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Development of The Stem of *Jatropha multifida* Linnas A New Antimalarial through Erythrocytes Test on *Mus musculus* Infected by *Plasmodium berghei*

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ABSTRACT

Introduction : *Jatropha multifida* Linne is known as Betadin plant by the Bengkulu. The stem extract of *J. multifida* has antibacterial activity. This study examined the potential of stem extract of *J. multifida* to be developed as an anti-malarial drugs through trials in *M. musculus* whose erythrocytes infected by *Plasmodium berghei*.

Methods : Phytochemical test of the stem of *J. multifida* to test the flavonoids, alkaloids, tannins, saponins, terpenoids and steroids. The stem extract of *J. multifida* obtained by maceration with 96% ethanol. Thirty *M. musculus* equally divided in 6 groups. P0 was the negative control group that was not infected by *P berghei*. P1 was the positive control group that was only infected by *P berghei*. P2 was a group that was infected with *P berghei* and chloroquine. P3, P4, and P5 groups were infected by *P berghei* and treated with stem extract of *J multifida* in dose of 0.028 g / kgbw, 0.056 g / kgbw and 0.084 g / kgbw, respectively. After 24 hours, the number of erythrocytes was observed using hemocytometer under a microscope at 1000x magnification in which the number of uninfected erythrocytes by *P berghei* was counted.

Results : The stem extract of *J. multifida* at doses of 0.028 g/kgbw, 0.056 g/kgbw and 0.084 g/kgbw were able to increase the number of *P berghei*-uninfected erythrocytes an average of 9.135 million cell eritrosit/mm³, 7.618 million cell eritrosit/mm³, and 9.856 million cell eritrosit/mm³, respectively. The ability of stem extract of *J. multifida* in increasing the number of erythrocytes uninfected with *P berghei* was much higher than the malaria drug chloroquine diphosphat. On one way ANOVA analysis of $F_{count} (13,2) > F_{table} (2.76)$, with ($\alpha = 0.05$), there are noticeable differences in the provision of treatment. The increasing number of uninfected erythrocytes by *P berghei* was due to the content of flavanol glycosides in *J multifida* stem. Flavanol glycosides was expected to form Flavanol glycosides-heme complex that could inhibit the formation of *P berghei* parasites Hemazoin

Conclusion: The stem extract of *J. multifida* might be a potential anti-malarial drugs better than chloroquine diphosphat based on the increase number of *P berghei*-uninfected erythrocytes of *M. musculus*.

Key words : *Jatropha multifida* Linn, antimalarial, *Plasmodium berghei*, flavanol glycosides, erythrocyte

INTRODUCTION

Malaria is a disease caused by the *Plasmodium*, a parasite which is transmitted by infected mosquito bites¹. WHO reported that an approximately 300-500 million cases of malaria each year and 1.5-1.7 million people died because of that². Indonesia is a malaria-endemic areas with quite high fatal cases caused by malaria. Malaria patients in Bengkulu province has reached 122-130 per thousand of the population in each year and is increasing annually. An accurate diagnosis and treatment are two important principles in controlling malaria. There are many attempts were made to prevent and conquer the disease. The development of antimalarial drugs and models of effective therapy is still needed in conquering malaria.

Indonesia is one country that is known to have an abundance amount of biodiversity. There are about 30,000 species of plants and 1,260 species of them have been found which have medicinal savor. For example in Bengkulu *Jatropha multifida* Lin plant which is widely grown as an ornamental plant can be used as a new wound healing drug, so that the plant is better known by the public as a *Betadin* plant. The extract of *J. multifida* stem improved to have a low bioactivity potential towards the shrimp larvae (*Artemia salina* Leach) but it has antibacterial activity (*Salmonella typhi*) which is characterized by the formation of lipid zone³. The study of *J. multifida* stem extract has also been proved to have the ability to increase the number of platelets in the *Mus musculus*⁴.

Because of the increasing threats against malaria, the purpose of this research is to develop a stem of *J. multifida* as an antimalarial. *M. musculus* inoculated with *P. berghei* is an animal model widely used for studying malaria pathogenesis⁵. *J. multifida* stem as an antimalarial drugs through trials was therefore performed in *M. musculus* in which the change number of erythrocytes infected with *P. berghei* was observed.

MATERIALS AND METHODS

Plant material and Experimental animals. The materials used are the *J. multifida* stems which were taken in Pematang Gubernur, Bengkulu and animal tested is male *M. musculus* Swiss strain, weighing 25-30 g, around 7-12 weeks old. Infected *M. musculus* males of *P. berghei* were imported from Health Research Center of Central Jakarta.

