Antiplasmodial Activity of Isolated Polyphenols from Alectryon serratus Leaves Against 3D7 Plasmodium falciparum

by Aty Widyawaruyanti
Antiplasmodial Activity of Isolated Polyphenols from Alectryon serratus Leaves Against 3D7 Plasmodium falciparum

Uswartan Khasana1–3, Aty WidyaWaruyanti1–4, Achmad Fuad Hadi1–4, Mulyadi Tanjung1

Post Graduate Program, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Department of Pharmacy, Faculty of Medicine, Brawijaya University, Malang, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Institute of Tropical Disease, Universitas Airlangga, Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT

Background: Alectryon serratus was selected from a screening program devoted to search naturally occurring antimalarial compound from plants in Alas Purwo National Park, Banyuwangi, East Java, Indonesia. The previous studies showed that ethanol extract of A. serratus leaves contains some polyphenol compounds. Objective: This study was designed to isolate and investigate antiplasmodial activity of polyphenol compounds. Method: Fractionation: The ethanol extract of A. serratus leaves was fractioned using liquid–liquid fractionation and column chromatography. Isolated compounds were identified using high-performance liquid chromatography, ultraviolet-visible, nuclear magnetic resonance, and compared with references. The isolates were tested in vitro for antimalarial activity against chloroquine-sensitive 3D7 strain of Plasmodium falciparum. Thin blood smears were used to assess the levels of parasitemia and growth inhibition of the isolates. Result: Half maximal inhibitory concentration of gallic acid (1), methyl gallate (2), and kaempferol-3-O-rhamnoside (3) were 0.0722 μM, 0.0238 μM, and 3.466 μM, respectively. Conclusion: The results suggest that gallic acid, methyl gallate, and kaempferol-3-O-rhamnoside isolated from A. serratus leaves have antimalarial activity and are potential to be developed as antimalarial drugs.

Key words: Alectryon serratus, antimalarial, polyphenol

SUMMARY

- The ethanol extract of Alectryon serratus leaves was successively fractionated in CHCl3, EtOAc, and n-butanol. EtOAc fraction was fractionated using column chromatography and purified using preparative thin-layer chromatography (TLC). Isolates were studied for their antimalarial activity on parasites culture of chloroquine-sensitive 3D7 strain of Plasmodium falciparum. Parasitemia percentages, growth percentages, and inhibition percentages of each group were calculated. The half maximal inhibitory concentration (IC50) values that represent the concentration required to inhibit 50% of Plasmodium growth were calculated from a calibration curve using linear regression. The results suggest that isolates have antimalarial activity and are responsible in the antimalarial activity of Alectryon serratus leaves.

Abbreviations Used: S.F: Subfraction, EGCg: Epigallocatechin gallate, EGC: Epigallocatechin

Correspondence: Dr. Aty WidyaWaruyanti, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia. E-mail: atywvw@yahoo.com

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INTRODUCTION

Malaria is a major parasitic infectious disease in many tropical and subtropical regions. Malaria incidence has been increasing since the emergence of drug-resistant Plasmodium falciparum. According to the WHO, as many as 207 million people suffer from malaria, with up to 627,000 deaths each year.10 Drug-resistant P. falciparum malaria is a major killer and becomes one of the most difficult obstacles to combat. Therefore, a development of a new class drug is an urgent matter. Screening plant extract for antimalarial activity is a useful way for discovering new leads.11 As a part of our study for novel antimalarial agents from plants, a screening program was undertaken on plants of Alas Purwo National Park, Banyuwangi, East Java, Indonesia. In our screening, leaves of Alectryon serratus were selected.4 This plant is widely distributed throughout the tropical region of Southeast Asia, and no traditional uses are reported.12 Ethyl acetate fraction of the leaves was found to exhibit an antimalarial activity (IC50:9.45 μg/mL) on chloroquine-sensitive 3D7 strain of P. falciparum.13 TLC profile of ethyl acetate fraction showed polyphenol compounds. In this paper, we report the isolation, structure elucidation, and antimalarial activity of polyphenol compounds.

MATERIALS AND METHODS

Collection of plant material

Leaves of A. serratus were collected from Alas Purwo National Park, Banyuwangi, East Java, Indonesia in August 2014. A voucher specimen was identified at Purwokerto Botanical Garden, Pasuruan, East Java, Indonesia, and a voucher specimen was deposited at the herbarium.

