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Antiplasmodial and cytotoxicity evaluation of *Artocarpus sericicarpus* leaves extracts and fractions as a potential source of antimalarial substances

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ABSTRACT: Malaria is one of the tropical diseases that become a health problem worldwide. The elimination of this disease faced various burdens, leading to the need for new antimalarial drugs. Based on the previously reported antimalarial activity of *Artocarpus* genus, *Artocarpus sericicarpus* has been selected as one of the candidates which are potential to be explored as a source of antimalarial substances. This study aimed to obtain active antimalarial fractions from *A. sericicarpus* leaves. Extraction of *A. sericicarpus* leaves was conducted by ultrasonic-assisted extraction using n-hexane, dichloromethane, and methanol as solvents. All extracts were tested for their antimalarial activity by Lactate Dehydrogenase (LDH) assay. The most active extract was fractionated by Vacuum Liquid Chromatography (VLC) and fractions were further tested by LDH assay as well. Cytotoxicity test conducted by resazurin assay on several cell lines to determine the active and nontoxic fractions which have the potential to be further purified and identified the active substances. The n-hexane, dichloromethane, and methanol extract showed antimalarial activity with IC_{50} values of 23.96±0.06 µg/mL, 2.72±0.08 µg/mL, and 23.39±0.05 µg/mL, respectively. Dichloromethane extract was chosen for further separation due to its highest antimalarial activity. The separation was obtained in nine fractions. Fractions 2-9 had an IC_{50} value of less than 10 µg/mL, indicating an active antimalarial substance. Meanwhile, fraction 1 has moderate antimalarial activity. Cytotoxicity test results considered all fractions to be non-toxic. The TLC profile of dichloromethane extract identified polyphenolic compounds, suggesting the active polyphenolic compounds can be further isolated from the active fractions (F2-F9).
KEYWORDS: *Artocarpus sericicarpus*; cytotoxicity; extracts; fraction; malaria

1. INTRODUCTION

Malaria is one of the tropical diseases that become a health problem worldwide. According to the World Malaria Report 2021, there were 241 million cases of malaria globally, increasing from 227 million in 2019 [1]. The elimination of this disease faced various burdens, leading to the need for new antimalarial drugs.

The history of antimalarial drug discovery from nature especially plants contributed to the search for antimalarial drug sources. The *Artocarpus* genus is reported to have antimalarial properties [2]. New prenylflavone namely Artocarpone A and new dihydrobenzoxanthone namely Artocarpone B, along with seven known prenylated flavones have been isolated from the bark of *A. champeden* and reported to be active as antimalarials [3]. Antimalarial active compounds, class of dihydrochalcone which identified as 1-(2,4-dihydroxy phenyl)-3-[8-hydroxy-2-methyl-2-(4-methyl-3-pentenyl)-2H-1-benzopyran-5-yl]-1-propanone have also been isolated from *A. altilis* leaves with a mechanism of action on inhibition of parasitic stage development. The in silico study predicted strong interaction of the compound with falcipain-2 receptor, as a cysteine protease inhibitor [4].

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In this study, the potential of *A. sericicarpus* as an antimalarial will be investigated. This plant was selected based on a chemotaxonomic approach with *Artocarpus* species, namely *A. champeden* and *A. altilis*. The approach considers that plants from the same taxa had close kinship relations, especially at the taxonomic level of family, genus, and species. The existence of this close relationship allows for the similarity of the substances or compounds contained in those plants [5].

The preliminary study also suggested *A. sericicarpus* as selected species from *Artocarpus* genus to be further explored, apart from the chemotaxonomic approach. In our previous studies, screening of antimalarial activity on several *Artocarpus* species was conducted and the results showed that n-hexane, dichloromethane, and methanol extract from *A. sericicarpus* leaves at a test concentration of 100 µg/mL could inhibit *P. falciparum* in vitro with a range of 99-100% average inhibition. Therefore, we aim to continue the investigation of the antiplasmodial activity of *A. sericicarpus* leaves. This study was conducted to determine the IC_{50} value of *A. sericicarpus* leaves extract and fractions.

