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Original Article

IN VITRO ANTIMALARIAL ACTIVITY SCREENING OF SEVERAL INDONESIAN PLANTS USING HRP2 ASSAY

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ABSTRACT

Objective: Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria disease control today. The spread of drug resistance and the limitation number of effective drugs for treatment indicated important to find a new antimalarial drugs. The aim of this study was to determine antimalarial activity several Indonesia plants.

Methods: Twenty leaves and stems of plants which were obtained from exploration from Alas Purwo National Park, East Java, Indonesia, were extracted with ethanol 80% by "maceration technique assisted ultrasonic". These 20 extracts were tested for in vitro antimalarial activity againt *P. falciparum* 3D7 strain (chloroquine-sensitive), using the histidine-rich protein II (HRP2) assay.

Results: Two leaves extracts were active as an antimalarial, *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) with the value of IC_{50} <14.8µg/mL and between 15.5-30.9 µg/mL respectively. These extracts contained rich chemical substances that considered for the antimalarial activity, such as terpenoids, polyphenol, flavonoids, and anthraquinone.

Conclusion: GFL and ASL were active as an antimalarial and potential to be developed as a new antimalarial drug.

Keywords: Antimalarial activity, *Plasmodium falciparum*, Indonesia plants, HRP2 in vitro antimalarial assay.

INTRODUCTION

Malaria was an infectious disease caused by protozoa parasites of the genus Plasmodium which were transmitted to humans during the bite of the female anopheles mosquito [1]. This disease was a major global public health concern. Annually, there were approximately 300 millions clinical cases and over one million of deaths worldwide due to malaria. Because a vaccine for malaria was not available, chemotherapy remain the main treatment [2,3]. However, new problem occured, it was the resistance of parasites to antimalarial drugs.

Resistance to antimalarial drugs increased the mortality rate associate with malaria [4]. The rationale studies, a new candidate drug based on the resistance of the parasites to conventional treatment as observed in the case of malaria [5]. History showed that the plant was a major source of drugs against malaria which has now developed into major malaria drugs throughout the world, namely quinine and artemisin, both of them were obtained from medicinal plants [6]. This fact was the reason that searching for antimalarial drugs from natural materials was important to do.

Indonesia was worldwide recognized as being the richest in the world in item of diversities and number of medicinal plants. It has 30.000 flowering plants, 7000 species of medicinal plants and 940 species have been identified of having medicinal properties. This tremendous potention needs to be explored and exploitated for the health and prosperity. Therefore, it was interesting to screen the antimalarial activity of medicinal plants.

Various methods have been developed to test antimalarial activity of drugs that allegedly sensitive to *P.falciparum* in vitro. One of them was the method of WHO microtest which routinely done in many laboratories, using morphological observation of *P. falciparum*. This method has high sensitivity, but high accuracy was required in microscopy observation to provide optimal observation took considerable experience [7]. This method required a lot of effort and takes a long time, especially if there were many samples.

Noedl have developed a new method for testing antimalarial activity in vitro, known as HRP2 measurement in a simple enzyme-linked immunosorbent assay (ELISA). HRP2 was naturally occurring histidine and alanine-rich protein localized in several cell compartments including the cytoplasm of *P. falciparum*. The amount of HRP2 found associated with the development and proliferation of the parasite and therefore was perfectly suited to reflect growth inhibition as a measure of drug susceptibility. HRP2 assay was more sensitive than any other in vitro anti malarial activity assay. This method required fewer technical tools. The implementation was easy and fast especially if done on many samples, and it was very suitable for screening the candidate of antimalarial drugs [8].

Antimalarial activity assay using HRP2 measurement has already done in some antimalarial drugs. Noedl *et al*. has conducted research on dihidroartemisin (DHA), meflokuin (MEF), quinine (QNN), and chloroquine (CHL) using fresh *P. falciparum* culture. The result, HRP2 measurement assay has a proximity test results to assay of modified WHO schizont maturation (R^2=0.96, P<0.001; IC_{50}=0.054) [8]. Antimalarial activity test was also conducted on artesunate (AS) and dihidroartemisin (DHA) by Rutvisuttinunt *et al*. in 2012 [7].

Therefore, this research aims to determine antimalarial activity of some Indonesian plants, obtained from exploration in Alas Purwo National Park, Banyuwangi, East Java using in vitro antimalarial ELISA-HRP2 assay.