1. Phytochemical test

Flavonoids, a total of 4 g of fresh *J. multifida* stems were cut into small pieces, then boiled in a beaker glass containing 30 ml of 96% technical ethanol using a

water bath. After that, filtering had done in hot conditions. The filtrate was concentrated by half, then added 1 drop of concentrated HCl 6 M and magnesium powder⁶.

Alkaloids, as much as 50 mg of powdered *J. multifida* stems was put into a test tube and diluted with 10 ml of HCl and then filtered. The filtrate was tested with Mayer reagents⁷, Wagner reagents and Dragendroff reagents⁶.

Tannin, as much as 500 mg of *J. multifida* stems was added 50 ml of distilled water, boiled for 15 minutes, after being cold down, dripped iron (III) chloride reagent, greenish black coloration indicates the presence of tannin.

Saponins, as much as 50 mg of powdered *J. multifida* stems were put into a test tube and were added 20 mL of distilled water, the mixture was shaken for 15 minutes. The formation of foam layer as high as 2 cm indicates saponin⁸.

Terpenoids and steroids, as much as 0.5 g of *J. multifida* stems were put into a test tube and were added 2 mL glacial acetic, then were added 3 mL of concentrated H₂SO₄. Green to blue coloration indicates a positive steroid and brownish red to purple indicates positive terpenoids⁷.

2. Preparation of *J. multifida* stem extract

J. multifida rods were cleaned, and subsequently were cut into small pieces. This was then dried aerated with no direct sunlight. This followed by finely grinded and macerated using 96% ethanol for 7 days. The maceration results were filtered. The filtrate obtained was evaporated using a rotary evaporator.

3. *Plasmodium berghei* cultivation

P. berghei transferred from *M. musculus* which has been infected by taking blood from the heart with 2.5 mL injection syringe that had previously been filled with the anti-coagulant heparin of 0.5 mL. Blood was injected into healthy *M. musculus* with volume ± 0 , 2 mL intraperitoneally.

Then, parasitemia of *M. musculus* was examined on blood-thin smear. This followed by fixation using absolute methanol, and then flooded with 10% Giemsa solution for 45 minutes. After that the blood-thin smears were washed and dried. This was then examined under a light microscope with a magnification of 100x with oil emersi given. Parasitemia was then calculated in percentage⁹. After parasitemia reached about 30-40%, the blood of *M. musculus* can be used as a source of *P. berghei* inoculum of experimental animals.

4. Animals assay

M. musculus as experimental animals were purchased from Bandung Institute of Technology and infected *M. musculus* were provided by laboratory testing of malaria Litbang Kesehatan Jakarta. Before the treatment, *M. musculus* were adapted for a week in Kebun Biologi, University of Bengkulu. Thirty of *M. musculus* then divided equally in 6 groups. The group was inoculated with *P. berghei* and treated either with chloroquine, or different dose of *J. multifida* as shown in **Table 1**. After 24 hours, the erythrocytes was counted by using a hemocytometer. The procedure mentioned as following. Mice tails poked with a sterile knife, first drop of blood was discarded, and the next drop was sucked by hemocytometer to the extent of 0.5 or 1. Suction of diluting solution until the number 101, the suspension then was shaken until completely

homogeneous (solution becomes to red in the tube). The cover glass was placed on the correct place in such way avoiding the cover glass from falling behind. Blood was dripped on the edge of the cover glass and then was counted for the number of infected and uninfected erythrocyte within ± 1000 erythrocyte representation (with a microscope magnification 1000x). The infected – uninfected erythrocytes were determined manually at several fields. Parasitemia percentage was the number of infected erythrocytes divided by the total number of erythrocytes multiplied by 100% ¹⁰.

RESULT

Based on the phytochemical test, *J. multifida* stems contain flavonoids, alkaloids, tannins and saponins, test results are presented in **Table 2**.

Table 1. Treatment for each group of *M. musculus*

Treatment group	Number of <i>M. musculus</i>	<i>P. berghei</i>	Chloroquine dose g/kgbw	<i>J. multifida</i> extracts dose (g/kgbw)
Negative control(P0)	5	-	0	0
Positive control (P1)	5	+	0	0
Comparison (P2)	5	+	0,6	0
Treatment one(P3)	5	+	0	0,028
Treatment two(P4)	5	+	0	0,056
Treatment three(P5)	5	+	0	0,084

Information: + infected, - uninfected

Table 2. The content of secondary metabolites in the *J. multifida* Linn stem

Sample	Test			Tanin	Saponin	Terpenoid	Steroid
	Flavonoid	Alkaloid					
<i>J. multifida</i> .L		Mayer	Wagner				
	+	+	+	+	+	-	-

