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In vitro Antiplasmodial Properties of Selected Plants of Sabah

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ABSTRACT

The antiplasmodial activity of the crude extracts of thirty plant species collected from Sabah was evaluated using chloroquine-sensitive strain (D10) and chloroquine-resistant strain (Gombak A) of *Plasmodium falciparum*. Significant activities were observed for the bark extract of *Polyalthia insignis* (IC_{50} 3.89 and 11.89 μg/ml against Gombak A and D10, respectively), the leaf extracts of *Kopsia dasyrachis* (4.62 μg/ml against Gombak A) and *Litsea elliptibacea* (IC_{50} 8.88 μg/ml against Gombak A), as well as the leaf and bark extracts of *Neouvaria acuminatissima* (IC_{50} 6.90-10.08 and 0.69 μg/ml against Gombak A and D10, respectively), and the bark extract of *Polyalthia microtus* (IC_{50} 9.0 and 12.12 μg/ml against Gombak A and D10, respectively).

Keywords: ***Plasmodium falciparum*, lactate dehydrogenase assay, *Polyalthia insignis, Kopsia dasyrachis, Neouvaria acuminatissima*, antimalarial**

INTRODUCTION

Malaria, one of the most important tropical diseases, is caused by pathogenic strains of *Plasmodium* (WHO, 2000). According to the latest statistics on the disease, half of the world population is at risk of malaria. In 2008 alone, an estimated 243 million cases were reported to have caused an estimated of 863,000 deaths (WHO, 2009). The emergence of multi-drug resistant strains of *Plasmodium* exacerbates the situation further, posing a major obstacle to successful chemprophylaxis and chemotherapy of the disease (Solomon *et al*., 2009). In Malaysia, several reports of the parasite resistance to common antimalarial drugs have been documented (*see* Montgomery and Eyles, 1963; Clyde *et al*., 1973; Dondero *et al*., 1976, Black *et al*., 1982; Ponnampalam, 1982). A more recent study has also reported the widespread resistance of falciparum malaria to both chloroquine and sulfadoxine/pyrimethamine (SDX/PYR) in several areas of Peninsular Malaysia (Hakim *et al.*, 1996; Cox-Singh *et al*., 2001). This rapid spread of parasite resistance has spurred a renewed interest in the search for new alternative antiplasmodial agents (Winstanley, 2000; Siti Najila *et al.*, 2002; Noor Rain *et al*., 2007; Wan Omar *et al*., 2007). To this end, tropical rain forest plants represent a fertile source of the new candidates for development into new alternative antimalarial drugs for therapy.

In the search for plants with anti-malarial properties, the authors have conducted an *in-vitro* screening for plants with activity against the malaria parasite, *Plasmodium falciparum*. For this

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purpose, thirty plant species collected from Tabun Wildlife Reserve in Sabah were evaluated for antiplasmodial activity using the lactate dehydrogenase (LDH) assay (Makler *et al*., 1993). The enzymatic assay is based on the observation that the LDH enzyme of *Plasmodium falciparum* has the ability to use 3-acetyl pyridine NAD as a coenzyme in the reaction leading to the formation of pyruvate from lactate. This assay has been tested for its applicability in assessing parasite density and drug sensitivity. In drug sensitivity testing, the inhibition profiles and IC_{50} values that were obtained using this assay were directly comparable to those that were determined by the radioactive uptake and microscope methods. The assay is relatively easy to perform and rapid compared to the radioactive uptake and microscopy methods. This paper reports the primary screening results obtained on the thirty species.

MATERIALS AND METHODS

Plant Materials

Plants were collected from the Tabun Wildlife Reserve located in Sabah, East Malaysia. Voucher specimens (*see* Table 1) were lodged at the Sepilok Forest Research Centre in Sandakan, Sabah.

The plant materials for testing were air-dried and ground to a fine mesh using a Wiley mill, and they were then directly soaked in dichloromethane:methanol 1:1. The extract that was obtained from 3 overnight soakings was collected, pooled and evaporated to dryness, lyophilized and stored at –4°C prior to testing.

