**P r o c e e d i n g I n t e r n a t i o n a l C o n f e r e n c e , 2 0 1 3 , * , * * - * *
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THE EFFECTIVITY OF ETANOLIC EXTRACT SUNFLOWER LEAVES (*Helianthus annus*) AS ANTI-MALARIAL AGENT AGAINST *Plasmodium berghei*

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ABSTRACT

Malaria, an infectious disease with a high mortality rate, encountered efficacy on the descreasing level since its firstline drug that is combination between artemisin and amodiaquine. A traditional herbal medicine, sunflowers (*Helianthus annus*), in fact, have been empirically used as antimalarial agent in Indonesia. Sunflowers leaves contain sesquiterpen lacton that have physiological function as an antimalarial agent. This study aimed to reveal the antimalaria effect of sunflower leaves on the degree of parasitemia of mice infected by *Plasmodium berghei*. Mice were peritoneal infected by 10^6 *Plasmodium berghei* ANKA and divided into 7 experimental groups: (1) negative control, (2) non-infection control, (3) positive control (chloroquine of dose 5.71 mg/kgBW), (4) Sunflower leaves of dose 0.1 mg /kgBW; (5) Sunflower leaves (henceforth ESF) of dose 1 mg/kgBW, (6) Sunflower leaves of dose 10 mg / kgBW, and (7) Sunflower leaves of dose 100 mg / kgBW. The treatments were started on day 0 when parasitemia degree reached 1-5% and continued for 5 days therapy. The parasitemia observation was carried out on day 0,1,2,3, and 4. The results showed that the extract of sunflower leaves could significantly inhibit the growth of *Plasmodium berghei* ($p<0.05$) with the result of ED_{50} is 4.64 mg/kgBW.

Key word : Anti-malarial agent, etanolic extract, Sunflowers leaves, *Plasmodium berghei*

INTRODUCTION

Malaria is considered as one of the infectious diseases spreaded all over the world. Approximately, 2.57 billions or 41% residents in the transmition area are susceptible of being infected by *Plasmodium falciparum*, including Indonesia (Gething *et al*, 2011; WHO, 2011). On the basis of WHO data 2010, 37% Indonesian reside in malaria endemic area, such as: Bengkulu, Kepulauan Bangka Belitung, Central Kalimantan, North Sulawesi, Gotontalo, Central Sulawesi, Maluku, West Nusa Tenggara, East Nusa Tenggara Timur, West Papua, and Papua, with high infectious trasmition level. Other provinces, on the other hand, belong to low-endemic area as well as transmition level of malaria. In addition, the number of cases encountered in 2010 reached 229.829 cases which mortality level was 432 people (WHO, 2010).

The difficulty to overcome malaria problem is closely related to the *Plasmodium's* parasitic ability in forming self-defension toward antimalarial medicine, so that it will be the resistant toward malaria medicine. To be more specific, *Plasmodium falciparum*, the most number of species in Indonesia (53%) (WHO, 2011), have been found resistant toward klorokuin. In 1993, it is reported that *Plasmodium falciparum* was klorokuin resistant in 22 provinces, and even antimalarial drug resistant in 11 provinces (Tjitra, 2004; Fidock *et al*, 2004)

Recently, it is reported that *Plasmodium falciparum* is also resistant toward artemisin. The sensitivity test of dehydroartemisin toward *Plasmodium falciparum* in the form of *in vitro* revealed that there is increasing of IC_{50} (Noedl, 2008; Pilai *et al*, 2012; Dondorp, 2009). Besides, the artemisin has half time which is very short so that there is recrudesention after the therapy (Sardjono and Fitri, 2007). Due to those recent facts, it is essential to conduct further research and development of antimalarial in order to solve the mentioned problems which one of them is by utilizing traditional *jamu*.

