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Non-Toxic Fractions of *Streptomyces hygroscopicus* Subsp. Hygroscopicus Metabolite Suppressed the Growth of *Plasmodium Falciparum in Vitro* Possibly through L-malate: Quinone Oxidoreductase (*Pf*MQO) Mitochondrial Enzyme Inhibition

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ABSTRACT

Introduction: Previous research has proven that ethyl acetate extract of *Streptomyces hygroscopicus* can inhibit *Plasmodium* growth *in vivo* and *in vitro* through inhibition of the Proteasome ubiquitin system. In this study, we analyzed the activity of several fractions *of S. hygroscopicus* toward the growth of *P. falciparum* culture with specific targets on the mitochondrial enzyme of *P. falciparum;* L-malate: quinone oxidoreductase (*Pf*MQO) and dihydroorotate dehydrogenase (*Pf*DHODH). Methodology: the fractionation process uses the *Flash Column Chromography* (FCC) BUCHI Reveleris® PREP, analyzed using thin layer chromatography (TLC). The antimalarial activity of the fractions was tested on the culture of *P. falciparum 3D7* using enzyme lactate dehydrogenase (*Pf*LDH) assay and their activity toward *Pf*MQO and *Pf*DHODH target enzymes. The fractions analyzed using HPLC and compared to the standard *dihydroeponemycin*. The toxicity of the fractions, the WST-8 assay was conducted. Results: of the 30 fractions produced, 6 fractions were able to suppress the *P. falciparum* and two of them were able to inhibit more than 50% of *P. falciparum* growth. Both of these fractions were proven to inhibit the activity of the *Pf*MQO enzyme but had no inhibition of the *Pf*DHODH enzyme. Both fractions reduced human cell viability by less than 50% in toxicity test. HPLC analysis indicated that besides *dehydroeponemycin,* there were other compounds presences in the extract. Conclusions: fractions of *S. hygroscopicus* metabolite contains non-toxic compounds in addition to *dihydroeponemycin* that could suppress the growth of *P. falciparum in vitro*. This potential effect might be related to the inhibition of *Pf*MQO enzyme.

Keywords: Fractions, *Streptomyces hygroscopicus*, *P. falciparum*, *Pf*DHODH, *Pf*MQO, LDH

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INTRODUCTION

Plasmodium falciparum is one of the types of malaria-causing parasites that can provide severe clinical manifestations of malaria. The mechanism of action of the drug toward *Plasmodium* determines the therapeutic success of malaria. Mitochondria are organelles that have an important role in several metabolic pathways for parasite survival. In the parasite metabolic system, glycolysis, the tri carboxyl acid (TCA) and mitochondrial electron transport chain (ETC) cycles are a potential target in the development of anti-parasitic drugs [1,2]. *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH) and *Plasmodium falciparum* L-malate: quinone oxidoreductase (*Pf*MQO) have proven to act as a very important enzymes in the life of asexual parasites [3, 4]. *Pf*DHODH which is needed by the *Plasmodium* in pyrimidine biosynthesis has become a potential target of malaria treatment [3], while *Pf*MQO is the one of dehydrogenase enzyme that has important role in ETC and TCA cycle only found in *Plasmodium* and lacked in humans thus *Pf*MQO could also be served as potential drug target for antimalaria [4].

Streptomyces hygroscopicus is a gram-positive bacterium found in soil. As part of the Actinomycetes family, *S. hygroscopicus* is known contains many secondary metabolites, one of them is *dihydroeponemycin* that can inhibit the *Plasmodium* Ubiquitin-Proteasome System (UPS). Our previous study using Balb-c mice shown that crude extract from *S. hygroscopicus* could inhibit *Plasmodium* growth in vivo [5]. Our recent study showed that crude extracts of *S. hygroscopicus also* had an effect of inhibition in *Plasmodium* growth in vitro. In fact, the ethyl acetate extract of *S. hygroscopicus* still have many kinds of compound, thus fractionation process is needed to reduce the variation of compounds contained in each fractionation result, we assume each fraction result will contain different compounds.

