The antiplasmodial activity of chalcone derivative through the inhibition of haemoglobin formation and the induction of stomatocytes formation

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ABSTRACT

Background: One of methoxy aminochalcone derivatives, (E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl)prop-2-en-1-one has been synthesized and proven its in vitro antiplasmodial activity. In this study, we reported in vivo antiplasmodial activity of this compound against Plasmodium berghei infected mice. Its effect on the haemoglobin and erythrocytes stomatocytes formations was also evaluated.

Methods: The in vivo antiplasmodial activity was evaluated on P. berghei infected mice by the classical 4-day suppressive test. The effect of the tested compound on the haemoglobin formation inhibition was evaluated by flowcytometry, whereas its effect on the stomatocytes formation was evaluated by microscopic examination of the thin blood smear. Doxycycline was used as positive control.

Results: The median effective dose (ED₅₀), which is the dose leading to 50% parasite growth inhibition or haemoglobin formation inhibition or stomatocytes formation of tested compound and doxycycline were determined using probit analysis and compared using t test.

Conclusion: The (E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl)prop-2-en-1-one exhibits potent antimalarial activity via inhibition of haemoglobin formation and induction of stomatocytes formation. This compound might be developed into a new antimalarial drug.

Keywords: antiplasmodial, chalcone, Plasmodium, haemoglobin

INTRODUCTION

Malaria is a parasitic disease caused by Plasmodium and transmitted by Anopheles mosquito. Malaria remains a major health problem in the world, especially in tropical and subtropical countries including Indonesia. In 2016, the World Health Organization (WHO) reported that of the approximately 3.2 billion people living in malarious countries, 1.2 billion were at high risk. Moreover, an estimated 216 million cases of malaria occurred worldwide with 445,000 deaths. In Indonesia, malaria morbidity from 2009 to 2016 tended to decline from 1.8 per 1,000 people in 2009 to 0.84 per 1,000 people in 2016. Papua is the province with the highest Annual Parasite Incidence (API), which is 45.85 per 1,000 people.

One of the problems in the elimination of malaria is the resistance of Plasmodium to antimalarial drugs. Artemisinin-based combination therapy (ACT) has been recommended by WHO as the first-line drug for the treatment of uncomplicated malaria since 2001. However, the emergence of ACT resistance has been reported in some countries in Southeast Asia, Africa, and South America. The ACT resistance was encouraged numerous studies to explore new antimalarial compounds. Natural products are one of the potential sources of new antimalarial compounds. Success stories in new antimalarial discovery came from quinine isolated from Cinchona pubescens and artemisinin isolated from Artemisia annua.

One of the natural products that potential to be developed as a new antimalarial drug is chalcone. Chalcones (1,3-diaryl-2-propen-1-ones) are secondary metabolites found in various plant species. Chalcones are abundant inedible plant and are intermediates in the biosynthesis of flavonoids and isoflavonoids. They are well known for their antibacterial, antimalarial, antifungal, antitumor, anti-inflammatory and antiprotozoal. Chalcones can be obtained both through isolation from a plant or synthesis using a relatively simple procedure. Among the five derivatives tested,
(E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl) prop-2-en-1-one showed the high activity with an inhibitory concentration 50% (IC\textsubscript{50}) of 4.21 ± 1.8 µg/mL and good selectivity with a selectivity index (SI) of 16.9. In this study, we reported \textit{in vivo} antiplasmodial activity of this compound against \textit{Plasmodium berghei} infected mice.

Haemozoin formation, a unique heme detoxification pathway found in the Plasmodium, is well known as an important antimalarial target. Antimalarial drugs such as quinine, chloroquine, and halofantrine have been demonstrated to act as haemozoin inhibitors.\textsuperscript{12,13} Recent studies showed an induction of stomatocytes formation through erythrocyte membrane modifications as a novel target for potential antimalarial drugs.\textsuperscript{14-16} In this study, we also reported the effect of this compound on the formations of haemozoin and stomatocytes of erythrocytes.

**MATERIALS AND METHODS**

**Materials**

Tested compound, i.e. (E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl) prop-2-en-1-one was synthesized by Dr. Hery Suwito from Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Surabaya (Figure 1). Doxycycline was used as positive control obtained from Sigma Chemical Co. (St. Louis Mo.).