Parasite Strain and Its Preparation

Two strains of *P. falciparum* were used. Gombak A is a Malaysian isolate and it is known to be resistant to chloroquine (Slamet Trijono *et al.*, 1991). It was originally isolated form an Orang Asli patient who was admitted to the Gombak Hospital in 1982. Meanwhile, the chloroquine-sensitive strain (D10) was obtained from the Institute of Medical Microbiology, University of Milan.

A continuous culture of the chloroquine sensitive (D10) and a Malaysian strain of the chloroquine resistant parasite (Gombak A) was maintained in a suspension consisting of RPMI 1640 culture medium that was supplemented with HEPES (25mM), sodium bicarbonate (0.2%) and gentamycin (40 μg/ml) at pH 7.4, and A or O type of red blood cells. The method of culturing is similar to that described by Trager and Jansen (1976: 1977).

Antiplasmodial Activity

The *in vitro* testing of the antiplasmodial activity was done by measuring the lactate dehydrogenase (LDH) activity of the parasite, as described by Makler *et al.* (1993) with slight modifications. Briefly, 190 μl of the *P. falciparum*-infected erythrocyte suspension, which had been prepared with 1.5% parasitemia and 2% haematocrit, were seeded into each well of the prepared test plate. The final concentration of the test ranged from 64 μg/ml to 0.006 μg/ml (lowest). Meanwhile, the highest concentration of DMSO that the parasites were exposed to, i.e. 0.3%, was shown to have no measurable effect on parasite viability. All the test samples were done in duplicate. Control readings of parasitized red blood cells devoid of plant extracts/drugs and non-parasitized red blood cells were done simultaneously. The positive and negative control wells containing 10 μl of diluted DMSO in 1:16 dilution which had been prepared earlier were added into 190 μl of each *P. falciparum*-infected and non-infected erythrocytes (2% hematocrit), respectively. Similarly, 190 μl of the parasite material were added into the different concentrations of the test extracts. Later, the test and the control plates were placed in a candle jar (with an approximate gas environment

TABLE 1
In vitro antiplasmodial activity of plant extracts against *Plasmodium falciparum*

No	Plant species	Family	Voucher specimen no:	Plant part tested	IC_{50} µg/ml	
					D10 (sensitive strain)	Gombak A (resistant strain)
1	*Alangium griffithii*	Alangiaceae	143521	Bark	1.54	*
2	*Alpinia fraseriana*	Zingiberaceae	145379	stem	4.40	13.68
3	*Borneodendron aenigmaticum*	Euphorbiaceae	145393	leaf	*	*
4	*Callicarpa havilandii var rispida*	Verbenaceae	145389	bark	1.88	*
5	*Calophyllum blancoi*	Guttiferae	145398	leaf	*	*
				bark	0.75	46.34
6	*Calophyllum gracilipes*	Guttiferae	145377	bark	*	*
7	*Calophyllum nodosum*	Guttiferae	145385	bark	*	*
8	*Chionantus crispus*	Oleaceae	145396	bark	*	*
9	*Chisoceton erythrocarpus*	Meliaceae	143512	bark	*	40.75
				fruit	14.85	19.03
10	*Clausena excavata*	Rutaceae	145366	leaf	*	40.32
11	*Canarium hirsutum*	Burseraceae	143522	leaf	*	*
12	*Decaspermum fruticosum*	Myrtaceae	145397	bark	*	*
13	*Dendrocnide elliptica*	Urticaceae	145375	bark	*	*
14	*Diospyros cauliflora*	Ebenaceae	143504	bark	*	*
15	*Diospyros tuberculata*	Ebenaceae	143519	bark	*	*
16	*Diplectria sp.*		143515	bark	*	*
17	*Kopsia dasyrachis*	Annonaceae	145362	leaf	*	4.62
				stem	*	*
18	*Leea indica*	Vitaceae	145370	bark	*	*
19	*Leucosyke winklerii*	Urticaceae	145387	bark	*	*
20	*Litsea elliptibacea*	Lauraceae	145380	leaf	*	8.88
				bark	*	*
21	*Neouvaria acuminatissima*	Annonaceae	143506	bark	0.69	10.08
				leaf	*	6.90
22	*Orophea corymbosa*	Annonaceae	143509	leaf	*	33.00
23	*Polyalthia insignis*	Annonaceae	143511	leaf	*	*
				bark	11.89	3.89
24	*Polyalthia microtus*	Annonaceae	145376	bark	9.01	12.12
25	*Polyalthia rumpii*	Annonaceae	143524	root	*	*
26	*Melicope subunifoliolata*	Rutaceae	145390	leaf	25.02	*
27	*Sauralia sp.*	Sauraliaceae	143505	bark	*	*
28	*Schima wallichii*	Theaceae	145383	bark	*	*
29	*Sterculia stipulata*	Sterculiaceae	145381	bark	49.96	*
30	*Xylopia malayana*	Annonaceae	143507	bark	30.58	*
-	Chloroquine	-	-	-	0.017 (±0.0009)	1.78 (±0.116)