Although it is well-known that *S. hygroscopicus* extract has the potential as a new candidate for malaria treatment, no toxicity test has been conducted to determine whether the drug candidate is toxic or not to human cells. Besides, a toxicity test needs to be carried out because the isolation of *S. hygroscopicus* from the soil may carry contaminants such as, heavy metals (e.g. cadmium) [6], aflatoxin which is a toxic substance [7] and potentially pathogenic microbes during the extraction process [8]. This study aims to explore the effectiveness of the *S. hygroscopicus* fractions as antimalarial and determine their cytotoxic activity *in vitro.*

MATERIAL AND METHODS

Research Design

This study uses explorative designs and true experimental laboratory *in vitro* method with posttest only controlled group design. The study compared the results obtained from the experimental group after being treated with the control group *in vitro.* In antimalarial activity test the *Plasmodium falciparum* 3D7 culture was divided into 5 groups, there were negative control group (group exposed with medium contains 2 µl DMSO 1% similar to the solvent of the fractions), positive control group (group treated with 2 µl atovaquone each well) and groups that treated with fractions of *S. hygroscopicus* dosage 50ppm, 5ppm, and 0.05ppm, respectively. For enzymes assay, we used 5 experiment groups, there were negative control group (2 µl DMSO 1% similar to the solvent of the fractions), positive control group (without reaction substrate) and fraction doses of 200ppm, 20ppm, and 2 ppm groups. In the cytotoxic activity test the fraction dose divided into ten different concentrations of 0.15625ppm, 0.3125ppm, 0.625ppm, 1.25ppm, 2.5ppm, 5 ppm, 10ppm, 20ppm, and 40 ppm. In an explorative study, we performed a High-performance liquid chromatography (HPLC) fraction compare with standard compound *dihydroeponemycin.*

The characterization of S. hygroscopicus subsp. Hygroscopicus

Streptomyces hygroscopicus subsp. Hygroscopicus isolate obtained from the Microbiology LIPI (LIPI-MC) Cibinong, which was subcultured in the microbiology laboratory of the Faculty of Medicine, Universitas Brawijaya. *Streptomyces* colonies were then characterized based on macroscopic colony morphology and microscopic bacterial morphology based on Gram staining.

Fermentation and extraction of the secondary metabolite of S. hygroscopicus subsp. Hygroscopicus

Fermentation was done using International *Streptomyces* Project 4 (ISP4) liquid media in aseptic conditions on erlenmeyer. Liquid media ISP4 was prepared in an erlenmeyer glass in aseptic (autoclaved) conditions of 1000 ml with medium conditions pH 5-7. Bacterial colonies were scraped from ISP4 culture medium and homogenized in ISP4 media. The ISP4 media was mixed with 100 ml of inoculum (comparison between media and inoculum 10: 1). The media then tightly closed with cotton and aluminum foil, to ensure that there is no air inside an erlenmeyer and then incubated for 5 days in a shaking incubator at 28^{o}C at 150 rpm. After 5 days, fermented bacteria centrifuged at 3000 rpm for 10 minutes and the supernatant was taken for the extraction process [9]. The fermented product is then mixed with 1:1 ethyl acetate (v/v) and shaken for 1 hour, then left for 1-2 hours in a separating funnel to form two layers. The bacterial metabolite will be carried to the solvent in the ethyl acetate phase. The water phase was removed and the solvent phase (ethyl acetate) was collected and evaporated in the rotary evaporator until it becomes a paste, then continued evaporated on a water bath at 40-50^{0} C until it turns into powder and weighed before keeping in refrigerator.

