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Stage Specificity of Eurycomanone Isolated from *Eurycoma longifolia* on *Plasmodium falciparum* Cycles

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Abstract: Eurycomanone is the most active compounds in the roots of *Eurycoma longifolia* and shown to have *in vitro* antiplasmodial activity. However, the stage of *Plasmodium falciparum* cycles which are sensitive to eurycomanone have not been investigated. This study was conducted to investigate stage specificity of eurycomanone at various stages of *P. falciparum* life cycles. Stage specificity of eurycomanone at various stages of *P. falciparum* was performed on *P. falciparum* culture *in vitro*. A total of 100 μL of solution containing *P. falciparum* at ring stage after synchronized with 1-2% parasitemia (hematocrit 3%) were included in 96 wells microcultures and then added 100 μL of solution containing eurycomanone with 6 various concentrations. The specificity of eurycomanone was evaluated microscopically by counting the percentage of each stage of *P. falciparum* after for 8, 16, 24, 32, 40, 48, 56, 64 and 72 h incubation time, compared with control without any compound. The results showed that eurycomanone can kill ring stage of *P. falciparum* and may inhibit the development of young schizont to mature schizont *in vitro*. However, it needs further investigations for the mechanism.

Keywords: Eurycomanone, *Eurycoma longifolia*, Antiplasmodial Activity, *Plasmodium falciparum*, Stage Specificity

Introduction

Malaria is still health problem in the world. Globally, an estimated 3.3 billion people in 97 countries and territories are at risk of being infected with malaria and developing disease and 1.2 billion are at high risk (WHO, 2014). The increasing resistance of *Plasmodium falciparum* strains to currently available anti-malarial has initiated numerous studies aimed at identifying new anti-malarial agents. One of the strategies in search for new anti-malarial compounds is a research of active plant constituents. Medicinal plants have been used traditionally to treat of malaria in some countries in the world. Significant success was achieved with the new compounds extracted from plants like Qinghaosu (artemisinin) (Li and Rieckmann, 1992) and it has stimulated the search for new plant derived drugs.

A part of our research program consists in the evaluation of the antiplasmodial activities of plants traditionally used in Indonesian regions to treat malaria, we have evaluated the antiplasmodial activity of some medicinal plants from South Kalimantan such as mahoni (*Swientenia mahagoni* Jack), brotowali (*Tinospora tuberculata* Beumee), mimba (*Azadirachta indica* A. Juss) and pasak bumi (*Eurycoma longifolia* Jack). Among aqueous extract of four plants tested, aqueous extract of *E. longifolia* showed strong antiplasmodial activity with an IC_{50} value ranged from 1.07-5.64 μg mL^{-1} on chloroquine-sensitive (D-10) and-resistant (FCR-3) strains (Qamariah, 2002). In order to know the most potent extract of *E. longifolia* further study have been conducted. Three extracts of *E. longifolia* i.e., aqueous, methanol and chloroform extracts have been evaluated for their *in vitro* antiplasmodial activity and cytotoxicity (Mustofa and Qamariah, 2004). Among three extracts of *E. longifolia* tested, methanol extract exhibited a highest antiplasmodial activity with the IC_{50} ranging from 0.6

to 1.9 μg mL^{-1} for the *P. falciparum* strains tested and its Cytotoxicity Index was higher (CI: 22.9-98.6) than chloroform extract (CI: 30.6-35.8) and lower than aqueous extract (CI: 132.6-142.6). The ethyl acetate soluble and insoluble fractions obtained from metahonic extract showed high antiplasmodial activity too (Mustofa and Sholikhah, 2007). Previous study of 5 isolates of methanol soluble fractions showed that isolate 4 showed a high *in vitro* antiplasmodial activity and high selectivity.

Phytochemical screening of *E. longifolia* extract showed that eurycumanone is the most active compounds contained in the plant's roots and potential as an antimalarial with IC_{50} value of 48.1 ng mL^{-1} on chloroquine-resistant *P. falciparum* (W-2) and 47.7 ng mL^{-1} on the chloroquine sensitive *P. falciparum* (D-6) (Kardono *et al*., 1991). However, the stage of *Plasmodium falciparum* cycles which are sensitive to eurycomanone have not been investigated. Based on our previous achievements above mentions, continuation study has been planned in order to know which stage of *P. falciparum* sensitive for the activity. This study was intended to evaluate the stage specificity of eurycomanone isolated from *E. longifolia* root on *P. falciparum* cycles.

