

Metabolite extract of *Streptomyces hygroscopicus* Hygroscopicus inhibit the growth of *Plasmodium berghei* through inhibition of ubiquitin – proteasome system

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Received 4 January 2013; received in revised form 19 March 2013; accepted 23 March 2013

Abstract. *Streptomyces hygroscopicus* Hygroscopicus, a member of family of *Actinomycetes* produces eponemycin a proteasome inhibitor that can inhibit Ubiquitin-Proteasome System (UPS) function in eukaryotic cell. Previous study showed that coronamycin, an active substrate isolated from *Streptomyces* sp. can act as anti-plasmodial, antibacterial, and antifungal, however the research did not show the mechanism of coronamycin in inhibiting the growth of *Plasmodium*. This research was done to reveal if eponemycin that is contained in metabolite extract of *S. hygroscopicus* can inhibit UPS function of *Plasmodium berghei*. This study was an experimental study using *P. berghei* infected Balb/C mice as malaria model. Samples were divided into 1 control group (group infected with *P. berghei* without treatment) and 3 treatment groups (mice infected with *P. berghei* and treated intra-peritoneal with metabolite extract of *S. hygroscopicus* dose 130 µg/kgBW, 580 µg/kgBW, and 2600 µg/kgBW for 5 days). The degree of parasitemia and morphology of the parasite were measured from the first day of malaria induction until the last treatment. The accumulation level of *polyubiquitin* was measured using *Western blot* and ELISA method. The degree of parasitemia on day 6 showed significant differences among treatment groups and control (p=0,000). Percentage of inhibition showed significant differences between control and group treated with metabolite extract of *S. hygroscopicus* 2600 µg/kgBW. An increasing dose of extract of *S. hygroscopicus* followed by an increasing of inhibition in parasite growth (r=0,850). Probit analysis showed that ED50 was 9.418 µg/kgBW. There was a change in morphology of the parasite after treatment. Parasite morphology became *crisis form*. There was an accumulation of *polyubiquitinated* protein in the group treated with metabolite extract of *S. hygroscopicus* 2600 µg/kgBW. It can be concluded that analog *eponemycin* in metabolite of *S. hygroscopicus* is a potential candidate for new malarial drug by inhibiting UPS function of the parasite and cause stress and dead of the parasite.

INTRODUCTION

Malaria, one of tropical infectious disease, is still included as an important health concern worldwide. In 2010, there were 216 million cases of malaria, 81% of these were in the WHO African Region. An estimated 3.3 billion people were at risk of acquiring malaria and 655 000 persons died of malaria. About 86%

of the victims were children under 5 years of age, and 91% of malaria deaths occurred in the WHO African Region (WHO, 2011). Snow *et al.* (2005) provide an empirical approach to estimating the number of clinical events caused by *P. falciparum* worldwide, by using a combination of epidemiological, geographical and demographic data and estimate that there were 515 (range 300-660)

million episodes of clinical *P. falciparum* malaria in 2002. These global estimates are up to 50% higher than those reported by the World Health Organization (WHO) and 200% higher for areas outside Africa (Snow *et al.*, 2005).

Effort to reduce the high mortality and morbidity of malaria is having problems due to the development of anti-malarial drug resistance. The greatest challenge recently is the emerging incidence of resistance to the treatment of malaria, especially resistance of *P. falciparum* to artemisinin, which is known as the first-line drugs in the treatment of malaria. Failures with oral artesunate monotherapy may be due not only to decreased sensitivity but also to high pretreatment parasitaemia. Therefore, in order to maximize the effectiveness of artemisinin and its derivatives and to protect them from the development of resistance, WHO has repeatedly recommended that they should be combined with other drugs that have different mechanisms of action and longer half-lives. However, resistance to the combination of artemisinin with other antimalarial drugs has also been known to develop. It has been shown that the combination of artemether-lumefantrin has decreasing efficacy in several areas. The increase in the proportions of patients parasitaemic at day 3 after ACT treatment observed in both Cambodia and Thailand indicate that the efficacy of artesunate is diminishing and failure of treatment with artesunate-amodiaquine combination was also found in four studies in Indonesia (WHO, 2010). Moreover, Artemisinin-based Combination Therapy is more expensive than the treatments currently being used (Garner & Graves, 2005). Based on malaria treatment resistance data shown above and the potential public health impact costs of reducing transmission, the development of new malaria drug is strongly required.