* - parasite inhibition > 50µg/ml

of about 3% O_2, 6% CO_2 and 91% N_2). The test samples were incubated for 72 hrs at 37°C for maximum parasite growth.

After 72 hours, the plates were placed at –20°C for a minimum of 24 hours. Then, an aliquot (20 μl) of the blood lysate, from every concentration, was added into 100 μl of Malstat (Flow Inc., Portland, OR, USA), which had been dispensed into a new microtitre plate. The mixtures were mixed well before any further addition of aliquots of 20 μl of a mixture of 1:1 Nitro Blue Tetrazolium (NBT) and Phenazine ethosulphate (PES) (Sigma Chemicals, USA). The plates were placed in the dark for 2 hours, after which the absorbance was read at 630 nm using an ELISA plate reader (MRX Microplate Reader, Dynex Technologies, USA). The 50% growth inhibition concentration or IC_{50} value was estimated from a dose response curve. The plants with IC_{50} values less than 8 μg/ml were considered as having potential antiplasmodial activities and were suggested for further investigations in an animal model. On the contrary, the plants with IC_{50} values > 50 μg/ml were considered as inactive.

RESULTS AND DISCUSSION

Of the thirty plant species tested, the crude extracts of fourteen species showed significant antiplasmodial activity, *in vitro*, with IC_{50} values ranging between 0.6 to 49 μg/ml (*see* Table 2). Of the fourteen, six species (namely *Alpinia fraseniana, Calophyllum blancoi, Chisocheton erythrocarpus, Neovaria acuminatissima, Polyalthia insignis*, and *Polyalthi microtus*) displayed varying degrees of activity towards both the sensitive and resistant *P. falciparum* strains. The other eight species (*Alangium griffithii, Callicarpa havilanda var rispida, Kopsia dasyrachis, Litsea elliptibacea, Orophea corymbosa, Melicope subunifoliolata, Sterculia stipulata* and *Xylopia malayana*) were active, with varying degrees of activity towards either one of the strains.

The bark extract of *Polyalthia insignis* and the leaf extract of *Kopsia dasyrachis* seemed to be the most potent species against the resistant Gombak A strain (IC_{50} 3.89 and 4.62 μg/ml, respectively). At the same time, *P. insignis* also exhibited a moderate inhibition of the sensitive D10 strain (IC_{50} 11.89 μg/ml), whereas *K. dasyrachis* was inactive towards the strain. A significant inhibition of the resistant strain was also shown by the leaf extracts of *Neouvaria acuminatissima* (IC_{50} 6.90 μg/ml) and *Litsea elliptibacea* (IC_{50} 8.88 μg/ml), while the bark extract of *Polyalthia microtus* inhibited both strains with the IC_{50} values between 9 – 12 μg/ml.

