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Original Article

IN VITRO ANTIPLASMODIAL ACTIVITYOF ETHANOL EXTRACTS OF BORNEO MEDICINAL PLANTS (*HYDROLEASPINOSA, AMPELOCISSUSRUBIGINOSA, URARIA CRINITE, ANGIOPTERISEVECTA*)

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ABSTRACT

Objective: The efforts to obtain new antimalarial medicine were among others to explore and to develop both natural substances and synthetic compounds whose activities have been established. An *in vitro* antiplasmodial activity test was carried out to several plants from Borneo to find out IC_{50} value in the effort to explore new antimalarial plants.

Methods: Dried materials of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinita* roots and *A. avecta* tubers were extracted using 96% ethanol with maceration method. An *in vitro* antiplasmodial activity test was carried out to the resulting ethanol extracts of the four plants using candle jar method. The resulting data were processed using probit analysis to give the IC_{50} value.

Results: The results of the study included parasitemia percentage, *P. falciparum* growth inhibition percentage and IC_{50} value. At low concentration (1 µg/ml) of the four tested materials gave the parasitemia percentage of *H. spinosa*, *A. rubiginosa*, *U. crinita* and *A. evecta* of 10.89±1.29%, 10.58±0.67%, 11.20±0.86%and 8.32±2.44 %, respectively. Meanwhile, *P. falciparum* growth inhibition percentage were 34.68±8.13%, 33.64±4.23%, 11.83±3.42%and 47.83±3.24 %, respectively. And, the biggest *P. falciparum* percentage of the four plants was 47.83±3.24% that took place to*A. evecta*. The IC_{50} values of the ethanol extract of the*H. spinosa,* the *A. rubiginosa,* the *U. crinita* and the *A. evecta*were more than 250, 9.38±8.26, 25.48±3.10, and 2.86±0.27µg/ml, respectively.

Conclusion: The *in vitro* antiplasmodial activity of the four ethanol extracts indicated that the *H. spinosa* leaves were classified to be inactive, the *A. rubiginosa* tubers and the *U. crinita* tubers were active and the *A. evecta* tubers were very active.

Keywords: *Hydroleaspinosa, Ampelocissusrubiginosa, Uraria crinite, Angiopterisevecta, In vitro*, Antiplasmodial, Malarial, Borneo.

INTRODUCTION

Anopheles mosquito has been resistant to insecticides as DDT that it caused the increase in the number of malaria cases in some tropical countries [1, 2]. The efforts to find new alternative antimalarial medicine have not essentially solved the problem of controlling malaria because of the presence of resistance to medicines [3]. The resistance of *Plasmodium* to artemisinin has been reported in Cambodia-Thailand [4, 5]. The resistance to chloroquine and sulfadoxyne-pyrimethamine spread rapidly and it gave the fig. of the possibility of the spread of the resistance to the artemisinin. It posed serious threat especially in the situation in which there was not any effective alternative for the resistance. The presence of the resistance of the *Plasmodium* to antimalarial medicine resulted in medicinal treatment failure. Therefore, the availability of new antimalarial medicine was very urgent in overcoming the resistance. Various strategies have been implemented by researchers in finding and in developing new antimalarial medicine, for example, by exploring and developing natural substance and by synthesizing compounds to improve the antispasmodic activity and to suppress the side effect. The antimalarial medicine must have high sensitivity as indicated *in vitro* by low IC_{50} value (i.e., inhibition concentration 50%), high effectiveness, safety, and ease in obtaining it and affordable [6].

