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Original Research Article

Bioassay-guided fractionation and *in vitro* antiplasmodial activity of ethyl acetate fractions from *Alectryon serratus* (Sapindaceae) leaf

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Abstract

Purpose: *To investigate the antimalarial activity of active fractions from Alectryon serratus leaves and identify their secondary metabolites.*
Methods: *Fractionation of ethyl acetate fraction from Alectryon serratus leaves was conducted using column chromatography. Five serial concentrations (10, 1, 0.1, 0.01 and 0.001 µg/mL) of each sub-fraction were tested in vitro against Plasmodium falciparum 3D7 (chloroquine-sensitive). The active sub-fractions were separated using preparative TLC. Crude isolates were administrated to P. falciparum culture and incubated for 48 h. The percentages of growth inhibition and IC_{50} were calculated. Secondary metabolites group of sub-fractions and the crude isolate were determined using TLC, FTIR, and UV-VIS spectrophotometry. Chloroquine was used as a standard drug for antiplasmodial testing.*
Results: *Four sub-fractions, ETA.4, ETA.8, ETA.9 and ETA.11, exhibited antimalarial activity with IC_{50} values at 0.035, 0.187, 0.015 and 0.017 µg/mL. Sub-fractions ETA.11 separated using preparative TLC yielded ETA.11.1 and ETA.11.2. Sub-fractions ETA.11.1 and ETA.11.2 showed antimalarial activity with IC_{50} values at 0.011 and 0.01 µg/mL. The TLC chromatogram of active sub-fractions showed flavonoids and phenolic.*
Conclusion: *Crude isolates ETA.11.1 and ETA.11.2 contain flavonoid compounds and have potential antiplasmodial activity.*

Keywords: *Alectryon serratus, Crude isolates, Plasmodium falciparum, Anti-malarial, Flavonoids*

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INTRODUCTION

Artemisinin resistance was first reported in western Cambodia and led to an increase in artemisinin-based therapeutic failure [1]. Local people have used some plants as anti-malaria, such as *Artocarpus communis* in Kalimantan, Indonesia. *Annona squamosa, Isolana hexaloba* in Cameroon and *Abrus precatorius, Andrographis paniculata, Caesalpinia crista* (Linn), *Tinospora cardifolia, Azadirachta indica* in India [2–4].

Secondary metabolites with high antimalarial efficacy include alkaloids and flavonoids [5,6]. *Cassia siamea* contains alkaloid compounds, cassiarins C-E, that exhibit moderate antiplasmodial activity against *Plasmodium falciparum* 3D7 [7]. Some Sapindaceae subfamilies that have been tested for antimalarial activity are *Dodonae angustifolia* and *Alectryon serratus* [8–10]. Previous studies have suggested that *Alectryon serratus* has antimalarial activity both *in vitro* and *in vivo* [11,12].

In this study, ethyl acetate fraction was separated using chromatography methods and the phytochemical profile of sub-fractions was analyzed using TLC. Each crude isolate was investigated for antimalarial activity testing against *Plasmodium falciparum* 3D7 chloroquine-sensitive and functional groups analysis using FTIR and UV-VIS spectrophotometry.

EXPERIMENTAL

Materials

The following reagents were used in this study: Dimethyl sulfoxide (DMSO) and chloroquine diphosphate (Sigma), RPMI medium, ethanol, dichloromethane, ethyl acetate, n-butanol and chloroform (Merck), and acetone (Smartlab).

Plant collection

Alectryon serratus leaves were obtained from Alas Purwo forest, Banyuwangi, East Java, Indonesia. The plant sample was taxonomically identified by Kebun Raya Bogor in Bogor Botanical Garden, Indonesia. Sample specimens were stored in the herbarium in Bogor Botanical Gardens with code AP.5 and letter of identification number 685/IPH.3.04/HM/IV/2018.

Extraction and fractionation

Alectryon serratus leaves were dried, powdered, and subjected to extraction using the sonification method with 80 % ethanol, and the liquid extract was concentrated using rotary vacuum evaporation at 40 °C. Liquid-liquid fractionation was performed for ethanol extract using dichloromethane, ethyl acetate, and n-butanol [11].

