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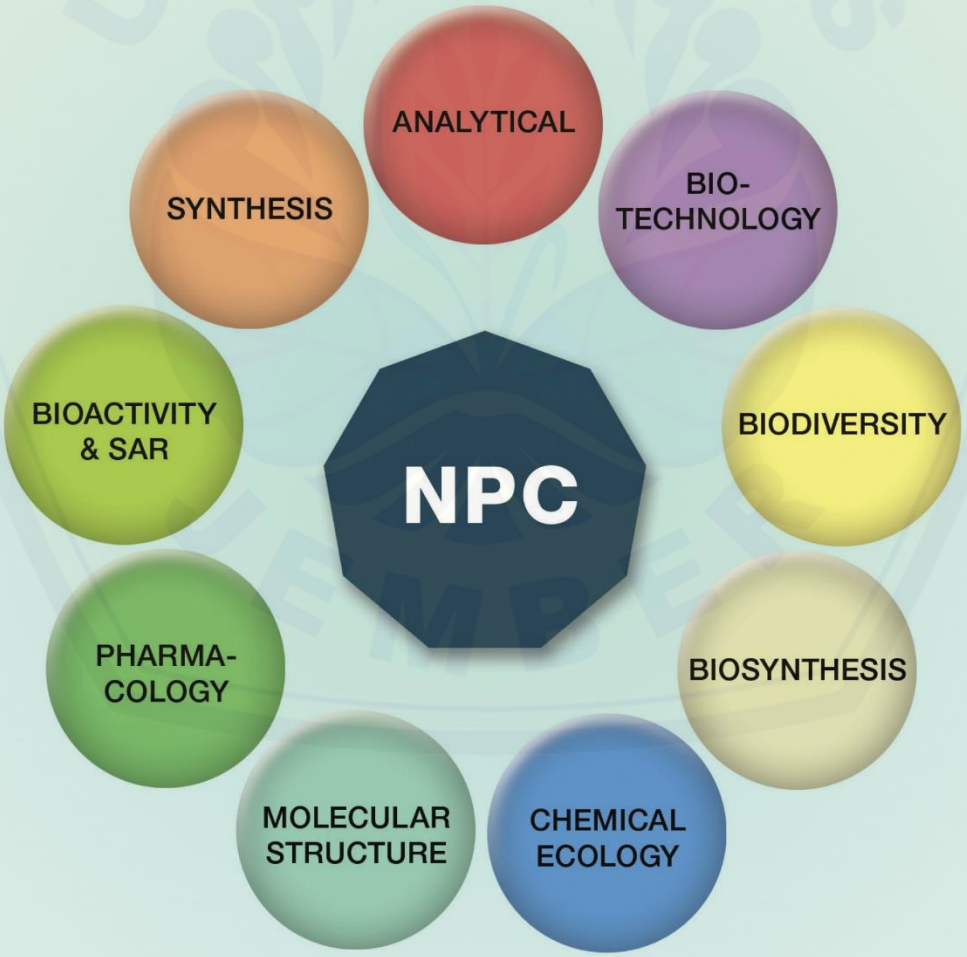

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Isolation of Antimalarial Agents From Indonesian Medicinal Plants: *Swietenia mahagoni* and *Pluchea indica*

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Abstract

Malaria is a neglected tropical disease that still demands serious efforts to tackle successfully, including the need for new antimalarial lead compounds to combat drug-resistant *Plasmodium*. Intensive phytochemical and pharmacological investigation into the Indonesian medicinal plants *Swietenia mahagoni* and *Pluchea indica* successfully revealed 5 constituents. Antimalarial bioassays indicated 34,5-tri-*O*-caffeoylquinic acid (**4**) to be the most active against *Plasmodium falciparum* 3D7 and Dd2 strains with IC_{50} values of 8.2 and 8.8 μM, respectively. No cytotoxicity was observed against Human Embryonic Kidney cells at a concentration of 40 μM.