The selection of the natural substance in an exploration was usually based on empirical use of the substance by people in treating the malaria. There were in Borneo several plants used by local people to treat it. Jeruju (*Hydroleaspinosa*L.) leaves were used by Banjar tribesmen in South Borneo to treat it. Cawathanoman (*Urariacrinita*) roots were used in Buntok area of Central Borneo. Tawas ut (*Ampelocissusrubiginosa* L.) tubers and hatitanah (*Angopterisevecta*) were used by Palangkaraya people of Central Borneo. Khan & Omosolo's [7] study reported that petroleum, dichloromethane, acetic ethyl, butanol and methanol extracts of the leaves, the stem bark and the roots of the *A. evecta* had wide spectrume antibacterial activity. The leaves and the roots of the *A. evecta* also had antifungal activity [8]. The methanol extracts of the roots of the *U. crinita* has proven to have antioxidant activity [9]. Though the plants have been empirically used to treat the malaria, there has not been any publication of the study of *in vitro* anti plasmodial activity. Therefore, the recent study gave scientific data and verified the biological activity of the ethanol extracts of the leaves of the *H. spinosa,* the tubers of the *A. rubiginosa,* the roots of the *U. crinita* and the tubers of the *A. evecta.* It was different from prior studies because it tested the *in vitro* antiplasmodial activity of the ethanol extract of*H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinita* roots and *A. avecta* tubers to give the IC_{50} value. The IC_{50} value served ad the standard in categorizing the *in vitro* anti plasmodial activity of the ethanol extract of each of the plants.

MATERIALS AND METHODS

Materials

The materials used were 96% ethanol (Bratacem), RPMI (Sigm), 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid (HEPES) (Sigma), $NaHCO_3$(Sigma), NaCl (Bratacem), dextrose (Bratacem), red blood cell (RBC) and serum of human blood to test the *in vitro* antiplasmodial activity.

Research subjects

The parts of the plants used in the study were the *H. spinosa* leaves, the *A. rubiginosa* tubers, the *U. crinata* roots, and the *A. evecta* tubers that was collected from Borneo. The used parasites were *P. falciparum strain* FCR3 obtained from the Pharmacology Laboratory of the Faculty of Medicine of Gadjah Mada University, Yogyakarta.

Plant determination and ethical clearance

The determination of the plants *H. spinosa, U. crinite, A. rubiginosa,* and *A. evecta* was conducted inJanuary 2012 in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science Bogor. The written evidence was maintained in the Study Program of Pharmacy of the Faculty of Mathematics and Natural Sciences of Lambung Mangkurat University of Borneo Province of the Republic of Indonesia numbered 1265/IPH.1.02/If.8/X/2012. Ethical clearance was issued by the ethical clearance committee of the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine of Gadjah Mada University with the approval number KE/FK/1029/EC. The Medical and Health Research Ethics Committee were pursuant to Helsinki Declaration 2008.

Extraction

The collected parts of the plants were washed using tap water, chopped into small pieces and dried in oven at 50°C. The simplisia were then grinded into powder and sifted using the fine sieve of 4/18. Dried powder of the leaves of the *H. spinosa,* the tubers of the *A. rubiginosa,* the stems of the *A. evecta* and the roots of the *U. crinita* was macerated using 96% ethanol for 24 h. The resulting filtrate was evaporated to obtain thick ethanol extract.

***In vitro* antiplasmodial activity test**

The *in vitro* antiplasmodial activity test was carried out to the ethanol extract of the leaves of the *H. spinosa,* the tubers of the *A. rubiginosa,* the roots of the *U. crinita,* and the tubers of the *A. evecta.* It was carried out using candle jar method [10]. Cryo tube containing the *P. falciparum* that was taken from liquit N_2 container was moved into conical tube and added 200 µl of 12% natrium cloridesolution drop by drop, left idly for 2 minutes and added 10 ml of 1.6% natrium cloridesolution. The solution was centrifuged at 7000 rpm for 10 minutes. Its supernatant was removed and added 10 ml 0.2% dextrose and 0.9% natrium cloridesolutions and then re-centrifuged. Again, its supernatant was removed and the residue was moved into the culture flask, added RPMI, serum and RBC. The culture flask was then capped and maintained in the candle jar in the standing position. The candle was enkindled and the candle jar was tightly closed and maintained in an incubator at 37 °C for 48 h. The culture was maintained by replacing the media every 24 h. If the parasitemia was too high (i.e., more than 10%), subculture was prepared by adding red blood cells so that it becomes lower. If the *Plasmodium* culture has grown and reached more than 2%, the test might be carried out by doing synchronization.

