Research Article
Antimalarial Activity of Piperine

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Abstract
Malaria remains a public health problem in tropical and subtropical regions. Resistance of Plasmodium falciparum to artemisinins in Southeast Asia is a great concern for disease control and research on discovery and development of new alternative antimalarial drugs is urgently required. In a previous study, the fruit of Piper chaba Hunt. was demonstrated to exhibit promising antimalarial activity against the asexual stage of 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant) P. falciparum clones. The aim of the present study was to further investigate the antimalarial activity of piperine, the major isolated constituent of Piper chaba Hunt. fruits against both P. falciparum clones. The antimalarial activity was determined using SYBR green-I-based assay and morphological change was observed under the light microscope with Giemsa staining. The median IC50 (concentration that inhibits parasite growth by 50%) values of piperine against 3D7 and K1 P. falciparum were 111.5 and 59 𝜇M, respectively. A marked change in parasite morphology was observed within 48 hours of piperine exposure. Results of real-time PCR showed no effect of piperine on modulating the expression of the three genes associated with antimalarial drug resistance in P. falciparum, i.e., pfcrt, pfmdr1, and pfmrp1. Piperine could be a promising candidate for further development as an antimalarial drug based on its antimalarial potency and low risk of resistance development.

1. Introduction
The emergence and spread of artemisinin-resistant Plasmodium falciparum is a major concern for malaria control in malaria-endemic areas particularly in areas bordering Myanmar and Cambodia [1, 2]. In Thailand and most countries in Southeast Asia, a three-day artesunate-mefloquine combination has been used as first-line treatment for acute uncomplicated P. falciparum malaria. Nevertheless, accumulating evidence of delayed parasite clearance with reduced parasite sensitivity has been reported in Cambodia and other countries in Greater Mekong Subregion since 2003 [3–9]. In the light of these reports, research on discovery and development of effective alternative antimalarial candidates are urgently required.

Piperine is a major amide isolated from the fruits of Piper chaba Hunt. This compound has been demonstrated to possess several biological activities including immunomodulatory, antioxidant, antiasthmatic, anticarcino-genic, antipyretic, anti-inflammatory, antiulcer, antidepressive, and antiamoebic activities [10]. Moderate antimalarial activity of the ethanolic extract of Piper chaba Hunt. (fruits) has been demonstrated in our previous study [11] with IC50 (concentration that inhibits parasite growth by 50%) of 5.3 and 4.1 𝜇g/ml in K1 and 3D7 P. falciparum clones, respectively. The aim of the present study was to further investigate the antimalarial activity of piperine, the major isolated constituent of Piper chaba Hunt. against both P. falciparum clones. In addition, its modulatory effects on the expression of the key genes associated with antimalarial drug resistance in P. falciparum, i.e., pfcrt, pfmdr1, and pfmrp1. Piperine could be a promising candidate for further development as an antimalarial drug based on its antimalarial potency and low risk of resistance development.
2. Materials and Methods

2.1. Parasites, Chemicals, and Reagents. Mefloquine, chloroquine, artesunate, and diethylpyrocarbonate (DEPC) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Piperine (98% purity) was purchased from Wako Pure Chemical Co., Ltd. (Tokyo, Japan). Roswell Park Memorial Institute (RPMI) 1640, HEPES, and gentamicin were supplied by Gibco BRL Life Technologies (Grand Island, NY, USA). SYBR Green I was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ethanol was purchased from Labscan Co. Ltd. (Bangkok, Thailand). All reference compounds and piperine were kindly provided by the Liverpool School of Tropical Medicine (Bangkok, Thailand). All reference compounds and piperine were kindly provided by the Liverpool School of Tropical Medicine (Bangkok, Thailand). Roswell Park Memorial Institute (RPMI) 1640, HEPES, and gentamicin were supplied by Gibco BRL Life Technologies (Grand Island, NY, USA). SYBR Green I was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ethanol was purchased from Labscan Co. Ltd. (Bangkok, Thailand). All reference compounds and piperine were kindly provided by the Liverpool School of Tropical Medicine (Bangkok, Thailand).

2.2. In Vitro Antimalarial Activity of Piperine, Chloroquine, Mefloquine, and Artesunate. Two P. falciparum parasite clones, i.e., 3D7 (chloroquine-sensitive) and K1 (chloroquine-resistant), were used in the study. These two parasite clones were kindly provided by the Liverpool School of Tropical Medicine, UK, and School of Public Health, Chulalongkorn University, Thailand, respectively. Both were maintained in continuous culture in O2 human erythrocytes suspended in RPMI culture medium supplemented with 10% human B serum and 25 mM HEPES (at 37°C under 5% CO2, 5% O2, and 90% N2 atmosphere) [12]. The level of parasitemia in the culture was maintained at 2-5%. Synchronization of the parasite culture to ring stage P. falciparum was obtained using 5% sorbitol [13].