Streptomyces hygroscopicus Hygroscopicus, the family of *Actinomycetes*, is commonly found in soil. *Streptomyces* is a gram positive rod, non-motile, produces spores, and is an obligate aerobic bacterium. It forms soft filaments called hyphae/mycelium which rarely fragments (Health

Protection Agency, 2009). The genus *Streptomyces* is one of the dominant genus of Actinomycetes and is known to produce antibiotics and the majority of bioactive molecules (Khamma *et al.*, 2008; Solanki & Khanna, 2008), higher than those produce by other microbes such as fungi and yeast. *Streptomyces hygroscopicus* Hygroscopicus is a species of *Streptomyces* sp. which potentially produces antibiotics as secondary metabolites. Formation of metabolites results from microbial fermentation process and is highly dependent on the filament biomass level, the morphological profile of the culture, as well as the environments such as tropical rainforests, the ocean, desert, and ice (Nurkanto *et al.*, 2010). *Streptomyces hygroscopicus* contain *eponemycin* that has antitumor and anti-plasmodial activities. Eponemycin is a linear peptide, anti-angiogenic α,β -epoxyketone isolated from *S. hygroscopicus* P247-271. Eponemycin affects the activity of 20S proteasome in Ubiquitin-Proteasome System (UPS) which forms an unusual six-membered morpholino ring with the amino terminal catalytic Thr-1 of the 20S proteasome (Kim, 2005).

Ubiquitin – Proteasome System (UPS) is the main metabolic route in cytosol and nuclear eukaryotes providing selective degradation of cellular proteins. Ubiquitin – Proteasome System plays important roles in a variety of fundamental cellular processes such as regulation of cell cycle progression, division, development and differentiation, apoptosis, cell trafficking, and modulation of the immune and inflammatory responses. The central element of this system is the covalent linkage of ubiquitin to targeted protein, called ubiquitylation (Wang & Maldonado, 2006). Ubiquitin – Proteasome System has an important role in protein quality control of *P. falciparum*. This is because: i) erythrocytic parasite phase has a high replication rate, ii) protein of *Plasmodium* sp. has a large size, iii) region with low complexity is abundant among and within the globular domain, and iv) proteins are under stress due to the increasing of patient's body temperature (fever) (Kreindenweiss, 2008). The proteasome inhibitor eponemycin (analog of eponemycin) effectively kill all

stages of intraerythrocytic parasites. Twenty-four hours after treatment, the total parasitemia, asexual parasites and gametocytes decreased significantly. Seventy-two hours after treatment, no viable parasites remained in treatment group. Epoxomicin also blocked oocyst production in the mosquito midgut (Czesny *et al.*, 2009). Based on data above, *S. hygroscopicus* Hygroscopicus has the potential to be developed for antimalarial therapy.

MATERIALS AND METHOD

Experimental Design

This study was an experimental study using *Plasmodium berghei* infected Balb/C mice as malaria model. Samples were divided into 1 control group (group infected with *P. berghei* without treatment) and 3 treatment groups, there were mice infected with *P. berghei* intra-peritoneally with metabolite extract of *S. hygroscopicus* dose 130 µg/kgBW (P1), 580 µg/kgBW (P2), and 2600 µg/kgBW (P3) for 5 days. The accumulation level of *polyubiquitin* was measured using *Western blot* and ELISA method. The degree of parasitemia and morphology of the parasite were measured from the first day of malaria induction until the last treatment.

Preparation of Bacterial Isolate Medium

One liter of medium ISP4 (International Streptomyces Project) needs 10g soluble starch, 1g K₂HPO₄, 1g MgSO₄.7H₂O, 1g NaCl, 2g (NH₄)₂SO₄, 2g CaCO₃, 0, 1g trace salt solution (0.1 g Fe₂SO₄.7H₂O, ZnSO₄.7H₂O, MnCl₂.4H₂O 0.1 g, distilled water 1ml), 20g agar, and one liter distilled water with pH 7.0 to 7.4. (Shepherd, 2010). The solution was autoclaved at 121°C for 15 minutes, then *S. hygroscopicus* isolates were streaked on the medium and stored at 28°C for optimal growth (Sharma & Parihar, 2010).