Materials and Methods

Materials

The *E. longifolia* roots were collected in Education Park Forest of Mulawarman University, South Kalimantan, Indonesia and were identified by comparison with authentic specimens. Eurycomanone was isolated in Department of Pharmacology and Therapy, parasite were obtained from the laboratory stock at the Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

In vitro Stage Specificity Testing on Plasmodium falciparum

The FCR-3 strain of *P. falciparum* was used in this study. Parasites were cultured continuously according to Trager and Jensen (1976) with modifications described by Van Huysenn and Rieckmann (1993). The parasites were maintained *in vitro* in human red blood cells (O±), diluted to 3% hematocrit in RPMI 1640 medium supplemented with 25 mM Hepes and 30 mM $NaHCO_3$ and complement with 10% human O serum. Before used, parasite cultures were synchronized by D-sorbitol in order to obtained ring stage of *P. falciparum* as reported by Lambros and Vanderberg (1979). The stage specificity of eurycomanone was evaluated microscopically by observing the percentage of each stages of *P. falciparum* after 8, 16, 24, 32, 40, 48, 56, 64 and 72 h. incubation periods with 6 various concentration of eurycomanone compared with control without any compound.

Results

The results showed that giving eurycomanone with all concentrations i.e., 10, 20, 40, 60, 80 and 100 ng mL^{-1} on *P. falciparum* showed that the difference percentage of *Plasmodium* stage started at 8 h incubation period. At 8 h of incubation periods, untreated control *Plasmodium* showed 77.2% at the ring stage, whereas in *Plasmodium* which is given eurycomanone in concentration 10, 20, 40, 60, 80 and 100 ng mL^{-1}, there was a decrease in the percentage of ring r to 62.9; 57.7; 67.7; 69.8; 64.7 and 45.3% respectively. These results suggested that giving eurycomanone in all concentrations in this study can kill ring stage of *Plasmodium.*

At the 24-h incubation period, control Plasmodium showed that 7.23% had been in mature schizonts stage, whereas in Plasmodium which is given eurycomanone in concentartion 10, 20, 40, 60, 80 and 100 ng mL^{-1} respectively, showed only 1.11; 0; 1.85; 1.56; 0 and 1.39% only which were on mature schizont stage (Table 1). *Plasmodium* should have been in the mature schizont stage, but the eurycomane inhibited the growth of young schizont to mature schizont (Fig. 1-6). Eurycomanone than can kill ring stage of *Plasmodium*, seems also inhibit the growth of young schizonts to mature schizonts. This condition caused the IC_{50} value in 32, 40, 48, 56, 64 and 72 h of incubation time were declined (Table 2).

Table 1. Percentage of each stage of *in vitro P. falciparum* after giving eurycomanone for 24 h

	Percentage of each stage (%)			
Concentration (ng/mL)	Ring	Trophozoites	Young schizonts	Mature schizonts
Control	2.09±1.82	80.59±9.19	10.08±2.83	7.22±5.43
100	2.08±3.68	79.17±9.55	15.60±3.92	1.38±2.40
80	8.55±7.83	72.91±11.45	17.33±3.36	0±0
60	3.06±1.79	76.56±2.48	18.79±0.81	1.58±1.38
40	5.51±7.15	76.16±5.47	16.58±3.26	0±0
20	2.23±1.99	79.38±5.57	18.38±7.54	0±0
10	0±0	73.59±9.33	25.30±7.53	1.11±1.92

Table 2. Means percentages of FCR-3 strain of *P. falciparum* growth inhibition *in vitro* after giving eurycomanone and IC_{50} values at nine various incubation periods