**In vivo antiplasmodial activity testing**

The \textit{in vivo} antiplasmodial activity was evaluated on \textit{P. berghei} infected mice by the classical 4-day suppressive test.\textsuperscript{17} The protocol of the study has been approved by the Health Research Ethics Committee, Dr. Moewardi General Hospital/ Faculty of Medicine, Universitas Sebelas Maret, Surakarta. \textit{Plasmodium berghei} strain was obtained from the Department of Parasitology, Faculty of Medicine, whereas Swiss mice strain used were obtained from the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta. Sixty-six male mice (20-25 g and 6-8 weeks) were inoculated intraperitoneally with $10^7$ \textit{P. berghei}-infected mice erythrocytes obtained from donor mice, resuspended in RPMI 1640 medium on day to a volume of 0.2 mL. The mice were then divided into 11 groups with six mice in each group. The first five groups were given 10, 20, 40, 80 and 160 mg/kg BW/d of tested compound, respectively. The second five groups as positive control were given 0.25, 0.5, 1, 2, 4 mg/kg BW of doxycycline, respectively. Another group as a negative control was given aquadest. Each dose of tested compound or doxycycline were given once daily for four consecutive days, beginning on the day of infection, starting two hours after inoculation until day three. In the day following the last treatment, Giemsa-stained thin blood smear from the tail vein was prepared. Parasitemia level was then determined microscopically by counting the number of parasitized erythrocytes of 200 erythrocytes in random fields of the microscope. Average percentage parasite growth inhibition was calculated as \([(A-B)/A]\) x 100, where A is the average parasitemia in the negative control group, and B is the average percentage parasitemia in the treatment groups. The median effective dose (ED\textsubscript{50}), which is the dose leading to 50% parasite growth inhibition was determined using probit analysis.

**In vivo haemozoin formation inhibition testing**

The effect of the tested compound on the haemozoin formation inhibition was evaluated by flowcytometry as conducted by Frita \textit{et al.}\textsuperscript{12} In the day following the last treatment after thin blood smear conducted, blood samples of the \textit{P. berghei} infected mice were incubated with Fc-block and then labeled with anti-TER119. After washing the blood samples, the DNA of intraerythrocytic parasites was stained with the DNA-specific dye SYBR green I (Invitrogen, Carlsbad, USA). After 20 minutes of incubation in the dark, the stained sample was immediately analyzed by flowcytometry using a 535/45 nm band pass filter in front of the detector. Flowcytometry results were analyzed using FlowJo software (version 9.0.2, Tree Star Inc., Oregon, USA). The median effective dose (ED\textsubscript{50}), which is the dose leading to 50% haemozoin formation inhibition was determined using probit analysis.

**Stomatocytes formation examination**

The effect of the tested compound on the stomatocytes formation was evaluated by microscopic examination of the thin blood smear as conducted for parasitemia examination. In the day following the last treatment, Giemsa-stained thin blood smear from the tail vein was prepared. Stomatocytes formation level was then determined.
microscopically using 1000x magnification by counting the number of stomatocytes out of 100 erythrocytes in random fields of the microscope. The median effective dose (ED$_{50}$), which is the dose leading to 50% stomatocytes formation was determined using probit analysis.

**Statistical analysis**

Data of parasitemia, haemozoin formation and stomatocytes formation were presented as mean ± standard error of the mean (SEM). The ED$_{50}$ of tested compound and doxycycline to parasite growth or haemozoin formation inhibition or stomatocytes formation was compared using t-test. A p-value < 0.05 was considered to be significant.

**RESULTS**

The results of the 4-day suppressive test demonstrated that the tested compound resulted in significant parasite growth inhibition in dose-dependent manner. In the lowest dose of tested compound resulted in 27.66 ± 7.39% of parasite growth inhibition, whereas in the highest dose resulted in 95.55 ± 24.06% (Table 1). Furthermore, the probit analysis results showed that the ED$_{50}$ of the tested compound against parasite growth (17.36 ± 4.59 mg/kg BW) was significantly higher that doxycycline as a positive control (<0.25 mg/kg BW) (p<0.05). The ED$_{50}$ of doxycycline was assumed <0.25 mg/kg BW due to in the lowest dose resulted in parasite growth inhibition less than 50%. This result indicated that the tested compound was less active than doxycycline against *P. berghei*.

The results of flow cytometry analysis also demonstrated that the tested compound resulted in significant inhibition of haemozoin formation in a dose-dependent manner. In the lowest dose of tested compound resulted in 15.31 ± 1.60% of haemozoin formation inhibition, whereas in the highest dose resulted in 98.96 ± 16.49% (Table 2). Furthermore, the ED$_{50}$ of tested compound to haemozoin formation inhibition of parasite (18.56 ± 5.19 mg/kg BW) was significantly higher that doxycycline as a positive control (2.56 ± 0.59 mg/kg BW) (p<0.05).

