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 IC₅₀ 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 vulgaris (Damong maria), Leuconotis eugeniifolia (Suatot), Sandoricum koetjape (Santol), Pericampylus glaucus (Kelempenang), Artabotrys crassifolius (Akar mempisang), Diospyros wallichii (Kaya baleh) and Aquilaria sinensis (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 -20ºC 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 37ºC in sealed T25 or T75 flasks (SPL Life Sciences, Korea) at 5% hematocrit and flushed with a gas mix of 5% O2, 5% CO2, and 90% N2 (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 antimalarial activity in triplicate in 96-well microtitre plates. A modified standard schizont maturation assay was used to determine antimalarial 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 CO₂ 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. IC₅₀ 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 (IC₅₀ < 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 wollichii, hexane fraction from fruit), KK1 (Artabotrys crassifolius, hexane fraction from leaf) and KK4 (Artabotrys crassifolius, hexane fraction from bark). Other plant extracts that exhibited IC₅₀ 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 IC₅₀ 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 integrity 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

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

\(^a\)Concentration required to induce 50% reduction of parasitemia after 48 h. IC50 >50µg/mL is considered to be inactive. NE, no effect.
Interestingly, the *Artabotrys* genus have been known to harbour antimalarial properties, especially for *A. hainanensis* (Han et al., 2005), *A. hexapetalus*, *A. monteiroae*, *A. oblancoelatus*, *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 with its IC$_{50}$ value of about 3.95 ng/mL, which is similar to those observed in another study (Muganga et al., 2010).

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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