# ANTIPLASMODIAL ACTIVITY AND MECHANISMS OF ACTION OF *EURYCOMA LONGIFOLIA* JACK ROOT ISOLATED COMPOUNDS

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**Abstract.** *Eurycoma longifolia* Jack is a medicinal plant had been traditionally used as an antimalarial agent in Indonesia. Antiplasmodial activity and action mechanisms of *E. longifolia* Jack root isolated compounds have not previously been evaluated. In this study we aimed to identify the activity and mechanisms of action of *E. longifolia* Jack against *Plasmodium falciparum* to determine its potential as an antimalarial agent. Five different isolated compounds of the ethyl acetate soluble fraction derived from *E. longifolia* Jack root methanol extract were tested. The antiplasmodial activity against *P. falciparum* FCR3 and D10 strains was tested in vitro. We used a modified fixed-ratio isobologram method to evaluate the activity of each of the 5 isolated compounds against the E64, an epoxide acting as a cysteine protease inhibitor. We used an in vitro Heme Polymerization Inhibition Assay (HPIA) to identify the ability of the isolated compounds to inhibit β-hematin formation of *P. falciparum*. The 50% inhibitory concentration ($IC_{50}$) values (±Standard Deviation (SD)) for the 5 isolated compounds against the *P. falciparum* FCR3 ranged from 2.1 (±0.1) to 808.8 (±26.8) μg/ml and the $IC_{50}$ values (±SD) against the *P. falciparum* D10 strain ranged from 55.9 (±28.2) to 1151.1 (±122.7) μg/ml. The selectivity index was in the range of 0.1-66.2. Compounds 4 and 5 had greater antimalarial activity than the other compounds, with an antagonistic and an additive effect, respectively. The activity of Compound 4, interfering with hematin polymerization, and the additive interaction between Compound 5 and E64 corresponding to inhibition of protease enzymes could be possible action mechanisms of Compounds 4 and 5 suggesting them as potential antimalarial drug candidates.

**Keywords**: *Eurycoma longifolia* Jack root; *in vitro* antiplasmodial activity; selectivity index; protease inhibitor; HPIA.

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## INTRODUCTION

Antimalarial drug resistance has been reported worldwide (Antony and Parija, 2016; WHO, 2010). The use of medicinal plants as antimalarial agents has been studied (Flannery *et al*, 2013; Sá *et al*, 2011). *Eurycoma longifolia* Jack is a medicinal plant used in some Southeast Asian

countries, such as Malaysia, Thailand, Vietnam and Indonesia (Khanijo and Jiraungkoorskul, 2016). The roots of *E. longifolia* have been used as an antipyretic, an anthelminth and an antihistamine (Mohd Effendy *et al*, 2012). In Indonesia, the roots of *E. longifolia* have also been used as an antimalarial agent (Mardisiswojo and Radjakmangunsudarso, 1975; Syamsuhidayat, 1997). The quassinoids of *E. longifolia* root extracts from Southeast Asia had been found to have antiplasmodial activities against *Plasmodium falciparum* (Ang *et al*, 1995; Kuo *et al*, 2004; Wernsdorfer *et al*, 2009). Longilactone, dehydrolongilactone, 11-dehydroclaineanone, 15β-hydroxyklaineanone, 14, 15β-dehydroxyklaineanone and 15β-O-acetyl-14-hydroxyklaineanon have been isolated from *E. longifolia* (Jiwajinda *et al*, 2001). These compounds have been reported to have cytotoxic and antiplasmodial activity (Jiwajinda *et al*, 2002).

Erythrocytic malaria parasites take up and degrade large quantities of hemoglobin to generate amino acids (Rosenthal, 2011). The inhibition of hemoglobin degradation by *Plasmodium* spp has been reported to be a target of the antimalarial drug chloroquine (Zhang, 1987). Various studies have reported the antimalarial properties of *E. longifolia* Jack against *P. falciparum* (Chan *et al*, 2004; Mustofa and Qamariah, 2004; Wernsdorfer *et al*, 2009); however, none of the studies demonstrated action mechanisms of *E. longifolia* Jack related to hemoglobin degradation of *P. falciparum*. In the current study we aimed to evaluate the inhibition of heme polymerization activity by *E. longifolia* Jack root's isolated compounds using a Heme Polymerization Inhibition Assay (HPIA).