**Table 1. In vivo antiplasmodial activity of tested compound and doxycycline on percentage parasitemia and growth inhibition of *P. berghei* infected mice in the 4-day suppressive test**

| Tested compound | Dose (mg/kg BW) | Parasitemia (%) | Inhibition growth (%) | Dose (mg/kg BW) | Parasitemia (%) | Inhibition growth (%) |
|-----------------|-----------------|-----------------|-----------------------|-----------------|-----------------|-----------------------|
|                 | 160             | 8.50 ± 2.14     | 95.55 ± 24.06         | 4               | 6.67 ± 1.26     | 96.51 ± 18.04         |
|                 | 80              | 11.33 ± 2.39    | 94.07 ± 19.84         | 2               | 11.33 ± 2.39    | 94.07 ± 19.84         |
|                 | 40              | 55.50 ± 7.09    | 70.94 ± 8.95          | 1               | 9.67 ± 2.33     | 94.94 ± 22.88         |
|                 | 20              | 65.83 ± 11.99   | 65.53 ± 11.99         | 0.5             | 12.33 ± 3.04    | 93.54 ± 23.06         |
|                 | 10              | 138.17 ± 7.39   | 27.66 ± 7.39          | 0.25            | 15.67 ± 3.61    | 91.80 ± 21.15         |
| Aquadest        | 191.00 ± 13.01  | 0.00            | Aquadest              | 191.00 ± 13.01  | 0.00            |

ED$_{50}$ (mg/kg BW) = 17.36 ± 4.59

**Table 2. Effect of tested compound and doxycycline on haemozoin formation and haemozoin formation inhibition of *P. berghei* infected mice in the 4-day suppressive test**

| Tested compound | Dose (mg/kg BW) | Haemozoin formation (%) | Haemozoin formation inhibition (%) | Dose (mg/kg BW) | Haemozoin formation (%) | Haemozoin formation inhibition (%) |
|-----------------|-----------------|-------------------------|-----------------------------------|-----------------|-------------------------|-----------------------------------|
|                 | 160             | 0.31 ± 0.05             | 98.96 ± 16.49                     | 4               | 3.20 ± 0.47             | 89.23 ± 13.11                     |
|                 | 80              | 4.93 ± 1.12             | 83.55 ± 18.98                     | 2               | 30.73 ± 5.79            | -2.78 ± 0.52                      |
|                 | 40              | 10.36 ± 3.39            | 65.35 ± 21.39                     | 1               | 29.80 ± 5.89            | 0.33 ± 0.07                       |
|                 | 20              | 14.19 ± 1.25            | 56.00 ± 25.66                     | 0.5             | 29.35 ± 2.28            | 1.84 ± 0.14                       |
|                 | 10              | 25.32 ± 2.65            | 15.31 ± 1.60                      | 0.25            | 29.36 ± 2.52            | 1.84 ± 0.15                       |
| Aquadest        | 29.90 ± 0.68    | 0.00                    | Aquadest                          | 29.90 ± 0.68    | 0.00                    |

ED$_{50}$ (mg/kg BW) = 18.56 ± 5.19

ED$_{50}$ (mg/kg BB) = 2.56 ± 0.59

Data presented as mean ± standard error of the mean (SEM); ED$_{50}$: the median effective dose (ED$_{50}$), which is the dose leading to 50% parasitemia growth inhibition.

Data presented as mean ± standard error of the mean (SEM); ED$_{50}$: the median effective dose (ED$_{50}$), which is the dose leading to 50% haemozoin formation.
The results of microscopic examination of the thin blood smear demonstrated that the tested compound resulted in significant induction of stomatocytes formation in a dose-dependent manner. In the lowest dose of tested compound resulted in 2.17 ± 0.12% of stomatocytes formation induction, whereas in the highest dose resulted in 37.83 ± 0.98% (Table 3). In the highest dose, both of tested compound and doxycycline did not result in induction of stomatocytes formation more than 50%. Therefore, it was assumed that the ED_{50} of both compounds to induction of stomatocytes formation was more than its highest dose, i.e. > 160 mg/kg BW for tested compound and > 4 mg/kg BW for doxycycline.