Incubation periods (h)	Means percentages of FCR-3 strain of *P. falciparum* growth inhibition *in vitro* Concentration (ng/mL)						Mean of IC_{50} (ng/mL)
	10	20	40	60	80	100	
8	0	0	0	10.93*	12.62± 6.18	14.84*	TD
16	20.42±9.07	23.88±31.83	12.41±2.77	20.58±15.11	26.94±15.83	40.18±6.45	TD
24	12.05±1.68	22.06*	0	0	22.89*	40.08±16.71	TD
32	0	15.99±19.17	6.35±4.51	35.03±6.05	38.84±10.28	54.30±1.80	93.19±8.32
40	0	14.92± 6.78	26.29±9.42	41.47± 7.98	48.08±22.64	67.93±2.98	76.82±9.05
48	14.15±5.80	23.58± 7.87	23.89±3.61	39.65±10.77	67.98±6.52	81.56±1.90	55.59±6.42
56	17.69±2.65	6.93± 5.50	31.76±10.84	42.07±6.77	66.94± 3.91	83.40± 2.94	53.56±2.82
64	9.09±5.82	21.53±6.82	28.65±4.27	46.42±6.93	73.75±2.33	85.68±2.58	50.29±3.25
72	9.62± 8.26	23.12± 1.69	41.10± 5.91	51.39± 3.77	70.72± 6.49	90.66± 0.71	44.17±4.73

ND = Not determined. *One result only, there was not any means

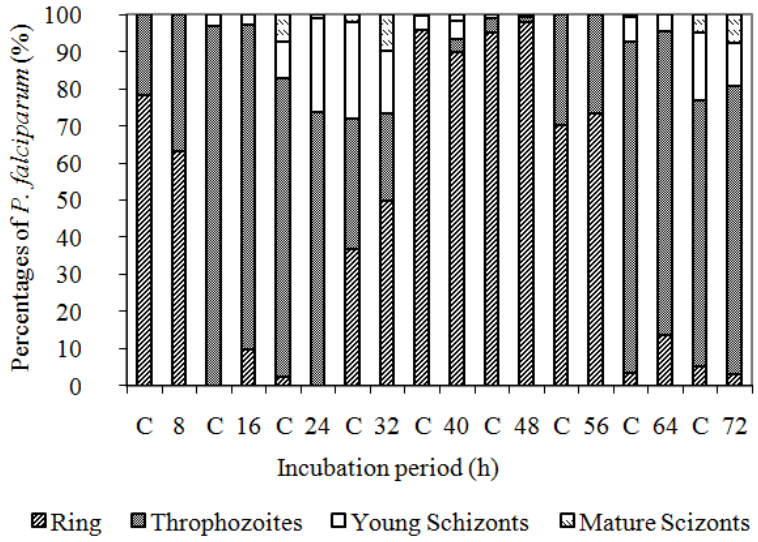

Fig. 1. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 10 ng mL^{-1} on various stage of incubation time periods, compared with control (C = control without any compound)

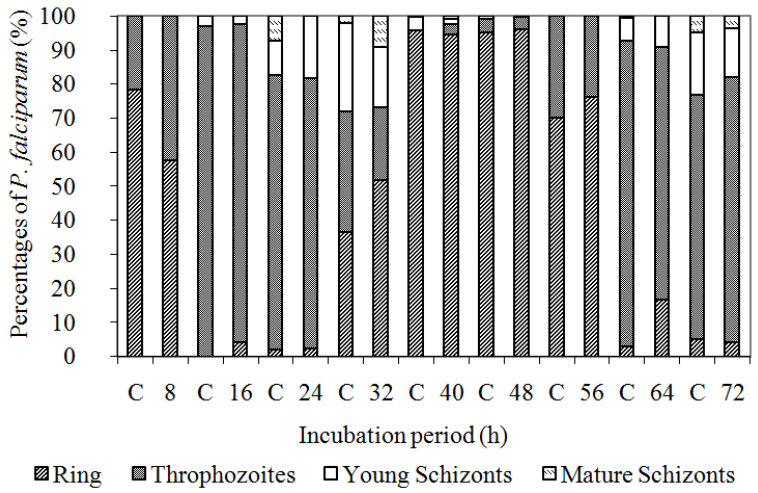

Fig. 2. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 20 ng mL^{-1} on various stage of incubation time periods, compared with control (C = control without any compound)

