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

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ORIGINAL ARTICLE

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

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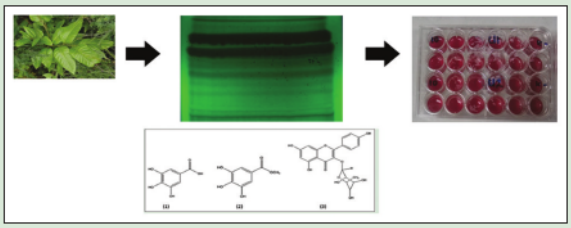
ABSTRACT

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. **Material and Methods:** 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 antiplasmodial 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 kempferol-3-O-rhamnoside (3) were 0.0722 µM, 0.0128 µM, and 3.4595 µM, respectively. **Conclusion:** The results suggest that gallic acid, methyl gallate, and kempferol-3-O-rhamnoside isolated from *A. serratus* leaves have antiplasmodial activity and are potential to be developed as antimalarial drugs.

Key words: *Alectryon serratus,* antiplasmodial, polyphenol

SUMMARY

- The ethanol extract of *Alectryon serratus* leaves was successively fractionated in CH_2Cl_2, EtOAc, and n-butanol. EtOAc fraction was fractionated using column chromatography and purified using preparative thin-layer chromatography (TLC). Isolates were studied for their antiplasmodial 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 (IC_{50}) 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 antiplasmodial activity and are responsible in the antimalarial activity of *Alectryon serratus* leaves.



Abbreviations Used: S.F: Subfraction, EGCG: Epigallocatechingallate, EGC: Epigallocatechin

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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.[1] 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 antiplasmodial activity is a useful way for discovering new leads.[2] 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.[3] This plant is widely distributed throughout the tropical region of Southeast Asia, and no traditional uses are reported.[4] Ethyl acetate fraction of the leaves was found to exhibit an antimalarial activity (IC_{50} 9.45 µg/mL) on chloroquine-sensitive 3D7 strain of *P. falciparum*.[5] TLC profile of ethyl acetate fraction showed polyphenol compounds.[5] In this paper, we report the isolation, structure elucidation, and antiplasmodial 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 Purwodadi 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

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then removed, and the residue was pressed and dried. The residue was re-macerated twice with ethanol 80%. The dried extract was suspended in distilled water and fractionated. Liquid fractionation was done successively in CH_2Cl_2, 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 CH3CN-MeOH-H2O (2:1:4 v/v) as the mobile phase to yield 12 subfractions (SF.1-SF.12). White powder was obtained as a precipitate from S.F.2. The precipitate was filtered using Kiriyama to yield compound 1 (12 mg). S.F.4 (30 mg) was purified using TLC preparative with $CHCl_3$-MeOH (9:1 v/v) as mobile phase and silica gel F_{254} as stationary phase to yield S.F.4.1 (4 mg) and S. F.4.2 (8 mg). S.F.4.2 was identified as compound 2. S.F.8 (40 mg) was purified using preparative TLC with $CHCl_3$-MeOH (8:2 v/v) as mobile phase and silica gel F_{254} as stationary phase to yield S.F.8.1 (5 mg), S.F.8.2 (7 mg), and S.F.8.3 (2 mg). S.F.8.3 was identified as compound 3.

Characterization of isolated polyphenols

TLC profiles of S.F.2 was done using RP-18 F_{254} as the stationary phase and CH_3CN-MeOH-H_2O (2:1:4 v/v) as the mobile phase. S.F.4.2 and S.F.8.3 were identified using TLC with silica gel F_{254} as stationary phase and $CHCl_3$-MeOH (9:1 v/v) as mobile phase. The spots were detected under ultraviolet (UV) (254 and 366 nm) before spraying using 10% H_2SO_4 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 Shim-pack column 4.6 mm × 250 mm as stationary phase, and CH_3CN-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 internal standard for ^1H, ^{13}C NMR, heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence. Compound 1 and 2 were measured using D6 methanol solvent; compound 3 was measured using D6 acetone solvent. The structural determination of compound 1, 2, and 3 was confirmed by comparing the results of NMR and UV data reported in the literature.[6-11]