The sunflower leaves (*Helianthus annus*) consist of sesquiterpen lacton compound (Macias *et al.,* 2002). In the previous study, it is known that dichlorometan extract is effective in cutting-off the life cycle of *Plasmodium berghei* in 5, 50, 500 mg/kg BB and parasitemia degree reach 0% in the third and forth day after having therapy in each dose (Muti'ah, *et al.*, 2011).The separation of sunflower leaves and KLTP, then, was continued by identifying isolate using spektrofotometer UV-Vis. FTIR contains steroid compound and seskuiterpen lacton (Bayyinah, 2013) in which sesquiterpen lacton was functionated against parasite in the erythrocyteical phase. The working mechanism of sesquiterpen lacton (artemisin) is through enzim ATPase inhibition depending on calsium (PfATP6). The free radical which is produced by artemisin tied and blocked PfATP6 irreversibly and specifically (Ridley, 2003). The function of ATPase in the complex system of pumping ion ion Na^+/K^+ is adjusting the degree of ion in the cell. The failure of PfATP6 function causes the drastic decrease of ion kalium in the cell which endangers the parasite. (Muti'ah, 2012). The current research, then, aimed to reveal the effect of anti-malaria from each ethanol extract of sunflower leaves.

MATERIAL & METHOD

Research Setting

This recent study was conducted in the laboratory of Chemistry Department (Organic Chemistry and Biotechnology Laboratory) and in the Biology laboratory (Animal Physiology, Biosystem, and Optical Laboratory) Faculty of Science and Technology, State Islamic University (UIN) Maulana Malik Ibrahim Malang in February to May 2013.

Plants

The leaves of sunflowers (*Helianthus annuus*) were taken from Punten, Sido Mulyo-Batu.. The powder of each sample was measured in 100 g. Then, it was macerated to get extract by using 80% ethanol solution. The filtrate was concentrated using *rotary evaporator* till the researcher achieved the consentrated extract which then was continued by giving N_2 gas.

Animal Model

This recent study employed animal testees which were forty-eight male mice

(*Mus musculus)*, particularly Balb/C j48 species, weight of 21–25 g, age of 8–12 weeks. They were divided into seven experimental groups and feeded using standard foods and drink of *ad libitum.*

Research Planning

The researcher used full random experimental planning. Mice were divided into 15 groups that had different treatment (for sunflower leaves and its combination); 8 treatment groups (for anting-anting plants with additional dosage 0,01 mg/kg BB), such as:

1. Negative control group was a group treated by giving *P. berghei* infection without therapy and solely given 0,5 mL CMC-Na 1%.
2. Positive control group was Chlorokuin group using 71 mg/kg BB dose once a day per oral (Sukandar, 2011).
3. ESF_1 was a group which was given infection and 80% ethanol extract sun flower leaves with 0,1 mg/kg BB dose once a day per-oral.
4. ESF_2 was a group which was given infection and 80% ethanol extract sun flower leaves with 1 mg/kg BB dose once a day per-oral.
5. ESF_3 was a group which was given infection and 80% ethanol extract sun flower leaves with 10 mg/kg BB dose once a day per-oral.
6. ESF_4 was a group which was given infection and 80% ethanol extract sun flower leaves with 100 mg/kg BB dose once a day per-oral.

The testing of antimalarial activity in vivo was conducted using Peter method (Phillipson and Wright, 1991 in Muti'ah, 2012). The therapy was conducted when parasitemia infection reached 1–5% that has been measured since the day 0. The therapy was given daily (morning/afternoon/evening depending on the % gaining of infection which was 1- 5% on the day of 0) in four days. The observation of parasitemia was started on the day 0, 1, 2, 3, and 4 (as the curative testing).

Donor Production

The treatment in producing donor was referred to the study conducted by Muti'ah *et al.* (2012). In producing donor system, erythrocyte which was infected by parasite was extruded to 200 µL using PBS. Then, it was injected to the mice *intraperitonially* (i.p). Furthermore, the researcher observed parasetimia degree in mice donor. If the percentage of parasitemia in a mouse donor reached 2,5 %, it means that it can infect other mice.

Freezing and Thawing Isolate *P. berghei*

The treatment of Freezing and Thawing isolate parasite in this current research referred to the study conducted by Coutrier (2009). The first thing done in freezing isolate parasite was by taking 0,8 mL heart blood from a mouse donor which had been infected, and then it was put in the vacum tube which contained EDTA. After that, the vacum tube containing heart blood and EDTA was added by 1,6 mL Alsever's solution containing 10 % glicerol. Next, vacum tube was covered and put in the liquid nitrogen tank about ± 1 menit. Then, it was moved to the freezer of -70 ˚C. When its parasite infected blood was taken to give infection treatment, vacum tube containing isolate parasite was taken out from freezer (thawing process). Thus, parasite was thawed and ready to be infected to the animal testees. All works related to the isolate *P. berghei* were conducted in *Laminar air flow vertical* and asepticable condition.