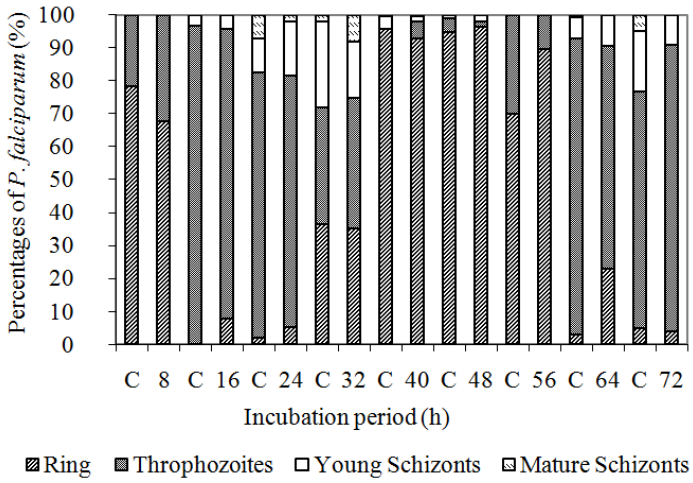

Fig. 3. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 40 ng mL^{-1} on various stage of incubation time periods, compared with control (C = control without any compound)

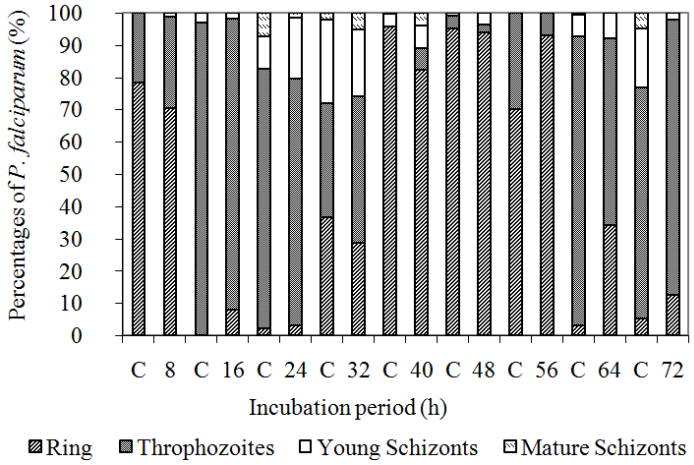

Fig. 4. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 60 ng mL^{-1} on various stage of incubation time periods, compared with control (C = control without any compound)

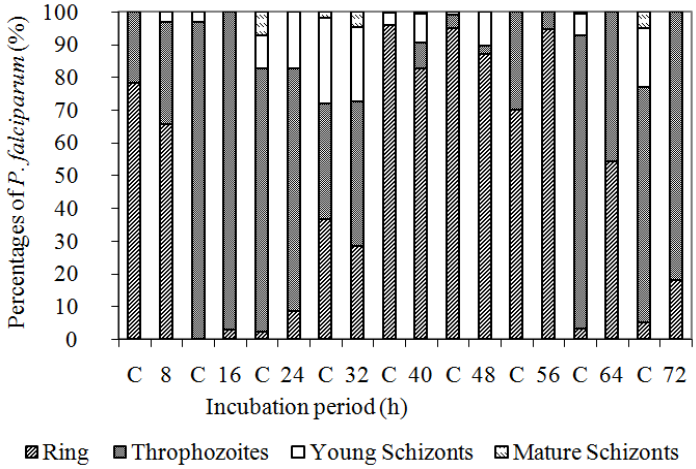

Fig. 5. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 80ng/mL on various stage of incubation time periods, compared with control (C = control without any compound)

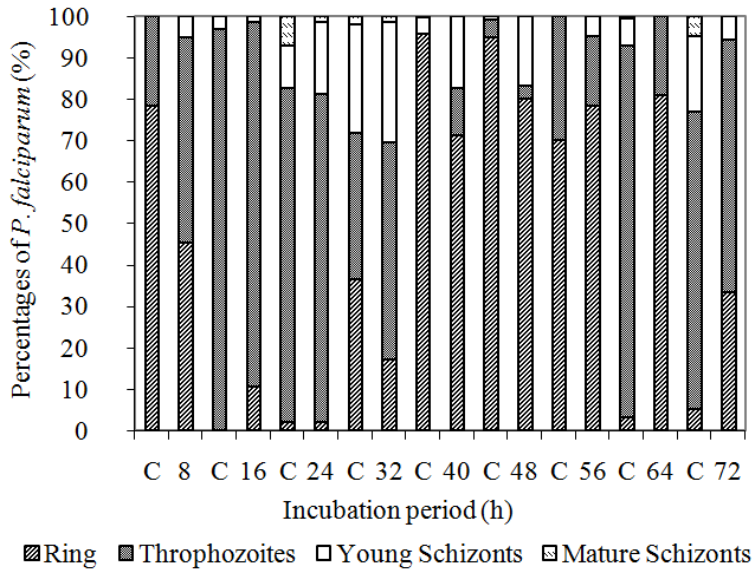

Fig. 6. Percentage of each stage of FCR-3 *P. falciparum* strain *in vitro* after giving eurycomanone 100 ng mL^{-1} on various stage of incubation time periods, compared with control (C = control without any compound)