Description: + detected, - : undetected

Table 3. The average number of uninfected erythrocytes of *M. musculus* for each treatment

Treatment	Loop (n)	Average of uninfected erythrocytes (10^6) \pm SD	% Average of uninfected erythrocytes
Negative control(P0)	5	9,320 \pm 3,36 ^b	100%
Positive control (P1)	5	2,300 \pm 1,12 ^a	21,60%
Comparison (P2)	5	3,500 \pm 1,51 ^a	37,50%
Treatment one (P3)	5	9,140 \pm 3,21 ^b	58,60%
Treatment two (P4)	5	7,600 \pm 3,86 ^a	58,70%
Treatment three(P5)	5	9,840 \pm 5,23 ^b	62,22%

Statements: P0 uninfected by *P. berghei*
 P1 infected by *P. berghei*
 P2 infected by *P. berghei* and administered chloroquine
 P3, P4, P5 infected by *P. berghei* and administered ethanol extract at dose 0,028, 0,056, 0,084 g/kgbw)

Based on the phytochemical test, it is known that *J. multifida* stem extracts contain secondary metabolites of the flavonoid, saponins, tannins, and alkaloids.

J. multifida stem ethanol extract has a great activity in increasing the number of uninfected erythrocytes towards *M. musculus* which are infected with *P. berghei*, the result of the increasing in non-infected erythrocytes can be seen in **Table 3**.

The difference in the condition of uninfected erythrocytes and infected erythrocytes under the microscope can be seen in **Figure 1**.

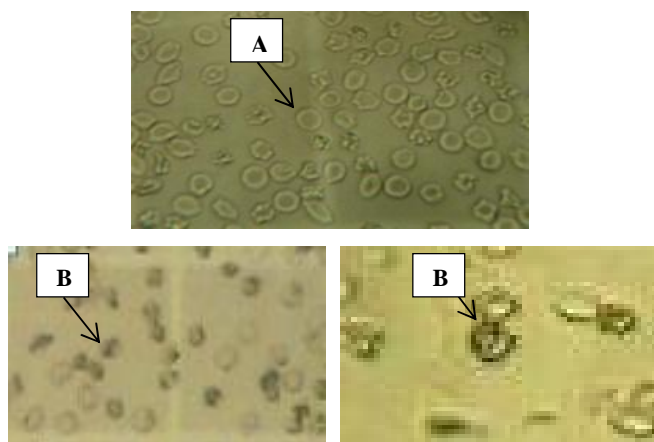


Figure 1. Erythrocytes uninfected with *P. berghei* and B. erythrocytes infected by *P. Berghei*

J. multifida stems extracts through IR and ¹H NMR spectroscopy were identified containing flavonol glycosides compounds structured as in **Figure 2**.



Figure 2. Flavonolglycosides in *J. multifida*. L stems extracts

DISCUSSION

J. multifida stem extract after evaporated with a rotary evaporator was insoluble in distilled water because of the possible presence of many sugar groups which are disconnected at the time of evaporation. Therefore, for the importance of oral administration by

gavage using the tool on the *M. musculus*, *J. multifida* stem extract dissolved in olive oil. The administration of *J. multifida* stem extract on *M. musculus*, each in treatment 1 (P3) with a dose of 0.028 g/kgbw and treatment 3 (P5) with a dose of 0.084 g/kgbw, continuously, are able to increase the average number of uninfected erythrocytes to 9.135 million erythrocytes cells/mm³ and 9.856 million uninfected erythrocytes cells/mm³ which closer to normal erythrocytes in *M. musculus*, whereas treatment 2 (P4), the administration of *J. multifida* stem extract with a dose 0.056 g/kgbw was able to increase the average number of uninfected erythrocytes around to 7.618 million erythrocytes cells/mm³. *J. multifida* stem extract has the higher ability to increase uninfected erythrocyte than the malaria drug chloroquine diphosphate. Full results of the increasing in non-infected erythrocytes can be seen in **Table 3**.

Conditions of uninfected and infected erythrocytes under the microscope showed clear differences. Infected erythrocytes have different forms compared to normal erythrocytes. The difference can be seen in **Figure 1**.