Preparation of *S. hygroscopicus* subspecies Hygroscopicus Isolate

Streptomyces hygroscopicus subspecies Hygroscopicus was obtained from the microbiology laboratory LIPI (LIPI-MC) Cibinong. Gram staining carried out to

examine the characteristics of *S. hygroscopicus* subspecies Hygroscopicus bacteria that have grown on medium in accordance with the submitted by LIPI-MC. In ISP4 agar medium, macroscopically, the bacterial culture colonies were round, hard, and black pigmented. Microscopically by gram staining it is a gram-positive rod bacterium which is purple, has endospores and produce hyphae that characterize the *Actinomycetes* group of bacteria.

Inoculation and Fermentation of *S. hygroscopicus*

A total of 25.8 x 10⁶ bacteria in two ml of liquid inoculum were inoculated in Erlenmeyer flask containing 50 ml ISP4 broth medium and allowed to stand 7 days a 28°C in a shaking incubator (Sharma & Parihar, 2010).

Extraction of *S. hygroscopicus* Metabolite

The fermented *S. hygroscopicus* was mixed with ethylacetate 1:5 (v/v), shaken for 1 hour, and deposited in a separate funnel for 4 hours. After that, the water phase was discarded and the solvent phase (ethylacetate) was evaporated in a water-bath 80 - 90°C (Sharma & Parihar, 2010).

Thin layered chromatography (TLC) of *S. hygroscopicus* metabolite extract

Extraction was dissolved in methanol. Silica plate 2x10 cm was dotted with 30 µl samples by a capillary tube. The eluent and the silica plate were placed in an upright state inside TLC chamber. The eluent was read under 254 nm and 365 nm UV light. Afterwards, the eluent was sprayed with anisaldehyde and heated until the brown color appeared (Sin *et al.*, 1998).

Animal model

Animal model used were male Balb/c mice weighing 30-40 grams, healthy, active, and had white fur, purchased from LPPT Gadjah Mada University. Mice were kept in cages 30x30 cm with 4-5 mice per cage. Mice were given standard food and drink every day. Treatment in experimental animals has been approved by the Ethics Commission of the

Faculty of Medicine, University of Brawijaya. Animals were acclimatised and adapted for 7 days.

Preparations and inoculation of *P. berghei*

Plasmodium berghei ANKA strain were obtained from Biomedical Laboratory of University of Brawijaya. Pellet of erythrocytes infected with *P. berghei* that were stored in liquid nitrogen tank -135°C temperature was thawed and centrifuged at 2000 rpm 5 minutes. Pellets were washed twice in RPMI medium and diluted as needed for inoculation intra-peritoneally (ip) at 10⁷ parasites in 0.2 ml of blood per mice (Blazquez *et al.*, 2008). Parasitemia was checked with thin blood smear stained with Giemsa. The degrees of parasitemia were measured by calculating infected erythrocyte per 1000 erythrocytes.

Metabolite extract of *S. hygroscopicus* Hygroscopicus therapy

Metabolite extracts of *S. hygroscopicus* Hygroscopicus dissolved in DMSO with sonicator. Dilution was made with RPMI medium to make concentration of 130 µg/kgBW, 580 µg/kgBW, and 2600 µg/kgBW per 200 µl. Therapy was given by injecting a total of 200 µl metabolite extracts of *S. hygroscopicus* Hygroscopicus in mice intra-peritoneal once daily for five days. Thin blood smear for evaluation of parasitemia was made after each treatment once daily for six days.

Western Blotting of ubiquitin

On sixth day after five times treatment, mice were euthanized using chloroform. Intra-cardiac blood was collected. Blood plasma was removed and blood pellet was separated using double ficoll 1077 and 1118. Erythrocyte layer was collected. Samples were washed with PBS and parasites were extracted with 0.15% saponin. Washing was then performed in PBS equipped with 20 mM NEM, 0.05 mM EDTA, 1 mM AEBSF, 0.02% sodium azide and protease inhibitors. Proteins were extracted from the parasite pellets by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM AEBSF, 20 mM NEM, 0.5 mM EDTA, 1% Triton X-100, Protease Inhibitor, 0.02% Na

azide). Protein concentration was determined by spectrophotometry with BSA (Bovine Serum Albumin) as standard. Then, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by semidry electro-blotter. Spots were examined with anti-ubiquitin monoclonal antibody (Biolegend) as primary antibody, goat anti-mouse IgG as secondary antibody, SAHRP, and DAB substrate.