Subsequently, the material was weighted, added 100 µl DMSO and 900 µl RPMI solutions and sterilized by filtering it using 0.20 µm membrane filter. Concentration ranks of the materials were then made, viz. 250, 50, 25, 10 and 0.5 µg/ml. Chloroquine was used as positive control and its concentration ranks were made, viz. 40, 20, 16, 12, 8 and 4µg/ml. The testing material, negative control (i.e., RPMI media) and 100 µl chloroquine were put into microplate (with 96 wells) and added 100 µl of *Plasmodium* resulting from the synchronization. The microplate was put into the candle jar, incubated at 37 °C for 72 h. Once the incubation period has completed, the microplate was taken out of the candle jar and harvesting was done by moving the mixture of each of the wells into micro tube. Subsequently, it was centrifuged and its supernatant was removed. The residue was used to prepare smears on glass object. Once the smears have dried, they were fixed using methanol. The dried smears were painted using 5% Giemsa paint, left idly for 30 minutes and washed using tap water. They were aerated till completely dry and then added emersion oil. The number of erythrocytes and parasitemia of the smears might be clearly observed and calculated under microscope. The percentage of the parasitemia was calculated by comparing the number of the infected erythrocyte of about 1000 erythrocytes, by employing the formula below:

$$\%Parasitaemia = \frac{\sum InfectedErythrocites}{\sum Erythrocites} x100\%$$

The data of the parasitemia percentage of each test was compared to that of the negative control that parasite growth inhibition percentage (%) was obtained, with the formula:

$$\%Inhibition = \frac{ParasitaemiaNegativeControl - ParasitaemiaTest}{\sum Erythrocites} x100\%$$

The resulting data was presented in the form of correlation curve between the testing compound concentration and the parasite growth inhibition percentage. The IC_{50} was determined using probit analysis of the inhibition percentage with testing concentration logarithm. The resulting IC_{50} data were classified on the criteria by Jennet-Siems*et al.* [11] that the extract and the fraction of the medicinial plants were considered to have no antiplasmodial activity if the IC_{50} value was more than 50 µg/ml.

Statistical analysis

The *in vitro* antiplasmodial activity test were executed in triplicate and the data is presented as mean±SEM in the results. The IC_{50}value was determined by ProbitAnalysis(95% confidence interval) on the percent inhibition with the logarithm of test concentration using SPSS version 16 for windows.

RESULTS AND DISCUSSION

Extract

The extraction results of the four medicinal plants were ethanol extract rendements. The ethanol extract rendement resulting from the leaves of the *H. spinosa* was 22.85%, that resulting from the tubers of the *A. rubiginosa* was 23.30%, that resulting from the roots of the *U. crinite* was 1.79% and that resulting from the *A. evecta* was 25.01%. The comparison results of the rendements of the four medicinal plants showed that the ethanol extract rendement of the roots of the *U. crinita* was the lowest. It indicated that there was only small part of the chemical content of the roots of the plant that dissolved in ethanol solution. It indicated that there was bigger part of the chemical content of the roots of the *U. crinita* that dissolved in more polar solvent (i.e., aqua destillata) than ethanol. Concerning with the polarity of the solvent, solvents of different polarity would dissolve compounds at different levels [12].

***In vitro* antiplasmodial activity**

The results of the *in vitro* antiplasmodial activity test of the ethanol extract of the leaves of the *H. spinosa,* the tubers of the *A. rubiginosa,* the tubers of the *A. evecta,* the roots of the *U. crinita* and chloroquine in triplet manner included mean parasitemia percentage, inhibition and IC_{50} value as summarized in table 1. It was clearly observed in the table that the parasitemia percentage was inversely proportional to the *Plasmodium* growth inhibition percentage. Increasingly bigger parasitemia percentages resulted from the low to the high concentration of each of the testing materials, while on the contrary the *Plasmodium* growth inhibition percentages were increasingly smaller.