2. RESULTS AND DISCUSSION

This study was part of our continuing study of the development of natural product medicine as antimalarial sources, especially for *Artocarpus* genus. Our preliminary study has screened *A. sericicarpus* leaves extracts and obtained data on the inhibition of *Plasmodium* growth but the effectivity level was not yet conducted. Therefore, the determination of IC_{50} value was important to perform.

In this present study, the extraction of *A. sericicarpus* leaves (800 g) yielded three extracts namely n-hexane extract (3.77 g), dichloromethane extract (16.07 g), and methanol extract (57.43 g). Thin Layer Chromatography (TLC) of extracts showed that extracts containing groups of polyphenolic compounds based on the TLC spot indicated brown to orange colors after spraying with sulfuric acid.

The antimalarial activity by LDH assay was conducted to determine the active extract. *Plasmodium falciparum* lactate dehydrogenase enzyme has been considered a potential molecular target for antimalarials due to this parasite's dependence on glycolysis for energy [6]. LDH assay was chosen because this assay has acceptable demand for equipment, labor, technical skills, and affordability and offers a good opportunity for scientists in low-and middle-income countries to participate in the global effort of discovering future antimalarial drugs [7]. The LDH assay of n-hexane, dichloromethane, and methanol extract of *A. sericicarpus* leaves revealed dichloromethane extract as the most active extract, with an IC_{50} value of less than 10 µg/ml. Meanwhile, n-hexane and methanol extract had IC_{50} values of more than 10 µg/mL (Table 1). These results were in accordance with the previously reported antimalarial activity of *A. sericicarpus* stem bark extracts in which dichloromethane had superior activity compared to n-hexane and methanol extract [8]. Dichloromethane extract from *A. sericicarpus* stem bark and leaves had a similar IC_{50} value which is 2.11 µg/ml and 2.72 µg/mL.

The criteria for the antimalarial activity of the extract according to Basco et al., 1994 and Dolabela et al., 2008 were as follows: IC_{50} <10 µg/mL was the active extract, IC_{50} 10-50 µg/mL was included in the moderate criteria, IC_{50} 50-100 µg /mL extract has low activity, and IC_{50} >100 µg/mL indicates the extract is inactive. Meanwhile, isolated compound(s) were classified as highly active if the IC50 value was ≤1 µg/mL [9, 10]. From the IC_{50} value of extracts, it is known that the dichloromethane extract was included in the criteria for the active extract while the n-hexane extract and methanol extract were included in the extract with moderate antimalarial activity. The dichloromethane extract showed the lowest IC_{50} value compared to n-hexane and methanol extract meaning that dichloromethane was the most active among the three extracts. This study conducted bioassay-guided separation and suggested that the most active extract will be chosen for further separation. Therefore, the dichloromethane extract was continued for further fractionation process.

The separation of dichloromethane extract by VLC resulted in nine fractions. The TLC profile showed that chlorophyll is present in all fractions, but mostly in fraction 2 and 3 (F2 and F3). Meanwhile, other compounds detected in fractions were terpenoids, flavonoids, and chalcones which were represented by violet, yellow-brown, and orange spots after spraying with sulfuric acid.

The fractions were further tested for their antimalarial activity by LDH assay. The test results showed that all fractions were active with an IC_{50} value of less than 10 µg/mL, except for fraction 1 (F1) as shown in Table 1. Fraction 1 (F1) had moderate antimalarial activity, meanwhile, fractions 2-9 (F2-F9) exhibited active antimalarial activity. The most active fraction was F8 with an IC_{50} value of 1.43±0.06 µg/mL. These results were similar to the antimalarial activity of dichloromethane fractions from *A. sericicarpus* stem bark which reported IC_{50} value of active fractions at a range of 1.53–3.65 µg/mL [8].

