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RESEARCH ARTICLE

Antimalarial Activity of Multiple Dose on *Plasmodium berghei* Infected Mice and Heme Detoxification Inhibitory Activity of *Helianthus annuus* L. Leaf Extract

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SUMMARY

Helianthus annuus L. (sunflower) is ¬traditionally used to treat malaria. Crude extracts of the plant leaves are found to be active against Plasmodium falciparum in in vitro antimalarial activity assay. In the present study, we aimed to investigate in vivo antimalarial activity against Plasmodium berghei in mice and heme detoxification inhibitory activity of 80% ethanol extract of H. annuus leaves. In vivo antimalarial activity was carried out using the Peters' 4-day suppressive test against P. berghei in BALB/c mice. Animals were treated orally twice a day for 4 days with 0.1, 1, 10, and 100 mg/kg of 80% ethanol extracts, respectively. Dihydroartemisinin-piperaquine was used as a positive control. Heme detoxification inhibitory activity was carried out by using the method of Basilico which had been modified and the absorbance was read by ELISA reader at a wavelength of 405 nm. Chloroquine was used as a positive control. The results showed that inhibition of in vivo antimalarial activity against P. berghei in mice increased along with the increasing dose. In repeated dose, ED_{50} *was found to be 1.489 mg/kg. Heme detoxification inhibitory activity results showed that* IC_{50} *values for 80% ethanol extract and positive control were 0.690 and 0.688 mg/mL, respectively. There was no statistically significant difference between 80% ethanol extract and positive control (*$p>0.05$*). The results showed that 80% ethanol extract of H. annuus L. leaf administered twice a day gives a strong activity both as an antimalarial in vivo and heme detoxification inhibitory.*

Key Words: *Helianthus annuus L., Antimalarial activity, Plasmodium berghei, Heme detoxification inhibitory activity, Asteraceae, Leaf extract*

Helianthus annuus L. Yaprak Ekstresinin Plasmodium berghei ile Enfekte Edilen Farelerde Çoklu Dozda Antimalaryal ve Hem Detoksifikasyon İnhibe Edici Aktivitesi

ÖZ

Helianthus annuus L. (ayçiçeği) geleneksel olarak sıtmayı tedavi etmek için kullanılır. Bitki yapraklarının ham ekstrelerinin in vitro antimalaryal aktivite deneyinde Plasmodium falciparum'a karşı aktif olduğu bulunmuştur. Bu çalışmada farelerde Plasmodium berghei'ye karşı in vivo antimalaryal aktiviteyi ve H. annuus yapraklarının % 80 etanol ekstresinin hem detoksifikasyon inhibe edici aktivitesini araştırmayı amaçlanmıştır. In vivo antimalaryal aktivite BALB / c farelerinde P. berghei'ye karşı Peters'in 4 günlük baskılayıcı testi kullanılarak gerçekleştirilmiştir. Hayvanlara oral olarak 0.1, 1, 10 ve 100 mg / kg % 80 etanol ekstresi ile 4 gün boyunca günde iki kez verilmiştir. Dihidroartemisin-piperakin pozitif kontrol olarak kullanılmıştır. Hem detoksifikasyon inhibitör aktivitesi Basilico'nun modifiye edilmiş yöntem kullanılarak yapılmış ve ELISA okuyucu tarafından 405 nm dalga boyunda absorbans okunmuştur Pozitif kontrol olarak klorokin kullanılmıştır. Sonuçlar P. berghei'ye karşı farelerde in vivo antimalaryal aktivitenin inhibisyonunda artan dozla birlikte artış göstermiştir. Tekrarlanan dozda ED50 1.489 mg / kg olarak bulunmuştur. Hem detoksifikasyon inhibisyon aktivite sonucu, % 80 etanol ekdtresi ve pozitif kontrol için IC50 değerlerinin sırasıyla 0.690 ve 0.688 mg / mL olarak bulunmuştur. % 80 etanol özütü ile pozitif kontrol arasında istatistiksel olarak bir fark görülmemiştir (p> 0.05). Sonuçlar H. annuus L. yaprağının % 80 etanol ekstresinin günde iki kez uygulandığında hem in vivo antimalaryal hem de hem detoksifikasyon inhibitörü olarak güçlü bir aktivite göstermiştir.