Extraction and fractionation

One kilogram of dried powder of A. serratus leaves was macerated using sonification method in ethanol 80% as the solvent. The extract was this is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

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then removed, and the residue was pressed and dried. The residue was re-crystallized twice with ethanol 80%. The dried extract was suspended in distilled water and fractionated. Liquid fractionation was done successively in CH₂Cl₂, EtOAc, and n-butanol.

**Isolation of polyphenol compounds**

The ethyl acetate fraction (2 g) was separated using a column chromatography method using RP-18 F254 as stationary phase and CH₂CN-MeOH-H₂O (2:1:4 v/v) as the mobile phase to yield 12 subfractions (SFE1-SF12). White powder was obtained as a precipitate from SFE2. The precipitate was filtered using Kariyuma to yield compound 1 (0.2 mg). SFE4 (30 mg) was purified using TLC preparative with CHCl₃-MeOH (9:1 v/v) as mobile phase and silica gel F₂۵۴ as stationary phase to yield SFE4.1 (4 mg) and SFE4.2 (8 mg). SFE4.2 was identified as compound 2. SFE8 (40 mg) was purified using preparative TLC with CHCl₃-MeOH (8:2 v/v) as mobile phase and silica gel F₂۵۴ as stationary phase to yield SFE8.1 (5 mg), SFE8.2 (7 mg), and SFE8.3 (2 mg). SFE8.3 was identified as compound 3.

**Characterization of isolated polyphenols**

TLC profiles of SFE2 were done using RP-18 F₂۵۴ as the stationary phase and CH₂CN-MeOH-H₂O (2:1:4 v/v) as the mobile phase. SFE8.2 and SFE8.3 were identified using TLC with silica gel F₂۵۴ as stationary phase and CHCl₃-MeOH (9:1 v/v) as mobile phase. The spots were detected under ultraviolet (UV) (254 and 366 nm) before spraying using 10% H₂SO₄ in EtOH, and followed by heating the plate at 120°C and then detected under UV 366 nm and visible light. High-performance liquid chromatography (HPLC) chromatogram profile was performed using Shimadzu LC-06, with RP Shimpack column 4.6 mm × 250 mm as stationary phase, and CH₂CN-MeOH (7:3 v/v) as mobile phase with a flow rate of 0.7 ml/min, and was detected using UV detector.

Nuclear magnetic resonance (NMR) spectra were performed using JEOL 400 spectrometer, with tetramethylsilane as an internal standard for ¹H, ¹³C NMR, heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence. Compound 1, 2, and 3 were confirmed by comparing the results of NMR and UV data reported in the literature.¹²⁻¹⁵

**Plasmodium falciparum (3D7 strain) culture and maintenance**

P. falciparum 3D7 strain (chloroquine sensitive) was obtained from the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. Plasmodium parasites were grown and maintained in culture using Trager and Jensen method with some modifications.¹²⁻¹⁵ The parasites were bred in vitro in human type O-positive red blood cells in a complete medium (RPMI 1640 supplemented with 5.96 g HEPES, 0.06 g hypoxanthine, 2.1 g NaHCO₃, 50 μg/ml gentamycin, and 10% human O + serum) in Petri dish with modified candle-jar method. The incubation was done at 37°C. The media was routinely changed daily, and the parasite growth was monitored through Giemsa staining in thin blood smears. The culture to be used for the experiment should be dominated by ring forms. Stock parasite cultures were further diluted with uninfected type O human erythrocytes and complete culture medium to achieve 1% parasitemia and 50% hematocrit. Those final parasite cultures were immediately used for the antimalarial assay.¹²⁻¹⁵

**In vitro antimalarial assay**

A stock solution of 10 μg/ml was prepared from the isolated compounds. Four-fold serial dilution was prepared from each stock solution and yielded five serial concentrations (10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml, and 0.001 μg/ml). To each microplate well, 500 μl diluted extract solution was added into 500 μl of final parasite culture. The plates were then incubated for 48 h at 37°C. Thin blood smears were prepared on labeled slides and air-dried and fixed with methanol. The dried slides were stained using Giemsa and observed under light microscope at 1,000 times magnification, and parasitemia percentage was calculated.