Table 1. Antimalarial activity (IC_{50}) of extracts and fractions

Samples	IC_{50} (µg/mL)*	Classification
n-Hexane extract	23.96±0.06	Moderate
Dichloromethane extract	2.72±0.08	Active
Methanol extract	23.39±0.05	Moderate
F1	28.13±0.07	Moderate
F2	7.43±0.04	Active
F3	2.84±0.07	Active
F4	2.39±0.07	Active
F5	2.38±0.08	Active
F6	1.75±0.06	Active
F7	1.97±0.06	Active
F8	1.43±0.06	Active
F9	2.49±0.06	Active
Chloroquine diphosphate	0.008±0.05	Highly Active

F1-F9 were fractions obtained from the separation of dichloromethane extract.
*Data represent Mean ± SD

The cytotoxicity test by Resazurin assay was also conducted for extracts and fractions. Resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. Cellular viability quantification based on resazurin has advantages, including rapidity, reliability, sensitivity, safety, and cost [11]. The cytotoxicity test was conducted on several cell lines namely two carcinoma cells (Huh7, HepG2), and two normal cells (BHK-21, Vero). Two kinds of cell lines, carcinoma, and normal cells were used in the cytotoxicity assay to assure the safety of extracts and fractions as antimalarial. The active and safe substances normally showed nontoxic criteria on both carcinoma and normal cell lines. İf substances showed cytotoxic on normal cell lines then their antimalarial activity should be considered whether the activity appeared due to toxic effect on cells. The cytotoxic effect of substances on carcinoma cells might be considered the substance's potential as an anticancer agent. The results showed that either extracts or fractions had CC_{50} values higher than 20 µg/mL on all tested cell lines (Table 2). According to the U.S. National Cancer Institute (NCI) plant screening program, plant extracts with IC_{50} values below 20 µg/mL or 10 µM following incubation between 48 and 72 h are recognized as potential cytotoxic substances [12]. In the present study, all extracts and fractions were considered nontoxic substances based on their CC_{50} values which are above 20 µg/mL.

Evaluating Selectivity Index (SI) for any research on herbal drugs is crucial for determining whether further work can be continued. SI can be defined as the ratio of the toxic concentration of a sample against its effective bioactive concentration. The ideal drug should have a relatively high toxic concentration but a very low active concentration [13, 14]. The acceptance criterion of SI ≥ 10 was recommended for the selected bioactive sample that is the potential to be further investigated [15, 16]. F8 showed the lowest IC_{50}, high CC_{50} values, and high SI amongst others. This data suggested that F8 was the potential fraction to be further studied to isolate the active antimalarial compounds.

Table 2. Cytotoxicity of extracts and fractions against several cell lines

Samples	Huh7		HepG2		BHK-21		Vero	
	CC_{50} (µg/mL)	SI	CC_{50} (µg/mL)	SI	CC_{50} (µg/mL)	SI	CC_{50} (µg/mL)	SI
n-Hexane extract	>100	4.17	>100	4.17	>100	4.17	>100	4.17
Dichloromethane extract	>100	36.76	>100	36.76	82.74 ± 0.02	30.42	90.00 ± 0.04	33.08
Metanol extract	>100	4.27	>100	4.27	>100	4.27	>100	4.27
F1	>100	3.55	>100	3.55	>100	3.55	>100	3.55
F2	>100	13.45	>100	13.45	>100	13.45	>100	13.45
F3	>100	35.21	93.98 ± 0.04	33.09	59.46 ± 0.04	20.94	58.72 ± 0.03	20.67
F4	>100	41.84	98.82 ± 0.03	41.34	73.99 ± 0.08	30.96	71.85 ± 0.05	30.06
F5	>100	42.02	>100	42.02	81.60 ± 0.02	34.28	86.70 ± 0.04	36.42
F6	77.02 ± 0.04	44.01	>100	57.14	68.79 ± 0.03	39.31	89.50 ± 0.02	51.14
F7	84.33 ± 0.05	42.81	93.98 ± 0.04	47.71	65.73 ± 0.06	33.36	90.95 ± 0.01	46.16

