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Original Article

ANTIMALARIAL ACTIVITY AND CYTOTOXICITY STUDY OF ETHANOL EXTRACT AND FRACTION FROM *ALECTRYON SERRATUS* LEAVES

ATY WIDYAWARUYANTI[1,2], USWATUN KHASANAH[4], LIDYA TUMEWU[2], HILKATUL ILMI[2], ACHMAD FUAD HAFID[1,2], INDAH S. TANTULAR[2,3]

[1]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Surabaya 60286, Indonesia, [2]Center for Natural Product Medicine Research and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia, [3]Department of Parasitology, Faculty of Medicine, Universitas Airlangga, Surabaya 60286, Indonesia, [4]Postgraduate student, Faculty of Pharmacy, Universitas Airlangga, Surabaya 60286, Indonesia
Email: aty_ww@yahoo.com

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ABSTRACT

Objective: The resistance of *Plasmodium falciparum* to standard antimalarial drugs has led the scientist to investigate medicinal plants as new potentials in the treatment or prevention of malaria. Previous study showed that the ethanol extract from *Alectryon serratus* leaves inhibited *P. falciparum in vitro*. The purpose of this study was to determine the *in vitro* and *in vivo* antimalarial activity and toxicity of ethanol extract and fractions of *A. serratus* leaves.

Methods: Ethanol extract of *A. serratus* leaves was partitioned with dichloromethane, ethyl acetate and n-butanol successively. Antimalarial activities were determined *in vitro* against *P. falciparum* 3D7 based on HRP2 measurement in a simple *enzyme-linked immunosorbent assay* (ELISA) and *in vivo* against *Plasmodium berghei* ANKA based on the 4-days suppressive test by Peters. The toxicity study was determined by MTT assay using Huh7it cells.

Results: Ethanol extract and fractions of *A. serratus* exhibited antimalarial activity and was proved to be nontoxic. Ethyl acetate (EA) and butanol (BuOH) fractions performed a higher antimalarial activity (IC_{50}<10 µg/ml) and lower toxicity (SI>10) compared with ethanol extract and dichloromethane (DCM) fraction. EA fraction had IC_{50}value of 9.45 µg/ml and SI of 10.58, while BuOH fraction had IC_{50}value of 7.69 µg/ml and SI of 130.04. *In vivo* antimalarial activity was conducted for ethanol extract and EA fraction. The result showed that EA fraction and ethanol extract had ED_{50} 5.92 mg/kg BW and 13.82 mg/kg BW, respectively.

Conclusion: Ethanol extract and EA fraction of *A. serratus* leaves showed *in vivo* and *in vitro* antimalarial activities and proved to be nontoxic. Both of them are a good candidate of new source in the development of new antimalarial drugs.

Keywords: *Alectron serratus* leaves, Antimalarial activity, Toxicity.

INTRODUCTION

Malaria is one of the most important infectious diseases in the tropics and sub-tropics region. Each year 300 to 500 million new cases are diagnosed and approximately 1.5 million people, mostly children [1], died because of the disease. *Plasmodium falciparum,* the most widespread etiological agent for human malaria, has become increasingly resistant to standard antimalarial drugs e. g. chloroquine and antifolates [2]. Consequently, new drugs are urgently needed today to treat malaria. These drugs should have novel modes of action or be chemically different from the drugs in current use.

Natural products, particularly plants, might become the strategy to find new antimalarial drugs since nature is a promising source of drugs. Plants have been sources of medicine throughout the history of medicine. For thousands of years natural compounds, mostly derived from plants, have been used for traditional medicine. Plant species have demonstrated their potential to provide effective drugs for the treatment of malaria. Two of the most effective antimalarial drugs available are quinine and artemisinin, which are derived from the plant [3]. Quinine is isolated from *Chinchona* species (Rubiaceae) and artemisinin from *Artemisia annua*. Artemisinin derivates are now recommended by the World Health Organization (WHO) worldwide in combination with other drugs, such as amodiaquine, mefloquine, lumefantrine as the first-line treatment of malaria [4].

Our preliminary study, the screening of antimalarial activity of some Indonesian plants using HRP2 method, indicated that extracts of *A. serratus* leaves had the highest activity (IC50 value of 12.3µg/ml) and was potential as a target for further study [5]. *A.-serratus* is included in the Sapindaceae family, which is rich in substances such as flavonoid, diterpenoid, glycoside, phenol, saponin, kaempferol, quercetin and β-sitosterol [6]. This information encouraged us to study the plant further. In this study, antimalarial activity and toxicity effect of the extract and fractions *A. serratus* leaves were assessed.

