Antimalarial Activity of Flavonoid Compound Isolated from Leaves of Artocarpus altilis

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ABSTRACT

Introduction: Artocarpus altilis leaves extract has previously been reported as a potential antimalarial drug. Inhibition concentration (IC50) against P. falciparum and effective dose values (ED50) against P. berghei have been reported at 1.32 μg/mL and 0.82 mg/kg, respectively. The aim of this study is to identify the active compound from the ethanol extract of A. altilis leaves against P. falciparum. Materials and Methods: The isolation of the active compound from the ethanol extract of A. altilis were conducted using chromatography methods, and the chemical structure of the isolated compounds was determined based on NMR and MS spectra data. Antimalarial assay was determined using microscopic method against P. falciparum 3D7 and molecular docking studies was performed using Molegro Virtual Docker version 5.5 program. Results: A flavonoid compound, class of dihydrochalcone was finally isolated from A. altilis and identified as 1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3-pentenyl)-2H-1-benzopyran-5-yl]-1-propanone (Compound-1). Antimalarial activity test revealed that the compound strongly inhibited P. falciparum growth, with IC50 value of 1.05 μM. An in silico study to determine the mechanism of action of the compound revealed the existence a 3.BPF receptor that possesses a cysteine protease inhibitor of falcipain-2. Conclusion: Compound-1 were isolated from the leaves of A. altilis is a good candidate of new source in the development of antimalarial drugs. An animal study using this compound is recommended before a clinical trial.

Key words: Artocarpus altilis, Cysteine protease inhibitor, Dihydrochalcones, P. falciparum 3D7.

INTRODUCTION

Malaria is a potentially fatal disease transmitted through the bite of female Anopheles mosquito and affects more than 106 countries worldwide. Mortality rates of malaria have increased over the past 20 years, primarily due to resistance of P. falciparum to antimalarial drugs, especially chloroquine, artemisinin and its derivatives. Efforts are therefore needed to develop new antimalarial as that are effective, safe and have fewer side effects while also being cheap and easy to obtain, one potential source are compounds derived from plants. Flavonoids are secondary metabolites that are widely found in plants, and the antimalarial activity of this group has been widely reported.

Moraceae, a family of flowering plants, consists of 60 genera that include 1,400 species. The largest genus of the Moraceae family is Artocarpus which contains 50 species, 40 of which are in Indonesia. The genus Artocarpus is widely used in traditional medicines. Generally, Artocarpus species are rich in phenolic compounds. The extracts and metabolites of Artocarpus, especially leaves, barks, stems, and fruits, have been shown to be useful bioactive compounds. Several pharmacological studies on Artocarpus have proven its utilization for various diseases such as inflammation, malarial fever, diarrhea, diabetes, and tapeworm infections.

Artocarpus altilis, which in Indonesia has a local name “sukun” referred to as breadfruit, is a tropical plant. The breadfruit tree produces fruit from March to September. The synonyms of A. altilis are A. communis, and A. incises. An ethanol extract of A. altilis leaves actively inhibited the growth of P. falciparum in vitro with IC50 values 1.32 μg/mL and was highly active against P. berghei in vivo with ED50 values 0.82 mg/kg body weight. While stem bark extract from A. altilis showed a very good in vivo activity against P. berghei, and weak in vitro activity against P. falciparum. The previous study showed that another species of genus Artocarpus reported that prenyllflavonoid compounds isolated from A. champeden stem bark extract. A. champeden stem bark extract contains artopacenes A, artocarpone B, artoindonesianin E, heteroflavanone C, artoindonesianin R, heterophyllin, artoindonesianin A-2, cyclohexeterophyllin, and artonin. Heteroflavanone C had the most active inhibition against P. falciparum with IC50 values 1 nmol/L. A prenylated chalcone, isolated from A. champeden stem bark extract, namely morachalcone A, was identified as a antimalarial active marker compound. Leaves and stem bark extract from another species, Artocarpus heterophyllus and Artocarpus camansi, also has been reported to have good antimalarial activity against P. falciparum and P. berghei.
While *A. altilis* leaves extract is efficacious against malarial parasite, their mechanism of action remains to be identified. This study was conducted to search for active antimalarial compounds contained in the leaves extract of *A. altilis*.