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] 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, 0.05 g hypoxanthine, 2.1 g $NaHCO_3$, 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. These final parasite cultures were immediately used for the antimalarial assay.[12,13]

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:

Growth percentage (%growth) = %Parasitemia – D_0

Inhibition percentage was calculated as follows:

Inhibition percentage (% inhibition) =100% – {Xu/Xk) × 100%}

Note:

- D_0 = Parasitemia percentage of infected red blood cell on day 0
- Xu = Growth percentage of each isolate
- Xk = 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 S.F.2 and S.F.4.2 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 S.F.2 and S.F.4.2 after being sprayed and observed under UV 366 nm. S.F.8.3 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% H_2SO_4 and was detected under UV 366 nm and visible light. Isolation of phenolic compounds was done for S.F.2 and S.F.4.

HPLC chromatogram of S.F.2 showed a single peak with Rt 4.3 min with a purity index of 0.98 and UV/Vis λ_{max} (MeOH) at 266 nm. S.F.4.2 also showed a single peak with Rt 4.5 min, purity index 0.97 and UV/Vis λ_{max} (MeOH) at 271 nm.

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

S.F.2 (1)

White powder

^1H NMR (400 MHz, Methanol-d_6): 7.02 (s).

^{13}C NMR (100 MHz, Methanol-d_6): 115.6 (C), 103.9 (CH), 140.1 (C), 133.3 (C), 140.1 (C), 164.1 (COOH).

S.F.4.2 (2)

Pale yellow powder

^1H NMR (400 MHz, Methanol-d_6): 7.02 (s), 3.75 (3H, s).

^{13}C NMR (100 MHz, Methanol-d_6): 116.0 (C), 104.0 (CH), 140.4 (C), 133.0 (C), 140.4 (C), 164.4 (COOH), 46.2 (OCH_3).

S.F.8.3 (3)

Yellow powder

^1H NMR (400 MHz, Acetone-d_6): 7.83 (2H, dd, J = 8,4), 6.99 (2H, dd, J = 8,4), 6.45 (d, J = 2,4 Hz, 1H), 6.24 (d, J = 2,4 Hz, 1H), 12.69 (1H, s), 5.51 (1H, d, J = 1,6), 3.30–3.60 (3H, m), 4.18 (1H, m), 1.16 (3H, s), CH_3).

^{13}C NMR (100 MHz, Acetone-d_6): 178.5 (C = O), 115.4 (CH), 130.1 (CH), 98.7 (CH), 93.5 (CH), 170.3, 164.4, 159.7, 163.8,

156.6 (oxyaryl group), 71.3, 70.6, 70.5, 70.2 (C-2", C-3", C-4", C-5"), 16.9 (CH_3).

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 IC_{50} 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 white powder. TLC profile showed that S. F.2 was a phenolic compound. UV spectrum (MeOH)λ_{max} 266 nm showed that S. F.2 had a chromophore group. ^{1}H NMR spectrum showed signal typical to aromatic proton (δ_H 7.02). ^{13}C NMR spectrum indicated the presence of 7 carbon atom signals including carboxylic carbon at δ_C 164.1. Further support for the structure (1) was also obtained from the comparison of NMR data with those reported for gallic acid.[6-8]

S. F.4.2 was identified as methyl gallate (2) that was isolated as yellowish-pale powder. UV spectrum (MeOH) λ_{max} 271 nm showed that S. F.4.2 had a chromophore group. ^{1}H NMR spectrum showed signal typical to aromatic proton (δ_H 7.07) and methoxy proton (δ_H 3.75). ^{13}C NMR spectrum indicated the presence of 8-carbon atoms including carboxylic carbon at δ_C 161.4 and methoxy carbon at δ_C 46.2. Further support for the structure (2) was also obtained from the comparison of NMR data with those reported for methyl gallate.[6-8]