MATERIALS AND METHODS

Plant material

Leaves of *A. serratus* were collected from Alas Purwo National Park, Banyuwangi, East Java, Indonesia. Authentication and identification of plant were carried out at the Purwodadi Botanical Garden, East Java, Indonesia.

Extraction and fractionation

1 kg of the sample was extracted using 80% ethanol by ultrasonic-assisted maceration technique for two minutes to achieve three-time replication. The ethanol extract were filtered, pooled, and dried at 40°C using rotary evaporator and weighed afterward. 100 g of ethanol extract was suspended in distilled water and partitioned with dichloromethane (DCM), ethyl acetate (EA) and n-butanol (BuOH) successively, which were then concentrated to dry in a rotary evaporator. The crude extract and fractions were kept in air tight containers and were stored at 4°C for use in phytochemical screening and antimalarial assay.

Phytochemical screening

Dried ethanol extract and fractions of DCM, EA, BuOH and water (10 mg) were diluted in methanol. The phytochemical screening was performed by Thin Layer Chromatography (TLC) method using stationary phase silica gel RP-18 and acetonitrile: methanol: water (2:1:4) mobile phase, as well as 10% sulphuric acid reagent. The spots were observed under UV 254 nm and 366 nm.

P. falciparum (3D7 strain) and *in vitro* culture

P. falciparum 3D7 strain was obtained from Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia and was maintained in our laboratory at 5% hematocrit (human type O-positive red blood cells) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 5.96 g HEPES, 0.05 g hypoxanthine, 2.1 g $NaHCO_3$, 50 µg/ml gentamycin and completed with 10% human O+serum) in petri dish by modified candle jar method. Incubations were done at 37 °C. The culture was routinely monitored through Geimsa staining of the thin blood smears. For the experiment, the parasit contain predominantly ring forms. Parasit of stock cultures were further diluted with uninfected type O+human erythrocytes and culture medium to achieve a starting parasitemia of 0.05% and a hematocrit of 1.5%. This final parasite culture was immediately used for antimalarial assay [7, 8].

In vitro antimalarial assay

Antimalarial activity assay used HRP2 (HRP2 Kit Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). The screening assay used single concentration of extract and fractions (average concentration of 100 µg/ml). To each well of a microplate, 100 µl diluted extract solution was added into 100 µl of final parasite culture. The plates were then incubated for 72 h at 37 °C. They were subsequently frozen-thawed twice to obtain complete hemolysis and stored at-30 °C until further processing and 100 µl of each of the hemolysis culture samples was transferred to the ELISA plates. The plates were precoated with monoclonal antibodies against *P. falciparum* HRP2 and incubated at room temperature for 1 hour in humidified chamber. The plates were washed five times with the washing solution (200 µl of each well) and 100 µl of the diluted antibody conjugate were added to each well. After incubation for an additional 1 hour in humidified chamber, the plates were washed with the washing solution (200 µl of each well) and 100 µl of diluted (1:20) chromogen TMB (tetramethylbenzidine) was added to each well. The plates were then incubated for another 15 min in the dark, and 50 µl of the stop solution was added. The optical density values were read with an ELISA plate reader at an absorbance maximum of 450 nm [9]. Inhibition percentage was calculated using the following formula:

$$\frac{(optical\ density\ of\ control\ well - optical\ density\ of\ sample\ well)}{optical\ density\ of\ control\ well}\ x\ 100\%$$

Samples which were revealed with inhibition percentage>80% will be further tested to determine the Inhibitory Concentration (IC_{50}) values using serial concentration of 100 µg/ml to 0.01 µg/ml. The IC_{50} values were determined graphically from dose-response curves (concentration versus percent inhibition curves) with non-linear analysis by SPSS probit.

Toxicity assay and analysis

Toxicity assay used MTT cell proliferation assay for several concentration 1000 µg/ml; 800 µg/ml; 400 µg/ml; 200 µg/ml; 100 µg/ml; 10 µg/ml; 1 µg/ml; 0.1 µg/ml; 0.01 µg/ml. Huh7it cells in 96 well plates were treated with serial dilution of the samples or control. Prepared wells were analyzed using ELISA reader at 720 nm and 650 nm wavelength.

Criteria of antimalarial activity *in vitro* and toxicity

Extract and fraction exhibiting IC_{50}<25 µg/ml was considered active. Extract showing IC_{50}<100 µg/ml was classified as follows: Marginally active at SI<4, partially active at SI 4-10 and active SI>10 [3].