*Plasmodium* spp feed on host hemoglobin and develop into the ring stage, then trophozoites, and finally into a multinucleated schizont. Cysteine, aspartic, metallo-, and serine protease activities are crucial for this cycle (Drag and Salvesen, 2010; Rosenthal, 2002). Hemoglobin degradation requires proteases (Rosenthal, 2002; Goldberg, 2013). Several studies have suggested these proteases might have potential as antimalarial drug targets (Alam, 2014; Rosenthal, 2002). To the best of our knowledge, no studies have evaluated the action mechanisms of *E. longifolia* Jack against *P. falciparum* proteases. We aimed to identify the action mechanisms of *E. longifolia* Jack root against the protease enzymes of *P. falciparum* using an *in vitro* drug combination assay in order to determine its potential as an antimalarial agent.

## MATERIALS AND METHODS

### Collection, identification and isolation of *E. longifolia* Jack root

The current study built on the preliminary research findings conducted by Mustofa and Qamariah (2004), in which the methanol fraction of *E. longifolia* Jack root was found to possess antiplasmodial activity. The roots of *E. longifolia* were obtained from South Kalimantan, Indonesia and identified by the Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Five isolated compounds of the ethyl acetate soluble fraction derived from the methanol extract of *E. longifolia* were prepared by maceration in methanol for 3 days. The compound was then evaporated to obtain the extract and further fractionated with ethyl acetate to obtain soluble and

insoluble fractions. The ethyl acetate soluble fraction was concentrated, and the five compounds were isolated using thin layer chromatography. The process of isolating *E. longifolia* Jack root is illustrated in Fig 1.

***In vitro* cultivation of *P. falciparum***

In the current study, a chloroquine (CQ)-resistant strain FCR3 and CQ-sensitive strain D10 of *P. falciparum* were cultured *in vitro* as described by Trager and Jensen (1976) with minor modification. Blood type O erythrocytes were suspended in a culture media consisting of filtered, sterilized RPMI 1640 solution supplemented with 500 mg of gentamycin, 2 g of sodium bicarbonate, 6.2 g of HEPES per liter and 10% blood type O human serum at a pH of 7.3. Incubation was performed in a candle jar at 37°C in 5% $CO_2$. The level of parasitemia in the culture was kept between 2% and 5% with a 5% hematocrit. Parasite synchronization

