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A preliminary screening of potentially antimalarial plants against *Plasmodium falciparum in vitro*

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Abstract. *Plasmodium* is a blood protozoan parasite that is responsible for malaria. To date, *Plasmodium falciparum* has shown multi-drug resistance, particularly in Thailand, Myanmar and Malaysia. The aim of the study is to screen the plant extracts that can effectively inhibit *P. falciparum* 3D7, a common lab strain malaria parasite. Nine plants were collected and processed through maceration using hexane, chloroform and ethanol, resulting in 24 crude plant extracts. Of these, extracts from *Artabotrys crassifolius*, *Pericampylus glacus* and *Leuconotis eugeniifolia* showed promising antiplasmodial activities at IC_{50} of 15.32 to 39.75 µg/mL in a modified schizont maturation assay. Further studies are warranted to explore its efficacies and lead compounds of these three plant extracts for the development of antiplasmodial drugs.

INTRODUCTION

Malaria is one of the most devastating diseases to affect mankind. Despite many advances in malaria biology and therapeutics, malaria still affects many countries in terms of morbidity and mortality. In 2012 alone, an estimated 207 million cases and 627 000 deaths due to malaria have occurred (WHO 2013). Forefront in the battle against malaria is the artemisinin-based combination therapies (ACTs).

Coming from the Chinese traditional medicinal plant, qinghaosu or *Artemisia annua*, artemisinin has exhibited potent antimalarial properties, acting on all developmental stages of the parasite (White 2008). Together with a slower eliminated antimalarial drug (White 2008), artemisinin and its derivatives constitute the backbone of an ACT. The use of ACT has resulted in substantial reduction of both morbidity and mortality associated with malaria (Bhattarai *et al.*, 2007). Currently, there are five ACTs recommended by WHO, such as artemether-lumenfantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate- sulfadoxine pyrimethamine and dihydroartemisinin-piperaquine (WHO 2013).

However, a recent worrying development has threatened to undermine ACTs as the frontline of malaria treatment. Like its predecessors, chloroquine (CQ) (Payne 1987) and sulfadoxine-pyrimethamine (SP) (Roper *et al.*, 2004), there have been reports of emerging artemisinin resistance in

Cambodia (Noedl *et al.*, 2008, Amaratunga *et al.*, 2012), Thailand (Phyo *et al.*, 2012) and Myanmar (Kyaw *et al.*, 2013). These countries, along with Laos and the province of Yunnan, China, constitute the Great Mekong Subregion (GMS). GMS, unfortunately, happened to be also the birthplace of the CQ (Payne 1987) and SP (Roper et al. 2004) drug-resistant strains of malaria, which has subsequently spread to other countries (Klein 2013).

With reports of emerging artemisinin resistance in Cambodia (Noedl *et al.*, 2008, Amaratunga, *et al.*, 2012), Thailand (Phyo *et al.*, 2012) and Myanmar (Kyaw *et al.*, 2013), novel antimalarials are needed to be discovered.

Hence, 25 crude extracts from nine plants, that were traditionally known to treat fever and assuage inflammatory conditions, which are *Leucaena leucocephala* (Ipil ipil), *Annona squamosa* (Atis), *Artemisia vulgari* (Damong maria), *Leuconotis eugeniifolia* (Suatot), *Sandoricum koetjape* (Santol), *Pericampylus glacus* (Kelempenang), *Artabotrys crassifolius* (Akar mempisang), *Diospyros wallichii* (Kaya baleh) and *Aqualaria sinensi* (Agarwood), respectively, were screened for their anti-plasmodial effect on the growth of *Plasmodium falciparum in vitro* in order to uncover novel compounds that will be able to contribute towards treatment of malaria.

MATERIALS AND METHODS

Plant materials

The plant leaves, barks and/or fruits were collected and identified, prior to being deposited at the University of Malaya. *Leucaena leucocephala* (Lam.) de Wit (Voucher no V1122), *Annona squamosa* L. (Voucher no V1123), *Sandoricum koetjape* (Burm. f.) Merr. (Voucher no V1124) and *Artemisia vulgaris* L. (Voucher no V1125) were collected from Tanay, Rizal, Philippines. In Perak, Malaysia, *Leuconotis eugeniifolia* (Wall. ex G. Don) A. DC. (Voucher no V1126), *Pericampylus glaucus* (Lam.) Merr. (Voucher no V1127) and *Artabotrys crassifolius* Hook. f. & Thomson. (Voucher no V1128), *Diospyros wallichii* King & Gamble (Voucher no V1129) and *Aquilaria sinensis* (Lour.) Spreng (Voucher no V1130) were collected. As shown in Figure 1, the leaves and/or barks and/or fruits from the plants were completely dried before reduced into a fine powder by maceration. The powder was then extracted with methanol for three days and the crude extracts were further fractionated with hexane, chloroform and finally ethanol to allow the extraction of non-polar, mid-polar and polar compounds (Figure 1), respectively. Liquid extracts were evaporated *in vacuo* and dry extract were kept at -20°C until *in vitro* testing.