Open column chromatography was accomplished to separate 1 g of ethyl fraction using reversed-phase C18 column as stationary phase and acetonitrile: methanol: water in a volumetric ratio of 2:1:4 (v/v) as mobile phase to yield 12 sub-fractions (ETA.1 – ETA.12). Sub-fraction ETA.11 was selected for further separation using preparative TLC with silica GF_{254nm} as stationary phase and chloroform: acetone: formic acid in a volumetric ratio of 6.5:3:0.5 (v/v) as mobile phase to yield four sub-fractions (ETA.11.1 – ETA.11.4).

TLC analysis

Ethyl acetate fraction (EA) and sub-fractions (ETA.1 – ETA.12) were examined for TLC profile using two stationary phases. The first condition, silica GF_{254nm}, was used as a stationary phase, and chloroform: methanol in a volumetric ratio of 8:2 (v/v) was used as mobile phase. The second condition, silica RP-18, was used as stationary phase, and acetonitrile: methanol: water in a volumetric ratio of 2:1:4 (v/v) was used as mobile phase. Thin Layer Chromatography (TLC) was also performed for sub-fraction ETA.11.1 – ETA.11.4, with silica GF_{254nm} as stationary phase and chloroform: acetone: formic acid in a volumetric ratio of 6:3.5:0.5 (v/v) as mobile phase.

Chromatogram profiles were observed under UV 254 and 366 nm, followed by spraying the chromatogram plates with sulfuric acid (H_2SO_4 10 %). The TLC plates were heated at 120 °C and observed under UV 366 nm and visible light. The color of the spots was noted, and the retardation factor (Rf) was calculated using Eq 1.

$$Rf = Da/Db \quad \ldots\ldots\ldots\ldots (1)$$

where Da and Db are the distance travelled by solute and solvent, respectively.

Ultraviolet and infrared spectroscopy

Absorption bands of subfraction ETA.11.1 and ETA.11.2 in the ultraviolet (UV-VIS) spectroscopy were performed using Shimadzu UV-1800 spectrophotometer. Infrared Spectroscopy of ETA.11.1 and ETA.11.2 was conducted using Shimadzu Prestige 21 FT-IR spectrophotometer, between 4000 – 400 cm^{-1}. The crude isolates were mixed with KBr salt and compressed into a thin pellet.

In vitro cultivation of *Plasmodium falciparum* CQ-sensitive (3D7)

Antiplasmodial activity of sub-fractions and crude isolates was performed against *Plasmodium falciparum* 3D7 chloroquine-sensitive. *Plasmodium falciparum* was cultivated in human O+ red blood cells using RPMI 1640 medium supplemented with O Rh+ serum (10 %), 0.05 g hypoxanthine, 2.1 g sodium bicarbonate, 5.96 g

HEPES, and 50 µg/mL gentamycin sulfate. The culture was incubated at 37 °C under a modified candle jar method. The culture was maintained at the Malaria Laboratory, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, using Trager and Jensen's modified method [13]. Initial parasitemia was maintained between 0.5 - 1.0 %. The growth of the parasite was observed daily through microscopic examination of thin blood smear treated with Giemsa stain. Calculation of parasitemia percentage was conducted by counting the infected erythrocytes in a total of 1000 erythrocytes. *Plasmodium falciparum* cultures in the ring phase were transferred to 24 microwells plates for antimalarial assay. Uninfected type O+ human erythrocytes and complete medium were used to dilute stock parasite culture to achieve 1 % parasitemia and 50 % hematocrit for antimalarial assay [11].

In vitro antimalarial assay

The antimalarial assay was performed in two steps. First, screening of antimalarial activity was conducted for ETA.1 – ETA.12 with a concentration of 10 mg/mL. Sub-fractions with a parasitemia inhibition percentage of more than 50 % (ETA.4, ETA.8, ETA.9, ETA.10) were determined for the antimalarial activity to analyze IC_{50}. Five serial concentrations (10, 1, 0.1, 0.01 and 0.001 µg/mL) were used for anti-malarial assay according to Khasanah [12].

Preparation of thin blood smears on the labelled slides was performed after incubation for 48 h. The slides were dried in methanol and stained with Giemsa. Slides were observed under a light microscope at 1000x magnification. Growth (G) and inhibition (H) were calculated using Eqs 2 and 3, respectively.

$$G\ (\%) = P - D_0 \quad \text{..... (2)}$$

$$H\ (\%) = 100\% - \{(Xu/Xk)\}100 \quad \text{..... (3)}$$

where D_0 = initial parasitemia (%) of infected RBC, Xu = growth (%) of each isolate, Xk = growth (%) of negative control, and IC_{50} values repress = concentration required to inhibit 50 % of plasmodium growth.

