capa, sapu.	Leaf, entire plant or the root is used as antiplasmodial and the decoction of the leaves is used for coughs, fever and influenza.
<i>Croton argyratum</i> Blume, Synonym, <i>Croton argyratum</i> Blume (Euphorbiaceae), (FRI43118)	Semangkok, Hujan panas, Cenderai, Melokan.	The decoction of the leaves are used to treat fever.
<i>Goniothalamus macrophyllus</i> (Blume) Hook.f.et Thomson synonym, <i>Unona macrophylla</i> , <i>Polyalthia macrophylla</i> (Blume) (Annonaceae), (MP38)	Selada, akar beranak gajah, penawar hitam, lada hutan	The decoction of the roots are used to relieve colds and to treat fever.
<i>Goniothalamus scortechinii</i> King (Annonaceae), (FRI45437)	Selada putih, Akar gajah beranak, Bunga chenang	The plant has been reported to treat fever.

(Burkill, 1935; Norhayati *et al.*, 1999; HMRC, 2000).

technique for *P. falciparum* by Trager & Jensen (1976). The culture was set up in a 6-wells plate (Costa, USA) and maintained in culture medium RPMI 1640 containing 25 mM HEPES, 0.2 % sodium carbonate supplemented with 10 % heat-inactivated human serum and 4 mg/ml of gentamycin. *P. falciparum* D10 strain was grown in 'O' type blood group. The initial culture was initiated with 1% parasitemia and 7% hematocrit. The parasite density was monitored daily by making thin blood smears stained with Giemsa solution and

observed under microscope using 1000 times magnification. When the parasitemia of the parasite culture reached to about 5 to 7%, the parasites were harvested and the percentage parasitemia of the *P. falciparum*-infected erythrocyte suspension was then adjusted to 1 to 2% with washed uninfected red blood cells with haematocrit of 2% for use in the pLDH assay.

***In vitro* antiplasmodial activity**

The pLDH assay was carried out according to the method by Makler *et al.* (1993)

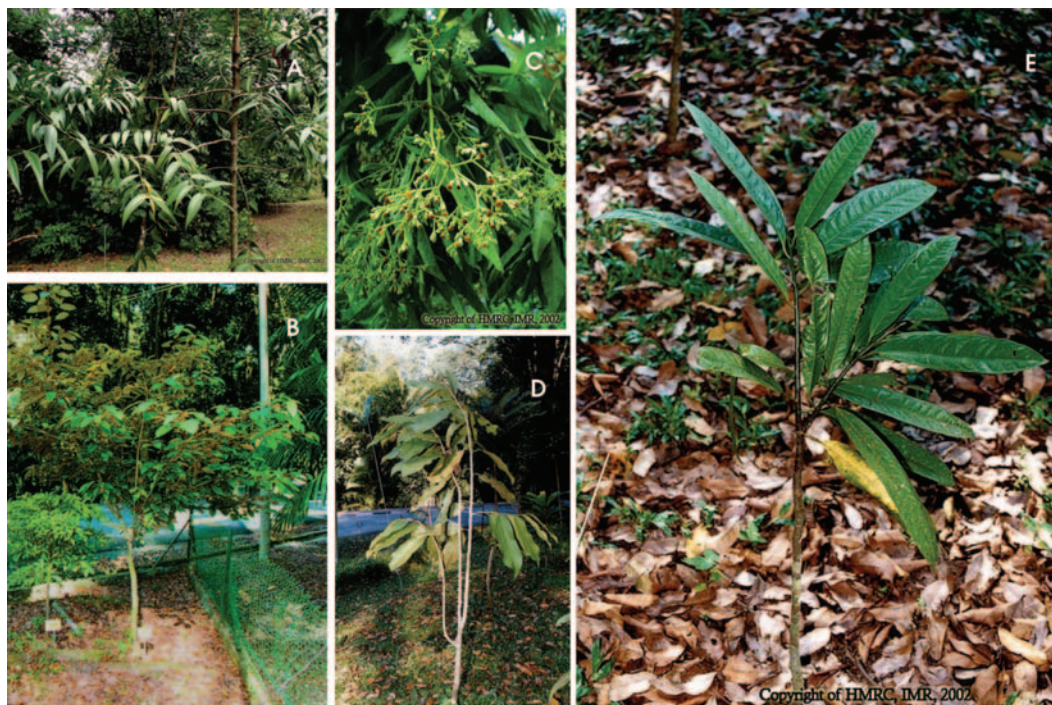


Figure 1. Medicinal plants used in the study. A: *Agathis borneensis*; B: *Croton argyратum*; C: *Blumea balsamifera*; D: *Goniothalamus microphyllus*; E: *Goniothalamus scortechinii*.