Fractionation of the secondary metabolite of S. hygroscopicus subsp. Hygroscopicus

Fractionation was conducted using flash column chromatography (FCC) BUCHI Reveleris® PREP Purification System with a gradient elution system using a UV detector of 254nm, 365nm, and 366nm as well as an evaporative light scattering detector (ELSD) to obtain all compounds detected at that wavelength. Total extract sample injection to the column with a total amount of 62 mg, using 33 grams of silica gel 60. The extract used was dissolved with methanol and then injected as much as 1 mL into the injector with a syringe without a needle that had been given filter paper on the inside of the syringe and the following steps were carried out as prepared previously as the gradient eluent system. There were two solvents used, according to the results of the previous TLC, namely solvent A (n-hexane pa) and solvent B (ethyl acetate pa) with a gradient mode from 100% n-hexane to 100% ethyl acetate for 120 minutes with a flow rate of 5 ml /minute. After that, the isocratic mode was changed to 100% ethyl acetate for 10 minutes to give time to replace n-hexane with methanol pa. Flushing with methanol was carried out with gradient 100% - 0% methanol pa (to water) for 30 minutes with a flow rate of 5 ml /min so that the compounds left /stuck in the tool could be accommodated. After finishing running, the collected samples were concentrated by evaporating them in a fume hood. The purity of the fraction was then validated using the normal phase Thin Layer Chromatography (TLC).

Plasmodium falciparum 3D7 culture

The parasitic culture in the TC flask which has a parasitic degree of 10% was taken using a 5 mL disposable pipette and put in a 15 ml centrifuge tube. The culture was centrifuged at a speed of 2000 rpm for 5 minutes and the supernatant was removed. The formed packets were ready to be used for an in vitro assay. An in vitro anti-malarial activity of *S. hygroscopicus* was carried out to determine the development of parasites using LDH *Assay.* A new Tissue Culture (TC) 96-well plate was prepared and laid out for the anti-malarial in vitro test mapping was arranged including controls that contain complete mediums and all group replication.

Plasmodium falciparum lactate dehydrogenase (PfLDH) enzyme assay

The fraction which has been given 10 ul 100% DMSO with the concentration 50ppm, 5ppm, and 0.05ppm put in the well of the 96-well plate *for the replication assay*. Then the plate which had a fraction was put into the concentrator for 1 hour at 900g. At the same time, *Plasmodium falciparum* culture which was prepared to achieve 0.3% parasitemia put into each well of 96- plates *assay* as much as 190µl, then store it in the incubator for 3 days. After that PBS was added as much as 200ul to all wells then

centrifuged at 1300g for 5 minutes, the supernatant was discarded, and the plate was put in the freezer -30˚C for 3 hours. LDH buffer which will be used for 30 minutes was made by mixing buffer reaction, nitro blue tetrazolium (NBT), and 3- acetyl pyridine NAD (APAD) to make Mix *Assay* and added to each well as much as 90µl, then placed on 650rpm shaker plate for 30 minutes and finally analyze using 650nm spectrophotometry the method has been optimized [10].

Plasmodium falciparum L-malate: quinone oxidoreductase (PfMQO) enzyme inhibitory assay

Fraction of *S. hygroscopicus* metabolite was mixed with 40 µl DMSO, then put 2µl into each well with concentrations of 200ppm, 20ppm and 2ppm, then added 193µL *Pf*MQO buffer assay (consisting of 50mM HEPES, 1mM KCN, 60 µM decylubiquinone, 120 µM, 120 µM DCIP, 2.5 µl / ml PfMQO membrane respectively). Then 5 µl of 400 mm sodium malate was added to each well in a dark room, so the final volume per well was 200 µl per well. Measurement was made using a spectrophotometer every 1 minute for 8 minutes, all procedures were carried out at 37º C.