For antimalarial activity evaluation, highly synchronous ring stage P. falciparum was used in each assay. An aliquot of parasite inoculum (50 μl) with 2% parasitemia and 1% hematocrit was added to each well of a 96-well microtiter plate. The plate was preseeded with the test compounds at eight final concentrations in the concentration ranges of 2.74-350 μM (piperine), 1.63-200 nM (mefloquine), 3.9-500 nM (chloroquine), and 0.39-50 nM (artesunate). The plates were incubated at 37°C under 5% CO2, 5% O2, and 90% N2 atmosphere for 48 h. The IC50 value (concentration that inhibits cell growth by 50%) was used as an indicator of the antimalarial potency of each compound and was determined by the log-concentration-response curve analysis using CalcuSyn™ software version 1.1 (BioSoft, Cambridge, UK). Data are presented as median (range) values of three independent experiments (triplicate each).

2.3. Morphological Change of Parasite Cells following Exposure to Piperine. Synchronized 3D7 P. falciparum was used in the experiment. The parasite was exposed to piperine at the concentrations of IC20 (111.5 μM) and IC50 (329 μM) at 37°C under 5% CO2, 5% O2, and 90% N2 atmosphere for 48 h. Blood film slides were prepared at the following time points 2, 4, 8, 12, 24, 36, and 48 h and stained with Giemsa (Biotechnical Thai, Bangkok, Thailand). Parasite cell morphology was observed under the light microscope (x100, Olympus, Tokyo, Japan).

2.4. Parasite Gene Expression following Exposure to Piperine

2.4.1. Preparation of RNA. The 3D7 P. falciparum clone was exposed to piperine as previously described in 2.3. Parasite cell pellets were separated from cell suspension following 2, 4, 8, 12, 24, 36, and 50 h of exposure. Total RNA was extracted using Trizol™ reagent according to the manufacturer's protocol (Ambion, California, USA). Briefly, the parasite was lysed with 0.5 ml of Trizol reagent in 500 μl parasite suspension, thoroughly mixed and incubated at 37°C under 5% CO2, 5% O2, and 90% N2 atmosphere for 5 min. Two ml of chloroform was added and parasite cell suspension was incubated at 25°C for 3 min. Cell debris was removed by centrifugation (1,372 xg for 30 min, 4°C) and the supernatant was transferred to a new tube. The parasite RNA was precipitated by adding isopropanol and incubated overnight at 4°C. RNA pellets were separated through centrifugation (21,952 xg for 30 min, 4°C) and washed with 800 μl of cold 75% ethanol for three times. The pellets were dried and reconstituted with 20 μl of DEPC-treated water. The concentration of RNA was determined using NanoDrop Spectrophotometry (NanoDrop Technologies, Wilmington DE, USA). The contaminating DNA was removed by treatment with RQ1 RNase-Free DNase according to the manufacturer's protocol (Promega, Mannheim, Germany).

2.4.2. Preparation of First-Strand cDNA Synthesis. The cDNA of each parasite clone was prepared using SuperScript® VILO™ cDNA Synthesis Kit according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). Briefly, total reaction volume was gently mixed and incubated at 25°C for 10 min, followed by 42°C for 60 min. The reaction was terminated by heating at 85°C for 5 min. The cDNA was synthesized in a total volume of 20 μl containing 5x VILO™ Reaction mix, 10x SuperScript® enzyme mix, DEPC water, and parasite RNA (2 μg).

2.4.3. Quantification of the Expression of pfmdr1, pfmrp1, and pfcr Genes. The expression of pfmdr1, pfmrp1, and pfcr genes of both P. falciparum clones was determined by SYBR Green I real-time PCR (iCycler, Bio-Rad, USA) using the default thermocycler program: denaturation at 95°C for 5 min followed by 40 cycles of amplification at 95°C for 15 sec and annealing at 60°C for 1 min. Individual real-time PCR reaction was carried out in a 25 μl reaction volume in a 96-well plate containing 10 μM each of sense and antisense primer, 12.5 μl of Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen, California, USA), and 50 ng cDNA. Each RT-PCR was performed in duplicate. Ct values (threshold cycle) which is the intersection between an amplification and threshold line was generated to reflect relative measure of the concentration of target in the RT-PCR reaction. The forward and reverse primers used in the experiment are shown in Table 1.