Briefly 190 μ l of *P. falciparum*-infected erythrocyte suspension which was prepared earlier, with 1.5% parasitemia and 2% haematocrit, were seeded into each well of the prepared test plate. The final concentration of the test range from 64 μ g/ml to the lowest 0.003 μ g/ml. The highest concentration of DMSO that the parasites were exposed was 0.3%, which was shown to have no measurable effect on parasite viability. All test samples were performed in duplicates. The positive and the negative control wells containing 10 μ l of diluted DMSO in water 1:16 dilution prepared earlier were added with 190 μ l of each *P. falciparum*-infected erythrocytes and noninfected erythrocytes (2% hematocrit) respectively. Similarly 190 μ l of the parasite material were added to the different concentrations of the chloroquine prepared similarly as the test substance. The test and the control plates were placed in a candle jar (with approximately gas environment of about 3% O₂, 6% CO₂ and 91% N₂). All tests were performed in

duplicates. The test samples were incubated for 72 hrs at 37°C for maximum parasite growth.

At the end of the incubation, the plates were placed at -20°C for a period of 24 hrs and thawed in 37°C-incubator to haemolyse. The parasite lactate dehydrogenase (LDH) activities from both test samples and the controls were evaluated by reaction of the blood lysate from each of the test wells with MALSTAT (TM) reagent in a new 96-well plate. The spectrophotometric assessment of the LDH activity was by addition of 20 μ l of a mixture of NBT (2 mg/ml) and phenazine ethosulphate (0.1 mg/ml) in the ratio of 1:1. The plates were placed in the dark for 2 hrs. The development of blue color was detected at 630 nm using an enzyme-linked immunosorbent assay reader (Dynatech, USA). The percentage of growth inhibition and the 50% growth inhibition concentration was estimated from a dose response curve.

Cytotoxicity study of extract on MDBK cells using MTT assay

Madin-Darby Bovine Kidney (MDBK) cells were obtained from ATCC. The cytotoxicity of the extracts was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). On the day of test, MDBK cells were harvested, counted and the number of cells were adjusted to 1×10^4 cells per ml. One hundred and ninety microliters of the cell suspension in culture medium supplemented with 5% fetal calf serum (complete culture medium) were seeded into each well of a 96-well plate and allowed to grow for 24 hrs. Then 10 μ l of test substance were added to each well accordingly. The final concentration of the extracts in test wells range from 64 μ g/ml to the lowest 0.03 μ g/ml. All tests were performed in triplicates. The control for cell growth was 190 μ l of cell suspension without test substance while the positive control was 190 μ l of cell suspension with 10 μ l of 1% Triton X 100. The culture was incubated at 37°C in 5% CO₂ incubator for 72 hrs. Then 50 μ l of MTT-PBS (5 mg/ml) solution in culture medium in a ratio of 1: 2.5 were added to each well. The plates were further incubated for 3 hrs at 37°C in 5% CO₂ incubator. The medium was then removed and replaced with 200 μ l of DMSO to solubilise the MTT formazan product. The solution was mixed over a belly dancer for 15 min and once for 30 sec before measuring the absorbance at 540 nm with a microplate reader. The percentage of growth inhibition and the 50% growth inhibition concentration was estimated from a dose response curve.