**MATERIALS AND METHODS**

**General**

NMR spectra were recorded on a JEOL ECS-400, using CDCl₃ as the solvent. The HPLC system also includes two LC-10AD pumps and a SCL-10A controller. An Agilent RP-18 XDB column 4.6 x 250 mm was eluted with CH₃CN-H₂O (7:3 v/v) at 2 mL/min of flow rate. Vacuum Liquid Chromatography (VLC) on silica gel GF₂₅₄ (Merck, Cat No. 1.07730.0500) and Thin Layer Chromatography (TLC) was carried out on silica gel 60 F₂₅₄ (Merck, Cat No. 1.05751.0001) and RP-18 silica gel plate (Merck, Cat No. 1.15389.0001). The identification of TLC profile was performed using TLC Visualizer (Camag).  

**Plant material**

The fresh leaves of *Artocarpus altilis* were collected from Purwodadi Botanical Garden, East Java, Indonesia in the rainy season, around September-December, and identification by a licensed botanist at Purwodadi Botanical Garden, East Java. A voucher specimen (No: B-107/IPH.06/AP01/II/2020) of this raw material stored at the Herbarium of the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

**Isolation and identification of active compounds**

The ethanol extract was fractionated using vacuum liquid chromatography (VLC) with a gradient of n-hexane and ethyl acetate (100%-0%). Based on thin layer chromatography (TLC) profiles, several fractions with the same profile were combined and yielded 6 fractions. Each fraction was identified with TLC methods and tested for its antimalarial activity. Fraction that contain yellow spot on its TLC profile and showed inhibition against *P. falciparum* was then separated by semi-preparative High Liquid Pressure Chromatography (HPLC) methods. The semi-preparative HPLC system was run on a Shimadzu, LC-06, included two LC-10AD pumps and SCL-10A controller, with an semi preparative column; Agilent Eclipse XDB-C18, 5 µm (9.4 x 250 mm). Fraction were eluted in a methanol:water (8:2, v/v) mixture at flow rate of 1 mL/min, which obtained three isolates.

The active isolate profile was analyzed using TLC and HPLC. TLC was analyzed using a silica gel 60 F₂₅₄ plates, scanned by TLC Visualizer, visualized on UV 254 nm and 366 nm, and sprayed with H₂SO₄:water (1:9, v/v) to highlight the flavonoid compounds. The HPLC analysis system included two LC-10AD pumps and SCL-10A controller, with an analytical column; LiChrospher 100 RP-18 analytical column, 5 µm, 4.6 x 250 mm. Compounds were eluted in a methanol:water (8:2, v/v) mixture at flow rate of 0.7 mL/min. The chemical structure of each isolate was determined using a Nuclear Magnetic Resonances (JEOL ECS 400) with CDCl₃ as the solvent. The spectra were then analyzed using MNova program. Mass spectra were identified by LC system coupled with Agilent Q-TOF/MS-MS for HRESIMS analysis using methanol:water (8:2, v/v), with Agilent Zorbax Extend-C18 column, 1.8 µm, 2.1 x 50 mm.

**In vitro cultivation of Plasmodium falciparum**

*P. falciparum* strain 3D7 was obtained from the Malaria Laboratory at The Centre of Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. The culture was established using the method described by Trager and Jensen with some modifications. Parasite were grown in fresh human erythrocytes (human type O red blood cell) at 5% haematocrit in complete RPMI-1640 medium supplemented with 10% (v/v) human type O-positive serum, 25 mM Hepes buffer, 2 µg/mL hypoxanthine, 2 µg/mL NaHCO₃, and 10 µg/mL gentamycin. The culture was incubated at 37°C in a modified candle jar. Parasitemia was determined by the examination of Giemsa's stained thin blood smears of infected erythrocytes. Human RBCs and serum were received from The Indonesian Red Cross, Surabaya, Indonesia.