MATERIALS AND METHODS

Plant Material and Extraction

Twenty samples (ten stems and ten leaves of plants) were collected from Alas PurwoNational Park, Banyuwangi, East Java Indonesia. Authentication and identification of plant were carried out at the Purwodadi Botanical Garden, East Java. For each plant, 50 g of powdered materials were extracted using 80% ethanol by maceration technique assisted ultrasonic for two minutes to three times replication. The ethanol extracts were filtered, pooled, dried at

40°C using a rotary evaporator and weighed afterwards. All the extracts were kept in air tight containers and were stored at 4°C for use in phytochemical screening and antimalarial bioassay.

Phytochemical screening

Dried extract (10 mg) was diluted in methanol. The phytochemical screening was performed by thin layer chromatography (TLC) method to determine the content of the group of chemical compounds of each extract, such as alkaloid, terpenoids, polyphenols, flavonoids, and anthraquinones using certain optimized mobile phase and particular reagent (dragendorf for alkaloids, anisaldehide-sulphuric acid for terpenoids, $FeCl_3$ for polyphenols, 10% sulphuric acid for flavonoids, and 10% potassium hydroxide in methanol for anthraquionones).

Parasite culture

Plasmodium falciparum 3D7 strain, were obtanined from Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, 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 NaHCO3, 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 [9, 10]. The culture was routinely monitored through Geimsa staining of the thin blood smears. For 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 [8].

Antimalarial Assay and analysis

Antimalarial activity assay has been done using HRP2 (HRP2 Kit Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). Single concentration of extract (average concentration 1000 µg/mL) was used for antimalarial screening assayed. 100 µL diluted extract solution and 100 µL of final parasite culture was added into the microplate. 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 hemolyzed culture samples was transferred to the ELISA plates, which were precoated with monoclonal antibodies against *P. falciparum* HRP2 and the plates were incubated at room temperature for 1 h in humidified chamber. The plates were washed five times with the washing solution (200 µL of each well) and 100 microliters of the diluted antibody conjugate was added to each well. After incubation for an additional 1 h in humidified chamber, the plates washed with washing solution (200 µL of each well) and 100 µL of diluted (1:20) chromogen TMB (tetramethyl benzidine) 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 read with an ELISA plate reader at an absorbance maximum of 450 nm [11]. Inhibition percentage was calculated using the following formula:

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

The extract with highest inhibition percentage tested again to determine the Inhibitory concentration $IC_{50)}$ values using serial concentration of 1000 µg/mL to 15 µg/mL. The IC_{50} values were determined graphically on dose-response curves (concentration versus percent inhibition curves) with non-linear analysis by SPSS probit. This activity was analysed in accordance with the norm of plants antimalarial activity of Chinchilla *et al* in 2012 [12]. According to this norm, strong active, active, weakly active, and inactive extract has IC_{50} < 5 µg/ml; IC_{50} 5 – 50 µg/ml; 50 µg/ml < IC_{50} <100 µg and IC_{50} > 100 µg/ml, respectively.

RESULTS AND DISCUSSION

Several authors have pursued the search for natural products with antimalarial effect in plants in the past and in recent years [6,13,14,15,16]. This study was also conducted to identify phytochemical constituent and investigate antimalarial activity of several plant obtained from exploration at Alas Purwo National Park, Banyuwangi, Indonesia.

The result of the phytochemical screening of 20 extracts indicated terpenoids were present in all extracts. Alkaloids was detected in three extracts, such as in *Ochrosia akkeringae* leaves (OAL), *Ochrosia akkeringae* stem (OAS) and *Tabernaemontana pandacaqui* stem (TPS) extract. The results summarized in Table 1. Very deep black coloration was observed in *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) extract. It was indicated that polyphenols were highly present in the leaves. A slight black coloration was observed in *Garuga floribunda* stem (GFS) and *Alectryon serratus* stem (ASS) extract and *Lepisanthes rubiginosum* leaves (LRL) and *Harpulia arborea* leaves (HAL) extracts which indicative that polyphenols was slightly present. Most of extracts contains of flavonoids, showed by yellow coloration spot and also anthraquinones showed by yellow or brownish yellow coloration.