In vivo antimalarial assay

The antimalarial activity of ethanol extract and EA fraction of *A. serratus* leaves were assessed by 4-day suppressive test. On the first day (D0) of the experiment, male BALB/C mice weighing 21-25 g were injected with intraperitoneal (i. p.) inoculation of 5% parasitemia infected by *P. berghei* (ANKA strain) erythrocytes in Alsever's suspension of 0.2 ml.

The mice were randomly divided into 7 groups, control group (no treatment), ethanol extract group (dose of 100 mg/kg BW; 10 mg/kg BW; 1 mg/kg BW) and EA group (dose of 100 mg/kg BW; 10 mg/kg BW; 1 mg/kg BW). Each group consisted of 5 mice.

Ethanol extract and EA fraction were suspended in 0.5% CMC-Na and administered orally for 4 d (day 0-day 3). Thin blood smears were made from the tail blood of the mice every day for 7 d (day-0 until day-6), stained with giemsa and then assessed by microscope. Percentage of parasitemia was counted based on infected erythrocytes calculated per 5000 erythrocytes. Percentage of inhibition growth of *P. berghei* was calculated using the formula:

$$100\% - (\frac{XE}{XK}\ x\ 100\%)$$

Xe: % parasitaemia growth of experimental group

Xk: % parasitaemia growth of negative control

Criteria of antimalarial activity *in vivo*

Criteria of antimalarial activity *in vivo* was based on Munoz *et al.* (2000), who suggests that an extract is highly active if it shows a value of ED_{50}<100 mg/kg BW, active if ED_{50} 100-250 mg/kg BW, and moderate if ED_{50}>500 mg/kg BW [10].

RESULTS AND DISCUSSION

Phytochemical screening

A. serratus from the Sapindaceae family has compounds such as flavonoids, diterpenoids, glicoside, phenol, saponin, kaempferol, quersetin and β-sitosterol [6]. Fractionation of *A. serratus* extract yielded 4 fractions, which were DCM fraction, EA fraction, BuOH fraction and an aqueous fraction. These four fractions from *A. serratus* extract were analyzed using TLC with a mobile phase of chloroform: methanol (3:1 v/v) and stationary phase of silica gel F254. The spot were observed under UV 254 nm and 366 nm, and derived by 10% of H_2SO_4. TLC result indicated that DCM fraction containing terpenoid compound, shown by red or brownish red coloration. EA fraction contained flavonoid compound, shown by yellow coloration. TLC analysis was also conducted using mobile phase of acetonitrile: methanol: water (2:1:4 v/v) and stationary phase of RP-18. BuOH fraction also contained flavonoid compound but was derived as glicoside compound. TLC profile from aqueous fraction showed polar compound.

Antimalarial *in vitro* and toxicity

Extract and fractions of *A. serratus* were screened for *in vitro* antimalarial activity against *P. falciparum* (3D7 strain) using HRP2 assay. The result of the antimalarial test indicated that ethanol extract, DCM fraction, EA fraction, and BuOH fraction had high activities because its inhibition percentage was more than 90%. The results are summarized in table 1.

Table 1: *In vitro* antimalarial activity of ethanol extract and fractions from *A. serratus* leaves

Sample	Conc (µg/ml)	% Inhibition*
Ethanol extract	100	95.30±0.203
DCM fraction	100	91.26±0.696
EA fraction	100	96.51±0.406
BuOH fraction	100	92.80±0.319
Aqueous fraction	100	54.18±6.349

*Values are mean±SD of triplicates

The result of table 1 became a basis to determine IC_{50} as a parameter of activity. To estimate the potential of extract to inhibit parasite growth without toxicity, the selectivity index (SI) was introduced. SI is defined as the ratio of the toxicity to antimalarial activity. Low SI indicates that the antiplasmodial activity is probably due to toxicity rather than activity against the parasite themselves. In contrast, higher selectivity (SI>10) indicates potentially safer therapy [3]. The IC_{50} values, CC_{50} values and SI values of ethanol extract and fraction of *A. serratus* are shown in table 2.

Table 2: IC_{50} values, CC_{50} values and SI of ethanol extract and fractions from *A. serratus*

Sample	CC_{50*}	IC_{50*}	SI*
Ethanol extract	>1000	10.27	97.37
DCM fraction	>100	14.81	6.75
EA fraction	>100	9.45	10.58
BuOH fraction	>1000	7.69	130.04

*Values are mean of duplicates.