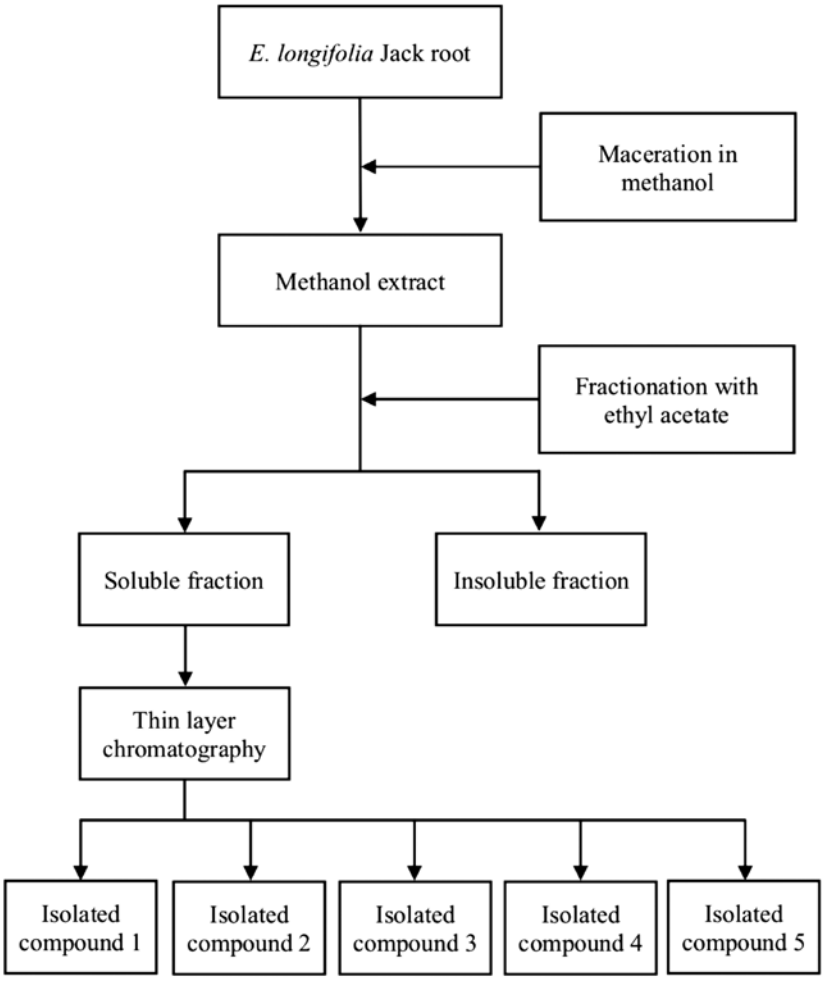


Fig 1-Isolation of the *E. longifolia* Jack root compounds from methanol extract used in this study.

was maintained by serial treatments with 5% sorbitol.

***In vitro* antiplasmodial assay**

*In vitro* antiplasmodial activity against *P. falciparum* was determined using standardized methods (Contreras *et al*, 2004; Lebbad, 2004). The assay was carried out in 96-well microplates. Isolated compounds of *E. longifolia* Jack root were dissolved in dimethyl sulfoxide prediluted with serum-free RPMI medium. A dose response assay was carried out to obtain the 50% inhibitory concentration ($IC_{50}$) of each compound. Microplates were preincubated with 100 µl of serially diluted test compounds. Ring stage-infected erythrocytes (100 µl per well with a 3% hematocrit and 2% parasitemia) were incubated in triplicate with twofold serial dilution for each compound and incubated for 72 hours. Each experiment was performed in duplicate. Parasitemia was measured microscopically on thin smears stained with 5% Giemsa. The number of parasitized red cells was counted per 1,000 red cells and divided by 10 to calculate the percent of parasitized cells. Red cells infected with any stages of *P. falciparum* were counted as infected red cells. Growth inhibition was expressed as percent parasitemia compared to the untreated control. The $IC_{50}$ value was calculated from the log of the compound concentration-response relationship (Wijayanti *et al*, 2010).

**Cytotoxic assay on Vero cells**

The *in vitro* cytotoxic assay was performed based on the MTT assay (Hughes and Mehmet, 2003). A Vero cell line was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cultures were maintained in a humidified incubator at 37°C with 5% $CO_2$. One hundred microliters of media containing $2x10^4$ cells was added into each well of the 96-well microplate and incubated for 24 hours. The isolated compounds of *E. longifolia* Jack root were added at the various concentrations and the study was performed in triplicate. After 24 hours incubation, the cells were gently washed with PBS and 100 µl of 0.5 mg/ml MTT was added to each well. The cells were then incubated for 24 hours at 37°C and the reaction was stopped by adding 100 µl of 10% SDS. The plates were then incubated overnight and the optical density was measured using an ELISA reader (Bio-Rad, Laboratories, Hercules, CA) at 595 nm. The data were then plotted in a dose-response curve, in which the $IC_{50}$ of each tested compound was determined. The selectivity of each tested compound (selectivity index) was calculated from the ratio of cytotoxicity ($IC_{50}$ on normal Vero cells) and the *in vitro* antiplasmodial activity ($IC_{50}$ of *P. falciparum*).

***In vitro* drug combination assay**

The analysis of the effect of the combination of *E. longifolia* compound and the protease inhibitor E64 (Sigma-Aldrich, St Louis, MO) was made using a modified fixed-ratio isobologram method (Fivelman *et al*, 2004; Semenov *et al*, 1998; Wiesner *et al*, 2002). E64 is an epoxide that is able to inhibit various cysteine proteases such as cathepsin B, cathepsin L, papain, staphopain and calpain (Varughese *et al*, 1989). *In vitro* antiplasmodial activity of each isolated compound and E64 against *P. falciparum* was determined prior to evaluating the drug combination.