RESULTS

Extract fractions

The result of open column chromatography from one gram ethyl acetate fraction was 12 sub-fractions (ETA.1 – ETA.12). The weight of each sub-fraction is 31.7, 172, 188, 108, 23, 20, 20, 15, 38, 14, 30 and 71 mg.

Chromatogram profile of ETA.1 – ETA.12 with silica reversed-phase C18 as stationary phase and acetonitrile: methanol: water (2:1:4 v/v) as mobile phase was shown in Figure 1. Spots were observed in ETA.2, ETA.3 at wavelength 254 nm with Rf 0.75 and ETA.4 with Rf 0.54. The blue spot was observed under 366 nm with Rf 0.62 for ETA.3. Yellow spots with Rf between 0.45 - 0.3 at a wavelength 366 nm were observed for ETA.4, ETA.5, and ETA.6. In ETA.7 and ETA.8, there was a yellow spot with Rf 0.3 at a wavelength of 366 nm. In ETA.9 – ETA.11, there were yellow spots with Rf between 0.21 and 0.3.

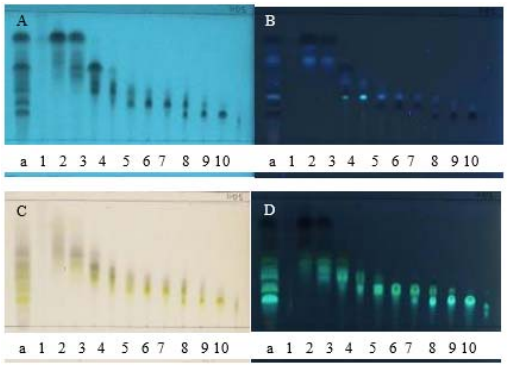

Figure 1: TLC profile of sub-fraction EA.1 – EA.12 (stationary phase: Silica RP-18). (a) EA fraction; (1 – 12) sub-fraction EA.1 – EA.12; (A) UV 254 nm; (B) UV 366 nm; (C) Visible; (D) UV 366 nm after spraying

The chromatograms of ETA.1 – ETA.12 with a stationary phase, silica GF_{254nm}, and mobile phase, chloroform/methanol (8:2 v/v), are shown in Figure 2. Fluorescent and tailing chromatograms were observed in ETA.2 and ETA.3 at 254 and 366 nm. Sub-fractions ETA.3 and ETA.4 also exhibited fluorescent spots at wavelength 254 nm with Rf 0.6. Observation at wavelength UV 366 nm and visible light for ETA.5, ETA.6, ETA.7, ETA.8, ETA.9, ETA.10, and ETA.11 showed yellow tailing spots (after derivatization using H_2SO_4 10 %). The yellow spot was also observed in ETA.6, ETA.7 and ETA.8 with Rf of 0.48 at wavelength 366 nm.

Preparative TLC yielded 4 sub-fractions, ETA.11.1 – ETA.11.4. Each of sub-fractions' weight was 3.9, 8.8, 1.9 and 1.5 mg. Figure 3 shows the TLC profile of ETA.11.1 – ETA.11.4. Yellow spots were observed in ETA.11.1 and ETA.11.2 with Rf 0.35 and 0.25 under UV 366 nm and visible light (after derivatization using H_2SO_4 10%).

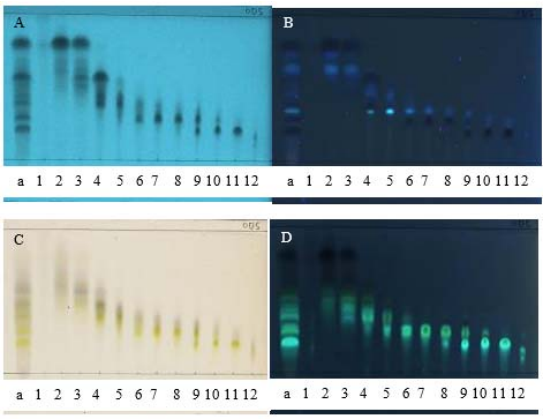

Figure 2: Chromatogram profile of sub-fractions ETA.1 – ETA.12 (stationary phase: Silica RP-18). (a) ETA fraction; (1 – 12) sub-fractions ETA.1 – ETA.12; (A) UV 254 nm; (B) UV 366 nm; (C) Visible; (D) UV 366 nm after spraying