Table 1: Phytochemical screening of plant extracts

Botanical name	Parts	Alkaloids	Terpenoids	Polyphenols	Flavonoids	Anthraquinones
Mitrephora polypyrena	Leaves	-	+	-	-	-
Mitrephora polypyrena	Stems	-	+	-	-	+
Lepisanthes rubiginoosum	Leaves	-	+	+	+	+
Lepisanthes rubiginoosum	Stems	-	+	-	-	-
Harpullia arborea	Leaves	-	+	+	+	+
Harpullia arborea	Stems	-	+	-	+	-
Garuga floribunda	Leaves	-	+	+	+	+
Garuga floribunda	Stems	-	+	+	+	+
Alectryon serratus	Leaves	-	+	+	+	+
Alectryon serratus	Stems	-	+	+	-	+
Ochrosia akkeringae	Leaves	+	+	-	+	+
Ochrosia akkeringae	Stems	+	+	-	+	+
Tabernaemontana pandacaqi	Leaves	-	+	-	+	+
Tabernaemontana pandacaqi	Stems	+	+	-	-	+
Diospyros javanica	Leaves	-	+	-	+	+
Diospyros javanica	Stems	-	+	-	-	+
Barringtonia aciatica	Leaves	-	+	-	-	-
Barringtonia aciatica	Stems	-	+	-	-	-
Dysoxylum gadichaudianum	Leaves	-	+	-	-	-
Dysoxylum gadichaudianum	Stems	-	+	-	-	-

* + : present; - : not present

Twenty of extracts plant, belonging to several families, screened for their potential antimalarial properties against cloroquine-sensitive *P. falciparum*3D7 strain using HRP2 assay. Three extracts of *Lepisanthes rubriginosum* stem (LRS), *Garuga floribunda* leaves (GFL) and *Alectryon serratus* leaves (ASL) have the highest activities, due to its higher inhibition percentage (92.4% ± 0.4%; 86.2% ± 0.8%; 88.1% ± 0.9% respectively). The results summarized in table 2. This result become a basis to the next assay to determine antimalarial activity of the potential extracts using IC_{50} as a parameter of activity.

The IC_{50} values from these three extract showed that LRS have the lower IC_{50} value than the other two (table 3). Criteria of antimalaria activity in vitro from Chinchilla *et al*. 2012, showed that LRS not active as an antimalaria, GFL and ASL active as antimalaria. IC_{50} values of the three extracts also indicated concentration of the extract did not have a linear relationship with the inhibition percentage, but rather to follow the curve of the sigmoid function [20]. The result of activity have remained constant at a certain concentration, proved by increasing the concentration of GFL, from 14.8 µg/mL to 475.0 µg/mL did not yield a significant increasing in inhibition percentage against *P. falciparum* growth, as well as increasing concentration of ASL, at concentration from 30.9 µg/mL to 495.0 µg/mL, which provided a range of percent inhibition remained, between 70% - 80%. Chemical substances of GFL and ASL as active extract were considered to take effect in antimalarial activity, such as terpenoids, polyphenol, flavonoids, and anthraquinone.

Table 2: Antimalarial activities of some Indonesian plants tested in this study againts *Plasmodium falciparum*

Botanical name	Parts	Family	%Inhibition*
Mitrephora polypyrena	Leaves	Annonaceae	58.5% ± 0.4%
Mitrephora polypyrena	Stems	Annonaceae	17.1% ± 10.4%
Lepisanthes rubiginoosum	Leaves	Sapindaceae	48.5% ± 2.4%
Lepisanthes rubiginoosum	Stems	Sapindaceae	**92.4% ± 0.4%**
Harpullia arborea	Leaves	Sapindaceae	53.8% ± 2.3%
Harpullia arborea	Stems	Sapindaceae	36.3% ± 0.1%
Garuga floribunda	Leaves	Burseraceae	**86.2% ± 0.8%**
Garuga floribunda	Stems	Burseraceae	67.6% ± 5.2%
Alectryon serratus	Leaves	Sapindaceae	**88.1% ± 0.9%**
Alectryon serratus	Stems	Sapindaceae	49.4% ± 2.8%
Ochrosia akkeringae	Leaves	Apocynaceae	26.0% ± 1.7%
Ochrosia akkeringae	Stems	Apocynaceae	14.8% ± 3.7%
Tabernaemontana pandacaqi	Leaves	Apocynaceae	38.6% ± 5.6%
Tabernaemontana pandacaqi	Stems	Apocynaceae	37.6% ± 4.0%
Diospyros javanica	Leaves	Ebenaceae	50.8% ± 12.9%
Diospyros javanica	Stems	Ebenaceae	51.3% ± 0.8%
Barringtonia aciatica	Leaves	Lecythidaceae	48.7% ± 4.4%
Barringtonia aciatica	Stems	Lecythidaceae	53.0% ± 0.4%
Dysoxylum gadichaudianum	Leaves	Meliaceae	40.1% ± 5.2%
Dysoxylum gadichaudianum	Stems	Meliaceae	27.4% ± 0.8%

*Percentage (%) Inhibition at the concentration of 1000 µg/ml

Table 3: Antimalarial activities (IC_{50}) againts *P. falciparum* of ethanol extract of *Lepisanthes rubiginosum* stem (LRS), *Garuga floribunda* leaves (GFL), and *Alectryon serratus* leaves (ASL)

