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ABSTRACT

Hyptolide and epoxy hyptolide were successfully isolated and synthesized from *Hyptis pectinata* extract that potential to cure malaria diseases. The previous studies showed that these compounds contained lactone groups associated with antibacterial and anticancer activities. However, the antimalarial activity remained unclear. This study aimed to investigate the antiplasmodial activity of *H. pectinata* extract, hyptolide, and epoxy hyptolide. These compounds were tested *in vitro* for antiplasmodial activity against chloroquinesensitive 3D7 strain of *Plasmodium falciparum*. Thin blood smears were used to assess the levels of parasitemia and growth inhibition of the isolates and the ClpP inhibition was analyzed under well diffusion assay. The results showed that hyptolide, epoxy hyptolide, and *H. pectinata* extracts potential inhibit proliferation of *P. falciparum* in doses-dependent manner with IC₅₀ value of 2.06; 2.75; and 11.84 µg/ml, respectively. Further inhibition assay toward Pk-ClpP revealed that hyptolide (δ-lactone) more potent than β-lactone and PMSF demonstrated remarkably inhibition activity in doses-dependent manner. The results suggested that hyptolide exhibited antiplasmodial activity through Pk-ClpP inhibition and is potential to be developed as antimalarial drugs.

Keywords: Antiplasmodial activity, caseinolytic protease, epoxy hyptolide, *Hyptis pectinata*, hyptolide

INTRODUCTION

Malaria is an extremely dangerous parasitic disease caused by the protozoa parasites including *Plasmodium falciparum* that transmitted from blood of an infected person and passed to a healthy human by female *Anopheles* mosquito.^[1] Malaria was widespread in the 20th century in more than 100 countries throughout the tropical and subtropical zones such as Indonesia.^[2] In 2019, the WHO reported that there were 228 million cases and 405,000 cases malaria related-death.^[3] This is mainly due to the parasites have developed resistance to a several chemical antimalarial treatment including chloroquine, artemisinin, and derivatives.^[4,5] The increasing global spread of drug resistance

to the most of antimalarial drugs is a major concern and requires innovative strategies to combat the disease.^[6] There is an urgent need to discover new compounds with novel mechanism of action. Recently, medicinal plants have a great potential to provide new antimalarial drug.

The previous studies have confirmed that caseinolytic protease (ClpP) proteins play a pivotal role in cell and organelle division, segregation, protein homeostasis, protein transport, and virulence.^[7,8] The previous study also reported that increases the ClpP expression correlated with increasing the *P. falciparum* life cycle and infects red blood cells.^[9] Recently, a group of lactones was identified to inhibit ClpP interfering with the progression of *P. falciparum*.^[9,10] However,

the relevance of ClpP protease in malaria pathogenesis offers an untapped targeted for antimalaria using the lactones groups. *Hyptis pectinata* L. Poit is one of δ -lactone resource with various pharmacological activity such as anticancer^[11,12] and antibacterial. In Indonesia, the dry herbs of *H. pectinata* widely used to treat malaria and other infections. However, the antiplasmodial activity of *H. pectinata* and their analog through inhibition of ClpP protein remains unclear. Therefore, this study aims to investigate antiplasmodial activity of *H. pectinata* and their analog against *P. falciparum* 3D7.

MATERIALS AND METHODS

Plant Material Collection

The plant sample *H. pectinata* was collected from Kanayaka village, Bandung, West Java, Indonesia (Latitude -7.0460282 and Longitude 107.7915393) in October 2014. For biological studies, the leaves were dried in a renewal air oven and circulated at 40°C until it is completely dehydrated. A Voucher specimen (MS 100562) was identified at the Faculty Science and Mathematics, Diponegoro University, Semarang, Indonesia.

Extraction and Isolation of Hyptolide, and Synthesis of Epoxy Hyptolide

Dried powdered of leaves (0.710 kg) were extracted by the maceration method using methanol according to the Suzery et al.^[11] Extract *H. pectinata* yield (7.89%, weight) was obtained in the methanol maceration. After extraction, the crystal of hyptolide isolation was carried out by the recrystallization method according to the previous study with slight modification.^[13] Hyptolide was isolated in 1.7% yield from extracts of methanol. Furthermore, the epoxidation procedure was prepared by meta-chloroperoxybenzoic acid epoxidation method. Our previous study reported that chemical characterization of hyptolide and epoxy hyptolide crystal was obtained using nuclear magnetic resonance and Fourier-transform infrared according to the Cahyono et al.^[14]