ELISA of Ubiquitin

After sample preparation on western blotting procedure above, lysate parasite were centrifuged 3000 rpm for 10 minutes. Supernatant were diluted (1:4) in coating buffer and coated at 96-well plates overnight. 96-well plates were incubated with blocking BSA, incubated with ubiquitin monoclonal antibody (Biolegend) as primary antibody (1:500), incubated with goat anti-mouse IgG as secondary antibody (1:1000), SAHRP, DAB substrate, and stop solution. After each step, wells were washed with PBS tween.

Data analysis

Data analysis was performed using SPSS 16 with Kruskal-Wallis test, one-way ANOVA test, post hoc test, Pearson correlation test, linear regression test, and the ED₅₀ probit analysis with p value <0.05.

RESULTS

The degree of parasitemia

Antimalarial activity of bacterial metabolites extracts were determined by measuring the degree of parasitemia. Examination of parasitemia day-1 (D-1) aims to prove that all the mice are in the same range of degrees of parasitemia on the day of treatment. The degree of parasitemia after intra-peritoneal injection of *S. hygroscopicus* Hygroscopicus metabolite extracts were measured on day 2, 3, 4, 5, and 6. Results of the degree of parasitemia are presented in the Figure 1A.

Kruskal-Wallis test on mean parasitemia degree of day-1 showed no significant differences among groups before treatment (p=0.192). Kruskal-Wallis test on mean parasitemia degree of day-2 showed no

significant differences among groups ($p=0.091$). One-way ANOVA test showed the data of parasitemia on day-3 differ significantly among groups ($p=0.000$). Post-hoc Tukey HSD test on day-3 showed a significant difference between control and P1, control and P2 as well as control and P3, in contrast there were no significant differences between P1 and P2, P1 and P3, P2 and P3. One-way ANOVA test showed that parasitemia on day-4 differed significantly among groups ($p=0.000$). Post-hoc Tukey HSD test on day-4 showed a significant difference between control and P1, control and P2, as well as control and P3. One-way ANOVA test of parasitemia on day-5 showed the data differ significantly among groups ($p=0.000$). Post-hoc Tukey HSD test on day-5 showed a significant difference between control and P1, control and P2, as well as control and P3. One-way ANOVA test of parasitemia on day-6 showed the data differ significantly among groups ($p=0.000$). Post-hoc Tukey HSD test on day-6 showed significant differences between control and P1, control and P2, as well as control and P3. Pearson correlation test showed a strong inversed relationship between concentration of the extract and the degree of parasitemia on day-3 ($p=0.000$, $r=-0.721$). Pearson correlation test showed a strong inversed relationship between concentration of the extract and the degree of parasitemia on day-4 ($p=0.000$, $r=-0.746$). Pearson correlation test also showed strong inversed relationship between concentration of the extract and the degree of parasitemia on day-5 ($p=0.000$ and $r=-0.734$). Pearson correlation test also showed strong inversed relationship between concentration of the extract and the degree of parasitemia on day-6 ($p=0.000$, $r=-0.751$).

Percentage inhibition of parasitemia

The percentage inhibition defines the inhibitory effect caused by the treatment/therapy given. Inhibition of the degree of parasitemia obtained by the formula: % inhibition = $(\% \text{ parasitemia positive control} - \% \text{ parasitemia treatment}) / \% \text{ parasitemia positive control} \times 100$. Percent inhibition is shown on the figure 1B. Treatments were begun on day-1 and the effects of treatment

were checked every day from day-2 onwards, so the inhibition percentage of day-1 is 0%. Parasitemia of all treatment groups on day-2 had not lead to significant differences from controls. After five times of the therapy, it could be seen that the largest inhibition percentage occurred on P3. One-way ANOVA test showed a significant difference among groups ($p=0.002$). Post-hoc test showed a significant difference between P1 and P3 ($p=0.002$) and between P2 and P3 ($p=0.018$). Meanwhile, P1 and P2 were not significantly different. Pearson correlation test showed that the increasing of the dose of *S. hygroscopicus* Hygroscopicus metabolite extract will increase the inhibitory effect ($p=0.000$; $r=0.850$). Values obtained by linear regression test $R^2=0.723$, which means of 72.3% inhibition of parasitemia caused by therapy and obtained regression equation is $Y=4.916 X + 57.006$.