Keywords

Indonesian medicinal plant, antimalaria, *Swietenia mahagoni*, *Pluchea indica*

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Introduction

Malaria is a serious health burden with the infective agent, *Plasmodium* species, transmitted by the mosquito, *Anopheles* sp. Five *Plasmodium* species, *P falciparum*, *P vivax, P malariae, P ovale*, and *P knowlesi*, are responsible for 229 million malarial cases globally, resulting in 409 000 deaths in 2019.[1] South East Asia is reported to contribute ~3% of global cases (5.6 million cases),[1] with Indonesia being one of the malaria-endemic countries in this region. The Indonesian authorities reported significant progress in controlling malaria in 2018, with more than 50% of districts in the archipelago claimed to be malaria-free.[2] Antimalarial drug-resistant *Plasmodium* has challenged global efforts targeting this disease, with no drug currently able to completely eradicate it. Several drugs, including proguanil and mefloquine, were reported to be ineffective within 5 years of their release onto the market.[3] Moreover, the discovery of *Plasmodium* resistance, or increased tolerance, to dihydroartemisinic piperaquine and artesunate-mefloquine combination therapies in Cambodia, Laos, and Thailand exacerbated the situation.[4] Chloroquine, sulfadoxine-pyrimethamine, and quinine resistant malaria have also been reported in the Indonesian archipelago.[5]

In the Global Technical Strategy for Malaria 2016 to 2030, the WHO constructed 3 pillars and 2 supporting elements in order to accelerate malaria eradication. The pillars involve universal access to malaria prevention, diagnosis and treatment, elimination and attainment of malaria-free status, and transformation of malaria surveillance into intervention. This strategic framework is supported by strengthening the enabling environment and harnessing innovation and expanding research[6] for the discovery of new antimalarial agents. As artemisinin was discovered from medicinal plants, natural products remain a major source in the search for new antimalarial agents.[7] Indonesian medicinal plants have been used by the indigenous people to treat an array of diseases, including malarial fever. Research surveys conducted by the Ministry of Health of the Republic

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of Indonesia with 12 226 participants in malaria-endemic regions reported that 1 in 5 participants used traditional medication to treat malarial fever.[8] The archipelagic country of Indonesia is covered by the second largest biodiversity in the world, including 6000 recorded medicinal plants.[9] Previous notable antimalarial discoveries from Indonesia include treatments arising from ethnopharmacology field trips from islands with malaria-endemic cases.[10] These studies provide foundations for the discovery of new antimalarial agents from Indonesian medicinal plants. The Indonesian Ministry of Health continues to support bioprospecting programs that investigate traditional medicinal plants—these explorations have successfully recorded more than 2700 medicinal plants and stored their voucher samples throughout the archipelago for further investigation.[11–13] These samples include the medicinal plants used in treating malarial fever, such as a decoction of *Lansium domesticum* Corr leaves.

Results and Discussion

Swietenia mahagoni is a woody tree distributed across the archipelago of Indonesia. To the indigenous people of the country, the mahagoni seeds (Figure 1) have been traditionally used to treat numerous ailments including malaria, diabetes, diarrhea, rheumatism, and hypertension.[14] Knowledge of the phytochemistry of the mahagoni seeds and its antimalarial potency is limited.[14] The perennial shrub, *Pluchea indica* is well-known to the people of Sumatra, Java, and Sulawesi Islands both as an edible vegetable (young leaves) and as a medicine. The leaves were traditionally decocted and used to alleviate cough, sweat odor, and *Plasmodia*-induced fever.[15] Reported in this current study, for the first time, is the isolation of the constituents of *S mahagoni* seeds and *P indica* leaves and the antimalarial evaluations of single isolates.