Anahtar Kelimeler: *Heliantus annuus L., Antimalaryal aktivite, Plasmodium berghei, Hem detoksifikasyon inhibitör aktivite, Astraceae, Yaprak ekstresi*

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INTRODUCTION

Malaria is an infectious disease that attacks red blood cells and can also cause anemia. Malaria is transmitted through the bite of *Anopheles* female mosquito with *Plasmodium* parasite. The obstacle faced today is the resistance of malaria drugs that have begun to spread. Therefore, it is necessary to have alternative treatment that can come from plants known as traditional medicine. In Indonesia, local people have already used medicinal plants to treat several diseases, including malaria. *Cassia siamea* leaf, a plant grows in Indonesia, is traditionally used as an antimalarial and its bioactive compound has been discovered as well (Morita et al., 2007; Ekasari, et al., 2009). One of the other plants that have long been used as traditional remedy for malaria is sunflower (*Helianthus annuus* L.). The *H. annuus* leaves also have been utilized to treat malarial fever in Caucasus region, Russia (Grieve, 1971). This plant in Indonesia has been used traditionally to treat various of diseases, especially the leaves of this plant are used to treat inflammation, analgesic as well as malaria infection. To treat malaria, the leaves are boiled and drink it when it become cooled (Sopi and Tallan, 2015). *H. annuus* leaves contain sesquiterpene lactone compounds suspected as active compounds that are efficacious as an antimalarial (Dwivedi & Sharma, 2014; Lim, 2014; Marsni et al., 2015). This compound is also contained in the antimalarial drugs of artemisinin synthesized from the plant *Artemisia annua* with the same family as the sunflower plants of Asteraceae (Arshad & Amjad, 2012). Several studies regarding antimalarial activity of sunflower leaf have been conducted, yet not much further research has been conducted to ensure its effectiveness as an antimalarial drug (Muti'ah et al., 2012).

Muti'ah et al. (2013) reports that *in vivo* antimalarial assay of 80% ethanol extract of *H. annuus* leaf administered in *P. berghei* infected mice exhibits active antimalarial agent with ED_{50} value of 4.64 mg/kg body weight. However, its percentage of inhibition effectiveness is still low with the inhibition percentage of 100 mg/kg and 10 mg/kg once daily as single doses that are 61.8% and 57.9%, respectively.

In order to extend therapeutic effect and enhance the bioactivity, most of the drugs are usually administered in repeated dose. Ethanol extract of *C. spectabilis* leaf in repeated dose administration shows better result than the single dose (Ekasari et al., 2018). Drug plasma concentration should be maintained on the therapeutic range to reach its maximum effectiveness. Therefore, repeated dose is recommended for oral administration to keep the drug plasma level within the narrow limits of the therapeutic window (Shargel et al., 2005).

Aside from its parasite growth inhibition, action mechanism of antimalarial agents is an important point to be investigated as well. One of the action mechanisms which play a major role as antimalarial drug is the inhibition of heme detoxification. It has already proved that 80% ethanol extract of sunflower leaf has *in vitro* and *in vivo* antimalarial activity nonetheless its mechanism of action of heme detoxification inhibition has not been investigated yet.

Based on the description above, this study aimed to test the *in vivo* antimalarial activity of 80% ethanol extract of *H. annuus* L. leaves administered in repeated dose in infected *P. berghei* mice and on the malarial inhibition of heme detoxification.

MATERIALS AND METHODS

Sample Collection

H. annuus leaves were obtained in November 2016 from Oro-Oro Ombo Village, Batu, East Java and determined in Materia Medica Batu, Batu, East Java, Indonesia. Specimen was deposited as the herbarium in Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, (Number: 04/WE/XI/2016).

Preparation of Crude Extracts

Leaf samples of plant were dried at room temperature under shade in the laboratory of Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga. The dried leaves were powdered using a grinder and sieved with 40-mesh sieve.

Crude extract was prepared by maceration techniques. Leaf powders (300 g) were soaked in 1500 mL of 80% ethanol in a sealed container. The maceration was repeated three times. The filtrate was collected, evaporated by using rotary evaporator, and put into the oven at 40°C until a stable weight of extract was obtained.

Animals

Adult male BALB/c mice with 20-30 g of bodyweight were obtained from *Pusat Veterinaria Farma* (PUSVETMA), Surabaya, East Java, Indonesia. The mice were fed with mice pellet diet and given free access to clean drinking water. The animals were allowed to acclimatize for two weeks before treated. The permission and approval for animal studies were obtained from Faculty of Veterinary Medicine, Universitas Airlangga, Ethical approval number No: 2.KE.120.07.2018.