F8	>100	69.93	98.82 ± 0.03	69.10	96.46 ± 0.04	67.45	>100	69.93
F9	89.83 ± 0.03	36.08	>100	40.16	73.73 ± 0.003	29.61	78.72 ± 0.01	31.61
Chloroquine diphosphate	93.73±0.15	>100	>100	>100	90.52±0.24	>100	>100	>100

F1-F9 were fractions obtained from the separation of dichloromethane extract.
*Data represent Mean ± SD. SI = CC_{50}/IC_{50}

F8 was subjected to HPLC analysis using Shimadzu HPLC system. Reverse-phase analytical HPLC experiment was performed on Lichrospher RP-18 column (4.6 x 250 mm, 5 µm). A wavelength ultraviolet-visible detector was set at 254 nm. Gradient mobile phase acetonitrile-water 50%-50% to 80%-20% at flowrate 0.5 mL/min was used as eluent. The HPLC profile of F8 is shown in Figure 1. Several major peaks contained in F8 were observed based on the % area of peaks at Retention time (Rt) of 2.4 min, 3.9 min, 6.0 min, and 14.6 min. The assignment UV spectrum of those major peaks which represent several substances was shown in Figure 2. The UV spectrum exhibited absorption maxima at 282 nm, 276 nm, 255 nm, and 278 nm (band II), which were suggestive of a chalconoid structure [17]. Based on TLC and HPLC data, it could be suggested that F8 possibly contains polyphenolic compounds with a chalconoid structure as one of the active compounds which take a role in its antimalarial activity [18, 19].

This study was focused on the finding of antimalarial substances from A. sericicarpus leaves extracts based on the bioassay-guided separation and determining the cytotoxicity as well. The results showed several fractions which exhibited antimalarial activity and were nontoxic. The HPLC profile of the most active fraction (F8) was performed, but the identification of substances contained in F8 was not conducted yet. This study was also limited to the active fractions activity and cytotoxicity data. Therefore, further studies need to be conducted to isolate and identify the active antimalarial compounds from active fractions, especially F8.

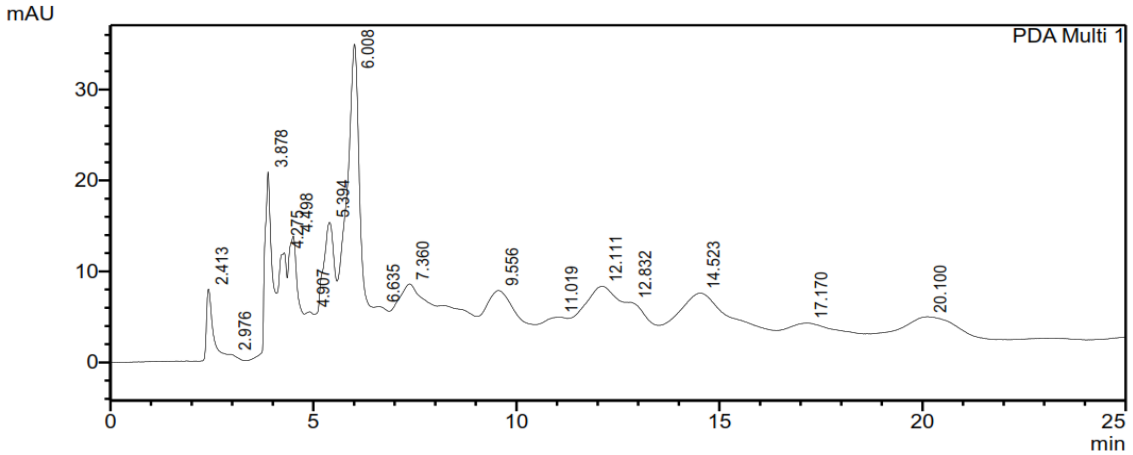

Figure 1. HPLC profile of F8. The analysis using Shimadzu HPLC system, Lichrospher RP-18 column (4.6 x 250 mm, 5 µm), gradient mobile phase acetonitrile-water 50%-50% to 80%-20%, flowrate 0.5 mL/min and observed at 254 nm.