Plasmodium falciparum (3D7 Strain) Culture

P. falciparum 3D7 strain was obtained from Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. *P. falciparum* was culture using the combination of trager and Jensen methods and candle-jar methods with slight modifications.^[15] Briefly, parasites were propagated in human type O-positive red blood cells in Roswell park memorial institute medium-1640 (RPMI-1640) (Gibco, USA catalog #11875093) enrich with 5.96 g HEPES, 2.1 g NaHCO_3 , 0.05 g hypoxanthine, 50 $\mu\text{g}/\text{ml}$ gentamycin, and 10% human O+serum. The parasites were cultivated at 37°C , culture media were renewed daily and the parasite growth was monitored through Giemsa staining in thin blood smears. The culture to be used for the experiment should be governed by ring shapes. Stock parasite cultures were further diluted with uninfected type O+ human erythrocyte and full culture medium to achieve 1% parasitemia and 50% hematocrit. These final parasite cultures were immediately used in the antimalarial assay.^[16] This study was approved and in accordance to the Commission on Health Research Ethics, Faculty of Pharmacy,

Universitas Airlangga, Surabaya, Indonesia under No. 196. KE.087.04. 2017.

Antiplasmodial Activity Assay

A 10 $\mu\text{g}/\text{ml}$ of stock solution was prepared from *H. pectinata* extract, hyptolide, and epoxy hyptolide, respectively. Six serial concentrations have been prepared from each stock solution (100 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$, 0.1 $\mu\text{g}/\text{ml}$, 0.01 $\mu\text{g}/\text{ml}$, and 0.001 $\mu\text{g}/\text{ml}$), respectively. For each microplate well, dilute 100 μL compounds solution was applied to 100 μL of final parasite culture and incubated at 37°C for 48 h. Thin blood tests were prepared on the labeled slides and air-dried and fixed with methanol. The dry slides were stained using Giemsa according to the Inbaneson and Ravikumar^[17] and observed under light microscope with 1000x magnifications and the percentage of parasitemia was determined.

The extract with the highest inhibition percentage tested again to determine the 50% inhibitory concentration (IC_{50}) values using serial concentration of 100 $\mu\text{g}/\text{mL}$ –0.001 $\mu\text{g}/\text{mL}$. The IC_{50} values were determined graphically on dose-response curves (concentration versus percent inhibition curves). This activity was analyzed in accordance with the norm of plants antimalarial activity according to the Chinchilla et al.^[18] Based on this norm, strong active, active, weakly active, and inactive extract has $\text{IC}_{50} < 5$ $\mu\text{g}/\text{ml}$; IC_{50} 5–50 $\mu\text{g}/\text{ml}$; 50 $\mu\text{g}/\text{ml}$ $< \text{IC}_{50} < 100$ $\mu\text{g}/\text{ml}$; and $\text{IC}_{50} > 100$ $\mu\text{g}/\text{ml}$, respectively.

Preparation and Quantification of Protein Content

A purified full-length recombinant of ClpP system of *P. knowlesi* (Pk-ClpP) was used a protein in this study. This recombinant protein was a kind gift of Angelesa Runin of Universiti Malaysia Sabah. The protein stock was dissolved in 20 mM phosphate buffer pH 8.0. First, the purity of the protein was analyzed using electrophoresis with 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gel. The sample was centrifuged at 30,000 g for 30 min at 4°C for the removal of aggregated proteins and other particles, followed by filtering the supernatant using 0.22 μm filters (Millipore Corp., Bedford, MA). Furthermore, the total protein content of samples was calculated by Lowry's methods with bovine serum albumin as protein standard.^[19]

To confirm the identity of the protein, the protein band on the unstained gel was first transferred to a nitrocellulose membrane (Amersham Biosciences, UK) using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., USA). The transfer was done for 20 min using 1x transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol) at room temperature. The membrane was then incubated for 1 h at room temperature with block ACE (Bio-Rad Laboratories, Inc., USA) to block non-specific binding. Primary antibody (Mouse Anti Penta Histidine Tag: Hrp) (Bio-Rad Laboratories, Inc., USA) was added into the mixture at a final ratio of 1:2000. The incubation was done for 2 h, which then was subsequently incubated with secondary antibody (Goat Anti Mouse IgG (H/L):Hrp (Multi Species Adsorbed) conjugate, Bio-Rad Laboratories, Inc., USA) in a ratio of 1:5000. The membrane was washed with 4x wash buffer and 2x PBS buffer for 5 min each. For detection of the signal, the

Clarity™ ECL substrate (Bio-Rad Laboratories, Inc., USA) was used according to the manufacturer protocol and the images of Western blot were visualized and analyzed using ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Inc., USA).