For the determination of ED50 (effective dose of 50% inhibition) in this study, probit analysis were used to obtain inhibition percentage of parasite growth on the 4th and 6th day of observation, followed by linear regression analysis (SPSS). This probit analysis compared log-doses and probit score converted from inhibition percentage. The test results obtained from the linear regression equation $Y = 0.269 X + 4.738$ with $R^2=0.929$. ED50 of the equation was obtained by 9.418 $\mu\text{g/kgBW}$ in mice.

Morphology of Plasmodium

The control group showed an increase in the development stage of *Plasmodium*. On day-2 parasites were found in immature and mature trophozoites with several young schizonts. On day-3 and day-4 erythrocytes contained several stages of trophozoites and mature schizonts. On day-5 mature schizonts were more dominant and on day-6 showed a lot of mature schizonts. For P1 group (130 $\mu\text{g/kgBW}$) in day-2 after treatment showed parasite in a ring-form. On day-3 and day-4 showed parasites on ring-form and early schizont with irregular shape and damage of the cytoplasm. On day-5 showed parasite in a ring-form with a magnification of the cytoplasm because of cell edema. On day-6 or at the end of therapy, there were many

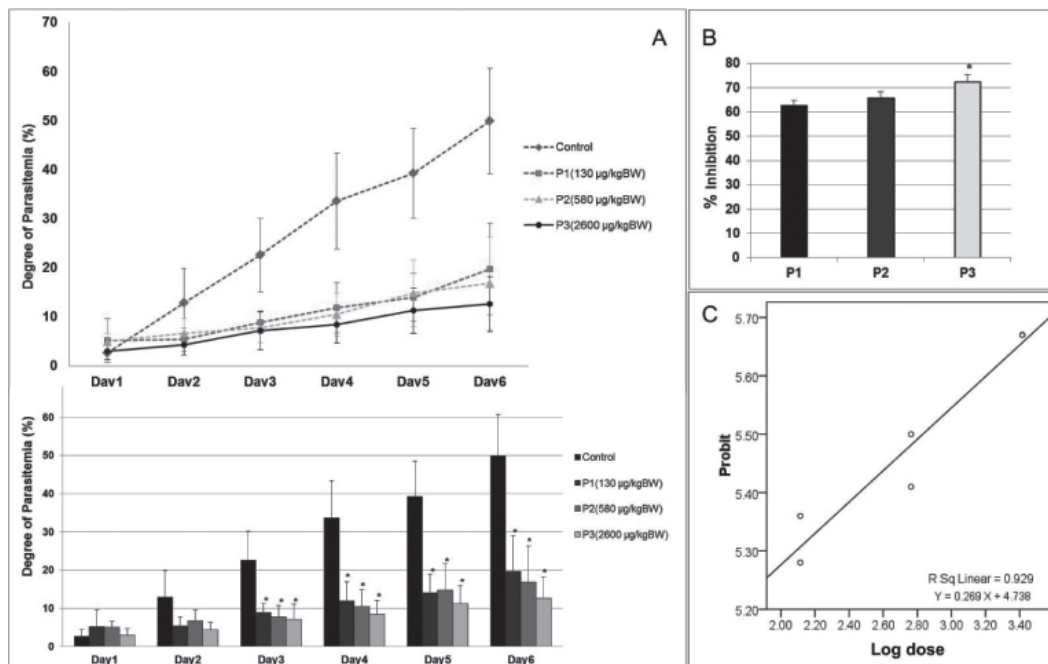


Figure 1. Activity of *S. hygroscopicus* metabolite extracts against *P. berghei*. Mice were treated by three doses of metabolite extracts five times (day 1 – day 5). A). The degrees of parasitemia were observed six times (day 1 – day 6). All doses showed significant decreasing of parasitemia compared to control ($p < 0.05$). B) Percentage of inhibition day 3 – day 6 increased significantly on P3 ($p < 0.05$). C) Effective dose of 50% of inhibition by probit analysis. *) Show significant different compared with control