Extract and fraction exhibited $IC_{50<}$100 µg/ml. Extract, EA fraction and BuOH fraction were classified as active with SI values of 97.37, 10.58, and 130.04 and one as partially active (DCM fraction) exhibiting SI of 6.75. Only two samples were found active: EA fraction and BuOH fraction, showing SI>10 and $IC_{50<}$10 µg/ml, respectively. EA and BuOH fractions also had IC_{50} values of 9.45 µg/ml and 7.69 µg/ml, respectively. Both of them were potential to be selected for further investigation.

***In vivo* antimalarial assay**

The average of parasitemia percentage resulted from ethanol extract and EA fraction group on first-day observation until fifth day (D0-D4) tended to increase, but the increase was not as high as in the control group. This showed that ethanol extract and EA fraction had an effect on the growth of *P. berghei* (ANKA strain) parasites in mice. The results are summarized in table 3 and 4.

Table 3: Activity of ethanol extract of *A. serratus* leaves on *P. berghei* infected mice

Dose	% Parasitemia					% Inhibition
(mg/kg BW)	D0	D1	D2	D3	D4	
Negative control	1.47±0.07	12.80±0.41	20.53±1.45	27.40±2.62	34.22±3.66	
100	1.04*±0.11	2.93*±0.32	6.30*±1.38	8.82*±1.54	12.55*±2.95	64.87±8.90
10	1.57±0.05	5.20*±0.47	10.24*±0.37	14.15*±0.58	18.03*±0.33	49.74±0.90
1	1.84*±0.22	7.01*±0.36	14.11*±0.42	18.07*±0.73	25.44*±0.35	27.95±0.73

Values are mean±SD (n=5). *Denotes significance at the level of P<0.05 versus the control group.

Tabel 4: Activity of EA fraction of *A. serratus* leaves on *P. berghei* infected mice

Dose	% Parasitemia					% Inhibition
(mg/kg BW)	D0	D1	D2	D3	D4	
Negative control	1.47±0.07	12.80±0.41	20.53±1.45	27.40±2.62	34.22±3.66	
100	1.02*±0.09	2.27*±0.18	4.66*±0.29	6.66*±0.62	8.91*±0.77	75.91±2.63
10	1.11*±0.09	4.30*±0.43	7.02*±0.71	10.60*±0.64	15.11*±0.77	57.29±2.17
1	1.21*±0.03	5.03*±0.17	12.75*±0.64	18.59*±0.58	23.71*±0.66	31.32±1.96

Values are mean±SD (n=5). *Denotes significance at the level of P<0.05 versus the control group.

Parasitemia percentage data were used to determine inhibition percentage. The highest inhibition of ethanol extract was found at a dose of 100 mg/kg BW with the value of 64.87%. Further percentage inhibition at a dose of 10 mg/kg BW was 49.74% and 27.95% at a dose of 1 mg/kg BW. The highest inhibition percentage on EA fraction was also found at a dose of 100 mg/kg BW with the value of 75.91%. Further inhibition at a dose of 10 mg/kg BW was 57.29% and at a dose of 1 mg/kg BW was 31.32%. Inhibition of *P. berghei* growth followed a dose-dependent manner. Higher doses applied to mice produced higher inhibition.

Inhibition percentage was analyzed using log-probit to determine ED_{50}. The result of the analysis showed that ethanol extract and EA fraction of *A. serratus* was highly active as an antimalarial agent with ED_{50} values of 13.82 mg/kg BW and 5.92 mg/kg BW, respectively. EA fraction had higher antimalarial activity and potential as a new source in the development of the antimalarial drug.

TLC profile showed that EA fraction from *A. serratus* leaves contained flavonoid compounds. The previous study showed that flavonoid compounds were active as antimalarial, e. g arto-indonesianin R, artoindonesianin A-2, and artokarpon A isolated from the bark of *Artocarpus champeden*; garcinia xanthone isolated from *Garcinia dulcis* was active as antimalarial with IC_{50} value of 0.96 µg/ml; biflavonoids isolated from *Selaginella bryopteris* were also active as antimalarial. The data of antimalarial activity of flavonoid had been reported, including exigua flavone A and exigua flavone B from the stem of *Artemisia indica*, which had antimalarial activity with IC_{50} value of 50 M and 50 µg/ml [11-13]. It was possible that flavonoid compounds have a contribution in antimalarial activity of EA fraction from *A. serratus* leaves.

CONCLUSION

Ethanol extract and ethyl acetate (EA) fraction *A. serratus* leaves showed antimalarial activities and proved to be non-toxic. Both of them are a good candidate of new source in the development of new antimalarial drugs. The result of TLC profile indicated that EA fraction contained flavonoid compounds. It was concluded that flavonoid compounds from EA fraction took effect on antimalarial activity.

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CONFLICT OF INTERESTS

Declared None

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