**In vitro antimalarial assay**

Antimalarial assay was conducted using giemsa stained slide method. Ten milligrams of tested compound and chloroquine was each diluted in 1 mL DMSO. The sample was then serially diluted in RPMI-1640 medium and prepared in dilution at a concentration of 0.01, 0.1, 1, 10, and 100 µg/mL in a 24 well plate. 500 µL parasite culture (±1% parasitemia, 5% hematoctrit) was added to each well and incubated for 48 hours at 37°C. After incubation, thin blood smears were made and stained using 10% (v/v) Giemsa dye to showed infected red blood cell. The percentage of parasitemia was determined by counting infected erythrocytes per 1000 total erythrocytes under a light microscope. The percentage of growth inhibition was calculated using the following equation:

\[
\text{100\% } \left( \frac{X_e}{X_k} < 100\% \right)
\]

Where Xₑ is the average parasitemia of the experimental group, and Xₖ is the average parasitemia of negative control.

The IC₅₀ values were calculated by applying four parameters logistic regression curve to the dose-response data using GraphPad Prism 7.0 software (GraphPad Co. Ltd., San Diego, CA, USA).

**Criteria of antimalarial activity in vitro**

In line with WHO guidelines and basic criteria for antiparasitic drug discovery activities of extracts were classified into four classes according to their IC₅₀ values: high activity (IC₅₀ ≤5 µg/mL); promising activity (5 µg/mL < IC₅₀ ≤15 µg/mL); moderate activity (15 µg/mL < IC₅₀ ≤50 µg/mL); weak activity (IC₅₀ >50 µg/mL). A pure compound is defined as highly active when its IC₅₀ <1 µg/mL.

**Docking analysis**

The molecular structure of the falcipain-2 enzyme was downloaded from the RCSB protein data bank website (http://www.rcsb.org/pdb/home/home.do). In this study, the falcipain-2 was chosen with the code PDB: 3BP.F. This receptor was chosen because showed strongest interaction with compound-1. The ligand (was prepared by making 2D, and 3D structures of compound-1 using the ChemBioOffice program Ultra 11.0 and its energy was minimized using MMFF94. The ligand with the minimum energy that has been measured is then saved in the form of anmol2 (SYBYL2 (*Mol2)). The docking process of the enzyme falcipain-2 pdb:3BP.F uses a Molegro Virtual Docker Version 5.5 program. The results obtained in the form of the Rerank Score (RS) value, which is the energy needed in the ligand-receptor interaction process, and from these values antimalarial activity can be predicted.

**RESULTS**

Isolation and identification of active compound

An ethanol extract of *A. altilis* was fractionated by the VLC method using the gradient solvent of n-hexane and ethyl acetate (100%-0%) yielded six fractions (fraction 1-6). Identification with TLC method showed that fraction-3 gave the yellow spot that indicated a flavonoid compound. Fraction-3 was further separated by semi-preparative HPLC using acetonitile:water (7:3 v/v) and yielded five isolates (isolate 1-5).
**In vitro antimalarial assay** showed that isolate-2 inhibited *P. falciparum* growth with IC\textsubscript{50} of 0.48 μg/ml. Isolate-2 was analysed by TLC method using silica gel 60 RP-18 F254, mobile phase acetonitrile:water (7:3 v/v), scanned on UV 254 and 366 nm, and sprayed with a specific reagent for flavonoid compound. The results showed that isolate-2 had the yellow spot, typical of a flavonoid group, with RF 0.4. Analysis of isolate-2 with HPLC method (Figure 1), at concentration 0.5 mg/ml, using isocratic solvent acetonitrile: water (7:3 v/v) showed that isolate-2 had a major peak, with retention time at 15.74 minutes. The UV spectrum of the peak showed maximum absorption with λ\textsubscript{max} 248, 275, 300, and 315 nm (Figure 2). The UV spectra of most flavonoids consists of two major absorption, for band II in the range of 240-285 nm and band I in the range of 300-400 nm. In general terms the band II absorption may be considered as having originated from the A-ring benzoyl system and band I from the B-ring cinnamoyl system. The UV spectrum of major peak showed a characteristic absorption peak of the flavonoid compound, class of dihydrochalcone.