thick and dark parasites with the picnotic cores. This condition is called crisis forms (James *et al.*, 1985). For P2 Group (580µg/kgBW) on day-2 showed irregular ring-forms with damaged cytoplasm. On day-3 and day-4 showed a disturbance of schizont development marked by enlarged infected red blood cells, the presence of vacuoles, and cytoplasmic damage. On day-5 and day-6 showed the crisis form of parasites marked by cytoplasmic disappearance, the core of chromatin enlarged, thickened, and compact. In P3 group, abnormalities could be seen from the observation of day-2 to day-6. In the day-2, the young schizonts failed to grow and the trophozoites were in crisis form. On day-3, the core of *Plasmodium* were pulled to the edge of cytoplasm, the dark cytoplasm began to experience damage. On day-4, day-5, and day-6 crisis parasites were dominant, marked by the disappearance of the cytoplasm, the nucleus was pulled over to the edge of parasite cytoplasm, and the chromatin was

thick, compact, and dark (James *et al.*, 1985). The viability of the crisis parasites from the highest dose had been tested, no crisis parasites survived and multiplied in this viability test (data not showed).

Polyubiquitinated protein

Activity of *S. hygroscopicus* metabolite extracts which contained analog eponemycin as a proteasome inhibitor on *P. berghei* *in vivo* was evaluated by Western blot and ELISA to see polyubiquitinated protein accumulation due to inhibition of the proteasome. Western blot result showed a thick band at P3 columns that are not found in other groups. It indicated the accumulation of polyubiquitinated protein on P3. Accumulation also showed in ELISA result. Optical density (OD) of the parasite polyubiquitinated protein which was measured by bar graph showed that increasing the dose leads to increased accumulation of parasite polyubiquitinated protein. One way ANOVA test

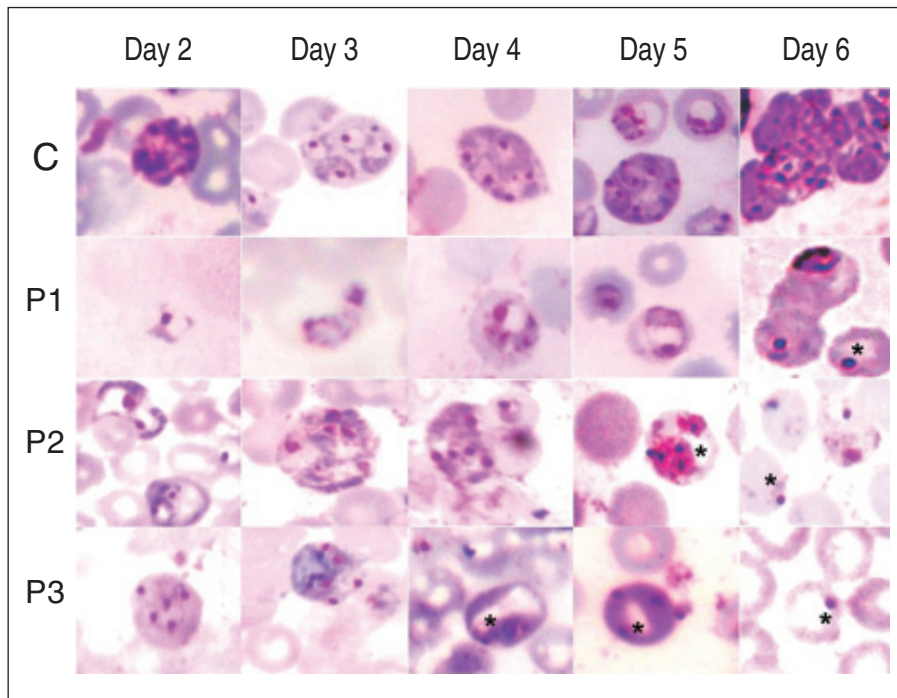


Figure 2. Effect of *S. hygrosopicus* Hygrosopicus metabolite extracts on *P. berghei* morphology. *P. berghei* morphology was observed five times (day 2 – day 6) as treatment results. Star mark showed crisis form of parasite