Plasmodium falciparum dihydroorotate dehydrogenase (PfDHODH) enzyme inhibitory assay

Fraction of *S. hygroscopicus* metabolite were given in each well with volume 2µl with concentrations of 200ppm, 20ppm, and 2ppm, then added 190µl *Pf*DHODH buffer assay (consisting of 100mM HEPES (pH 8.0), 150mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) Triton X-100, 20nM *Pf*DHODH, 18 µM decylubiquinone, 120µM DCIP), then 8ul of 5mM L-DHO was added each well in a dark room and the plate could be read using spectrophotometry with a wavelength of 600 nm every 1 minute, in 20 minutes, all procedure was performed at 25ºC.

Analysis of dihydroeponemycin compound using HPLC

The analysis was carried out on a sample of fractions that had activity on the LDH assay. HPLC uses Shimadzu Prominence HPLC system (binary LC-20AB pump, UV SPD-20A detector), YMC-Pack ODS-A (YMC) chromatography column 250x4.6mm ID, S-10µm, 12 nm, eluent acetonitrile (MeCN) and standard compounds *Dihydroeponemycin* obtained from SIGMA-ALDRICH USA.

Cytotoxicity analysis using WST-8 assay

Cell suspension of DLD-1cell line culture was prepared using DMEM media with the concentrations 1.25 x 105 cells / mL (2.5x 104 cells / 200 µL). Then 100 µL of cell suspension was inserted into each well of 96-well plates. The plate was placed in a 37°C incubator for 24 hours (overnight). A volume 0.4 µL of each fraction (dissolved in 100% DMSO) was added with various concentrations 80; 40; 20; 10; 5; 2.5; 1.25; 0.625; 0.3125; and 0.15625 ppm with three replicates for each concentration. The plate then placed in the incubator (37°C, 5% CO2) for 48 hours. After 48 hours, the media was removed from the plate with the aspirator. Cells were washed with 100µL PBS for each well with suction method then a mixture of 1 mL CCK-8 and 10 mL DMEM media was made in a sterile flask. 100 µL DMEM containing CCK-8 then was added to each well. Next, the plate was placed in a 37°C incubator for 3 hours. The absorbance of each well was measured at a wavelength of 450nm by SpectraMax Paradigm.

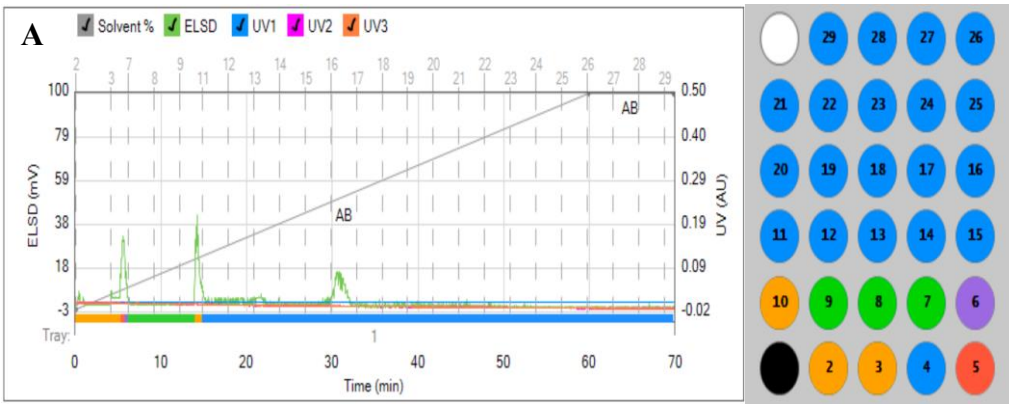
Statistical Analysis

The statistical analysis two-way repeated-measures analysis of variances (ANOVA) and Tukey's multiple comparisons test was used to perform the difference among treatment and control groups to determine the difference in inhibition of *P. falciparum 3D7* culture cells, *Pf*MQO and *Pf*DHODH enzyme using Graphpad Prism 8 software.