The 2−ΔΔCt method of relative quantification was adapted to estimate gene expression in P. falciparum. The ΔΔCt method was used to calculate MDR-1, CRT, and MRPI gene expression levels relative to control and the housekeeping gene β-actin was used for normalization of MDR-1
Table 1: The forward and reverse primers used for quantification of pfmdr, pfmrp, and pfcr genes expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>pfmdr1</td>
<td>Forward CAAGTGAGTTCAGGAATTGGTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse AGTAAGAGAAGAGACATTGGTCAACAT</td>
</tr>
<tr>
<td>pfmrp</td>
<td>Forward GAAAAGAGATTTGCGATAAAATACCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse CCAAGAATACGTCGACACAC</td>
</tr>
<tr>
<td>pfcr</td>
<td>Forward CCAAGAATACGTCGACACAC</td>
</tr>
<tr>
<td></td>
<td>Reverse AATTTATCTCGGAGCAGTTC</td>
</tr>
<tr>
<td>Pβ-actin</td>
<td>Forward CCAGCTATGTATTGGTCTATC</td>
</tr>
<tr>
<td></td>
<td>Reverse CTCACATCTCAACACAATTC</td>
</tr>
</tbody>
</table>

Table 2: Antimalarial activities expressed as IC_{50} values of piperine, chloroquine, mefloquine, and artesunate in 3D7 and K1 P. falciparum clones. Data are presented as median (range) value of three independent experiments, triplicate each.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Median IC_{50} (range)</th>
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<tbody>
<tr>
<td>Piperine (μM)</td>
<td>(111.5 (103.3-117.0))</td>
</tr>
<tr>
<td>Chloroquine (nM)</td>
<td>3.9 (3.5-4.0)</td>
</tr>
<tr>
<td>Mefloquine (nM)</td>
<td>14.6 (13.8-17.3)</td>
</tr>
<tr>
<td>Artesunate (nM)</td>
<td>2.48 (1.84-3.01)</td>
</tr>
<tr>
<td>K1</td>
<td></td>
</tr>
<tr>
<td>Chloroquine (nM)</td>
<td>115.0 (114.9-135.4)</td>
</tr>
<tr>
<td>Mefloquine (nM)</td>
<td>4.22 (3.93-4.83)</td>
</tr>
<tr>
<td>Artesunate (nM)</td>
<td>1.06 (1.06-1.17)</td>
</tr>
</tbody>
</table>

expression. The delta-delta Ct calculation for the relative quantification of the target gene was as follows:

\[ \Delta \text{Ct}(1) = [\text{Ct} \text{ (target gene)} - \text{Ct} \text{ (β-actin)}] \]

\[ \Delta \text{Ct}(2) = [\text{Ct control for MDR} - 1) - \text{Ct (control for β-actin)}] \]

\[ \Delta \Delta \text{Ct} = \Delta \text{Ct}(1) - \Delta \text{Ct}(2) \]

Relative expression = \(2^{-\Delta \Delta \text{Ct}}\)

Each individual sample was analyzed in triplicate and the Ct of each well was recorded at the end of the reaction.

3. Results

3.1. Antimalarial Activities of Piperine, Chloroquine, Mefloquine, and Artesunate. The median IC_{50} (range) values of piperine, chloroquine, mefloquine, and artesunate for 3D7 and K1 P. falciparum are summarized in Table 2 and the concentration-response curves of each compound for both P. falciparum clones are shown in Figures 1(a)-1(h).

3.2. Morphological Change of Parasite following Exposure to Piperine. The time- and stage-specific antimalarial actions of piperine were investigated using 3D7 P. falciparum clone. The morphological changes of 3D7 P. falciparum following exposure to piperine at 111.5 μM (IC_{50}) and 329 μM (IC_{90}) were observed compared with untreated control parasite during the period of 0 to 48 hours. The change started from 8 hours but the effect was clearly seen at 12 hours of piperine exposure (Figure 2). The parasite growth was slowed down and the cytoplasm was condensed compared with untreated cells. In addition, some of the surviving parasites showed a slower growth rate. At IC_{90}, almost all parasites died after 8 hours of piperine exposure.

3.3. Expression of pfmdr1, pfmrp, and pfcr Genes following Exposure to Piperine. The expression of pfmdr1, pfmrp, and pfcr of 3D7 P. falciparum following exposure to piperine at the IC_{50} (111.5 μM) and IC_{90} (329 μM) was observed at 2, 4, 8, 12, 24, 36, and 48 hours. No change in the expression of all genes was found in both piperine-treated and untreated control parasites at each exposure time point.