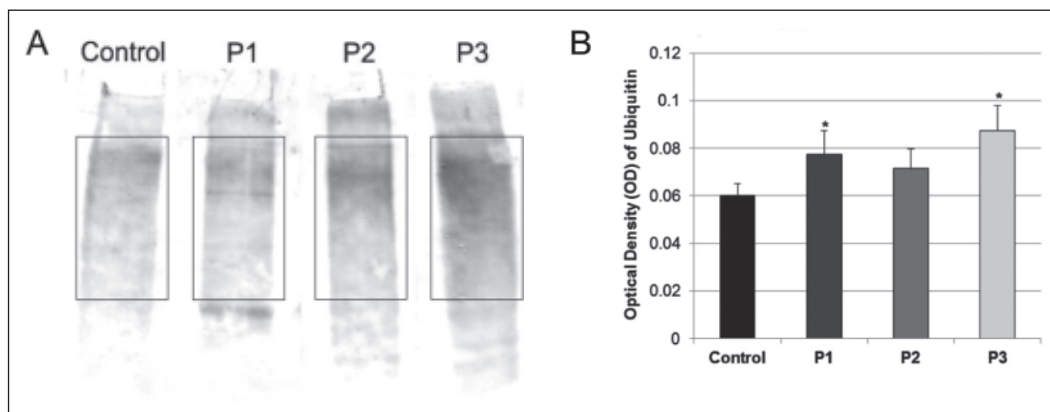


Figure 3. Polyubiquitinated protein of *P. berghei*. A) Western blot result showed accumulation of polyubiquitinated protein of *P. berghei* in smear bands inside square marks. P3 showed more intensity and thicker smear band that means more accumulation of polyubiquitinated protein. B) Immunoassay also showed increased optical density (OD) level of polyubiquitinated protein of *P. berghei* on P3. *) shows significant difference compared with control

showed a significant difference among groups ($p=0.006$). Post hoc test showed that P1 and P3 differed significantly with control ($p=0.015$ and $p=0.001$ respectively). P3 also differed significantly with P2 ($p=0.022$),

however P2 and P3 did not differ from P1 ($p=0.345$ and $p=0.127$ respectively). Pearson correlations test showed that increase doses correlated with increase polyubiquitinated protein ($p=0.003$; $r=0.684$).

DISCUSSION

This study has demonstrated the antimalarial activity of *S. hygroscopicus* Hygroscopicus metabolites extracts through UPS mechanism. Ubiquitin - proteasome system (UPS) is a protein degradation system for protein regulation and structural error protein. Eukaryotic cell, including *Plasmodium* sp, have a major non-lyzosomal UPS consisting of 26S proteasome and ubiquitin. Ubiquitin-proteasome system consist of two major biological functions, ubiquitination and proteasome-mediated proteolysis, which acts to control the regulation of cell protein receptor signaling via regulation and degradation of cyclins, cyclin-dependent kinase, cyclin-dependent kinase inhibitors during mitosis, and regulate the expression gene transcription and cell cycle progression. This function is carried out in synergy between ubiquitin that mark protein targets through polyubiquitination process and protein degradation by the proteasome. Therefore, the inhibition of UPS function resulted in decreasing of protein translation initiation and protein degradation leading to failure of cell stress response and ended in the death of cells. 26S proteasome consists of a central catalytic core particle (20S proteasome) and two regulatory particles (19S caps) which are at both ends of the core particle. 20S proteasome consists of four rings with seven polypeptide and subunits (SU) on each ring. However, only subunit 1, 2, and 5 which are proteolytically active (Piwko, 2007; Kisselev *et al.*, 2012).

Based on the results of TLC, extracts of bacterial metabolites *S. hygroscopicus* Hygroscopicus (Actinomycetes group) contains eponemycin analog, one of proteasome inhibitor. Eponemycin inhibits UPS function due to its special structure of the "war-head" C-terminus which can deactivate proteasome intermittently and irreversibly (Meng *et al.*, 1999). C-terminus has a α',β' -epoxyketone group (C=O) which can bind to the N-terminus of β SU proteasome by forming a stable six-ring (Fonovic & Bogyo, 2008). Eponemycin inhibits the proteasome specifically and does not inhibit the other non-proteosomal