**Statistical analysis of data**

Growth percentage was calculated using formula as follows:

\[
\text{Growth percentage} = \frac{\text{Plasmodium growth in isolated compounds}}{\text{Plasmodium growth in control}} \times 100\%
\]

Inhibition percentage was calculated as follows:

\[
\text{Inhibition percentage} = \frac{\text{Growth percentage of control}}{\text{Growth percentage of isolated compounds}} \times 100\%
\]

Note:

- \(D_{50}\) = Parasitemia percentage of infected red blood cell on day 0
- \(X_u\) = Growth percentage of each isolate
- \(X_c\) = Growth percentage of negative control
- \(IC_{50}\) values that represent the concentration required to inhibit 50% of Plasmodium growth were calculated from a calibration curve by linear analysis using SPSS. \(IC_{50}\) values were expressed as mean value (±standard deviation).

**RESULTS AND DISCUSSION**

TLC profile of SFE2 and SFE4.2 showed the presence of dark fluorescent spots under UV 254 nm and UV 366 nm with Rf 0.75 and Rf 0.6. Dark spots were also detected on SFE2 and SFE4.2 after being sprayed and observed under UV 366 nm. SFE8.3 also showed dark spot under UV 254 and UV 366 nm with Rf 0.48, and yellowish spots after the plate was being sprayed using 10% H₂SO₄ and was detected under UV 366 nm and visible light. Isolation of phenolic compounds was done for SFE2 and SFE4.

HPLC chromatogram of SFE2 showed a single peak with Rₜ 4.3 min with a purity index of 0.98 and UV/Vis λₘₐₓ (MeOH) at 266 nm. SFE4.2 also showed a single peak with Rₜ 4.5 min, purity index 0.97 and UV/Vis λₘₐₓ (MeOH) at 271 nm.

Results of NMR spectra of each isolate are described as follows:

- SFE2
  1. White powder
  2. IC NMR (400 MHz, Methanol-d₄): 7.02 (s).
  3. IC NMR (400 MHz, Methanol-d₄): 115.6 (C), 103.9 (CH), 140.1 (C), 133.5 (C), 164.1 (COOH).
  4. SFE4.2
  1. Pale yellow powder
  2. IC NMR (400 MHz, Methanol-d₄): 7.02 (s), 3.75 (2H, s).
  3. IC NMR (400 MHz, Methanol-d₄): 116.0 (C), 140.4 (C), 133.0 (C), 164.4 (COOH), 46.2 (OCH₃).
  4. SFE8.3
  1. Yellow powder
  2. IC NMR (400 MHz, Acetone-d₆): 7.83 (2H, dd, J = 8.4), 6.99 (2H, dd, J = 8.4), 6.45 (d, J = 2.4 Hz, H), 6.24 (d, J = 2.4 Hz, H), 12.69 (1H, s), 5.51 (1H, d, J = 1.6), 3.30–3.60 (3H, m), 4.16 (1H, m), 1.16 (3H, s, CH₃).
  3. IC NMR (100 MHz, Acetone-d₆): 178.5 (C = O), 115.4 (CH), 130.1 (CH), 98.7 (CH), 95.5 (CH), 170.3, 164.4, 159.7, 163.8,
156.6 (oxaryl group), 71.3, 70.6, 70.5, 70.2 (C-2', C-3', C-4', C-5'),
16.9 (CH3).
Antiplasmodial activity of each isolate concentration was shown in
Figure 1. Maximum inhibition percentage of 96.7%, 80.9%, and
62.8% were obtained for S.F.2, S.F.4.2, and S.F.8.3 at 10 µg/ml
concentration, respectively. Concentration below 10 µg/ml
exhibited lower inhibition percentage. The IC50 values of each
isolates were calculated and found to be 0.013, 0.0025, and 1.495 µg/ml
[Table 1].
S. F.2 was identified as Gallic acid (1) that was isolated as a white
powder. TLC profile showed that S. F.2 was a phenolic compound. UV
spectrum (MeOH) λmax 266 nm showed that S. F.2 had a chromophore
group. 1H NMR spectrum showed signal typical to aromatic proton
(δH 7.02). 13C NMR spectrum indicated the presence of 7 carbon atoms
signals including carbonylic carbon at δC 164.1. Further support for the
structure (1) was also obtained from the comparison of NMR data with
those reported for gallic acid.[4,5]
S. F.4.2 was identified as methyl gallate (2) that was isolated as a
yellowish-pale powder. UV spectrum (MeOH) λmax 273 nm showed that
S. F.4.2 had a chromophore group. 1H NMR spectrum showed signal
typical to aromatic proton (δH 7.07) and methoxy proton (δH 3.75).
13C NMR spectrum indicated the presence of 8-carbon atoms including
carbonylic carbon at δC 161.4 and methoxy carbon at δC 46.2. Further
support for the structure (2) was also obtained from the comparison of
NMR data with those reported for methyl gallate.[6,7]
S. F.8.3 was identified as Kempferol-3-O-rhamnoside (3) that was isolated
as yellow powder. 1H NMR spectrum indicated the presence of 2 aromatic
hydrogen signals with "meta-coupling" at δH 6.24 (1H, δd, δJ = 1.6 Hz)
and 6.45 (1H, δd, δJ = 2.4 Hz), which were predicted by hydrogen in C-6 and
C-8 of the B ring of the flavone skeleton. The signal at δH 12.69 was
predicted as hydroxyl group at C-5. Accordingly, this compound was
suggested to have a hydroxyl group at C-5 and C-7. Furthermore, 1H
NMR spectrum revealed two signals with "ortho-coupling" at δH 6.99 (2H,
δd, δJ = 8.4 Hz) and 7.83 (2H, δd, δJ = 8.4 Hz), the signals were predicted
as the hydrogen at C-2', C-3', C-5' and C-6' of the B ring. The absence of
a specific signal for an olefinic hydrogen at C-3 and the presence of an
anomeric hydrogen signal at δH 5.31 suggested that the compound was
a flavonol glycoside. The appearance of an anomeric carbon signal at δC
101.8 in 13C NMR spectrum indicated the presence of sugar moiety. Due
to a correlation between the anomeric hydrogen signal (δH 5.31) and C-3