RESULTS AND DISCUSSION

Fractionation of S. hygroscopicus ethyl acetate extract

The weight of the fraction obtained from the fractionation was different for each tube with a total fraction was 47 tubes. Only 30 fractions have sufficient weight to be tested in this study. The weighed stock fraction was dissolved in methanol with the same concentration in each fraction, by making a concentration of 20,000ppm in each fraction. In this study, fractionation was carried out using column chromatography with hexane and ethyl acetate solvents. The purification step was carried out with ethyl acetate solvent and using the column chromatography to obtain pure fractions of compounds contained in crude extracts from the bacterium *Streptomyces coelicoflavus* [11,12]. Combination of solvent using hexane: ethyl acetate aimed to obtain and produce bioactive compounds from extracts of these bacteria, as well as the use of solvent gradients to obtain fractions of bacterial extracts *Actinomycetes* [13]. Purification of bacterial *Streptomyces* has been carried out using fractionation of column chromatography in the normal phase using silica gel and gradients of solvents with a 0.0: 100% system [14]. In this study fractionation was ended using methanol as flushing solvent shows in figure 1.



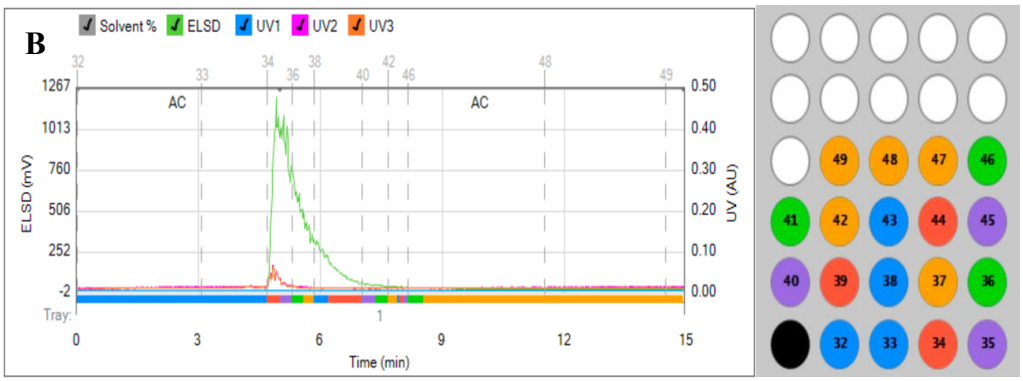


Figure 1. *Graph of fractionation results using the FCC BUCHI Reveleris® PREP Purification System*
(A) The total number of fractions collected was 29 fractions using n-hexane and ethyl acetate solvents; (B) The total number of fractions collected was 18 fractions using a Methanol (MeOH) solvent, the color of each fractionation tube based on the detector used in detecting the peak (Green-ELSD; Blue-UV1; Pink-UV2; orange-UV3).

Anti-malaria activity test of S. hygroscopicus metabolite fraction in culture of Plasmodium falciparum 3D7 using enzyme lactate dehydrogenase (LDH) assay

The result of inhibition activity on *Plasmodium falciparum* culture of 30 fractions showed only 6 fractions were able to suppress the growth of *P. falciparum* culture and two of them were able to inhibit more than 50% as shows in figure 2 below from Graphpad Prism 8 analysis.