Well Diffusion Assay

First, the casein agar plates were prepared according to the Manorma *et al.*^[20] with slight modification. The Pk-ClpP (0.5 mg/mL at final concentration) was combined with different concentrations of hyptolide, ranging from 1 to 30 nM. The mixture was then incubated at 37°C for 15 min and then deposited in casein agar plates at the well. The plates were then incubated at 37°C and observed for the forming of the halo zone for 2 days. The activity was classified as unit activity (U) in which 1 U is defined as the amount of enzyme required to generate 1 mm of halo zone in 1 h, indicating the hydrolysis of casein. In the negative control groups, the activity of Pk-ClpP was modified as 100% of the activity. The relative activity (Rel. activity) of Pk-ClpP in the presence of hyptolide was measured as the ratio of control activity. The IC₅₀ value was calculated using a four-parameter logistic curve under the SigmaPlot 14.0 statistical program (CA, USA). The test was performed in a triplicate. Inhibition percentage was calculated as follows: Inhibition percentage (inhibition %) = 100% - ((Xu/Xk) × 100%), Note: Xu = Growth percentage of each isolate and Xk = Growth percentage of negative control.

Statistical Analysis

The data presented the mean ± standard error of the mean ± (SE). The statistical analysis was done using SPSS (version 22.0). Group comparisons were performed using one-way ANOVA followed by Duncan *post hoc* test. Statistically significant was $P < 0.05$.

RESULTS AND DISCUSSION

The emergence of *P. falciparum* parasite resistance due to the use of chemical compounds leading to the exploration of natural plant that potent to inhibit the protease for the discovery of antimalarial compounds that function by inhibiting essential proteases of parasites. The lactone groups provide strong antiplasmodial activity by binding to the ClpP proteases. ClpP proteases are one of the new target protein groups that have not yet experienced resistance.^[9] Therefore, ClpP proteases have the potential to develop new antiplasmodium-targeted therapy. Previously, we successfully isolate a major compound of *H. pectinata* extract, namely, hyptolide and synthesize an analog compound of hyptolide. Hyptolide and epoxy hyptolide have been shown to have a lactone groups as a main structure [Figure 1]. Hyptolide also reported that have a various pharmacological effects including anticancer and antibacterial activity.^[11] However, the effect of hyptolide and its analog in inhibiting the growth of the *P. falciparum* is still unclear. Therefore, this study aims to explore the antiplasmodium activity of *H. pectinata* extract, hyptolide, and epoxy hyptolide against *P. falciparum* 3D7 strain *in vitro* and evaluate the molecular mechanism targeted on ClpP protease.

In vitro Antiplasmodial Activity

In the present study, the *H. pectinata* methanolic extract, hyptolide, and epoxy hyptolide were determined for their

antiplasmodial potencies against *P. falciparum* 3D7 strain. All of the compounds possess antiplasmodial activity in a dose-dependent manner [Figure 2]. The hyptolide and epoxy hyptolide were found to be more potent with an IC₅₀ value of 2.06 µg/ml and 2.75 µg/ml, respectively, than the crude extract of *H. pectinata* with an IC₅₀ value of 11.84 µg/ml [Table 1]. Based on the IC₅₀ value, it can be classified that the hyptolide and epoxy hyptolide have a strong activity and *H. pectinata* extract has moderate activity inhibiting the growth of *P. falciparum*.^[18] The antiplasmodial activity of *H. pectinata* extract and their isolate analog appeared and was associated with the δ-lactone group.^[21] Antiplasmodial activity of some lactone group derived from δ-lactone quassilactone was also shown to exhibit the potent antiplasmodial activity 40-fold greater than quassin.^[22] A previous study also reported that the lactone group was responsible for the antiplasmodial activity