Isolate-2 was then identified based on proton and carbon signals in the \textsuperscript{1}H and \textsuperscript{13}C-NMR spectrum, HMBC, HMQC, and COSY. The \textsuperscript{1}H NMR spectrum shows the presence of 2 aromatic rings, ring A and ring B. Ring A structure can be confirmed from the aromatic protons which showed ABX system at δ\textsubscript{H} 6.32 (1 H, d, J = 2.4 Hz, H-3”), δ\textsubscript{H} 6.35 (1 H, dd, J = 8.4 Hz, H-5’), and δ\textsubscript{H} 7.58 (1H, d, J = 9.2 Hz, H-6’). In ring B the aromatic proton signal shows ortho-coupled at δ\textsubscript{H} 6.62 (1 H, d, J = 8.0 Hz, H-6) and δ\textsubscript{H} 6.72 (1 H, d, J = 8.0 Hz, H-5). Singlet methyl signals at δ\textsubscript{H} 1.38 and 2 cis-olefinic proton doublets at δ\textsubscript{H} 6.55 (1 H, d, J = 10.0 Hz, H-1”) and δ\textsubscript{H} 5.64 (1 H, d, J = 10.0 Hz, H-2”), and carbon quartener at δ\textsubscript{C} 78.8 (C-3”) indicates a 2,2-dimethylchromene ring which is substituted in ring B. A chelated hydroxyl group at δ\textsubscript{H} 12.74 (1H, s, 2’-OH), and two hydroxyl signal showed at aromatic ring at δ\textsubscript{H} 7.25 (4’-OH) and δ\textsubscript{H} 5.58 (4-OH). The methylene group signal at δ\textsubscript{H} 3.10 (2H, q, H-α) and δ\textsubscript{H} 2.97 (2H, m, H-β) substituted to C7 shows the correlation between ring A and ring B. The characteristics of chalcone pyrano-O-prenylated are shown by the methylene group signal at δ\textsubscript{H} 3.1 (2H, q, H-alfa) and δ\textsubscript{H} 2.97 (2H, m, H-beta) which have a correlation with carbonyl at δ\textsubscript{C} 203.8. The prenyl group signal at δ\textsubscript{C} 5.08 (1H, t, H-6”), δ\textsubscript{C} 1.66 (3H, s, H-9”) and δ\textsubscript{C} 1.56 (3H, s, H-8”) substituted with C6”. The \textsuperscript{13}C NMR spectrum of isolate-2 shows 25 well-separated signals for 25 carbon atoms, consisting of one conjugated carbonyl carbon at δ\textsubscript{C} 203.8 (C=O), four oxyaryl carbon bond with two aromatic ring at δ\textsubscript{C} 139.6 (C-3), δ\textsubscript{C} 143.2 (C-4), δ\textsubscript{C} 165.3 (C-2’), δ\textsubscript{C} 162.3 (C-4’), one 2,2 dimethylchromen ring at δ\textsubscript{C} 78.7 (C-3”), and one isoprenyl at δ\textsubscript{C} 132.1 (C-7’). By conducting a references study on compounds that have been isolated from *A. altilis*, and comparing the NMR spectrum with the references, it is concluded that isolate-2 (Compound-1) is a flavonoid compound, class of dihydrochalcone.

The molecular structur of Compound-1 has been confirmed it mass spectra which were identified by Agilent LC system coupled with Q-TOF/MS for HRESIMS analysis. HR-MS measurement revealed an ion peak [M+H]\textsuperscript{+} at m/z 409.1998 (calculated 409.210), consistent with molecular formula of C\textsubscript{25}H\textsubscript{30}O\textsubscript{5}.

**Figure 1:** HPLC Chromatogram of compound 1.

**Figure 2:** UV-VIS chromatogram of compound-1.
Compound-1 was identified as 1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3-pentenyl)-2H-1-benzopyran-5-yl]-1-propanone (Figure 3).

Yellow gum, m/z 409.2100, the 1H-NMR spectral data were identical to those in reference: 1H-NMR (CDCl$_3$, 400 MHz) 1.66 (s), 1.56 (s), 1.38 (s), 1.79 (m), 2.09 (m), 2.97 (m), 3.10 (q), 5.08 (t), 5.58 (bs), 5.64 (d, J = 10 Hz), 6.32 (d, J = 2.4 Hz), 6.35 (dd, J = 8.4, 2.4 Hz), 6.55 (d, J = 10 Hz), 6.62 (d, J = 8.0 Hz), 6.72 (d, J = 10.0 Hz), 7.25 (d, J = 9.2 Hz), 7.58 (d, J = 9.2 Hz) and 12.74 (s).