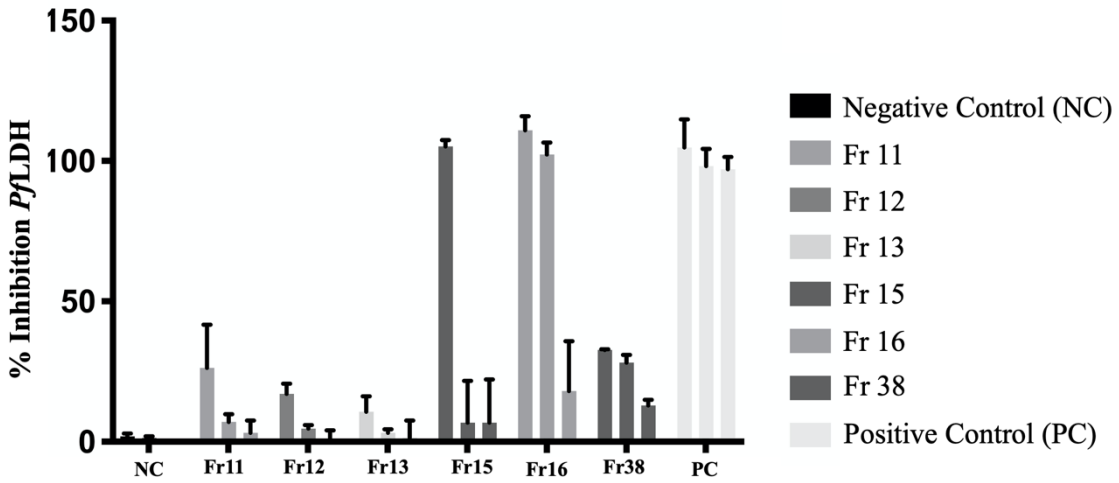


Figure 2. *Six fractions of S. hygroscopicus* metabolite that have inhibitory activity against *P. falciparum* culture using lactate dehydrogenase (*Pf*LDH) enzyme assay

Based on figure 2, fraction fr15 and fr16 showed inhibition more than 50% with a significant different (p=0,0019). In Tukey's multiple comparisons test, in fraction (fr) 15, a dose of 50ppm vs 5ppm and 50ppm vs 0.05ppm showed a significant difference (p=0.0001; p=0,0001) whereas a dose of 5ppm vs 0.05ppm showed no significant difference (p=0.9999). Further statistical analysis of fr16 showed that a dose of 50ppm vs 5ppm had no significant difference (p = 0.1827) whereas at 50ppm vs 0.05ppm and 5ppm vs 0.05ppm showed significant results (p = 0,0001: p=0,0001). The LDH assay test is useful to measure the viability of a parasite against its susceptibility to drugs because the activity of *p*LDH (*Plasmodium* LDH) is closely related to the number of parasites [10]. LDH enzyme assay detects pLDH antigen as a specific marker of the presence of *Plasmodium*, in principle this test will specifically detect *Plasmodium* *p*LDH that uses 3-acetylpyridine nicotinamide as an NAD analogue and nicotinamide adenine dinucleotide analogue. In the other hand, LDH erythrocyte human cannot use 3-acetylpyridine nicotinamide as an NAD analog and nicotinamide adenine dinucleotide analog [15]. In this study, *P. falciparum* 3D7 culture that exposed with fractions 15 and 16 decreased LDH levels by more than 50%, as in the positive control that given Atovaquon which is an antimalarial drug with similar mechanism through *Plasmodium* mitochondria.

Activity test of S. hygroscopicus metabolite fraction against PfDHODH and PfMQO

The activity tested for the recombinant enzymes *Pf*DHODH and *Pf*MQO of *P. falciparum* was carried out only in fraction samples fr15 and fr16 because both of those fractions have the highest results in inhibitory effects against *P. falciparum* 3D7 growth based on the LDH assay. In *Pf*DHODH test, it was found that the two fractions did not show a difference in inhibition of the activity of

dihydroorotate dehydrogenase compare to the control group. Whereas in *Pf*MQO test, both fractions showed significant inhibitory activity on L-malate: quinone oxidoreductase (*Pf*MQO) enzyme compared to the negative control group which can be seen in Figure 3.