S. F.8.3 was identified as Kempferol-3-O-rhamnoside (3) that was isolated as yellow powder. ^{1}H NMR spectrum indicated the presence of 2 aromatic hydrogen signals with "meta-coupling" at δ 6.24 (1H, *d*, *J* = 1.6 Hz) and 6.45 (1H, *d*, *J* = 2.4 Hz), which were predicted by hydrogen in C-6 and C-8 of the A ring of the flavone skeleton. The signal at δ 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, ^{1}H NMR spectrum revealed two signals with "ortho-coupling" at δ 6.99 (2H, *d*, *J* = 8.4 Hz) and 7.83 (2H, *d*, *J* = 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 δ 5.51 suggested that the compound was a flavonol glycoside. The appearance of an anomeric carbon signal at δ 101.8 in ^{13}C NMR spectrum indicated the presence of sugar moiety. Due to a correlation between the anomeric hydrogen signal (δ 5.51) and C-3 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, *s*) in 1H NMR spectrum and at δ 16.9 in ^{13}C 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.[9-11]

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.[9] The previous study showed that methyl gallate isolated from *Swintonia foxworthyi* had antiplasmodial activity against chloroquine-sensitive 3D7 strain of *P. falciparum* with IC_{50} 3.5 µg/mL.[14] The result of this study showed that methyl gallate exhibited antiplasmodial activity with IC_{50} 0.0025 µg/mL against chloroquine-sensitive 3D7 strain of *P. falciparum*. Kempferol-3-O-rhamnoside isolated from *Schima wallichii* also showed antiplasmodial properties with IC_{50} 106 µM.[15] In this study, Kempferol-3-O-rhamnoside showed antimalarial activity with IC_{50} 3.4595 µM [Table 1]. The difference of IC_{50} value might be caused by the type of parasite strain. Antiplasmodial activity of some gallic acid derived from green tea was also tested by Sannela *et al.*, and the result showed that both epigallocatechingallate and epigallocatechin have potential antiplasmodial activity.[16]

Oxidative stress through the generation of reactive oxygen species plays important role in the pathogenesis of malaria infection that causes hemoglobin degradation.[17] As phenolic compounds, gallic acid, methyl gallate, and kempferol-3-O-rhamnoside have antioxidant properties that may be responsible for antiplasmodial activity. According to Fidock *et al.*, a compound has potential antimalarial activity if IC_{50} <1–5 µM.[18] It can be concluded from the data that *A. serratus* leaves contain gallic acid, methyl gallate, and kempferol-3-O-rhamnoside that have antiplasmodial activity against chloroquine-sensitive 3D7 strain of *P. falciparum*

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Table 1: Antimalarial activity of isolate compounds

Compounds	IC_{50}	
	µg/ml±SD	µm±SD
Gallic acid	0.0130±0.0014	0.0722±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

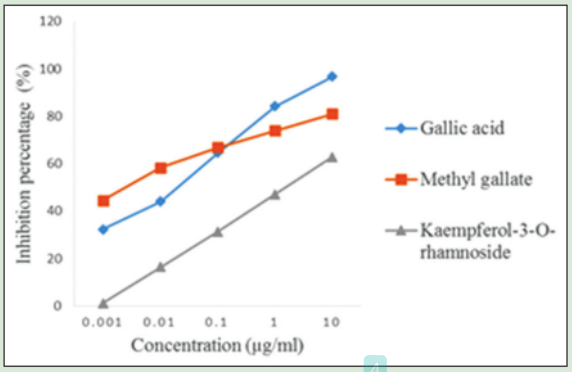

Figure 1: Various concentration of compounds determining the IC_{50} value against *Plasmodium falciparum* after 48 h of incubation. Data are the mean value of double-independent experiments

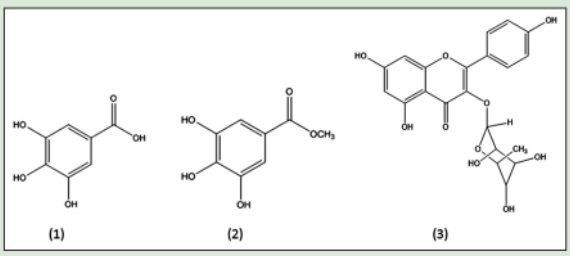

Figure 2: Chemical structure of the antiplasmodial polyphenols from *Alectryon serratus* leaves. (1) Gallic acid; (2) Methyl gallate; (3) Kempferol-3-O-rhamnoside

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Conflicts of interest

There are no conflicts of interest.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4