Experimental Animals and Parasite Inoculation

Male BALB/c mice (6-8 weeks old and 25-30 g of body weight) were obtained from PUSVETMA, Surabaya, East Java, Indonesia and also were used for the assay. The mice were maintained in the animal house of Faculty of Pharmacy, Universitas Airlangga, under standard condition at room temperature by exposing them to 12 hour light and 12 hour dark, with food and water *ad libitum*. Morever, the mice were handled based on internationally accepted guideline (National Academy of Sciences, 2011).

Chloroquine sensitive *Plasmodium berghei* (ANKA strain) was obtained from the Institute of Biomolecular Eijkmann, Jakarta, Indonesia. The parasites were maintained by serial passage of blood from infected mice to non-infected ones. Donor mice infected with a rising parasitemia of 20-30% were used to infect mice in the test. The donor mice were sacrificed, and their blood was pooled together in a tube containing 1% (w/v) EDTA as anticoagulant to avoid variability in parasitaemia. The blood was then diluted with phosphate buffer saline (PBS) (Sigma) so that each 0.2 mL of blood contained $1x10^7$ *P. berghei* infected erythrocytes. Each mouse used in the experiment was then inoculated intraperitoneally with 0.2 mL of the diluted blood.

***In Vivo* Antimalarial Screening**

In vivo antimalarial activity evaluation of the 80% ethanol extracts of *H. annuus* leaves was carried out against *P. berghei* according to the method described by Peters et al. (1975) which modifies the sample administration. Thirty-six male mice were randomly divided into six groups (four treatment groups and two control groups). The four treatment groups received 0.1, 1, 10, and 100 mg/kg of the 80% ethanol extracts, respectively, twice daily for 4 days. The two controls (negative and positive) received the vehicle (0.5% CMC Na) and dihydroartemisinin-piperaquine (DHA-P) (20.8 mg/kg + 166.4 mg/kg, standard drug), respectively. The vehicle, the plant extracts, and the standard drug were administered orally.

The treatment was started after the mice had been inoculated intraperitoneally with 0.2 mL of infected blood containing about $1x10^7$ parasites at day 0 by using a hypodermic needle and then continued for additional 3 days (from day 1 to day 3). On the day 4, thin blood smears were made from the tail blood of each mice and smeared onto a microscope slide, fixed with methanol, and stained with 10% Giemsa at pH 7.2 for 1 minute. The parasitaemia was examined microscopically to determine parasitaemia level and parasite suppression percentage. Effective dose which was able to inhibit parasite growth by 50% (ED_{50}) was calculated with probit analysis based on the relationship between dose and the percentage of parasite growth inhibition.

Heme Polymerization Inhibitory Activity Assay

Heme polymerization inhibitory activity test was conducted by Basilico et al. (1998) which modified the concentration of hematin solution, and the sample used. A total of 100 µL of 1 mM hematin in 0.2 M NaOH was put into the microtube, then added 50 µL of test solution with various concentration levels, such as 4, 2, 1, 0.5, 0.25, and 0.1 mg/mL. Each concentration was tested in triplicate. In order to initiate the polymerization reaction heme, 50 µL glacial ace-

tic acid (pH 2.6) was added in the microtube which already contained hematin solution and sample, then was incubated for 24 hours at 37°C. After the incubation, the microtube was centrifuged for 10 min at 8000 rpm. Supernatant was removed and the precipitate was washed 3 times with 200 µL of DMSO. Each microtube was washed by centrifugation speed 8000 rpm for 10 minutes. The precipitate obtained was dissolved in 200 µL of 0.1 M NaOH. Each 100 µL of the solution was put into a 96-wells microplate to read its absorbance value using an ELISA reader at a wavelength of 405 nm. Positive control was chloroquine diphosphate and negative control was DMSO 10%.

The standard curve of hematin was prepared by making a hematin solution with a series of concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 mM in 0.2 M NaOH. Furthermore, as much as 100 µL of each solution was put into a 96-wells microplate and read its absorbance using an ELISA reader at a wavelength of 405 nm. The absorbance values obtained, and the β-hematin concentration values were plotted into linear regression which would be the reference for calculating the β-hematin concentration formed in each test material. To get the value of IC_{50}, probit analysis was applied.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). IC50 values were calculated using Probit analysis. Statistical significance for heme polymerization inhibitory activity assay was determined by ANOVA (*One Way*) at significance level of $p < 0.05$

RESULTS AND DISCUSSION

In Vivo Antimalarial Activity of 80% Ethanol Extracts of *H. annuus* Leaves on Parasitaemia

The results of the study are listed in Table 1. The level of suppression of 80% ethanol extract of *H. annuus* leaves at concentrations of 0.1 mg/kg/day, 1 mg/kg/day, 10 mg/kg/day, and 100 mg/kg/day following the test is 19.82, 48.56, 76.00, and 82.05%, respectively. The results show that inhibition will increase along with the increasing doses. Then, the inhibition percentage is analyzed by probit analysis to find out the value of ED_{50}. In repeated dosing, ED_{50} is found to be 1.489 mg/kg.