4. Discussion

The IC_{50} of the crude ethanolic extract of Piper chaba Hunt. (fruits) reported in our previous study in K1 and 3D7 P. falciparum clones were 5.3 and 4.1 μg/ml, respectively [11]. The antimalarial potencies of piperine against both chloroquine-sensitive (3D7) and chloroquine-resistant (K1) P. falciparum clones are considered low compared with the standard antimalarial drugs under investigation. Results of the morphological study suggest that the window of activity of piperine is likely to be the late ring to trophozoite stages (8-12 h). Further investigation to elucidate the mechanism of action of piperine at molecular and cellular levels is underway. It was noted however that the potencies of activity of the isolated compound piperine (IC_{50}: 59 and 111.5 μM for K1 and 3D7, respectively) were relatively low compared with the crude ethanolic extract in both clones. This suggests that antimalarial activity of the extract is a result of additive or synergistic interaction of various constituents in the plant extract. The reported isolated compounds from Piper chaba Hunt. include piperine, N-isobutyl amide of octadeca-trans-2-cis-4-dienoic acid [14], isopiperolein B [15], piperchabamide D, dehydropipermolanine, and dehydropopermoline. Moreover, pipernonaline, guineensine, isobutylamide of 11-(3,4-methylenedioxy-phenyl)undeca-2,4,10-trienoic acid, piperchabamides A, piperchabamides B, piperchabamides
Figure 1: Concentration-response curves of 3D7 and K1 P. falciparum clones following exposure to piperine (a, b), chloroquine (c, d), mefloquine (e, f), and artesunate (g, h). Data are presented as the median value of three independent experiments, triplicate each.
C, piperlongumine, retrofractamide B, N-isobutyl-(2E,4E,14Z)-eicosatrienamide, and methylpiperate were also identified [16]. The in vivo antimalarial activity of piperine, when given in combination with curcumin, was reported in Plasmodium chabaudi-infected mouse model. Piperine (oral dosing of 20 mg/kg body weight/day for 15 days) used as an enhancer for improving the bioavailability of curcumin (oral dosing of 300 mg/kg body weight/day for 15 days) showed moderate antimalarial activity. Combining piperine (20 mg/kg body weight/day) with curcumin (300 mg/kg body weight/day) and artemisinin (150 mg/kg body weight/day) for 15 days provided no additional benefit on efficacy improvement [17]. In another in vivo study in AS-3CQ (chloroquine-resistant) strain of P. chabaudi, the combination of piperine (20 mg) with curcumin (250 mg) and chloroquine (2.5 mg) was given for 4 days and followed up for 7 days and was shown to produce additive effect in reducing the parasite load 7 days after treatment.
The combination was effective in reducing parasitemia to 45% in mice infected with chloroquine-resistant AS-3CQ P. chabaudi and to 44% in mice infected with artemisinin-resistant AS-ART P. chabaudi [18]. Altogether, these results suggest low to moderate antimalarial activity of piperine both in vitro and in animal models.

Pfmrp1, pfmdrl, and pfcrf genes of P. falciparum have been reported to be associated with the decrease in clinical efficacy of several antimalarial drugs. Compounds or drugs that modulate the expression of these genes may pose the risk of resistance development in the treatment of P. falciparum. The P. falciparum multidrug resistance protein 1 (pfmrp1) gene that encodes the PfMRP1 protein is a member of the ABC transporter superfamily located on chromosome 1 [19]. Increased expression of pfmrp1 gene has been associated with mefloquine, quinine, and chloroquine resistance. Moreover, this protein has been reported to be involved in the export of folate from malaria parasites into red blood cells [20]. The P. falciparum multidrug resistance gene 1 (pfmdr1) that encodes a P-glycoprotein homologue is also located on the membrane of parasite digestive vacuole, the main target of action of most antimalarial drugs. It is thought to play a role in modulation of the level of antimalarial drug resistance [21] by transporting drugs from parasite cytosol to digestive vacuole. The introduction of gene polymorphisms results in chloroquine and quinine-resistant phenotypes [22]. Finally, the P. falciparum chloroquine resistance transporter (pfcrf) encodes a protein localized on the membrane of parasite digestive vacuole and contains 10 predicted membrane-spanning domains in the erythrocytic stage of P. falciparum [23]. The K76T mutation has been linked to chloroquine resistance in parasite isolates collected worldwide [24]. Based on the results of the present study, no effects of piperine were found on modulating (inducing or inhibiting) the expression of all P. falciparum resistance genes under investigation including pfmrp1, pfmdrl, and pfcrf. This may imply a low risk of resistance development of P. falciparum by piperine.

5. Conclusions
Piperine could be a promising candidate for further development as an antimalarial drug with regard to its antimalarial potency and low tendency to modulate P. falciparum resistance genes and thus resistance development.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure
This article had presented in the 37th Congress on Pharmacology of Thailand 2015.

Conflicts of Interest
The authors have no conflicts of interest related to this study.

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