A carbon signal (δ 141.0) revealed by HMBC spectral data analysis, the
position of sugar moiety was assigned to C-3 hydroxyl group. The methyl
signal observed at δ 1.16 (3H, δ3) in 1H NMR spectrum and at δ 16.9
in 13C NMR spectrum indicated that the sugar moiety was rhamnose.
Based on the accumulated data above and data comparison, compound (3)
was identified as kaempferol-3-O-rhamnoside. [8,9]
The structure of gallic acid, methyl gallate, and kempferol-3-O-rhamnoside
were shown in Figure 2.
Antiplasmodial activity of compound 1 and 2 appeared and was associated
with gallate group. [10] The previous study showed that methyl gallate
isolated from Swertiafoxy sheh had antiparasitic activity against
chloroquine-sensitive 3D7 strain of Plasmodium falciparum with IC50 3.5 µg/ml.[11]
The result of this study showed that methyl gallate exhibited antiparasitic
activity with IC50 0.0025 µg/ml against chloroquine-sensitive 3D7 strain of
Plasmodium falciparum. Kempferol-3-O-rhamnoside isolated from Schima
wallichii also showed antiparasitic properties with IC50 106 µM.[12]
In this study, Kempferol-3-O-rhamnoside showed antimalarial activity
with IC50 3.4595 µM [Table 1]. The difference of IC50 value might be
carried by the type of parasite strain. Antiplasmodial activity of some
gallic acid derived from green tea was also tested by Sannella et al., and
the result showed that both epigallocatechin gallate and epigallocatechin
gallate have potential antiparasitic activity. [13]
Oxidative stress through the generation of reactive oxygen species plays
an important role in the pathogenesis of malaria infection that causes
elevated hemoglobin degradation. [14] As phenolic compounds, gallic acid, methyl
gallate, and kempferol-3-O-rhamnoside have antioxidant properties
that may be responsible for antiparasitic activity. According to
Fidock et al., a compound has potential antimalarial activity if IC50 < 1-5
µM. [15] It can be concluded from the data that A. serratus leaves contain
gallic acid, methyl gallate, and kempferol-3-O-rhamnoside that have
antiparasitic activity against chloroquine-sensitive 3D7 strain of
Plasmodium falciparum.

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Table 1: Antimalarial activity of isolate compounds

| Compounds            | IC50 μg/ml±SD | μm±SD |
|----------------------|--------------|-------|
| Gallic acid           | 0.0130±0.0014| 0.0752±0.0078 |
| Methyl gallate        | 0.0025±0.0007| 0.0128±0.0036 |
| Kempferol-3-O-rhamnoside| 1.495±0.007| 3.4595±0.0180 |

SD: Standard deviation

Figure 1: Various concentration of compounds determining the IC50 value against Plasmodium falciparum after 48 h of incubation. Data are the mean value of double-independent experiments.

Figure 2: Chemical structure of the antimalarial polyphenols from Alatecyron serratus leaves. (1) Gallic acid; (2) Methyl gallate; (3) Kempferol-3-O-rhamnoside.
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Conflicts of interest
There are no conflicts of interest.

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