The bark extract of *Neouvaria acuminatissima* exhibited the greatest potency towards the sensitive *P. falciparum* strain (D10, IC_{50} 0.69 μg/ml), while showing significant, albeit lower, potency against the resistant strain (Gombak A, IC_{50} 10.08 μg/ml) at the same time. On the other hand, the bark extract of *Calophyllum blancoi* was almost equally potent towards D10 (IC_{50} 0.75 μg/ml) but showed a weak activity towards Gombak A (IC_{50} 46.34 μg/ml). The bark extracts of *Alangium griffithi* and *Callicarpa havilanda var rispida* significantly inhibited the sensitive strain (IC_{50} 1.54 and 1.88, respectively) but did not inhibit the resistant strain. The other active species exhibited a moderate to weak activities against both or either one of the parasite strains. The only fruit extract in the collection exhibited a moderate inhibition of both strains with the IC_{50} values between 14 – 19 μg/ml.

In conclusion, this preliminary screening has identified several species with potential for further investigations of their bioactive constituents. In particular, *Polyalthia insignis, Kopsia dasyrachis* and *Neouvaria acuminatissima* deserve special attention due to their potent activity against both resistant and sensitive strains of *P. falciparum*. *Polyalthia microtus, Litsea elliptibacea* and *Alpinia frasesiana* are also of interest for further study since they have shown a quite significant inhibition of the resistant strain. Although they have no significant activity towards the resistant strain, *Alangium griffithii, Callicarpa havilanda* var *rispida* and *Calophyllym blancoi* should also be investigated further in view of their very potent activity towards the sensitive strain.

It is interesting to note that three of the active species (*P. insginis, P. microtus* and *K. dasyrachis*) are of the genera *Polyalthia* and *Kopsia*, with both belonging to the Annonaceae family. From the literature, it seems that the two genera are rich sources of isoquinoline and indole type alkaloids. The compounds belonging to these two classes of alkaloids have previously been reported to exhibit potent antiplasmodial activities (Xiao *et al*., 2002; Addae-Kyereme *et al*., 2001; Iwasa *et al*., 2001; Long *et al*., 1999). *Polyalthia insignis* contains seco-benzyltetrahydroisoquinoline alkaloids as the major constituents (Kee-Huat Lee *et al*., 1997). The species is used by the Murut tribe in Sabah as an antipyretic (Fasihuddin and Hasmah, 2001). Although no ethnomedicinal use was recorded for *P. microtus*, the phytochemical investigation on the species showed the major constituents to be alkaloids of the aporphinoid type (Lee *et al*., 1997). Meanwhile, the *Kopsia* species was not traditionally used to treat fever but it came into use as a treatment for ulcers and syphilitic sores (Mat Saleh and Latif, 2002). *K. dasyrachis* has been found to be rich in indole and monoterpene alkaloids (Kam *et al*., 1999a, b). *Litsea,* belonging to the family of Lauraceae, is also known to yield aporphine alkaloids (Borthakur and Rastogi *et al*., 1979; Uprety *et al*., 1972; Tewari *et al*., 1972).

Apart from alkaloids, it is also possible that the antiplasmodial activity is exerted by the other components in the bioactive plants. In particular, *diterpenoids* is known to co-occur with alkaloids in several *Polyalthia* species (Hao *et al.*, 1995; Hara *et al*., 1995; Ma *et al*., 1994; Kijjoa *et al.*, 1993) while *Neouvaria acuminatissima* has also been reported to elaborate labdane diterpenoids although it is not known whether the alkaloids is also present in the species (Lee *et al.*, 1995). Similarly, an Annonaceae, *Neouvaria* is a chemically understudied genus of this family. It is of interest to examine the class of alkaloids and other chemical constituents present in this particular Annonaceae. The isolation of the antiplasmodial components must be carried out for identification and bioactivity evaluation.

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