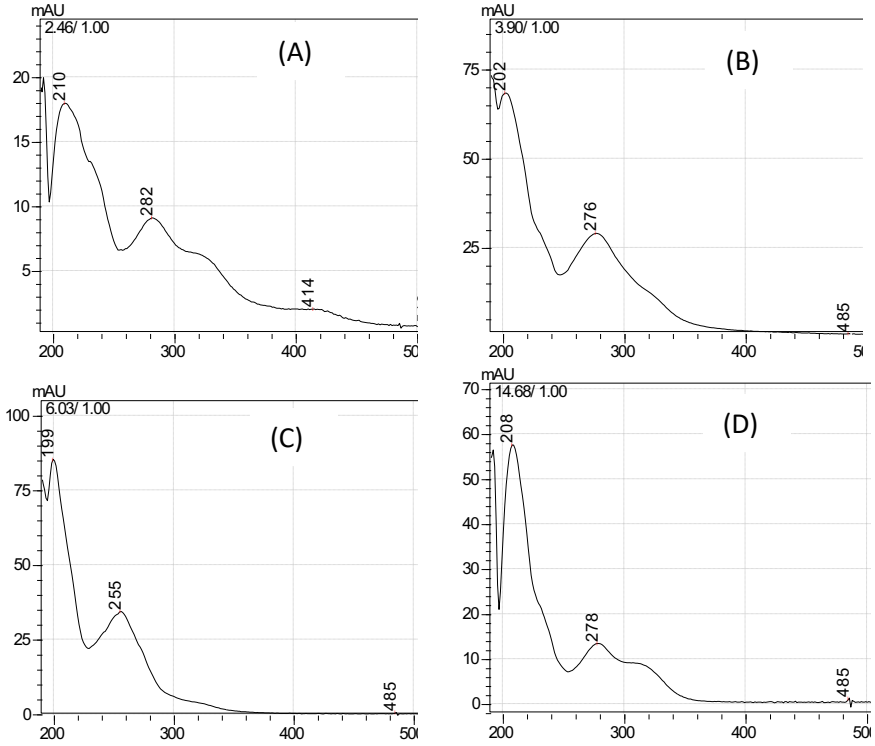

Figure 2. The UV spectra assignment of several major peaks contained in F8 was detected at Retention time (Rt) of 2.4 min (A), 3.9 min (B), 6.0 min (C), and 14.6 min (D). A chalconoid structure's characteristics were detected from absorption maxima at 282 nm, 276 nm, 255 nm, and 278 nm (band II).

3. CONCLUSION

The study results described that dichloromethane extract and eight fractions (F2-F9), of which the most active fraction is F8 of *Artocarpus sericicarpus* leaves contain antimalarial active substances so this plant becomes one of the potential sources of antimalarial compounds. In this study, the active compounds can be further isolated from the active fractions, which are possibly belonging to polyphenolic compounds.

4. MATERIALS AND METHODS

4.1 Plant material

Artocarpus sericicarpus leave was obtained from Balikpapan Botanical Garden in East Kalimantan, Indonesia. The plant was identified at Purwodadi Botanical Garden, East Java with determination letter No.0074 IPH.06/HM/XII/2015.