The *in vitro* combined drug susceptibility test was performed to determine whether the isolated compounds had a synergistic effect using the fractional inhibitory concentrations (FIC) (Huang *et*

*al*, 2019). The FIC = $IC_{50}$ of the compound combined with the E64 ($E64/IC_{50}$) for each tested compound was calculated and plotted as an isobologram. Isobologram analysis evaluated the nature of the interaction between the two substances when used as a single agent to produce a defined effect. The concentrations were plotted on the X and Y axes in a two-coordinate plot, corresponding to (CA, 0) and (0, CB), respectively. The line connecting these two points is the line demonstrating an additive nature. The concentrations of the two drugs used in combination to provide the same effect, denoted as (CA, CB), were placed in the same plot. The synergistic effect between the two compounds was determined by the position of the corresponding FIC on the contour map. Points located above the line were considered antagonistic, points below the line were considered synergistic and points on the line were considered addition (Huang *et al*, 2019). The combination of the *E. longifolia* Jack compounds and E64 was expressed as the sum of the fractional inhibitory concentration (Σ FIC) (Zhao *et al*, 2004). Based on the Σ FIC values, the interactions between the drugs was determined as synergistic (Σ FIC ≤0.5), additive (Σ FIC of 0.5-4.0) and antagonistic (Σ FIC ≥4) (Pattanapanyasat *et al*, 2001; Huang *et al*, 2019).

The $CA_x$ and $CB_x$ were the concentrations of drugs A and B, respectively, used in combination to achieve x% of the drug effect. $IC_xA$ and $IC_xB$ were the concentrations of the single agents required to achieve similar effects. Each combination was serially diluted and processed similar to the sensitivity assay, allowing $IC_{50}$ to be calculated. The ratios of Compound 4 and E64 and Compound 5 and E64 were 6:0, 5:1, 4:2, 3:3, 2:4, 1:5 and 0:6.

***In vitro* Heme Polymerization Inhibition Assay (HPIA)**

The ability of tested isolated compounds to inhibit heme polymerization by *P. falciparum* was assessed using a protocol modified from Basilico *et al* (1998). The current study used 1.5 ml microtubes (Eppendorf®) instead of 96-well U-bottom microplates. Hematin was freshly dissolved to 4 mM in 0.2 M NaOH solution. One hundred microliters of this solution was mixed in a microtube with 50 µl of glacial acetic acid and 50 µl of the various concentrations of *E. longifolia* compounds. Aquadest or dimethylsulfoxide (DMSO) were used as the negative control. The final pH ranged from 2.88 to 3.60. After 24-hours incubation at 37°C, the tubes were centrifuged at 3000 rpm for 15 minutes. Afterwards, the supernatant was discarded and the pellet was washed three times with 200 µl DMSO. The pellet was then completely dissolved in 200 µl 0.1 M NaOH and 100 µl of each sample was placed into the well of a 96-well microplate. The optical density was read at 405 nm with a micro-ELISA reader (Bio-Rad, Laboratories, Hercules, CA) and the β-hematin concentration was calculated using the standard curve for hematin concentration plotted against the optical density value. Prior to the assay, the standard curve was prepared by dissolving 4 mM hematin in 0.1 M NaOH and the optical density was read with a micro-ELISA reader. The result was plotted as a standard curve by plotting the mean optical density for each of the concentrations on the ordinate against the hematin concentration on the abscissa. The heme polymerization inhibition activity was expressed as the percent inhibition using the following equation: % inhibition = 100 x [1 - (A/B)], where A = the concentration of the β-hematin in the

tested compound; B = the concentration of β-hematin in the negative control. The data were expressed in μg/ml as equivalents of the tested compound required to inhibit heme polymerization by 50% ($IC_{50}$).

## RESULTS

Table 1 shows the antiplasmodial activity of CQ-resistant *P. falciparum* FCR3 ($IC_{50}$), cytotoxicity ($IC_{50}$) in Vero cells and the selectivity index of the five isolated compounds of *E. longifolia* Jack root after incubation for 24 and 72 hours.