Medicinal plant bioprospecting in the search for new antimalarial agents involves the use of several *Plasmodium* strains for screening. This includes the use of *P falciparum* and Dd2 parasite strains. The 3D7 strain was homologous to the NF54 strain and it was suggested to have originated from Africa in which this parasite strain is sensitive to a standard drug, chloroquine.[16,17] Compared to the 3D7 strain, the origins of the Dd2 strain were geographically apart, in Indochina. The Dd2 strain is considered to be resistant to chloroquine.[17]

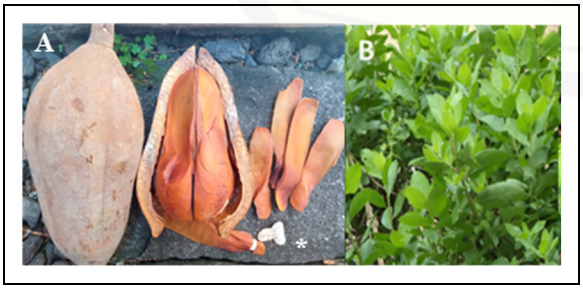

Figure 1. *S mahagoni* fruit showing cleaned seeds* (A); *P indica* herbs showing growing leaves (B).

Phytochemical study of *S mahagoni* and *P indica* led to the successful isolation of 5 compounds (Figure 2). The major component of the seeds of *S mahagoni* was obtained as white amorphous crystals. Intensive 1D and 2D NMR spectral analysis suggested the molecular structure as a typical limonoid, swietenolide (**1**), commonly present in the Rutaceae and Meliaceae plant families.[18] When tested for antimalarial activity, compound **1** was inactive against *P falciparum* 3D7 and showed only 42% inhibition of *P falciparum* Dd2 at a concentration of 40 μM. In addition, and of significance, this compound showed no cytotoxicity against Human Embryonic Kidney cells (HEK293) at a concentration of 40 μM.

Also isolated from *P indica* were quercetin (**2**), 4,5-di-*O*-caffeoylquinic acid (**3**), 34,5-tri-*O*-caffeoylquinic acid (**4**), and 1,3,4,5-tetra-*O*-caffeoylquinic acid (**5**). These phenolic compounds were previously reported from *P indica*, but with no information regarding their activity as either antimicrobial or antimalarial agents.[19–21] This medicinal plant is also used in malarial therapy, and the crude methanol extract was tested against *P falciparum* 3D7 and showed significant activity with an IC_{50} value of 4.12 μg/mL. Compound **2** was previously reported to lack antimalarial properties, and so was not tested again. However, compounds **3**, **4**, and **5** were individually tested against *P falciparum* (*Pf*) 3D7 and Dd2 strains. Compounds **3** and **4** possessed comparable moderate anti-plasmodial activities against *Pf* 3D7 with IC_{50} values of 8.9 and 8.2 μM, respectively. Compounds **3** and **4** were also active against the drug-resistant strain, *Pf* Dd2, with IC_{50} values of 13.8 and 8.8 μM, respectively. Compound **5** was less active against both *Pf* 3D7 and *Pf* Dd2, with 93% and 73% inhibition, respectively, at a concentration of 40 μM. While a previous study reported that the free carboxylic acid moiety was unnecessary for anti-plasmodial activity,[22] in our study, caffeoyl saturation at the R1 position contributes to an anti-plasmodial reduction with molecular bulkiness likely detrimental to

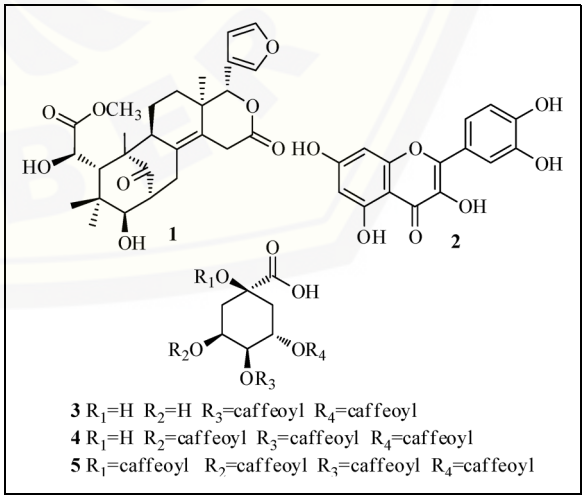

Figure 2. Limonoid, flavonoid, and caffeoylquinic acids isolated from *S mahagoni* seeds and *P indica* leaves.