4.2 Extraction and fractionation methods

Artocarpus sericicarpus leaves were dried and ground into powder form. The powder (800 g) was extracted successively using n-hexane, dichloromethane, and methanol as solvents by ultrasonic-assisted extraction. The extract was then filtered and evaporated in vacuo to obtain dried extracts. All extracts were tested against *Plasmodium falciparum* 3D7 strain. The most active extract was fractionated by vacuum liquid chromatography (VLC) using silica gel as a stationary phase, gradient n-hexane-ethyl acetate (70%-30% to 60%-40% v/v), and chloroform-methanol (95%-5% to 50%-50% v/v) as mobile phase. The fractions were further tested for their antimalarial activity to determine the active fractions.

4.3 *Plasmodium falciparum* culture

P. falciparum 3D7 strain was cultured in 2% hematocrit type O human red blood cell (RBC) in RPMI-1640 (Gibco, ThermoFisher Scientific, Waltham, MA, USA), supplemented with 25 mM HEPES buffer, 2 g/L sodium hydrogen bicarbonate, 50 µg/mL hypoxanthine, 50 µg/mL gentamicin sulfate, and 0.5% (w/v) Albumax II (Gibco, ThermoFisher Scientific, Waltham, MA, USA) under 5% O_2, 90% N_2, and 5% CO_2 at 37 °C. Human RBCs were received from The Indonesian Red Cross. The medium was replaced daily and parasitemia was maintained below 5% for a routine subculture [20]. Parasitemia was determined by the examination of Giemsa's stained thin blood smears of infected erythrocytes. The percentage of parasitemia was determined by counting infected erythrocytes per 1000 total erythrocytes under a light microscope.

4.4 Antimalarial activity test by LDH assay

Antimalarial activity of extracts and fractions was determined by *lactate dehydrogenase* (LDH) assay against *P. falciparum* 3D7 strain. Parasite culture was synchronized with 5% (w/v) d-sorbitol as previously described [21]. Ring-stage parasites (100 µL/well) were placed in a 96-well plate at 0.3% parasitemia. The 50% growth inhibition concentrations of extracts and fractions were determined by the addition of 0.4 µL of serial dilutions of each sample to the wells. As a control, wells containing culture medium and RBCs alone were prepared. After 72 hours of incubation, parasite growth was determined by diaphorase-coupled lactate dehydrogenase (LDH) assay, as previously described [22]. The absorbance of each well was measured at 650 nm using Multiskan Skyhigh microplate spectrophotometer (Thermo Fisher Scientific, USA). The inhibition rate was calculated with the absorbance of uninfected wells defined as 100% inhibition. The IC_{50} values were analyzed and calculated with GraphPad PRISM 6.0 by applying the "log (inhibitor) vs. response – Variable slope (four parameters)" in the "Dose-response – inhibition" equation family.

4.5 Cytotoxicity test by Resazurin assay

To measure cell viability based on metabolic activity in mammalian culture and primary cells, resazurin can be reduced resorufin by NAD(P)H has been widely used [23]. The carcinoma cell (Huh7, HepG2) and normal cell (BHK-21, Vero) lines were performed on D-MEM (High Glucose) media with L-Glutamine and Phenol Red (Wako, Fujifilm) and $NaHCO_3$, supplemented with 10% (v/v) Fetal Bovine Serum (Gibco) and 1% (v/v) Penicillium streptomycin (Sigma) as an antibiotic. Plant extracts and fractions were diluted with DMEM media to obtain the serial concentration: 100; 50; 25; 25;12.5; 6.25 µg/mL and seeded in a micro-96 well plate. Following the extracts and fractions, the $3x10^4$, $1x10^4$, $5x10^3$, and $1x10^4$ cells were added into each well for Huh7, HepG2, BHK-21, and Vero cell lines, respectively, and incubated for 44 hours in a 5% CO_2 incubator at 37 °C. After 44h incubation, 10 µL of 0.5mM Resazurin was added to each well and incubated for 4h. After the completion of incubation, the fluorescence data were measured at wavelengths ex 530 nm and em 595 nm using a Nivo plate reader (PerkinElmer). The CC_{50} values were analyzed and calculated with GraphPad PRISM 6.0.

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