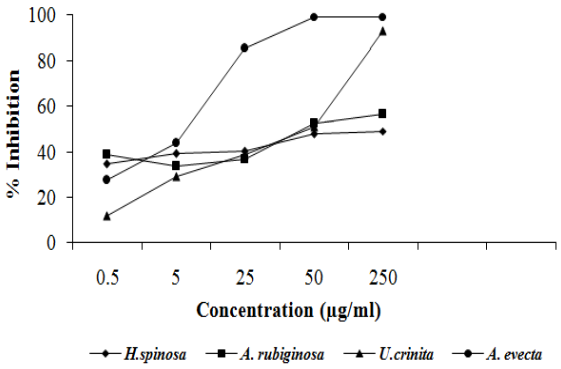

Fig. 1: The relationship between the concentration of extracts and the percentage of *P. falciparum* culture inhibition after the incubation for 72 h

The lowest concentrations (5 µg/ml) of the ethanol extract of the leaves of the *H. rubiginosa,* the roots of the *U. crinite* and the

tubers of the *A. evecta* were able to respectively inhibit 39.29±2.11%, 36.71±8.83%, 28.81±4.85%, and 66.15±4.33% of the *Plasmodium* growth. The comparison of the *Plasmodium* growth inhibitions of the four ethanol extracts of the testing materials showed that the ethanol extracts of the *A. evecta* at the concentration of 5 µg/ml has been able to inhibit more than 50% of the *Plasmodium* growth. It meant that it had the stronger inhibition of the *Plasmodium* growth than that of the ethanol extracts of the leaves of the *H. spinosa*, the tuber of the *A. rubiginosa*, and the roots of the *U. crinita* (fig. 1).

Table 1: The percentages of parasitaemia, of *Plasmodium* growth inhibition and IC_{50} values of the addition of ethanolic extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinite* roots, *A. evecta* tubers, Chloroquine on *P. Falciparum* (FCR3 strain) with incubation duration of 72 h

Ethanolic extracts	Concentration(µg/ml)	Parasitaemia±SD (%)	Inhibition±SD (%)	IC_{50}value±SD(µg/ml)
H. spinosa leaves	250	8.13±0.68	49.01±4.27	more than 250
	50	8.30±0.58	47.93±3.63	
	25	8.68±0.19	40.27±5.82	
	5	8.70±0.34	39.29±2.11	
	0.5	10.89±1.29	34.68±8.13	
A. rubiginosa tubers	250	0.07±0.07	99.56±0.41	9.38±8.26
	50	6.09±0.76	56.41±4.74	
	25	7.61±0.88	52.27±5.53	
	5	10.09±1.41	36.71±8.83	
	0.5	10.58±0.67	33.64±4.23	
U. crinite roots	250	1.13±0.45	92.89±2.80	25.48±3.10
	50	8.04±0.64	50.89±4.02	
	25	9.78±1.08	38.66±6.82	
	5	10.89±0.77	28.81±4.85	
	0.5	11.20±0.86	11.83±5.42	
A. evecta tubers	250	0.00±0.00	100±0.00	2.86±0.27
	50	0.00±0.00	100±0.00	
	25	1.28±0.16	91.99±1.03	
	5	5.40±0.69	66.15±4.33	
	0.5	8.32±2.44	47.83±8.23	
Chloroquine	$40x10^{-3}$	0.00±0.00	100±0	$4.81x10^{-3}$±0.10
	$20x10^{-3}$	0.04±0.08	99.45±0.95	
	$16x10^{-3}$	0.16±0.28	98.02±3.43	
	$8x10^{-3}$	0.21±0.29	97.48±3.51	
	$6x10^{-3}$	0.30±0.34	96.26±1.22	
	$4x10^{-3}$	5.96±0.53	26.61±2.56	
Negative control	200 µl	15.94±0.50	0.00±0.00	

The antiplasmodial activity of the ethanol extracts of the leaves of the *H. spinosa,* the tubers of the *A. rubiginosa,* the roots of the *U. crinite,* and the tubers of the *A. evecta* was considered to be at the inhibition concentration of 50% (IC_{50}), which were calculated using probit analysis. The probit analysis examined the correlation between the concentration of the testing materials and the *P. falciparum* growth inhibition percentage. The *in vitro* antiplasmodial activity was inversely proportional to the IC_{50} value. It meant that the smaller was the IC_{50} value, the bigger the *in vitro* antiplasmodial activity would be. The IC_{50} values of the ethanol extract of *H. spinosa* leaves, *A. rubiginos* tubers, *U. crinite* roots and *A. avecta* tubers and the chloroquine were more than 250, 9.38±8.26, 25.48±3.10, 2.86±0.27 and $4.809x10^{-3}$±0.10µg/ml, respectively. The resulting IC_{50} values were statistically analyzed using *Anova* to find out the significant difference in the antiplasmodial activity among the ethanol extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinite* roots and *A. avecta* tubers.