**DISCUSSION**

Chalcones (1,3-diphenyl-2 propen-1-on) is a compound containing two aryl rings connected with α, β unsaturated ketones. Chalcones are important intermediates in the flavonoids and isoflavonoids biosynthesis. They are well known for their broad spectrum of biological activities including antibacterial, antimicrobial, antifungal, antitumor, anti-inflammatory and antiprotozoal.

The *in vitro* antiplasmodial activity of chalcone derivatives was reported in the several studies. However, the study of *in vivo* antiplasmodial activity of chalcone derivatives is limited.

In our previous study, we synthesized chalcone derivatives that can inhibit the interaction between ferredoxin and ferredoxin – NADP⁺ reductase of *P. falciparum* on *in the silico* study. Furthermore, *in vitro* study showed among five chalcone derivatives tested, (E) -1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl) prop-2-en- 1-one exhibited the high potential antiplasmodial activity. This study showed that this tested compound exhibited *in vivo* antiplasmodial activity with an ED_{50} of 17.36 ± 4.59 mg/kg BW. The ED_{50} of this tested compound was lower than that of doxycycline (0.25 mg/kg BW) (Table 1). However, the *in vivo* antiplasmodial activity of this tested compound was similar or more active than that chalcones derivatives evaluated in the previous study.

Xiang synthesized and evaluated the *in vitro* and *in vivo* antiplasmodial activity of a series of ferrocenyl chalcones derivatives. Some of the ferronyl chalcones derivatives showed *in vitro* antiplasmodial activity with IC_{50} values less than 20 μM. However, further *in vivo* study in *P. berghei* ANKA infected mice at the tested dose of 100 mg/kg BW, these compound did not possess antiplasmodial activity. Chen et al. investigated the *in vivo* antiplasmodial activity of novel oxygenated chalcone, i.e. 2,4-dimethoxy-4’-butoxychalcone in *P. berghei* ANKA infected rats. The results showed that this compound inhibited parasite growth much higher than the control group at a dose of 50 mg/kg BW. Dominguez et al. evaluated *in vivo* antiplasmodial activity of the most active phenylurenyl chalcone derivatives obtained from the *in vitro* study. These compounds also were active against *P. berghei* infected mice. However, they’re *in vivo* activity were modest.

In this study, the effect of the tested compound on haemozoin formation was also reported. The tested compound exhibited inhibition of the haemozoin formation with the ED_{50} of 18.56±5.19 mg/kg BW (Table 2). It is suggested that this compound act through inhibition of haemozoin formation. Haemozoin is a biocrystal formed by *Plasmodium* to avoid the toxicity of ferriprotoporphyrin IX derived from the digestion of hemoglobin during the invasion of the erythrocytes. Some antimalarial drugs such as 4-aminoquinoline are well-known to act through inhibition of the haemozoin formation.
The effect of chalcone derivatives on haemozoin formation has been reported by some authors. Dominguez et al. reported that among phenylurenyl chalcones tested, the most active compound 1-[3′-N-(N′-phenylurenyl)phenyl]-3(3,4,5-trimethoxyphenyl)-2-propen-1-one exert its antimalarial activity via multiple mechanisms including inhibition of protease falcipain-2, globin hydrolysis, and haemozoin formation. Furthermore, Sinha et al. also reported that chalcones derivatives supposed to have antimalarial activity by inhibiting the haemozoin formation. Also, chalcone derivatives supposed to inhibit either Plasmodium aspartate proteases or cysteine proteases, enzymes involved in hemoglobin degradation. This inhibition also indirectly decreases the haemozoin formation.

In this study the effect of the tested compound on stomatocytes formation was also reported, although in the highest dose resulted in 37.83 ± 0.98% (< 50%) of stomatocytes formation (Table 3). The invasion and Plasmodium growth in erythrocytes depend on the integrity and normal function of erythrocyte membrane. Change of the erythrocyte membrane provides an unfavorable condition for Plasmodium growth.

A correlation between changes of the erythrocyte membrane shape towards stomatocytes or echinocytes observed microscopically, and the inhibition of Plasmodium growth caused by antimalarial agents has been reported. Zieger et al. reported Licochalcone A as powerful tissue layer active agent that change normal erythrocytes to echinocytes concurrent with antiplasmodial activity in rapid and concentration-dependent manner.

CONCLUSION

In conclusion, the E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl)prop-2-en-1-one exhibits potent antimalarial activity via inhibition of haemozoin formation and induction of stomatocytes formation. This compound might be developed into a new antimalarial drug.

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CONFLICT OF INTEREST

The author declares there is no conflict of interest regarding all aspect of the study.

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