Inoculation of *P. berghei*

The treatment of inoculation *P. berghei* referred to the study conducted by Muti'ah *et al.* (2012) which was *P. berghei* inoculation conducted *intraperitonially* (i.p) with the number of parasite which was infected was 1×10^6. In terms of mice examination which had been infected by the parasite, it was assumed that normal mencit had hematrocrite level (a number which shows solid percentage in the blood toward blood liquid was 60 %, and mice donor had 6×10^9 eritocyte /mL in the blood. If the parasitemia degree of mice donor was 2,5%,

its blood will be taken as much as 6,7 µL, then it was re-extruded to 200 µL using PBS solution. After infection process, the researcher conducted daily observation till the parasitemia reach 1–5% since the day 0 of therapy. Next, the researcher gave medicational therapy or extract testing till the day 4.

Parasitemia Degree Measurement

Blazquez et al (2008) explained the technique in measuring parasitemia degree by creating blood deletion track. It was conducted by taking a drip of a mouse's blood through cutting its tail and it is dripped to the glass object and dried. Next, the result of blood deletion was evenly spreaded by methanol and dried. The next process is coloring Giemsa by mixing Giemsa fluka and buffer Giemsa using 1:9 ratio. The Giemsa coloring was dripped to the deletion track and waited for 20 minutes. Next, it is washed using flowing water so that there is no remaining color and then dried. Next, the deletion of blood track which had been colored were examined its parasitemia using microscope with 1000x magnifition by calculating the number of erythrocyte which was infected by malaria out of 1000 erythrocyte. The parasitemia degree percentage was the number of erythrocyte infected by *P. berghei* in 1000 erythrocyte. The parasitemia degree percentage was measured using this formula:

$$\text{Percentage of parasitemia degree} = \frac{\text{The number of infected erythrocyte}}{\text{1000 erythrocytes}} \text{ x } 100\%$$

On the other hand, the percentage of parasite growth inhibition was measured using the formula below:

$$\%\ inhibition = \frac{(negative\ control\ parasitemia - medicine/extract\ paracitemia)}{negative\ control\ of\ parasitemia} \text{x}100\%$$

After that, it was determined the value of ED_{50} using probity analysis from % inhibition on the day 4.

RESULT AND DISCUSSION

The Effectiveness of 80% Ethanol Extract of sunflowers Leaves as the anti-malaria.

The observation of parasitemia degree was conducted on the day 0, 1, 2, 3, and 4. The deletion of blood was examined under microscope using 1000x magnifition by calculating the number of erythrocyte out of 1000 erythrocyte which was infected by malaria. The examination of parasitemia on the day 0 aimed to prove that all mice were in the equal parasitemia degree range on the day when the researcher gave medication. The result of parasitemia degree examination and deviation standard are shown in the Table 1, table 2, and table 3, below:

Table 1. The mean of parasitemia degree of 80% ethanol extract of sunflower and deviation standard

Experimental Group (mg/kg BB)	Mean of parasitemia degree (%) ± Deviation Standard				
	Day 0	Day 1	Day 2	Day 3	Day 4
Negative Control	1,5 ±0,31	4,3 ±1,03	5 ±1,24	5,5 ±1,46	5,6 ±1,14
Positive Control	2,9 ±0,86	1,4 ±0,33	1,2 ±0,3	0,38 ±0,31	0 ±0,00
ESF_1	1,7 ±0,38	2,5 ±0,4	3,33 ±0,99	3,5 ±0,47	3,90 ±0,8
ESF_2	3 ±1,09	3,1 ±0,5	2,9 ±1,18	3,8 ±0,54	3,90 ±0,26
ESF_3	1,9 ±0,66	3 ±0,74	3,4 ±0,49	3,5 ±1,12	2,4 ±0,48
ESF_4	2,6 ±1,51	2,7 ±0,86	3,20 ±0,38	3,20 ±0,71	2,1 ±0,74

Table 1 showed that the mean of parasitemia in the day 1, 2, 3, and 4 in all experimental groups which was treated by 80% ethanol extract was lower than negative control group. Then, the deviation standard showed that the mean percentage of parasitemia degree deviated (Sulisetijono, 2006). Normally, the deviation standard for in

vivo test was not quite slight; however the upper and lower limit should not exceed the mean of the paracitemia degree percentage of the six mice on the daily treatment. The more deviation standard means the more various mice, in sum.