Table 1. Effect of 80% ethanol extract of *H. annuus* leaves on parasitaemia and percent suppression of *P. berghei* infected mice

Test substances	Dose (mg/kg)	Mean of % parasitaemia ± SD		% suppression
		Day 0	Day 4	
80% ethanol extracts of *H. annuus* leaves	0.1	1.76 ± 0.74	17.66 ± 11.79	19.82
	1	1.80 ± 0.72	12.00 ± 3.71	48.56
	10	1.06 ± 0.45	5.82 ± 2.88	76.00
	100	1.50 ± 0.44	5.06 ± 2.06	82.05
Dihydroartemisinin-Piperaquine (DHA-P)	20.8 (DHA) 166.4 (P)	1.83 ± 2.04	0	100
CMC Na	-	1.40 ± 0.76	21.23 ± 10.94	-

A drug is considered prospective to be developed as a malarial drug if it has ED_{50} < 5-25 mg/kg on *in vivo* test (Fidock et al., 2004). The smaller the ED_{50}, the greater the effectiveness of the *in vivo* assay on *P. berghei*. ED_{50} value in repeated dose is smaller than a single dose. Thus, it can be argued that repeated dose is more effective in inhibiting parasite growth than a single dose.

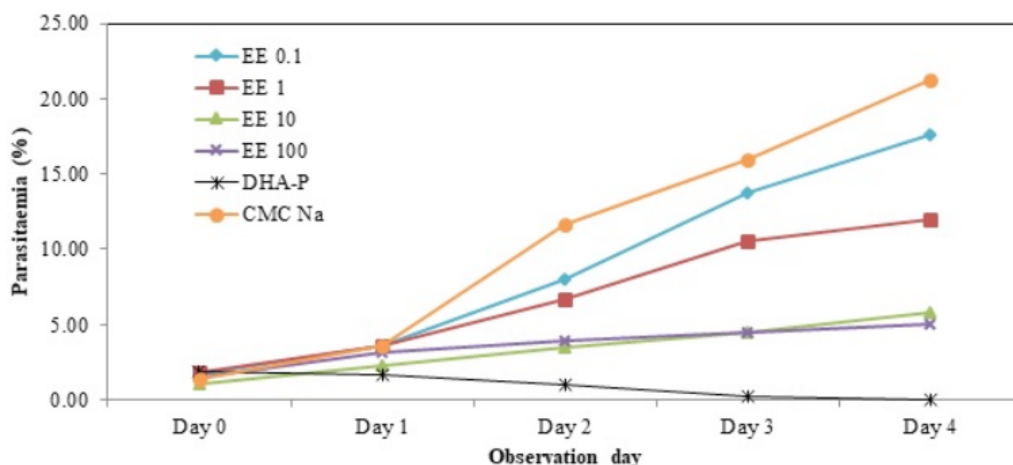

Figure 1. The effect of 80% ethanol extract of *H. annuus* leaves on parasitaemia of *P. berghei* infected mice on day 0 to day 4, data are mean ± SEM, *n* =6, DHA-P = dihydroartemisin-piperaquine, EE = 80% ethanol extract of *H. annuus* leaves, the numbers refer to doses in mg/kg/day

In order to obtain the optimal therapeutic effect, the factors that are closely related to the therapeutic effect should be considered during the rational doses and drug interval regulation. The factors are including the resulted activity of the drug used (Shargel, 2005).

The therapeutic effect of antimalarial drug depends on the availability of the drug in the blood that able to inhibit the growth of *Plasmodium* maximally. The various doses of antimalarial drug, including the repeated doses are usually applied during the development of antimalarial drug (Ndiaye et al., 2011). The effect of plant extract is less effective and the bioavailability of drugs in the blood is faster to disappear when it is applied as short administration (once a day). Twice a day administration can extend the act of extract in the blood (long acting), therefore repeated dose is better to obtain its optimum effectiveness.