Of the 5 isolated compounds of *E. longifolia* Jack root, Compounds 4 and 5 had the greatest antiplasmodial activity against *P. falciparum* FCR3. The $IC_{50}$ (±SD) for Compound 4 was 3.5 (±0.1) mg/ml at 24 hours and 2.1 ± (0.1) mg/ml at 72 hours. Compound 5 was 13.5 (±1.7) mg/ml at 24 hours and 17.4 (±0.2) mg/ml at 72 hours. The selectivity index for Compound 4 had a range of 44.8-63.5 and for Compound 5 was 66.2-315.6. Compounds 1 and 2 had moderate antiplasmodial activity at 72 hours and Compound 3 had no antiplasmodium activity.

The antiplasmodial activity of *E. longifolia* Jack root against CQ-sensitive *P. falciparum* D10 ($IC_{50}$), its cytotoxicity ($IC_{50}$) in Vero cells and its selectivity index at 24 and 72 hours are shown in Table 2.

Of the 5 isolated compounds of *E. longifolia* Jack root, Compound 4 had the greatest antiplasmodial activity against *P. falciparum* D10. The $IC_{50}$ (±SD) was 19.0 (±0.2) mg/ml at 24 hours and 1.0 (±0.1) mg/ml at 72 hours. These values show a high level of antiplasmodial activity. Compound 5 had an $IC_{50}$ (±SD) of 63.7 (±4.7) mg/ml at 24 hours and 16.7 (±0.1) mg/ml at 72 hours. Compound 4 had a selectivity index of 11.8-91.2 and Compound 5 had a selectivity index of 66.0-69.0 against *P. falciparum* D10. Compounds 1 and 2 had low to moderate antiplasmodial activity and Compound 3 had no antiplasmodial activity against *P. falciparum* D10. The action mechanisms of Compounds 4 and 5 were evaluated for their ability to inhibit the protease enzyme and heme polymerization.

The mechanisms of Compounds 4 and 5 to inhibit protease enzymes of *P.*

Table 1
Antiplasmodial activity of 5 isolated compounds of *E. longifolia* Jack root against *P. falciparum* FCR3 ($IC_{50}$), the cytotoxicity in Vero cells ($IC_{50}$) and the selectivity index at 24 and 72 hours incubation

| Isolated compounds | $IC_{50}$ on *P. falciparum* FCR3 (μg/ml)(±SD) | | $IC_{50}$ in Vero cell (μg/ml)(±SD) | | Selectivity index[a] | |
|---|---|---|---|---|---|---|
| | 24 h | 72 h | 24 h | 72 h | 24 h | 72 h |
| 1 | 178.9 (±14.3) | 24.3 (±1.5) | 695.7 (±34.6) | 191.9 (±116.1) | 3.9 | 7.9 |
| 2 | 64.1 (±7.9) | 13.3 (±1.1) | 141.3 (±55.6) | 107.4 (±4.8) | 2.2 | 8.1 |
| 3 | 117.9 (±9.5) | 808.8 (±26.8) | 22.7 (±53.8) | 55.9 (±28.2) | 1.9 | 0.1 |
| 4 | 3.5 (±0.1) | 2.1 ( ±0.1) | 223.7 (±81.2) | 94.0 (±8.3) | 63.5 | 44.8 |
| 5 | 13.5 (±1.7) | 17.4 (±0.2) | 4,273.5 (±351.1) | 1,151.1 (±122.7) | 315.6 | 66.2 |

$IC_{50}$: concentration required to inhibit *P. falciparum* or Vero cell growth by 50% *in vitro*; [a]Ratio between $IC_{50}$ on *P. falciparum* FCR3 to $IC_{50}$ on Vero cell.

*falciparum* D10 were evaluated with a protease inhibitor trans-epoxysuccinyl-L-leucylamido (4-guanido) butane (E64, Sigma-Aldrich, St Luis, MO). The CI value (±SD) of Compound 4 was 2.1(±0.6) and for Compound 5 was 1.1(±0.2). The isobolograms for Compound 4 are in Fig 2 and Compound 5 are in Fig 3.