protease such. High selectivity is dependent on a unique mechanism of two-step pharmacophore reaction with the hydroxyl and amino group of catalytic threonine residue. This occurs through the carbonyl group catalysis by the catalytic hydroxyl in epoxyketone group, followed by epoxy ring opening by free α -amino group to form a cyclical morpholino ring. The crystal structural complex of the 20S proteasome with epoxyketone in yeast can demonstrate the formation of morpholino ring between epoxyketonepharmacopore with the active side of Thr-1 N-terminal. Cysteine and serine proteases activities are not via this pathway because they don't have the free N-terminal (Delcros *et al.*, 2003). Eponemycin can inhibit all three proteolytic sides in varying degrees. Eponemycin can bind both $\beta 1$ and $\beta 5$ SU well (caspase-like activity and chymotrypsin-like). Based on Kreidenweiss *et al.* (2008), epoxyketone group has a narrow dose range of activities so that it has a low potential for resistant occurrence. α',β' -epoxyketone peptide is the most potent and specific proteasome inhibitor in mammals today. Proteasome $\beta 2$ and $\beta 5$ SU in *P. falciparum* are 55% and 53% identical to the human. Active side residues is the side that is maintained in *Plasmodium*, but the amino acids in the central peptides is different with amino acids in humans so that the inhibitor analog of the enzyme can also be developed in *Plasmodium* (Czesny *et al.*, 2009).

20S proteasome has an active CP SU $\beta 1$, $\beta 2$, and $\beta 5$ with caspase-like activity, trypsin-like, and chymotrypsin-like respectively. Parasites treated with proteasome inhibitor suffer inhibition in DNA replication phase because this phase is the peak of ubiquitinated protein. In this study, the accumulation of ubiquitinated proteins obtained from parasite lysate in the western blot analysis. Other studies have reported that the use of proteasome inhibitors showed inhibitory activity on liver phase (hipnozoite). Epoxyketone also showed inhibitory effect in gametocidal activity ranging from early phase to mature gametocytes. Therefore, proteasome inhibitor, eponemycin, is a multi-stage antimalarial drug candidate. Eponemycin demonstrates effective treat-

ment especially for acute phase of malaria (Aminake, 2012).

Proteasome inactivation caused by eponemycin induces morphological changes such as spindle cells, crisis forms, and apoptosis. Inhibition of UPS function results in the accumulation of polyubiquitinated protein in the *P. berghei* lysate (Prodhomme *et al.*, 2008; Aminake *et al.*, 2012). Western blot and ELISA results showed that there was polyubiquitinated protein accumulation, especially in P3. In the control, non-treatment group, showed only a few accumulation of polyubiquitinated protein that normally happened. These results prove that the polyubiquitinated protein accumulation in the parasite lysate increases with increasing dose of eponemycin analog on *S. hygroscopicus* metabolites extract causing inhibition of *P. berghei* growth. The probit analysis also showed the effective dose (ED50) of metabolite extracts of *S. hygroscopicus* which cause 50% inhibition in the growth of *P. berghei* are 9.418 µg/kgBW/mice. Multiplication with the dose conversion coefficient of 387.9 in human obtained ED50 dose of 1.04 µg/kgBW. An antimalarial compound is potent to have good activity and prospectively as an antimalarial drug while it has ED50 of less than 10 mg/kgBW on human (Kusumawardhani *et al.*, 2005). Based on this analysis, *S. hygroscopicus* metabolite extract is potential as antimalarial candidate drug with the ED50 of 9.418 µg/kgBW in mice and 1.04 µg/kgBW on human.

The results showed that there was an increase of inhibition level of parasitemia for all treatment groups with increasing doses of therapy. This inhibition level is significantly different as compared to positive control. Metabolite extract of *S. hygroscopicus* *Hygroscopicus* at doses of 2600 mg/BW decrease the degree of parasitemia of mice infected with *P. berghei* with ED50 9.418 µg/kgBW, and causes morphological changes and damage to *P. berghei* that suggested through inhibition of UPS revealed by increasing the density of the accumulation of ubiquitinated protein of *P. berghei*. The correlation test results indicate a drug relationship - dose dependent

with a negative correlation coefficient. Further research is needed to see the effect of metabolite extracts of *S. hygroscopicus* *Hygroscopicus* against proteasome activity *in vitro*, using *P. falciparum* culture.

Acknowledgements. This work was funded by The Ministry of Pendidikan & Kebudayaan Republic of Indonesia and Faculty of Medicine University of Brawijaya. We thank Dr. Hidayat Sujuti, SpM, PhD. for his technical help in western blot. Yudha Ngatiril Lady Ssi, Ferida, and Ali Sabet, Amd for their assistance.

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