In vitro culture

Plasmodium falciparum cultures were maintained using methods modified from those described by a previous study (Trager & Jensen 1976). In brief, parasites were grown in human erythrocytes (group A positive) in Malaria Complete Medium (MCM) consisting of RPMI 1640 fortified with 2 mmol/ L glutamine (Thermo Fisher Scientific, USA), glucose (10 mmol/L, Thermo Fisher Scientific, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (25 mmol/L, Thermo Fisher Scientific, USA), sodium bicarbonate (32 mmol/L, Thermo Fisher Scientific, USA) and albumax II (0.5%, w/v, Life Technologies, USA). Cultures were incubated at 37°C in sealed T25 or T75 flasks (SPL Life Sciences, Korea) at 5% hematocrit and flushed with a gas mix of 5% O_2, 5% CO_2, and 90% N_2 (Well Solutions Sdn Bhd, Malaysia). The *P. falciparum* line used in this study was the 3D7, tested negative for mycoplasma with a PCR detection kit (Minerva BioLabs, Germany).

Antiplasmodial activity

Twenty-four extracts of selected plants were screened for potential antiplasmodial activity in triplicate in 96-well microtitre plates. A modified standard schizont maturation assay was used to determine antiplasmodial activity. Briefly, The plant extracts were dissolved with 100% DMSO (Sigma-Aldrich, USA) before being diluted with MCM to achieve concentrations of 100, 10, 1, 0.1µg/mL. Chloroquine (Sigma-Aldrich, USA)

was also prepared and concentrations ranging from 40µg/mL to 6.25ng/mL were used. Final concentration of DMSO in the assays were at 0.08% and was found to have no effect on untreated infected red blood cells. Asynchronised parasites were used in the study, with starting parasitaemias at 2%, obtained by diluting with freshly washed RBCs. These were also mixed with MCM to achieve a hematocrit of 5%. Cultures were then incubated under similar conditions as the *P. falciparum* culture for 48 h in a CO_2 incubator (Thermo Fisher Scientific, USA). After incubation, contents of the wells were harvested and stained for 45 min in a 10% Giemsa solution (Sigma-Aldrich, USA), in accordance to manufacturer's specifications. Parasitised red blood cells were counted against the total number of at least 1,000 red blood cells. Parasitaemias at the end of the assays normally ranged between 3-7% parasitaemias. IC_{50} values, indicating the concentration of the extract required to obtain 50% inhibition of parasite growth, were calculated by non-linear regression analysis via GraphPad Prism (GraphPad, USA).

Chemical injury to erythrocytes

To ascertain whether the antiplasmodial activity of the plant extracts were due to its toxicity to red blood cells, experiments of similar nature as the previous section were performed. Instead of parasitised red blood cells, fresh red blood cells were incubated together with the plant extracts. Both DMSO-treated and non DMSO-treated controls were included, in order to rule out possibility of the solvent being toxic to the red blood cells, on its own. At the end of the experiment, red blood cells were harvested, stained and observed under light microscopy, at 1000x magnification. Morphology of the red blood cells treated by the plant extracts were then compared to the negative controls.