Antimalarial activity

*In vitro* antimalarial test was conducted using a synchronized *P. falciparum* strain 3D7, which is sensitive to chloroquine. This antimalarial activity test was conducted to determine the potential inhibition of *P. falciparum* growth in general, meaning that no inhibition was observed in certain phases. Chloroquine was use as a positive control.

Antimalarial assay of the Compound-1 (Table 1) revealed that this compound inhibited the growth of *P. falciparum* with the IC$_{50}$ value of 0.48 μg/ml (calculated 1.05 μM). The IC$_{50}$ value indicated that this compound possesses a potent antimalarial activity. This evidence suggests for further biochemical assay of the compound to determine the mechanism of action. The fact that the compound was originated from the leave extract also indicate its high availability and sustainability as we do not need to cut the tree source.

**Docking analysis**

To predict the mechanism of action, an *in silico* study was performed using the Molegro Virtual Docking (5.5) program to determine the possible interaction of compounds with the protein target. We evaluated several proteins from Protein Data Base, which have been reported to have an interaction with falcipain (www.rcsb.org). As a result, the isolated compound was predicted to have strong interaction with receptor 3BP, falcipain-2, a protein that can be found in the food vacuole of *P. falciparum* involved in hemoglobin hydrolysis. The *in silico* study was performed by comparing Compound-1 with drug references, chloroquine. The re-rank score of Compound-1 was -71.2844 kcal/mol, while the rerank score of chloroquine was -66.3610 kcal/mol. A lower value of the re-rank score indicates a stronger interaction to a receptor. Hydrogen bond interaction was predicted between falcipain 2 and Compound-1 group with Gln 36* (2 bonds), Asn 173*, and Gly 83. In addition, van der Walls interactions were predicted between falcipain-2 and Compound-1 group with Gln 36, Cys 39, Ser 41, Gly 40, Cys 42, Leu 172, Asn 173, Trp 206, His 174, and Val 152. It seems to contribute to the binding interaction of 3BP and Compound-1 (Figure 4). While, chloroquine revealed hydrogen binding to Gly 83, Gln 36, Cys 42, and His 174, and steric van der Walls to Cys 42, Ser 41, and Gly 82 (Table 2). The interaction is described in the 3D profile (Figure 5).

### Table 1: The IC$_{50}$ value of all samples against *P. falciparum* 3D7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC$_{50}$ (µg/mL)</th>
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<tr>
<td>Compound-1</td>
<td>0.43 ± 0.05</td>
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<tr>
<td>Chloroquine</td>
<td>0.004 ± 0.00</td>
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</tbody>
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Data are reported as Mean ± SD.

### Table 2: Hydrogen bond and van der Walls Interaction of Compound-1 and Chloroquine.

<table>
<thead>
<tr>
<th>Hydrogen Bond</th>
<th>Compound-1</th>
<th>Chloroquine</th>
<th>van der Walls</th>
<th>Chloroquine</th>
</tr>
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<tbody>
<tr>
<td>Gln 36 (two bonds)</td>
<td>Gln 36</td>
<td>Cys 39</td>
<td>Gly 40</td>
<td>Cys 42</td>
</tr>
<tr>
<td>Asn 173</td>
<td>Gly 83</td>
<td>Cys 42</td>
<td>Cys 42</td>
<td>Ser 41</td>
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<tr>
<td>Gly 83</td>
<td>Cys 42</td>
<td>Leu 172</td>
<td>Asn 173</td>
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<td></td>
<td>His 174</td>
<td>Trp 206</td>
<td>Val 152</td>
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Figure 4: Hydrogen bond interaction (dashed blue-line) and Steric-Van der Walls bond interaction (dashed red-line) between Chloroquine (a and b) and Compound-1 on the active site of *P. falciparum* protein 3BPF (c and d).