Muti'ah et al. (2013) reported that inhibition percentage of 80% ethanol extract of *H. annuus* leaves administer 100 mg/kg once daily as a single dose (61.8%) increases to 82.05% in twice a day administration. Inhibition percentage of 10 mg/kg once daily administration increases from 57.9% to 76% in twice a day treatment as well.

Based on the discussion above, it can be concluded that 80 % ethanol extract of *H. annuus* leaves with twice daily administration (repeated dose) for 4 days treatment can inhibit the growth of *P. berghei* higher than once a day administration (single dose).

Heme Polymerization Inhibitory Activity Assay

This *in vitro* antimalarial activity assay is performed by heme detoxification inhibition method. In the intraerythrocytic phase, the parasite will degrade hemoglobin into free heme and globin. Globin will then be converted into a small peptide to be used for enzyme synthesis. Free heme is toxic to parasites as it contains reactive oxygen species (ROS) which can lyse the parasitic membrane and disrupt the parasitic enzymatic activity. Thus, the parasite will convert the free heme into a non-toxic form, hemozoin (malaria pigment), with a special mechanism called heme detoxification mechanism (Kumar et al., 2007).

The mechanism of heme detoxification has been identified as: 1) Biocrystallization of heme spontaneously, 2) Heme degradation in food vacuole facilitated by hydrogen peroxide (H_2O_2), 3) Glutathione mediated heme degradation, 4) Heme oxygenation

was found in *P. berghei* (rodent malaria) and *P. knowlesi* (simian malaria) but not in *P. falciparum* (human malaria). Gluthatione-dependent and enzymatic heme degradation occur in the outside of food vacuole of parasite. Among the above mechanisms, biocrystallization is the major mechanism occurs in the malaria parasite (Sherman, 1998; Ziegler *et al.*, 2002; Tekwani and Walker, 2005). This process is an important target for the development of antimalaria drug (Fidock *et al.*, 2004).

The use of hematin in this study as the main ingredient will react to β-hematin as well as hemozoin. Glacial acetic acid is used to create an acidic atmosphere which adapts to the condition of the parasitic food vacuole. Washing uses DMSO solution is done to remove residual hematin which is still mixed with β-hematin crystals (Tekwani and Walker, 2005).

The result (see Table 2) shows that IC_{50} values for 80% ethanol extract and positive control are 0.690 and 0.688 mg/mL.

Table 2. The IC_{50} values of 80% ethanol extract of *H. annuus* leaves, negative, and positive controls based on heme polymerization inhibitory activity assay

Test substances	Concentration (mg/mL)	Average dose of hemozoin (mM) ± SD	Average inhibition percentage ± SD	IC_{50} (mg/mL)
80% ethanol extracts of *H. annuus* leaves	4	27.10 ± 1.31	75.74 ± 1.18	0.690
	2	38.85 ± 1.92	65.23 ± 1.72	
	1	48.15 ± 2.93	56.91 ± 2.63	
	0.5	59.13 ± 1.22	45.21 ± 1.69	
	0.25	72.00 ± 3.00	35.56 ± 2.68	
	0.1	88.54 ± 1.42	20.77 ± 1.27	
Chloroquine diphosphate	4	32.55	70.87	0.688
	2	42.00	62.41	
	1	53.72	51.93	
	0.5	59.27	46.96	
	0.25	67.23	39.83	
	0.1	80.98	27.52	
DMSO	-	111.7 ± 13.88	-	-

Compounds that have percentage of β-hematin inhibition greater than 60% are considered to have good potential as a β-hematin inhibitor. On the other hand, if the percentage of inhibition is less than 40%, the activity is weak in inhibiting the formation of β-hematin (Frolich et al., 2005). Baelsman et al. (2000) stated that if IC_{50} obtained from inhibition test of heme detoxification more than chloroquine sulfate is 37.5 mM (12 mg/mL), then the test sample can be categorized as having no activity in inhibiting heme detoxification. From this statement, it can be concluded that 80% ethanol extract of *H. annuus* L. leaves is declared active in the process of inhibition of heme detoxification.

From the statistical analysis that has been done, it is known that between the positive control and 80% ethanol extract, it does not differ significantly since the value of p = 0.561 (p >0.05), which means that 80% ethanol extract activity and positive control can be said to be comparable.

CONCLUSION

The present study indicates promising *in vivo* antimalarial activity of 80% ethanol extract of *H. annuus* leaves with repeated dose/administration. The extracts are also found to be active in inhibiting the heme detoxification process. Therefore, the extracts can be potentially used as a new source for the development of new plant-based antimalarial agent.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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