RESULTS AND DISCUSSION

The antiplasmodial activities of the plant extracts are summarized in Table 2. The thresholds for the *in vitro* antiplasmodial activity of antimalarial extracts were according to Rosoanaivo *et al.* (2004). It is classified as extracts with IC₅₀ value less than 0.1 μ g/ml is considered very good, 0.1

to 1.0 μ g/ml is good (active), 1.1 to 10 μ g/ml is good to moderate active, 11 to 25 μ g/ml is weak, 26 to 50 μ g/ml is considered very weak while more than 100 μ g/ml is inactive. To estimate the potential of a given extract to inhibit parasite growth without host toxicity, selectivity index was determined and defined as the ratio of IC₅₀ in cytotoxicity to IC₅₀ in *P. falciparum*. The higher the selectivity index, the higher is the selective antiplasmodial activity of a given extract (Rasoanaivo *et al.*, 2004). The IC₅₀ of chloroquine was 0.011 ± 0.02 (within the laboratory range and in line with reference values) (Siti Najila *et al.*, 2002; Elandalloussi *et al.*, 2005).

Out of 7 plants used in the study, 4 plants, *Goniothalamus macrophyllus*, *Goniothalamus scortechinii*, *Ardisia crispa*, and *Croton argyratus* showed very good antiplasmodial activities to *P. falciparum* sensitive strain D10 *in vitro*. Their percentage inhibition were more than 50% inhibition (Table 2) to parasite growth at the 0.03 μ g/ml extract concentrations (last concentration tested), therefore we could not get the exact IC₅₀ values. Nevertheless the IC₅₀ values could be at concentration lower than 0.03 μ g/ml, and therefore we classified them as has having very good anti malarial properties, according the the referred threshold (Rasoanaivo *et al.*, 2004). Their selectivity indices were high, indicating that they do not demonstrate harmful effect to normal cells *in vitro*. All these plants have traditional claims (Table 1) as to relieve fever and this study provides a preliminary scientific evidence for the claims. However further evaluation in animal model using rodent malaria parasite are being evaluated to further proof of the claims.

Croton argyratus demonstrated a very good antiplasmodial activity to *P. falciparum* D10 strain. Our findings were similar to earlier reported antiplasmodial activities elsewhere in the same genera. *Croton gratissimus* Burch (Family Euphorbiaceae), a medicinal plant native to South Africa, has been reported to have good antimalarial activity to *P. falciparum* D10 strain (Prozesky *et al.*, 2001; Clarkson

Table 2. Antiplasmodial and cytotoxic activities of the plant extracts

Plant species names	Plant part	Anti plasmodial activity *The % of growth inhibition or IC ₅₀	Cytotoxicity to MDBK cells (IC ₅₀)
<i>Agathis borneensis</i>	Leaves	11.00 ± 1.41	19.00 ± 1.00
<i>Aralidium pinnatifidum</i>	Leaves	Not active	Not active
<i>Ardissia crispa</i>	Leaves	5.90 ± 0.14	Not active
<i>Blumea balsamifera</i>	Root	26.25 ± 2.47	Not active
	Stem	7.75 ± 0.35	Not active
<i>Croton argyratus</i>	Leaves	> 60%	14.66 ± 1.154
<i>Goniothalamus macrophyllus</i>	Stem	> 60%	18.38 ± 0.577
<i>Goniothalamus scortechinii</i>	Root	> 90%	NA
	Stem	> 80%	25.33 ± 1.154
	Leaves	8.53	Not active

* Results are recorded as the % of growth inhibition or IC₅₀. The results for *C. argyratus*, *G. macrophyllus* and *G. scortechinii* (root and stem extract) were given in % of growth inhibition because the growth inhibition were beyond the 50% level at the last concentration tested that is at 0.03 µg/ml, therefore the IC₅₀ could be smaller than 0.03 µg/ml.

Not active = there was no growth inhibition,
NA = not available

et al., 2004). The two plant families most commonly reported as having antiplasmodial activities are the Euphorbiaceae and Annonaceae. *G. macrophyllus* and *G. scortechinii* (Family Annonaceae) and *C. argyratus* (Family Euphorbiaceae) indicated as having antiplasmodial activities have also been reported earlier (Siti Najila *et al.*, 2002; Clarkson *et al.*, 2004). From these initial findings, the plant extracts of *G. scortechinii* was selected for further investigation for their antimalarial activities.

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