antimalarial activity. Cytotoxicity screening against HEK293 cells indicated that at the concentrations tested, the compounds were not cytotoxic. This finding provided scientific support to the ethnopharmacological claim that decocted seeds used traditionally in treating malarial fever by the Indigenous people of Indonesia were effective.

Conclusions

Phytochemical and pharmacological study of the 2 Indonesian medicinal plants, *S mahagoni* and *P indica* resulted in the successful isolation of 5 compounds. These were nontoxic against HEK293 cell lines at 40 μM. Compound **4** was the most active compound against both 3D7 and Dd2 *P falciparum* strains.

Experimental

General Experimental Procedure

Short column chromatography utilized silica gel (C_{18} silica gel spherical, 40-75 μm, 70 Å pore size). Analytical HPLC was performed using a Waters HPLC system consisting of a 1525 binary HPLC pump, Waters 2487 dual λ absorbance detector, and a symmetry® C_{18} column (4.9 × 150 mm, 5 μm). Analytical HPLC was also conducted on a Shimadzu LC-203° C-3D (main unit) PDA with a Cooler HPLC system controlled by LabSolutions LC Workstation Ver.5.73 Multi-LC-PDA with a symmetry C_{18} RS column (5 μm, 4.6 × 150 mm). Semipreparative HPLC was conducted on a Waters 150 LC system with a Waters 2489 UV/visible detector and an OBD SunfireTM C_{18} semi-preparative HPLC column (19 × 150 mm, 5 μm). Preparative HPLC was also conducted using a Shimadzu Binary Preparative HPLC comprised of an LC-20AP Preparative HPLC pump, CBM-20A System Controller, SIL-10AP Preparative Auto-sampler, and SPD-M20A UV–vis spectrophotometric detector, controlled by Lab Solutions LC Workstation Ver.5.3 and equipped with a Luna C_{18} RS column (5 μm, 19 × 150 mm, 100 Å) protected with a Security Guard PREP C_{18} (5 μm, 19 × 10 mm, 100 Å). ESIMS were generated from a Shimadzu LC-2010 mass spectrometer in both electrospray positive and negative ionization modes (ESI-MS). NMR spectra were obtained from a Varian Unity Inova-500 MHz NMR spectrometer.

Plant Samples Collection and Extraction

Seeds of *S mahagoni* (L.) Jacq. and leaves of *P indica* (L.) Less. were collected from Karanglor, Klaten, Central Java-Indonesia by Mr Sugiya on April 20, 2019. Sample vouchers were transported to the Drug Discovery and Research Group, Faculty of Pharmacy, University of Jember for botanical determination, and sample vouchers were deposited under the accession codes SM and PI, respectively. Scientific names of the plant species were checked in http://www.theplantlist.org/ accepted names.[23] Samples were separately air-dried and ground into powder.

The powders of *S mahagoni* (116 g) and *P indica* (195 g), were separately soaked in methanol (500 mL), followed by stirring for 24 h prior to filtration. The process was repeated twice. Separately, pooled filtrates were vacuum dried to produce dried crude methanol extracts of *S mahagoni* (4 g) and *P indica* (28 g).

Semipreparative HPLC Separation and Isolation

For the *S mahagoni* sample, the crude methanol extract (4 g) was stirred with methanol (150 mL) and treated with n-hexane (10 × 50 mL) in order to remove the oil (0.83 g), which produced a defatted fraction (1.93 g). This was volume reduced to 5 mL, followed by filtration using an HPLC filter cartridge (0.45 μm). The filtered solution was injected into a Shimadzu preparative HPLC system into 6 blocks of injection with gradient elution from 50% to 40% solvent A within 30 min at a flow-rate of 50 mL/min (solvent A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile) to produce white amorphous crystalline swietenolide **1** (88.2 mg)[24,25] at t_R 10 min.