The deviation standard was categorized as the normal range as stated by Pasaribu (1975). The range which was got by the mean was in the interval $\bar{x}$ – 2s and $\bar{x}$ + 2s which was using 95,45% percentage. The result of investigation result of parasitemia degree and the value of deviation standard in the **Table 1** above, was explained using **Fig. 1** below:

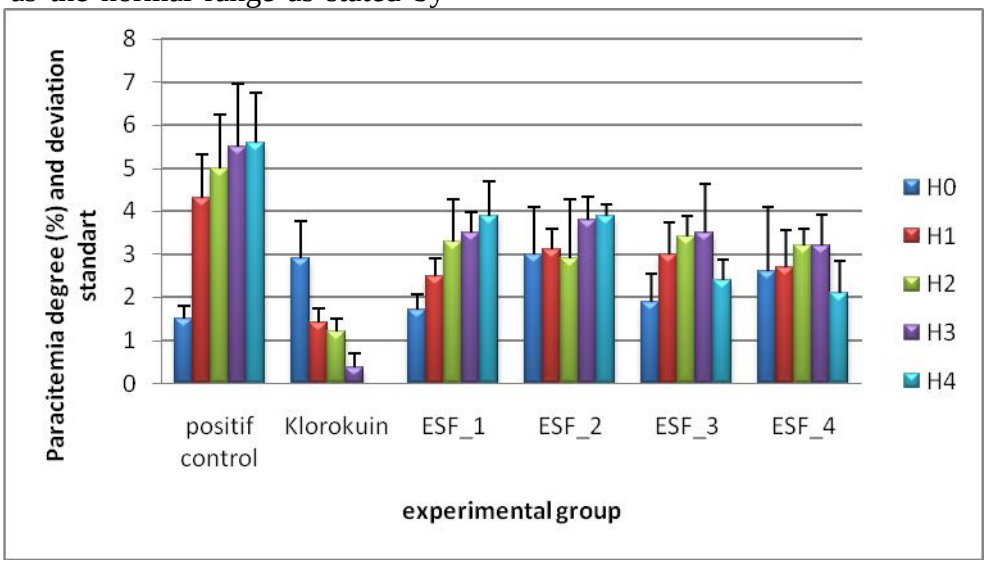

Figure 1. Graphic of Parasitemia Degree of 80% Ethanol Extract of Sunflower Leaves

The increasing degree of parasitemia in the negative control indicated that the number of erythrocyte infected by *Plasmodium berghei* is increasing as the more number of treatement days.

Next, the graphic of positive control group showed the opposite of the negative control one, in which it was concluded that the parasitemia degree on the day 4 is 0%. It showed that positive control is 100% effective for inhibiting the growth of parasite of *P.berghei*. It was also supported by deviation standard 0 which showed the high level of data validity and reliability.

After that, to the dosage of 80% ethanol extract of sunflowers leaves, alter in terms of parasitemia number in each mouse's body. However, it was clearly seen on the last day (test of Peter-*The 4-day suppressive test of blood schizontocidal action)* as stated by Philipson and Wright (1991), the number of parasitemia degree of sunflowers to the dosage of 1 and 2 increased on the day 4 after therapy treatment compared to the previous days. On the other hand, to the dosage of 3 and 4, it was shown that the parasitemia degree decreased particularly on the day 4. Those two statements were not absolutely stated that the dosage of 3 and 4 gave effective therapy which was better than dosage of 1 and 2. Those are due to the extensive effects which were not related to the parasite which infected, some of them due to the existence of mice's immune system, consumption of food and drink, and wound got as the result of quarrelling among mice so that it affects the increasing of parasitemia degree among dosage.