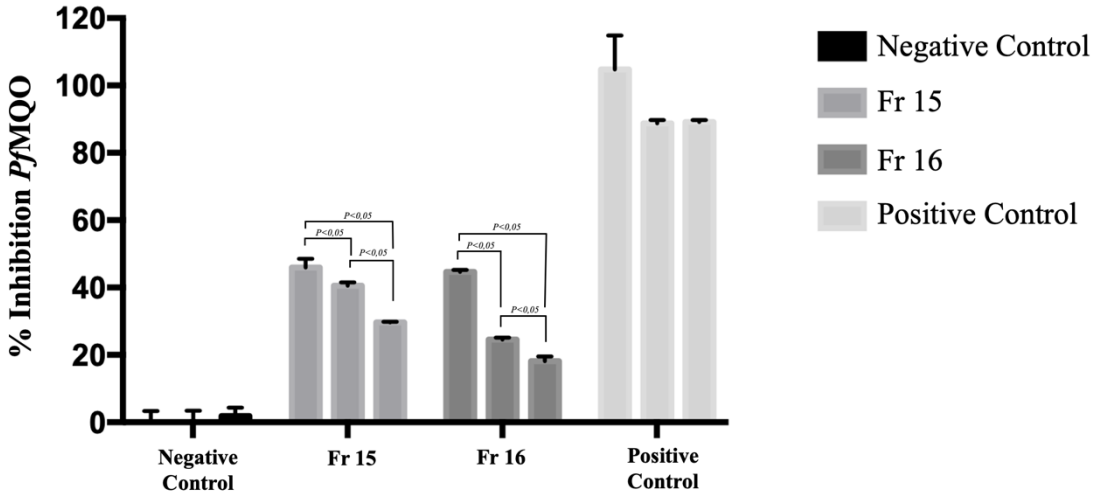


Figure 3. *Inhibitory activity of the P. falciparum L-malate enzyme: quinone oxidoreductase (PfMQO) enzyme assay.* In fraction 15 and 16 shows significant difference among doses of 200ppm, 20ppm, and 0.2ppm ($p<0.05$)

All doses of fraction 15 and 16 significantly differed with the control group ($p < 0.05$). In the multiple comparison results showed a significant difference of inhibition of *Pf*MQO enzymes in fraction 15 between doses of 200ppm and 20ppm (p=0,0007), 200ppm and 0,2ppm (p=0,0001), as well as between 20 ppm and 0,2 ppm (p=0,0001). Similar inhibition result showed in fraction 16, between doses of 200ppm and 20ppm (p=0,0001), 200ppm and 0,2ppm (p=0,0001), and between doses of 20 ppm and 0,2 ppm (p=0,0002) (Figure 3). In this study, fractions that had an inhibitory effect on parasitic viability of more than 50% on the previous evaluation using the *p*LDH assay were used on *Pf*DHODH and *Pf*MQO assays. The results of the *Pf*DHODH inhibition test did not give any inhibitory effect. This is based on the results of absorbance measurements in the *Pf*DHODH test which showed no inhibition of the enzyme reaction of the fraction. As it was known DHODH is one of the potential targets in the development of anti-malaria drugs that are quite potential [16]. At present the development of antimalarial drugs that work on the DHODH enzyme has entered into phase 2 clinical trials using the DSM265 compound obtained from the results of high-throughput screening (HTS) which showed the specific inhibitory effect on the mitochondrial DHODH enzyme [17].

High-performance liquid chromatography (HPLC) analysis of active fraction of S. hygroscopicus secondary metabolite

The samples analyzed by HPLC were fr15 and fr16, and a standard *dihydroeponemycin* compound. HPLC aimed to determine whether the anti-malaria activity of the fraction is due to the content of *dihydroeponemycin* or other compounds that have the potential as antimalarials.