Table 2
Antiplasmodial activity of 5 isolated compounds of *E. longifolia* Jack root against *P. falciparum* D10 ($IC_{50}$), the cytotoxicity in Vero cell ($IC_{50}$) and the selectivity index at 24 and 72 hours incubation.

| Isolated compounds | $IC_{50}$ on *P. falciparum* D10 (μg/ml)(±SD) | | $IC_{50}$ in Vero cells (μg/ml)(±SD) | | Selectivity index[a] | |
|---|---|---|---|---|---|---|
| | 24 h | 72 h | 24 h | 72 h | 24 h | 72 h |
| 1 | 36.5 (±4.2) | 62.2 (±2.4) | 695.7 (±34.5) | 191.9 (±116.1) | 19.0 | 3.1 |
| 2 | 41.2 (±3.1) | 26.5 (±2.4) | 141.3 (±55.6) | 107.4 (±4.8) | 3.4 | 4.0 |
| 3 | 721.2 (±43.7) | 657.4 (±34.2) | 223.7 (±53.8) | 55.9 (±28.2) | 0.3 | 0.1 |
| 4 | 19.0 (±0.2) | 1.0 (±0.1) | 223.7 (±81.2) | 94.0 (±8.3) | 11.8 | 91.2 |
| 5 | 63.7 (±4.7) | 16.7 (±0.1) | 4,273.5 (±351.1) | 1,151.1 (±122.7) | 66.0 | 69.0 |

$IC_{50}$: concentration required to inhibit *P. falciparum* or Vero cell growth by 50% *in vitro*; [a]Ratio between $IC_{50}$ on *P. Falciparum* D10 to $IC_{50}$ in Vero cell.

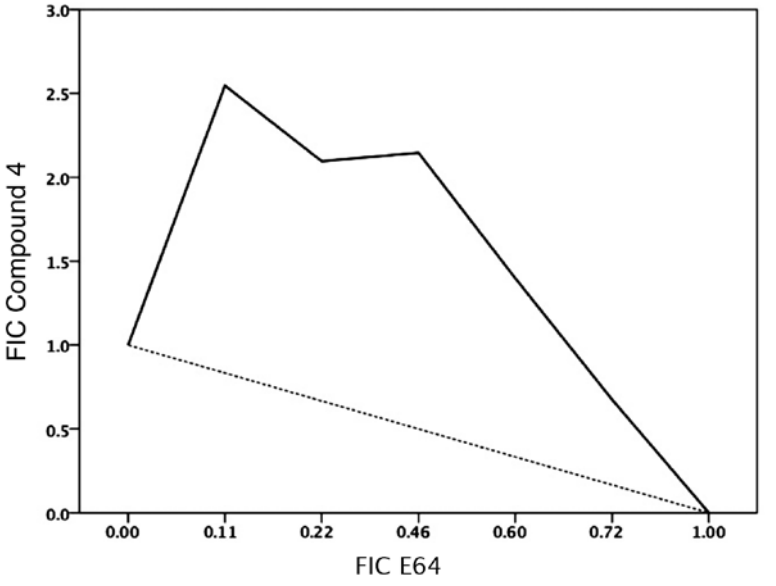


Fig 2-Isobologram showing the antagonistic interaction between fractional inhibitory concentrations (FIC) of E64 cysteine protease inhibitor and the FIC of Compound 4 for *E. longifolia* Jack root. The resulting concave line of FIC of both substances is located above the additive line (dotted line), showing antagonistic effect of the two substances.