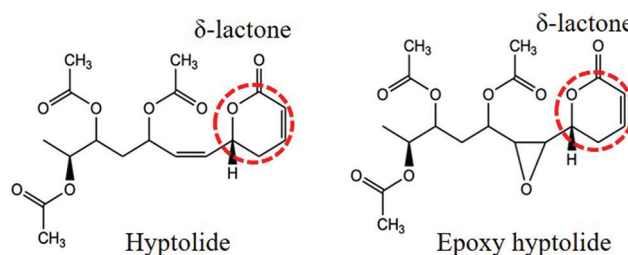


Figure 1: Lactone groups on hyptolide and epoxy hyptolide chemical structure

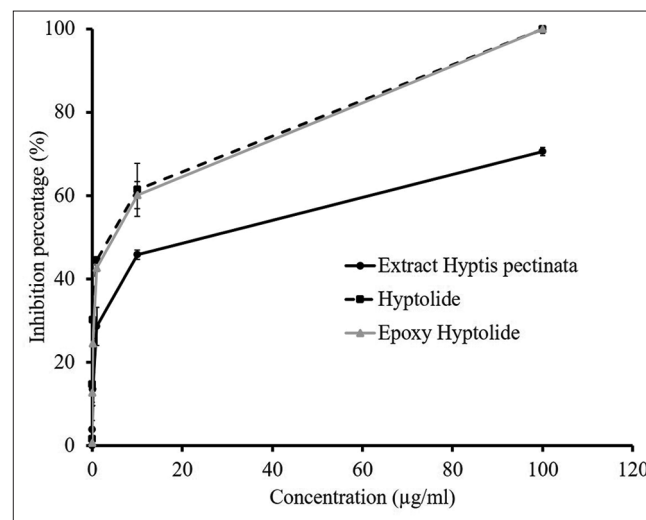


Figure 2: Antiplasmodial activity of each compound for 48 h incubation against *P. falciparum* 3D7 strain. Data represent mean value ± SE of triple-independent experiment

Table 1: IC₅₀ value of *H. pectinata* extract, hyptolide, and epoxy hyptolide against 3D7 *Plasmodium falciparum*

Compound	IC ₅₀ value (µg/mL)	P-value
<i>H. pectinata</i> extract	11.84 ± 0.41*	0.00
Hyptolide	2.06 ± 0.13*	0.00
Epoxy hyptolide	2.75 ± 0.24*	0.014

Values shown by * are significant at $P < 0.05$. *H. pectinata*: *Hyptis pectinata*

against *P. falciparum* through interaction with the active site of prokaryotic ClpP.^[9] Based on this phenomenon, it is interesting to confirm whether these compounds exhibit inhibition effect toward Pk-ClpP

Recombinant Pk-ClpP

The high purity recombinant Pk-ClpP recombinant was used for the inhibition assay. The high purity of the protein is the essential requirement for the inhibition assay to avoid unspecific inhibition due to the presence of contaminants. The purity of recombinant Pk-ClpP was confirmed by the presence of a single band at the apparent size of ~28 kDa [Figure 3]. The absence of the other protein bands suggested that the Pk-ClpP used in this study was almost free from the contaminant. This apparent size of Pk-ClpP on the gel corresponded to its theoretical size which confirmed that the apparent band was indeed Pk-ClpP. The ClpP from the other organisms was also reported to have comparable sizes (26–28 kDa).^[22,23] Further, the identity of Pk-ClpP was also confirmed using Western blotting which showed a positive signal at the position corresponding to the Pk-ClpP band. To note, Pk-ClpP was produced through recombinant production using *Escherichia coli* BL21 (DE3) system. For this production, the gene encoding was inserted into pET28a plasmid, expressed under lac operon system, and purified using affinity chromatography. Details of the expression and purification of Pk-ClpP will be published somewhere else. The use of pET28 yielded Pk-ClpP to have a 6His-tag at its N-terminal and, therefore, was able to bind to the anti-6x-tag primary antibody used in the blotting. The contaminant proteins, if exist, should not be able to bind due to the lack of the tag.