To emphasize the dosage effectiveness of extraction toward parasite malaria growth infected mice's blood cell, it could be directly seen from the % inhibition in each treatment. The value of % of 80% ethanol extract inhibition of sunflowers leaves could be achieved using formula % of inhibition:

$$\%\ inhibition = \frac{(negative\ control\ of\ parasitemia - medicine/extract\ parasitemia)}{negative\ control\ of\ parasitemia} \times 100\%$$

The result of percentage calculation of parasite growth inhibition of 80% ethanol extract of sunflowers leaves on the day of 4 after therapy are shown in the **Table 2,** below:

Table 2 Percentage and Probity of inhibition of parasite growth on the average of 80% ethanol extract of sunflowers leaves on the day 4

Dosage (mg/kg BB)	Percentage of Parasite Growth Inhibition	Probity % of Inhibition
0,1	30	4,48
1	30,7	4,49
10	57,9	5,18
100	61,8	5,31

Based on the percentage of parasite inhibition, it was known that the effectiveness of sunflowers extracts dosage is absolute. As showed on Table 4, the percentage of parasite inhibition increased as the increasing of dosage concentration given, the effectiveness in inhibiting the parasite growth is increasing. It is assumed that the more dosage given, the process of tropozoit changes to the early skizon is inhibited as well.

Then, in this current study, in order to decide the effective dosage to inhibit 50% of parasite growth (ED_{50}) was by utilizing probity % inhibiting the parasite growth in four days which was then continued through regression analysis using Microsoft Office Excel.

Based on the Table 4, it was continued by determining the correlation between log dosage of 80% ethanol extract used and the percentage of probity value of the inhibition as shown in the **Fig. 2**

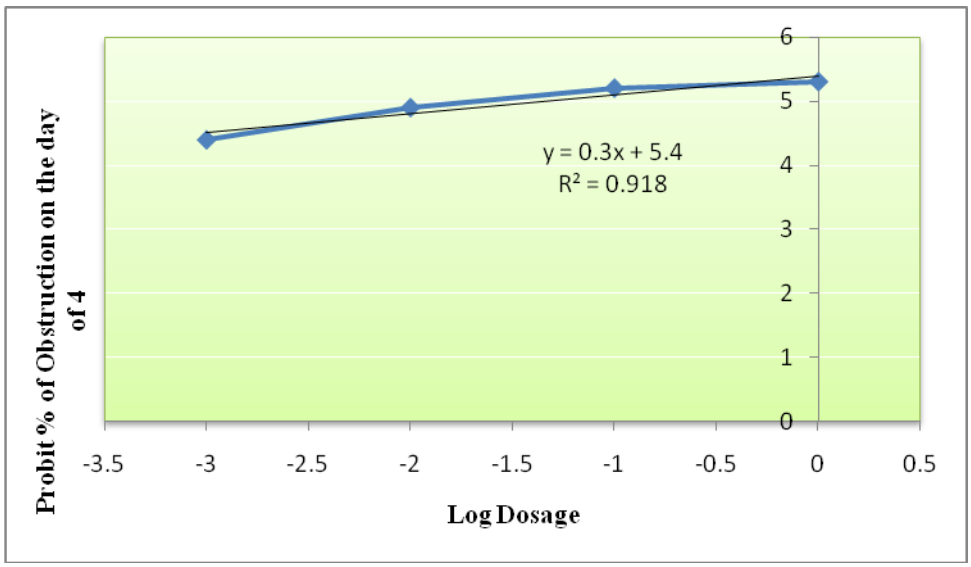

Figure 2. The curve of correlation between Log Dosage and Probity % of Inhibition of Sunflowers leaves

Figure 2 showed that the effective dosage which inhibits the growth of 50% parasite was in the dosage range of 1 mg/kg BB and 10 mg/kg BB, which was in the point of 4,64 mg/kg BB. The effectiveness point of 50% dosage was assumed as the effective point in inhibiting 50% of parasite growth as stated by Herintsoa *et al.* (2005) which is < 10 mg/kg BB.

CONCLUSION

The 80% ethanol extract of sunflowers leaves is effective as the antimalarial agent toward the animal testees which are infected by *P.berghei* parasite on the ED_{50} values of 4,64 mg/kg BB.

SUGGESTION

1. It is needed to separated and purify the compound used in the following step, for instance from KLTA which is then followed by KLTP and Kromatography coloumn, so that the researcher would get the pure isolate.
2. It is needed to have more identification using HPLC instruments to know the specification of active compound group and utilizing spectroscopy *Nuclear Magnetic Resonance* (NMR) to reveal the structure of antimalarial active compound.

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