P. berghei is widely used as an inducer of malaria in *M. musculus* to study the molecular basis of antimalarial for having similarities with *P. falciparum*. *P. berghei* parasite is transmitted by the Anopheles mosquito and are able infect the liver after entering the blood vessels. Parasite growth requires food taken from the erythrocyte cytoplasm, the parasite digests hemoglobin, byproduct of heme degradation of hemoglobin is a substance that is toxic to parasit^{11,12,13}. Heme is a compound [IX ferriprotoporphyrin FP, FP Fe (II)], This compound then detoxified by the parasite itself into hemozoin^{1,13,14}. Hemozoin is a synthesis that is unique to the malaria parasite, which is regarded as a polymer compound, which is associated with a unit of Fe (III) PPIX or known to the polymer compound of heme. The development of research at this time was hemozoin is a cyclic dimer compounds of Fe (III) PPIX in which the propionate group of each molecule Fe (III) PPIX binds coordination with Fe (III) as the central atom, whereas dimers linked through hydrogen bonding of the acid groups propionat^{12,15}.

Chloroquine shown to inhibit heme detoxification into hemozoin by inhibiting polymerization of hemozoin through-oxo bonds into dimers, so that hemozoin formation is not occurred. This process is also thought to be other molecular targets in the development of anti-malaria^{11,16}. Many have written evidence that antimalarial drugs like chloroquine acts forming a complex with heme [FP Fe (II)] and haematin or aqua complex [ferriprotoporphyrin IX, Fe (III) FP], which is derived from the proteolysis of hemoglobin by parasit¹³.

J. multifida stem extract has the ability to increase the number of uninfected erythrocytes by *P berghei* in *M. Musculus*. It is possibly that flavonol glycosides of *J. multifida* stem extract inhibits heme detoxification. If the flavonoid glycoside compound is not present then *P berghei* is able to detoxify the heme by turning it into hemazoin that are not toxic to *P berghei*. The presence of flavonol glycosides compounds is able to inhibit heme detoxification by forming flavonolglycosides-hemecompleses. This was subsequently unable the formation of *P berghei* hemazoin. A mixture of unreacted heme and flavonolglycosides-hemecompleses is a toxic compound for *P berghei* which then reduce the ability of *P berghei* to infect erythrocytes. The formation of complex compounds flavonolglycosides-heme estimated through such means as presented in **Figure 3**. Administering stem extract of *J. multifida* in *M. musculus* is able to increase the number of uninfected erythrocytes ability even higher than the malaria drug chloroquine diposphat for comparison (see again Table 3)

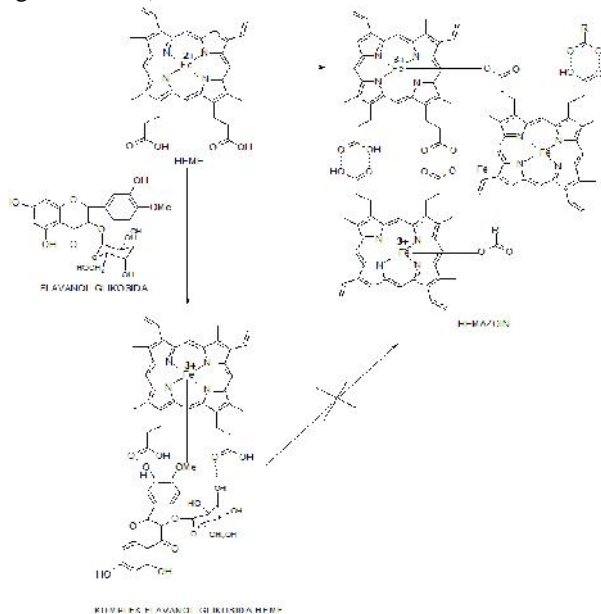


Figure 3. The Formation Hemazoin and Flavonolglycosides-heme complex

CONCLUSION

J. multifida stem extract at a dose of 0.028 g/kgbw, 0.056 g/kgbw, and a dose of 0.084 g/kg bw is able to increase the number of uninfected erythrocytes on *M. musculus* infected by *P berghei*. The ability of stem extract of *J. multifida* in increasing the number of erythrocytes infected with *P berghei* is not much higher than the malaria drug chloroquine diposphat. Based on this study, the ethanol stem extract of *J. multifida* can be developed as a potential new anti-malarial.

SUGGESTION

Research needs to be conducted in vitro to determine the ability of the active compounds on stem *J. multifida*: the flavonol glycosides in inhibiting *P berghei* so it will be able to obtain more detailed information will be the ability of the stem extract of *J. multifida* as an anti-malarial. Identification of flavonol glycosides compounds need to be completed using ¹³C NMR spectroscopy and 2D NMR to ensure the truth of the alleged structure identified flavonol glycosides.

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SINOPSIS

Stem extract of *Jatropha multifida* potentially be developed as a new antimalarial drug, because of the ability in increasing the number of uninfected erythrocytes by *Plasmodium berghei* in mice is much higher than the malaria drug chloroquine.