Discussion

The results showed that administration of eurycomanone in concentration 20, 40, 60, 80 and 100 ng mL^{-1} showed growth inhibition of young schizonts to mature scizont that it could be seen on a 24-h incubation time. The IC_{50} values in the 24-h incubation period could not be calculated. However since the incubation time of 32 h until the end of the study (72 h), the IC_{50} were getting down (Table 2). Theoritically, at the 24th h, almost 50% *Plasmodium* grow into trophozoites and schizonts and at 32 h began to re-invasion into erythrocytes were not infected previously. At 48th h re-invasion has increased to about 40% (Srinivas and Puri, 2002). In this study, giving eurycumanone for 24h showed inhibit maturation of young schizont to mature schizont. If re-invasion started at 32 h, the observation could be performed at 40 h incubation period. At 40th h, control *Plasmodium* without any compound showed almost (95.78%) at ring stage. When compared with eurycomanone *Plasmodium* treated at concentration 10, 20, 40, 60, 80 and 100 ng mL^{-1}, percentage of ring stage of Plasmodium were lower (89.8; 94.54; 92.87; 82.26; 82.76 and 71.26% respectively). The greater the concentration of eurycomanone, the greater the inhibition.

Determination of the stage specificity of antimalarial against *Plasmodium* cycles is important to estimate the therapeutic response. It can also help a consideration in designing dosage (frequency, dose and duration), predict treatment failure and also inhibits malarial resistance (White, 1997). The effect of existing antimalarial e.g., dihidrofolat reductase inhibitors (cycloguanil, pyrimethamine) and quinolone group (quinine, mefloquine) are weak against asexual stage malaria parasites in the first 24 h (Dieckman and Jung, 1986; Geary *et al*., 1989; Rieckman *et al*., 1987; Ter Kuile *et al*., 1993). Other antimalarial such as chloroquine, halofantrine and artemisinine inhibit younger parasite growth *in vitro* and lower-stage ring *in vivo* (Geary *et al*., 1989; Alin *et al*., 1990; Ter Kuile *et al*., 1993; Udomsangpetch *et al*., 1996; Yayon *et al*., 1983; Zhang *et al*., 1986), thus decrease parasitaemia faster (White *et al*., 1989).

In this study, eurycomanone can kill ring stage of *Plasmodium* and also inhibits the growth of young schizonts to mature schizonts. These results suggested that eurycomanone can reduce parasitemia faster as chloroquine, halofantrin and artemisinin (Geary *et al*., 1989; Alin *et al*., 1990; Ter Kuile *et al*., 1993; Udomsangpetch *et al*., 1996; Yayon *et al*., 1983; Zhang *et al*., 1986).

Conclusion

Eurycomanone can kill ring stage of *P. falciparum* and may inhibit the development of young schizont to mature schizont *in vitro.* However, it needs further investigations for the mechanism.

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Author's Contributions

Eti Nurwening Sholikhah: Research coordinator, counted and identified the stages of *Plasmodium falciparum* microscopically, analyzed data, drafted the manuscript and revised the manuscript.

Mahardika Agus Wijayanti: Counted and identified the stages of *Plasmodium falciparum* microscopically, revised the manuscript

Ratna Asmah Susidarti: Analyzed data, revised the manuscript.

Indah Purwantini: Prepared the culture of *Plasmodium falciparum*, revised the manuscript.

Rani Afifah Nur Hestiyani: Counted and identified the stages of Plasmodium falciparum microscopically, revised the manuscript.

Hanifah Yusuf: Isolated the eurycumanone from *Eurycoma longifolia*, revised the manuscript.

Mustofa: Scientific supervisor and consultant, revised the manuscript, give final approval for the latest version of the manuscript.

Ethics

This research was conducted after the approval of Medical and Health Research Ethics Committee of Faculty of Medicine Universitas Gadjah Mada-Dr. Sardjito General Hospital, Yogyakarta, Indonesia. The author declare that there is no potential competing interest of this manuscript.

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