RESULTS AND DISCUSSION

In this preliminary study, 24 different crude extracts from nine plant species, were examined for their capacity to inhibit plasmodial growth. Antiplasmodial activity of extracts was defined in accordance to WHO guidelines, with highly active extracts having an IC50 of less than 5µg/mL, promising activity at 5–15 µg/mL, followed by moderate activity at 15–50µg/mL and lastly, inactivity at >50 µg/mL (Lusakibanza *et al.*, 2010). In table 1, 8 crude plant extracts displayed potential anti-plasmodial activity (IC_{50} < 50µg/mL), which are BE (*Leuconotis eugeniifolia*, ethanol fraction from bark), BH (*Leuconotis eugeniifolia*, hexane fraction from bark), F1a (*Pericampylus glaucus*, hexane fraction from bark), F2a (*Pericampylus glaucus*, chloroform fraction from bark), F2b (*Pericampylus glaucus*, chloroform fraction from leaf), F4 (*Diospyros wallichii*, hexane fraction from fruit), KK1 (*Artabotrys crassifolius*, hexane fraction from leaf) and KK4 (*Artabotrys crassifolius*, hexane fraction from bark). Other plant extracts that exhibited IC_{50} of more than 50µg/mL, considered as having low activity were LC (*Leuconotis eugeniifolia*, chloroform fraction from leaf), LE (*Leuconotis eugeniifolia*, ethanol fraction from leaf), BC (*Leuconotis eugeniifolia*, chloroform fraction from bark), F3a (*Pericampylus glaucus*, ethanol fraction from bark), F5 (*Aquilaria sinensis*, hexane fraction from leaf) and KK3 (*Artabotrys crassifolius*, ethanol fraction from leaf). No reduction of parasitaemias were observed by the other 11 plant extracts, hence the absence of IC_{50} values in Table 1.

Like any other early drug discovery programmes, the safeness of the plant extracts used is crucial (Hughes *et al.*, 2011). Hence, as a follow up to the antiplasmodial activity study, the effect of the crude plant extracts on the morphology of red blood cells was examined (Figure 1) and listed in Table 1. These were done in comparison to the DMSO control and non-treated control (Figure 1). Out of the 8 crude plant extracts examined, only 3 of the extracts (BE, KK4 and F1a) have no effect on red blood cell membrane intergrity as no lysis was apparent.

Artabotrys genus are known to be mainly used for both medicinal and non-medicinal purposes, which have been reviewed extensively (Tan & Wiart 2014).

Table 1. List of the 24 crude extracts from 9 plants, its IC50 values and effect on RBC morphology

Scientific name	Plant Part	Solvent	Designation	IC 50 (µg/ml)[a]	Effect on RBC morphology
Leuconotis eugeniifolia (Wall. ex G. Don) A. DC. (Voucher no V1126)	Leaf	Hexane	LH	NE	NE
	Leaf	Chloroform	LC	501.2	NE
	Leaf	Ethanol	LE	71.9	NE
	Bark	Hexane	BH	14.0	Lysis
	Bark	Chloroform	BC	98.97	NE
	Bark	Ethanol	BE	15.32	NE
Pericampylus glaucus (Lam.) Merr. (Voucher no V1127)	Leaf	Hexane	F1b	NE	NE
	Leaf	Chloroform	F2b	1.9	Lysis
	Leaf	Ethanol	F3b	NE	NE
	Bark	Hexane	F1a	39.75	NE
	Bark	Chloroform	F2a	17.88	Lysis
	Bark	Ethanol	F3a	98.85	Lysis
Diospyros wallichii King & Gamble (Voucher no V1129)	Fruits	Hexane	F4	1.6	Lysis
Aquilaria sinensis (Lour.) Spreng (Voucher no V1130)	Fruits	Hexane	F5	81.44	NE
Artabotrys crassifolius Hook. f. & Thomson. (Voucher no V1128)	Leaf	Hexane	KK1	14.45	Lysis
	Leaf	Chloroform	KK2	NE	NE
	Leaf	Ethanol	KK3	104.0	NE
	Bark	Hexane	KK4	29.40	NE
	Bark	Chloroform	KK5	NE	NE
	Bark	Ethanol	KK6	NE	NE
Annona squamosa L. (Voucher no V1123)	Leaf	Ethanol	Atis	NE	NE
Artemisia vulgaris L. (Voucher no V1125)	Leaf	Ethanol	Damong	NE	NE
Leucaena leucocephala (Lam.) de Wit (Voucher no V1122)	Leaf	Ethanol	IPIL	NE	NE
Sandoricum koetjape (Burm. f.) Merr. (Voucher no V1124)	Leaf	Ethanol	Santol	NE	NE
Chloroquine				3.9 ng/mL	NE

[a]Concentration required to induce 50% reduction of parasitemia after 48 h. IC50 >50µg/mL is considered to be inactive. NE, no effect.