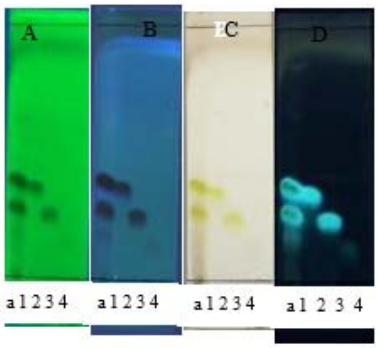

Figure 3: Chromatogram profile of sub-fractions ETA.11.1 – ETA.11.2 (stationary phase: Silica GF_{254nm}). (a) ETA.11; (1 – 4) sub-fractions ETA.11.1 – ETA.11.4 (A) UV 254 nm; (B) UV 366 nm; (C) Visible; (D) UV 366 nm after spraying

UV and FT-IR spectra

The results for the UV spectroscopy of sub-fractions ETA.11.1 and ETA.11.2 show that each sub-fraction has absorption bands corresponding to the flavonoid. Sample ETA.11.1 exhibited maximum absorption at wavelengths 265 and 339 nm. Sample ETA.11.2 exhibited maximum absorption at wavelength 266 and 341 nm.

The results of the FT-IR spectroscopy analysis of ETA.11.1 and ETA.11.2 revealed the presence of many functional groups (Figure 4 and Figure 5). Infra-Red spectrum of ETA.11.1 (Figure 4) exhibited peaks at 3402.96 and 3264.09 cm^{-1} (OH groups), 1601.57 cm^{-1} (C=O), 1451.13 – 1508.99 cm^{-1} (C= C), 1358.56 cm^{-1} (C-O), and 764.52 cm^{-1} (C-H).

The spectrum of EA 11.2 (Figure 5) showed a strong peak at 3436.2 and 3256.38 cm^{-1} (OH), 1599.67 cm^{-1} (C=O), 1453.06 and 1510.92 cm^{-1} (C=C), 1358.56 cm^{-1} (C-O), and 762.59 cm^{-1} (C-H).

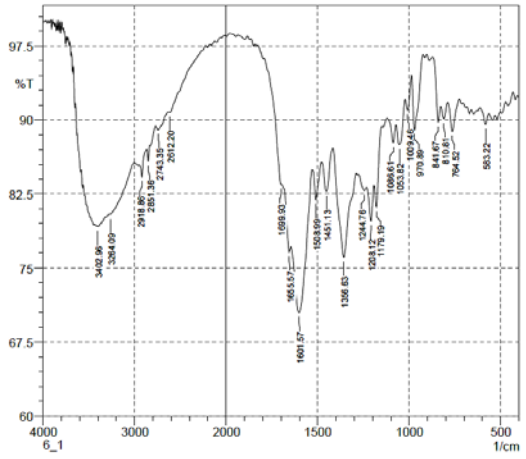

Figure 4: FTIR spectra of ETA.11.1

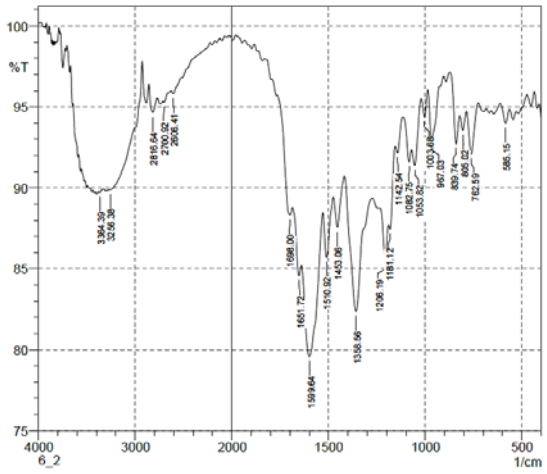

Figure 5: FTIR spectra of ETA.11.2

In vitro antimalarial activity

Antiplasmodial activity screening was performed on ETA.1 – ETA.12 using a single concentration of 10 µg/mL, and Table 1 shows the inhibition percentage of each subfraction. Sub-fractions ETA.4, ETA.8, ETA.9, ETA.10 and ETA.11 showed inhibition percentage higher than 50 % (60.70, 82.36, 79.89, 58.10 and 77.04 %). Sub-fractions ETA.4, ETA.8, ETA.9, and ETA.11 were subjected to further antimalarial assay to determine the IC_{50} value and are described in Table 2. Sub-fractions ETA.9 and ETA.11 exhibited IC_{50} values (0.015 and 0.017 µg/mL). Antiplasmodial activities of sub-fractions ETA.11.1 and ETA.11.2 were compared to chloroquine and are shown in Table 2.