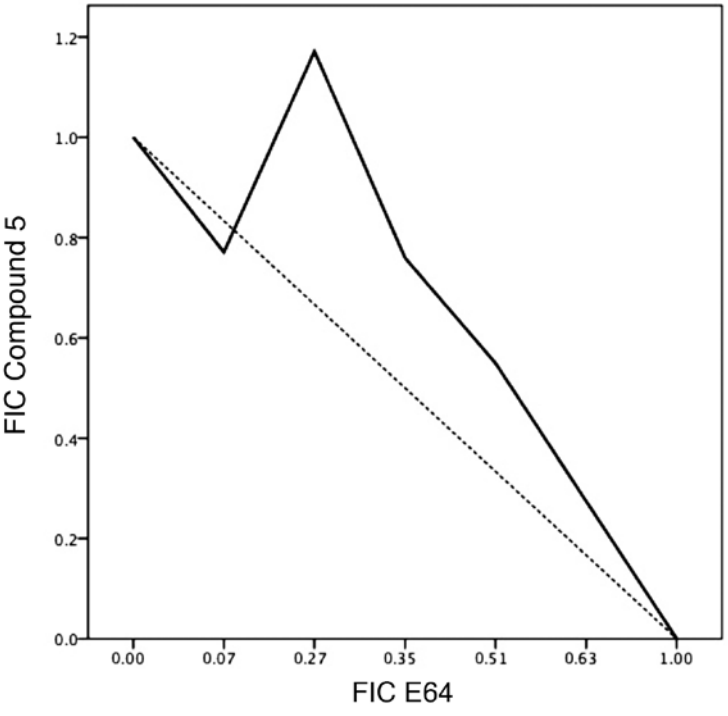


Fig 3-Isobologram showing the additive interaction between fractional inhibitory concentrations (FIC) of E64 cysteine protease inhibitor and the FIC of Compound 5 for *E. longifolia* Jack root. The relatively flat line of FIC of both substances is located on the additive line (dotted line), showing additive effect of the two substances.

Table 3
Inhibitory effect of Compounds 4 and 5 of *E. longifolia* Jack root against β-hematin formation.

| Isolated compound | Minimum inhibition (%)(±SD) | Maximum inhibition (%)(±SD) | $IC_{50}$ β-hematin formation (mM)(±SD) |
|---|---|---|---|
| Compound 4 | 31.8 (±15.4) | 73.5 (±4.5) | 445.6 (±146.3) |
| Compound 5 | 31.9 (±4.2) | 77.8 (±9.9) | 5,877.0 (±1,905.2) |

$IC_{50}$: concentration required to inhibit β-hematin formation of *P. falciparum* by 50% *in vitro*.

Compound 4 had an $IC_{50}$ value (±SD) for inhibiting β-hematin formation of 445.6 (±146.3) μg/ml and for Compound 5 of 5,877.0 (±1,905.2) (Table 3). These results show Compounds 4 and 5 had minimal inhibitory activity against β-hematin formation.

## DISCUSSION

*Plasmodium* species have a limited ability to synthesize amino acids. The majority of amino acids required for protein biosynthesis and energy metabolism are provided by hemoglobin

degradation products (Roy, 2017). Toxic byproducts of hemoglobin degradation are crystalized into a nontoxic biomineral identical to β-hematin, known as hemozoin. Massive degradation of hemoglobin results in the release of a large amount of toxic heme (Tekwani and Walker, 2005). Some antimalarial drugs bind to hematin, resulting in a higher concentration of free heme and antimalarial drug-heme complexes in food vacuoles, leading to parasite death (Ridley *et al*, 1997; Tekwani and Walker, 2005). In the current study, Compounds 4 and 5 had a mild inhibitory effect on β-hematin formation; this potential was lower than CQ which was found to have an $IC_{50}$ Heme Polymerization Inhibition Assay (HPIA) of 3.49 mM in a previous study (Wijayanti *et al*, 2007).

A previous *in vitro* study of the cytotoxic antiplasmodial activity of the aqueous, methanol and chloroform extracts of *E. longifolia* Jack root (Mustofa and Qamariah, 2004) showed the methanol extract had an $IC_{50}$ value at a concentration range of 0.6-1.9 mg/ml for *P. falciparum* FCR3 and D10 strains. The methanol extract had a cytotoxic effect against HeLa cells with a $IC_{50}$ value at a concentration range of 46.9-58.6 mg/ml. The methanol extract had a selectivity index range of 22.9-98.6 while the chloroform extract had a selectivity index of (30.6-35.8). Another study found *E. longifolia* Jack root reduced *P. berghei* parasitemia in infected mice (Kahtan *et al*, 2018).

Eurycomanone has been found to be the most active compound in *E. longifolia* Jack root (Rehman *et al*, 2016). Eurycromanone has been found in an *in vitro* study to have activity against *P. falciparum*; it has been found to kill the parasites in the ring stage and inhibit development of young schizonts (Sholikhah *et al*, 2016). In the current study, eurycromanone content in Compounds 4 and 5 might have interfered with the hematin polymerization and proteases enzymes.

In conclusion, the activity of Compound 4, interfering with hematin polymerization, and the additive interaction between Compound 5 and E64 corresponding to inhibition of protease enzymes could be possible action mechanisms for Compounds 4 and 5 suggesting them as potential antimalarial drug candidates.

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