***L. rubiginosum* stem (LRS)**		***G. floribunda* leaves (GFL)**		***A. serratus* leaves (ASL)**	
Concentration	**%Inhibition**	**Concentration**	**%Inhibition**	**Concentration**	**%Inhibition**
0.0	0.0%	0.0	0.0%	0.0	0.0%
15.0	4.1% ± 0.1%	14.8	76.3% ± 2.4%	15.5	39.2% ± 16.4%
30.0	2.8% ± 1.3%	29.7	74.0% ± 0.1%	30.9	76.8% ± 0.6%
60.0	3.0% ± 0.3%	59.4	75.7% ± 0.3%	61.9	76.4% ± 1.8%
120.0	12.2% ± 6.4%	118.8	76.3% ± 2.2%	123.8	79.0% ± 2.4%
240.0	34.8% ± 3.7%	237.5	79.1% ± 0.4%	247.5	81.6% ± 3.2%
480.0	88.8% ± 1.1%	475.0	80.6% ± 0.4%	495.0	84.8% ± 1.4%
960.0	94.3% ± 1.1%	950.0	91.2% ± 2.2%	990.0	95.6% ± 0.1%
IC_{50}	**252.2**		**< 14.8**		**12.3**

*Concentration and IC_{50} is in µg/mL

Terpenoids have an important role in producing antimalarial activity, proved by inhibition percentage of DJS and BAS which only contained terpenoids, however, result in inhibiton percentage greater than 50%. Terpenoids as an antimalarial acts by inhibiting the growth phase of the plasmodium parasite from ring to trophozoites and inhibited nutrient intake by the parasite by inhibiting the permeation pathway [21]. Inhibition percentage of DJS and BAS also showed that high inhibition percentage should not be produced by a variety of chemical compounds content. The presence of one class of compounds alone could generate activity. Other evidence appeared on the leaves and stem extracts of *Ochrosia akkeringae*, which contained almost all of chemical compounds, but had the lowest value of inhibition percentage, which was 26.0% ± 1.7% and 14.8% ± 3.7%.

Alkaloids compounds were have great potential antimalarial activity in some parts of Apocynaceae plants [15]. In this study, Apocynaceae plants (*Ochrosia akkeringae* and *Tabernaemontana pandacaqui*) had known containing alkaloids, except TPL. The content of alkaloids from two extracts plants indicated the potential antimalarial activity From the results of screening of antimalarial activity in both leaves and stems extract, it has known that *Ochrosia akkeringae* have potential antimalarial activity with relatively low inhibition percentage (26.0 % ± 1.7 % for the leaf extract and 14.8 % ± 3.7 % for the stem extract). While *Tabernaemontana pandacaqui* have a higher potential activity with inhibition percentage are 38.6 % ± 5.6 % in TPL and 37.6 % ± 4.0% in TPS. The low activity in both plants thought to be in consequence of alkaloids did not work against *P. falciparum*. Therefore, it required special extraction of alkaloids (ie,

chloroform extraction) in order to dissolve and force alkaloid producing antimalarial activity.

Flavonoids was thought playing an important role in generating antimalarial activity. The proof was the inhibition percentage of extract containing flavonoids higher, especially in ASL. ASL contains flavonoids and has inhibition percentage 88.1% ± 0.9%, whereas in the stem extract contained no flavonoids yield the inhibition percentage 49.4% ± 2.8%. Flavonoids worked by inhibiting the influx of L-glutamine and myoinositol into infected red blood cells [21].

Diospyrin, a quinone derivative compounds acts producing antimalarial activity of some plants from a genus Diospyros, Ebenaceae [22]. Extracts from the leaves and stems of *Diospyros javanica* which was containing anthraquinone, thereby potentially having antimalarial activity. Screening of the antimalarial activity of this plant extract showed antimalarial activity higher than *Ochrosia akkeringae* and *Tabernaemontana pandacaqui*, indicated the potential of anthraquinone in producing antimalarial activity. Anthraquinone could kill the parasite through various mechanisms, resulting from aldehyde at C-2 [21].

CONCLUSION

Screening of antimalaria activity with HRP2 from some Indonesian plants showed varying results, the highest yield was found in extracts of *Garuga floribunda* leaves and *Alectryon serratus* leaves. Both were rich of chemicals substances, such as terpenoids, polyphenol, flavonoids and anthraquinone. It concluded that *Garuga floribunda* and *Alectryon serratus* plants were contain antimalarial active substances that potential to be developed as a new antimalarial drugs.

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