The results of the statistical test of the IC_{50} values of the ethanol extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinite* roots and *A. avecta* tubers and the chloroquine showed that there was significant difference in the *in vitro* antiplasmodial activity among the four testing materials. The statistical test was followed by post hoc test to find out the difference among extract groups. The results of the post hoc test showed that there was significant difference among the ethanol extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinita* roots and *A. avecta* tubers and the chloroquine. It indicated that there was significant difference in the IC_{50}values of the four ethanol extracts so that the *in vitro* antiplasmodial activity of each of the extracts was different. Fig. 2 summarized the IC_{50} values of the use of the ethanol extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinita* roots and *A. avecta* tubers for the *P. falciparum* strain FCR3 of the incubation period of 72 h.

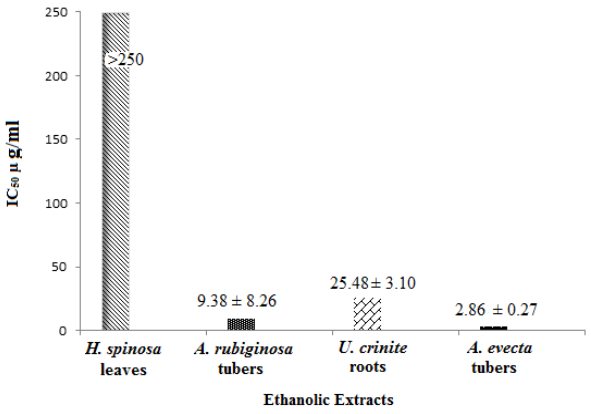

Fig. 2: The *in vitro* antiplasmodial activity (IC_{50}±SD µg/ml) of the ethanol extracts of *H. spinosa* leaves*; A. rubiginosa* rubers*; U. crinite* roots*; A. evecta* rubers, chloroquine in *P. Falciparum* FCR3 strain culture after the incubation for 72 h

The *in vitro* antiplasmodial activity of the ethanol extracts of *H. spinosa* leaves, *A. rubiginosa* tubers, *U. crinita* roots and *A. avecta* tubers was categorized on the basis of the resulting IC_{50} values. It was considered to be very active or very potent if it had the IC_{50}value less than 5 µg/ml [13-16], active if the IC_{50}value was in the range of 5-50 µg/ml [15, 16], less active if the IC_{50}value was in the range of 50-100 µg/ml and inactive if the IC_{50}value more than 100 µg/ml [16]. Based on the discussion above the *in vitro* antiplasmodial activity of the ethanol extracts of the *A. evecta* was classified to be very active, that of the *A. rubiginosa* and the *U. crinite* was classified to be active, and that of the *H. spinosa* was classified to

be inactive. It was necessary to compare the *in vitro* antiplasmodial activity of the ethanol extract of the *A. evecta* tubers and the positive control to find out how potential the *in vitro* antiplasmodial activity was. Therefore, sensitive chloroquine was used as comparator. The IC_{50} value of the chloroquine was±4.820 ng/ml. The value was categorized to be sensitive (IC_{50}more than 100 ng/ml) [17]. If the IC_{50} values of the chloriquine and the ethanol extract of the *A. evecta* tubers were compared, they were very different, but there *in vitro* antiplasmodial activity was categorized to be the same, which was very active. However, they might have the significant difference because of the difference in the working mechanism of the medicine and other influencing factors that must be scientifically proven in other studies.

CONCLUSION

The *in vitro* anti plasmodial activities of the four ethanol extracts was as follows: that of the *H. spinosa* leaves (IC_{50}more than 250 µg/ml) were categorized to be inactive, that of the *A. rubiginosa* tubers (IC_{50} = 9.38±8.26 µg/ml) and the *U. crinite*roots (IC_{50} =25.48±3.10µg/ml)was categorized to be active, and that of the *A. evecta* tubers (IC_{50} = 2.86±0.27 µg/ml) was categorized to be very active.

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CONFLICT OF INTERESTS

Declared None

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