Figure 5: The 3D profile of Secondary Structure View of active compound-1 (green color) with 3BPF.PDB protein.
Antimarial activity of the dihydrochalcone have been widely reported. 2,4′,6′-tri-hydroxy-4′-methoxy dihydrochalcone (asbogenin) from Piper hispidum and 2,6′-dihydroxy-4′-methoxydihydrochalcone was reported to have potential antimarial activity.17,18 Two dihydrochalcone compounds were isolated from Metrodorea stipularis [1-(5,7-dihydroxy-2,2′-dimethylchroman-6-yl)propan-1-1 and 1-(5,7-dihydroxy-2,2′-dimethylchroman-6-yl)-3-(1,1a-trimethoxy-2,3,4,9-hexahydro-1H-xanthene-7-yl)propan-1] showed significant growth inhibition against parasite 3D7, Dd2, and W2 strains.19 Carrol et al (2008) reported that prenylated dihydrochalcones from Boronina bipinnata inhibited the enzyme Hemoglobinase II in malaria parasites.20 Hemoglobinase II, also known as plasmspein II, is an aspartic protease that is found in the P. falciparum. The enzyme is involved in the digestion of hemoglobin from host cells within the acidic food vacuole of the parasite.21 Based on this evidence, we then performed an in silico study to predict the mechanism of action of compound-1.

We evaluated several proteins from Protein Data Base which reported having interaction with 3.BPF activity of falcipain-2 enzyme, hence disabling the degradation of large fragments into amino acids.

CONCLUSION
1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3-pentenyl)-2H-1-benzopyran-5-yl]-1-propanone were isolated from the leaves of Artocarpus altiss which is a good candidate of new source in the development of antimalarial drugs. The in silico study predicted that the mechanism of action of this compound had a strong interaction with 3BP, falcipain-2 receptor, as a cysteine protease inhibitor. Further study on animal model using this compound is also recommended before a clinical trial.

CONFLICTS OF INTEREST
The authors declare that they have no competing interest.
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GRAPHICAL ABSTRACT

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Mulyadi Tanjung is a lecturer at Faculty of Science and Technology, Universitas Airlangga. He was graduated as Bachelor of Chemistry from Andalas University. He was further obtain his Master and Doctoral degree at Institute of Technology Bandung. His major achievements are in the field of isolation and identification of compounds from endemic plants of Indonesia especially East area of Indonesia.

Tri Widiandani is a lecturer at Faculty of Pharmacy, Universitas Airlangga. She obtained her bachelor degree in Pharmacy at Faculty of Pharmacy. She was further pursued her Specialist of Hospital Pharmacy and Doctoral degree at Faculty of Pharmacy, Universitas Airlangga. Her main courses is Medicinal Chemistry, Physical Chemistry. Her research is focuses on virtual modeling, synthesis of biologically active compounds especially for cancer targeted.

Siswandono is currently head of the Graduate Program in Pharmaceutical Sciences at the Faculty of Pharmacy, Universitas Airlangga. He obtained his bachelor’s degree in Pharmacy at the Faculty of Pharmacy, Universitas Airlangga. He was further pursued his Master’s and Doctoral degree at the Faculty of Pharmacy, Universitas Airlangga. He acquired a Professor with expertise in the fields of medicinal Chemistry. His research is focused on drug design and development from synthetic compound or bioactive natural products especially for anticancer and CNS depressant agents.

Din Syafruddin was trained in medicine and doctoral degree in the parasite cell biology, he is currently a senior research fellow at the Malaria and Vector Resistance Laboratory at the Eijkman Institute for Molecular Biology, and Professor and chairman of the Department of Parasitology, Faculty of Medicine, Hasanuddin University. His research was to elucidate the role of the extrachromosomal DNAs in the assembly of the mitochondrial energy transducing membrane of the malarial parasite. His current research include molecular mechanisms underlying *Plasmodium* resistance to antimalarial drug, antimalarial drug discovery, molecular taxonomy of the mosquito vector and studies on the vector resistance to insecticides.

Achmad Fuad Hafid is currently vice-director of Institute of Tropical Disease, Universitas Airlangga. He is a lecturer at Faculty of Pharmacy, Universitas Airlangga and a researcher as well. His acquired a Professor with expertise in the fields of phytochemistry and bioactive natural product discovery for therapeutic applications.