The Relative Activity of Pk-ClpP Under Hyptolide Administration

A group of lactones was identified potentially inhibit ClpP protease leading to robust ability to decrease *P. falciparum* viability.^[9] In this study, we evaluate the effect of hyptolide (δ -lactone), β -lactone, and PMSF (serine protease inhibitor) on ClpP inhibition using well diffusion assay. These compounds were proven to inhibit Pk-ClpP in doses-dependent manner, hyptolide had the strongest inhibitory activity toward Pk-ClpP due to at a concentration of 30 nM of hyptolide had a percent residual activity of up to 4.32%, compared to β -lactone and PMSF which had a residual activity at the same dose of 20.11% and 44.32%, respectively [Figure 4]. Based on these findings, IC₅₀ values can be calculated for hyptolide, β -lactone, and PMSF [Table 2]. Based on the IC₅₀ value, the inhibitory properties of the extract against Pk-ClpP were in the following order hyptolide < β -lactone < PMSF. The hyptolide inhibits ClpP through competitive inhibition, whereby this compound exhibits higher affinity to the ClpP than the substrate, and might be the structure of the δ -lactone in hyptolide correlates with the ability to inhibit the activity of Pk-ClpP. These results supported the previous studies that the δ -lactone compound has a strong interaction with the ClpP protease, resulting in ClpP inactivation that associated with decreased plasmodium proliferation.^[22]

The δ -lactone is a novel inhibitor for the specific and selective targeting of the key parasite regulator ClpP in

P. falciparum and *P. knowlesi* strains. In fact, our strongest hyptolide was capable of dramatic reductions in ClpP expression which correlated with decreased viability of *P. falciparum* in infecting blood. Therefore, targeting these parasite regulators may be an attractive strategy to counteract the harmful effects of malaria and aid the immune response to eliminate the disarmed parasites. Its due to the ClpP is essential for survival and very highly conserved in many pathogens, our strategy can represent a global approach to infectious disease treatment that minimizes selective stress on malaria and the development of resistance.

Table 2: IC₅₀ value of hyptolide, β -lactone, and PMSF against Pk-ClpP

Compound	IC ₅₀ value (nM)	P-value
Hyptolide	2.4±0.11*	0.00
β -lactone	6.2±0.30*	0.00
PMSF (serine protease inhibitor)	18.2±2.16*	0.00

Values shown by * are significant at $P < 0.05$

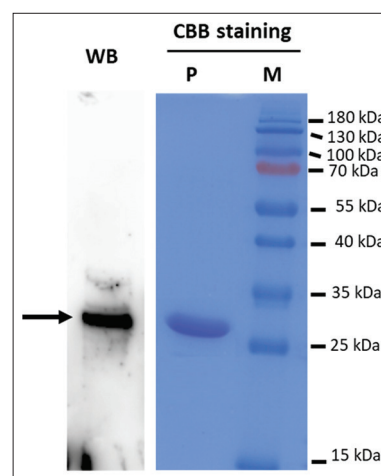


Figure 3: 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot (WB) analysis of purified Pk-ClpP. The gel was stained using CBB (Coomassie brilliant blue). P lane refers to the well where the purified protein was load onto the gel. Lane M refers to the well where the protein markers were loaded onto the gel. The band that corresponds to Pk-ClpP is indicated by the arrow

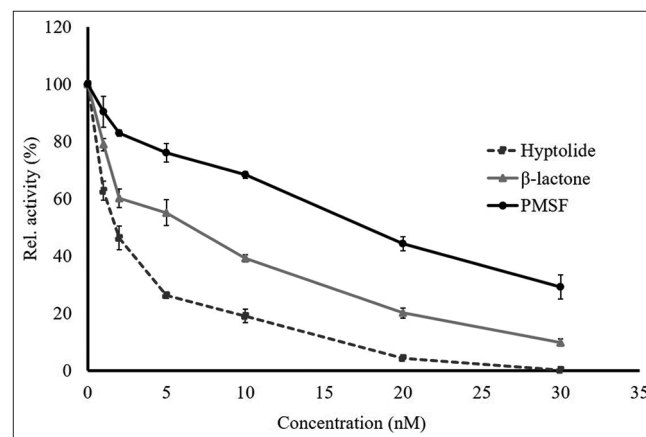


Figure 4: Pk-ClpP catalytic activity under various concentrations of hyptolide, β -lactone, and phenylmethylsulfonyl fluoride (PMSF). Data represent mean value \pm SE of triplicate experiment

However, in this study, we did not analyze in drug-resistant *P. falciparum* malaria, therefore, the detailed mechanism of *H. pectinata* extract, hyptolide, and epoxy hyptolide as an antimalarial in antimalarial drug-resistant remains unclear.

CONCLUSION

Taken together, we have identified synthetic δ -lactones as novel antiplasmodial treatment for malaria disease and inhibitors for specific and selective targeting of the key parasite regulator ClpP in *Plasmodium sp.* In addition, this study for the first time scientifically validates the traditional claim of the plant for its antimalarial property. Further the mechanism of the antimalarial activity is recommended to explore.

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