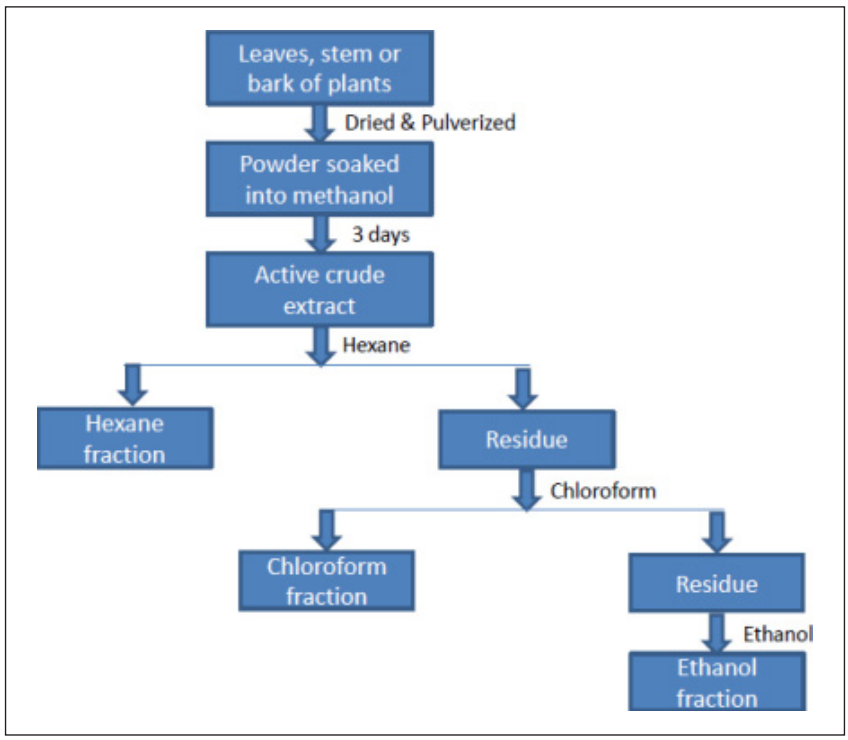

Figure 1. Flow chart of generating the plant extract

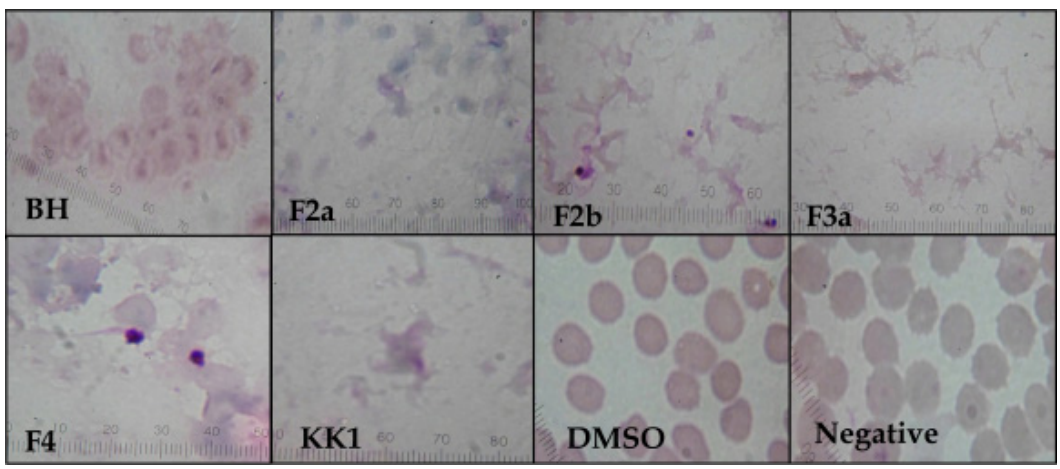

Figure 2. Effect of 8 plant extracts (BH, F2a, F2b, F3a, F4, KK1) at 1µg/mL on the morphology of the red blood cells after 48 h of incubation. Rulers indicate length in microns. BH- Hexane fraction from the bark of *Leuconotis eugeniifolia*, F2a- Chloroform fraction from the bark of *Pericampylus glaucus*, F2b- Chloroform fraction from the leaf of *Pericampylus glaucus*, F3a- Ethanol fraction from the bark of *Pericampylus glaucus*, KK1- Hexane fraction from the leaf of *Artabotrys crassifolius*

Interestingly, the *Artabotrys* genus have been known to harbour antimalarial properties, especially for *A. hainanensis* (Han *et al.*, 2005), *A. hexapetalus*, *A. monteiroae*, *A. oblanceolatus*, *A. odoratissimus*, *A. pilosus*, and *A. uncinatus* (Tan & Wiart 2014). In particular to *A. uncinatus*, also known as Yingzhao, there has been extensive efforts in utilising its modified active ingredient, the arteflene as an oral

antimalarial in clinical trials in the 1990s (Salako *et al.*, 1994, Somo-Moyou *et al.*, 1994). However, due to its lack of efficacy compared to mefloquine (Radloff *et al.*, 1996) as well as safety issues (Wells, 2011), this was abandoned.