For the *P indica* sample, a portion of crude extract (10 g) was dissolved in methanol (200 mL) and treated with n-hexane (3 × 100 mL). The methanol solution was separated and passed through a small C_{18} cartridge column (2 × 3 cm). The collected filtrate was reduced to 50 mL and filtered using an HPLC filter (0.45 μm). Each sample was injected into a semipreparative HPLC system with each injection of 5 mL and a gradient eluent from 90% to 60% solvent A within 20 min producing quercetin (**2**) (101.8 mg),[26] 4,5-di-*O*-caffeoylquinic acid (**3**) (26.8 mg),[27] 34,5-tri-*O*-caffeoylquinic acid (**4**) (41.0 mg),[27] and 1,3,4,5-tetra-*O*-caffeoylquinic acid (**5**) (51.0 mg)[27] at t_R 20, 25, 31, and 34 min, respectively.

Bioactivity Testing

The antiplasmodial activity of compounds was determined against *P falciparum* 3D7 (chloroquine-sensitive) and Dd2 (drug-resistant) parasite strains, as previously described.[28]

In brief, *P falciparum* 3D7 (chloroquine-sensitive) and Dd2 (drug-resistant) parasite strains were maintained in RPMI 1640 supplemented with HEPES (25 mM), AB human male serum (5%), Albumax II (2.5 mg/mL), and hypoxanthine (0.37 mM). Following sorbitol synchronization, ring-stage parasites (2% parasitemia) in 0.3% hematocrit were treated with the experimental compounds in 384-well imaging microplates (PerkinElmer). Plates were incubated for 72 h at 37°C, in 90% N_2, 5% CO_2, and 5% O_2, then the parasites were stained with 2-(4-amidinophenyl)-1*H*-indole-6-carboxamidine (DAPI) in permeabilization buffer (PBS, 5 mM EDTA, 0.5 μg/mL DAPI, 0.01% Triton X-100, and 0.001% saponin). Images were acquired with an Opera QEHS microplate confocal imaging system (PerkinElmer) using 20 × water-immersion objective, and 405 nm excitation, and 450/50 nm

emission filters. Images were analyzed using a custom Acapella spot detection script, to quantify DAPI-stained parasites in each well. The compounds were tested in a 22-point concentration-response, ranging from 80 μM to 0.01 nM, whereas dihydroartemisinin (DHA), puromycin, and chloroquine were tested in a 22-point concentration-response range of 40 μM to 0.01 nM (10 μM-0.003 nM for DHA). DMSO (0.4%) and puromycin (5 μM) were used as negative and positive in-plate controls, respectively. The experiment used DMSO at a final concentration of 0.4%.

A preliminary cytotoxicity test was performed against Human Embryonic Kidney (HEK293) cells. As described previously,[29] cells were maintained in DMEM medium supplemented with 10% FBS, and seeded at 2000 cells/well in TC-treated 384-well clear-bottom plates (Greiner), 24 h before the addition of compounds. Upon addition of compounds, plates were incubated for 72 h at 37°C in 5% CO_2. The media was removed from the wells and replaced with an equal volume of 44 μM resazurin. After an additional 5 to 6 h of incubation, the total fluorescence (excitation/emission: 530 nm/595 nm) was recorded using an Envision plate reader (PerkinElmer).

Raw data were normalized using the in-plate positive and negative controls to generate normalized % inhibition data, which were then used to calculate IC_{50} values, through a 4 parameters logistic curve fitting in Prism v 6.0 (GraphPad). The experiments were carried out in 2 biological replicates, each consisting of 2 technical repeats.

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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