Table 1: Inhibitory activity of sub-fractions at 10 µg/mL

Sub-fraction	Inhibition (%) (mean ± SD)
ETA.1	49.28±5.32
ETA.2	39.42±2.39
ETA.3	27.49±0.92
ETA.4	60.70±5.69
ETA.5	10.89±3.12
ETA.6	40.59±0.37
ETA.7	42.54±4.22
ETA.8	82.36±2.39
ETA.9	79.89±3.85
ETA.10	58.10±1.28
ETA.11	77.04±3.85
ETA.12	42.67±4.04

Table 2: IC_{50} value of sub-fractions at five serial concentrations

Subfraction	IC_{50} (µg/mL, mean ± SD)
ETA.4	0.035±0.003
ETA.8	0.187±0.018
ETA.9	0.015±0.000
ETA.11	0.017±0.004
ETA.11.1	0.011±0.014
ETA.11.2	0.01±0.009
Chloroquine	0.001±0.00

DISCUSSION

Sub-fractions ETA.4, ETA.9, ETA.10, and ETA.11 inhibit *in vitro Plasmodium falciparum* growth more than 50 %. According to the TLC profiles, yellow spots and fluorescent bands were observed in sub-fractions ETA.9, ETA.10, ETA.11, ETA.4, and they were identified as flavonoids and phenolics [11].

Crude isolates ETA.11.1 and ETA.11.2 also exhibited antimalarial activity with IC_{50} values of 0.01 and 0.011 µg/mL. According to chromatogram profiles, the antiplasmodial activity of crude isolates was due to the presence of flavonoids (yellow spots with Rf 0.4 and 0.27).

A spectrum of IR from both crude isolates ETA.11.1 and ETA.11.2 show characteristic peaks of flavonoid compounds. Carbonyl group (C=O) gives rise to strong absorption, OH groups, double bond (C=C) from aromatic ring (medium to strong absorption at 1600 - 1450 cm^{-1}). Hydrocarbon (=C-H) from the aromatic ring and vinyl ring occurs to the left of 3000 cm^{-1}. The intense peak at 1358.56 cm^{-1} indicates alcohol (C-O) [14–16].

The presence of yellow spots strongly suggests that antimalarial constituents of sub-fractions ETA.8, ETA.9, ETA.11, ETA.11.1, and ETA.11.2 are flavonoids. Previous studies from genus Moraceae, *Artocarpus chempeden* exhibited *in vitro* anti-plasmodial activity of arthocarpon A against *Plasmodium falciparum* 3D7 [17]. Dyprenilated flavanone was also isolated from *Erythrina fusca* extract and showed potential antimalarial activity against the multi-drug-resistant *P. falciparum* (K1 strain) [6].

Some dietary flavonoids also exhibited antimalarial activity, such as acetin, genistein, hesperidin, isoquercetin, kaempferol, luteolin, myricetin, and naringenin [5]. Luteolin inhibited 50 % of *Plasmodium falciparum* 3D7 and 7G8 growth at concentrations of 11 and 12 µM, respectively, and quercetin (at 15 and 14 µM, respectively) [18]. The antimalarial mechanism of flavonoids is not fully comprehended. Previous research displayed that flavonoids inhibited fatty acid biosynthesis and inhibited the influx of L-glutamine into infected erythrocytes [5]. Artemisinin described synergic effects when combined with some flavonoids, such as chrysosphenol-D, quercetin-glucoside, flaviolin, rhamnetin, and pillion [19].

CONCLUSION

Based on the results of the present study, phenolics and flavonoids compounds are responsible for the antimalarial activity of sub-fractions and crude isolates from the ethyl acetate fraction of *Alectryon serratus* leave.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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