On the other hand, only a handful of studies have been done on *Pericampylus glaucus* or xi yuan teng (Yan *et al.*, 2008). Most notably, its alkaloids, periglaucines in its ethanolic form, have been demonstrated to have antiviral effects against HIV and HBV (Yan *et al.*, 2008). Here, we show for the first time, a potential use of *Pericampylus glaucus* as a potential antimalarial.

The *Leuconotis* genus, synonym to *Melodinus* genus is commonly found in tropics and constitutes a part of the dogbane family, Apocynaceae (Endress & Bruyns 2000). Of the species, *Melodinus yunnanensis* thus far, have been the most studied, particularly on its alkaloids (Cai *et al.*, 2012), biosynthesis of the compound (Feldman & Antoline 2013) and the study of its alkaloid crystal structure (Wang *et al.*, 2013). It has only been mentioned to possess antimalarial (Wang *et al.*, 2013) and anticancer (Liu *et al.*, 2013) properties, as well as treating both meningitis and rheumatic heart disease (Goldberg & Stoltz 2011).

Not surprisingly, these crude plant extracts were much inferior to chloroquine in their capacity to inhibit *plasmodial* growth (Table 1), given the difference between crude extracts and active compounds. Chloroquine, in this study was used to validate the 3D7 malaria parasites with its IC_{50} value of about 3.95 ng/mL, which is similar to those observed in another study (Muganga *et al.*, 2010).

In our study, the ethanol fraction of *Artemisia vulgaris* did not show any effect on 3D7 malaria parasites. However, this is a contradiction to recent studies in Sri Lanka on the same fraction from *Artemisia vulgaris*, in treating murine malaria (Bamunuarachchi *et al.*, 2013, 2014). This may be due to the plants being harvested from different geographical locations. It has been described that different geographical regions and seasons could lead to changes in the levels of artemisinin in *Artemisia annua* (Wallaart *et al.*, 2000). Further improvements with the crude extract could be done as artemisinin was described to be efficiently extracted with hexane or petroleum ether from the leaves of the plant (Van Geldre *et al.*, 1997). Perhaps further studies with different extracts from the same plant may shed light on improving artemisinin yield as well as to reconcile the differences between the two conflicting studies.

While IC_{50} of our more promising plant extracts are considered only promising to moderate accordingly to WHO guidelines, this may be due to our methods being different to other similar studies (Lusakibanza *et al.*, 2010), in which plasmodial Lactate dehydrogenase (pLDH) activity was measured. In comparison to the microscopic method that we employed, it may be that the pLDH activity measurements were more sensitive. Other methods to measure plasmodial growth with greater sensitivity and specificity exist, such as radioisotope incorporation assays (Golenser *et al.*, 1981), SYBR Green 1 fluorescent based assays (Quashie *et al.*, 2013) and ELISAs for quantifying plasmodial proteins, such as pLDH and plasmodial histidine rich protein 2 (Noedl *et al.*, 2002). These methods are currently being adapted to be utilised later in our future work.

Not to mention, there is a possibility of the different phytochemicals in the crude extracts interfering with the antiplasmodial activity. As part of our ongoing efforts in isolating and determining the phytochemical profiles of these plant extracts, we must also consider the possibility of synergism and other interactions by the different components, in respect of the plant extract's antiplasmodial activity (Deharo & Ginsburg 2011, Rasoanaivo *et al.*, 2011).

To conclude, in this preliminary study, we show that Artabotrys *crassifolius*, *Leuconotis eugeniifolia* and *Pericampylus glaucus* possess potential antimalarial activity. While some parts of the plants may exhibit toxicity to red blood cells, it is still pertinent and feasible to identify the antimalarial compound, considering the dire situation of the potential rise of artemisinin

resistance in GMS. Future work would also involve testing on CQ resistant malaria parasite strains as well as human cell lines.

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