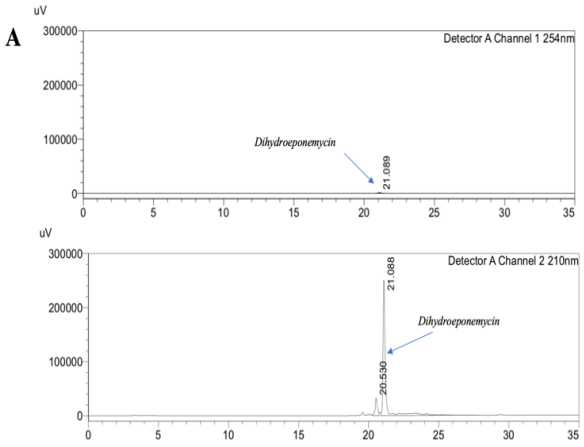


Figure 4A. The peak of dihydroeponemycin standard

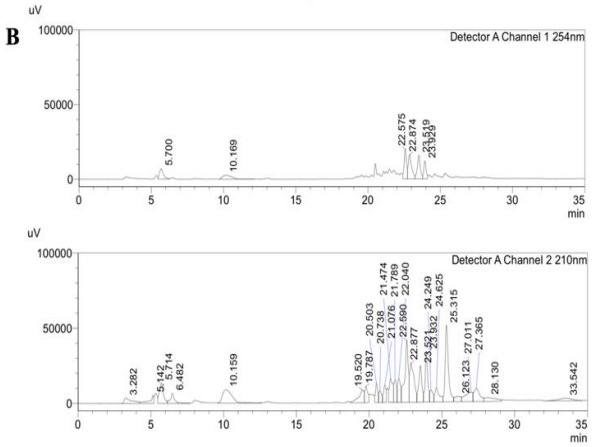
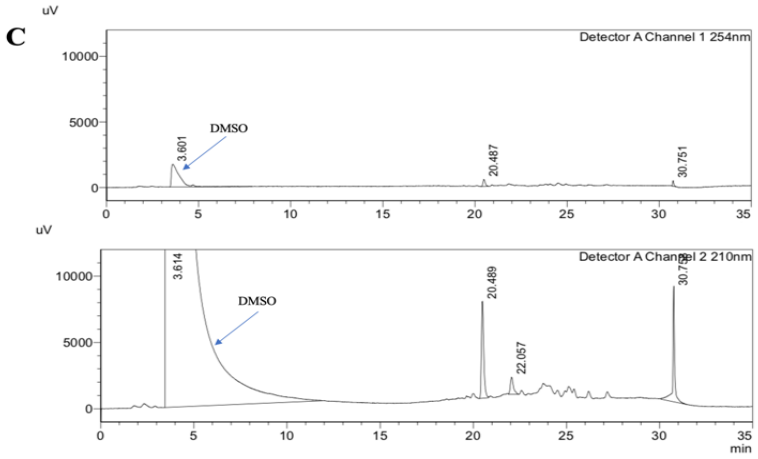


Figure 4B. The peak characteristic of fraction 15



The analysis results of standard *dihydroeponemycin* compound using MeCN (acetonitrile) as eluent with a gradient of 5% -10% - 100% for 35 minutes and an injected sample volume of 1µL showed a peak at *retention time*(rt) of 21,089 minutes (an area of 20,339 and a peak height of 1,387) on a detector with a wavelength of 254nm. Furthermore, it was analyzed at a detector with a wavelength of 210nm and the result showed a peak at rt of 21,088 minutes (an area of 2,859,790 and a peak height of 250,625) (Figure 4A). In the results of HPLC analysis on fraction 15 using a detector with a wavelength of 254nm, there were 6 peaks detected and at a wavelength of 210nm, there were 25 peaks detected by the detectors (Figure 4B). In the results of HPLC analysis on fraction 16 using a detector with a wavelength of 254nm, there were 3 peaks detected with the highest concentration at 3.601 minutes peak with a total concentration of 88.601%, which appeared because the peak is a DMSO solution. The other two peaks were detected on detectors with rt of 20,487 minutes and 30,751 minutes. In the results of HPLC analysis on fraction 16 using a detector with a wavelength of 210nm, there were 4 peaks detected as well as on the detector 254nm at the first peak with rt of 3,601 which was a DMSO (solvent before drying the sample), so that only the other 3 peaks will be analyzed (Figure 4C)**.** In HPLC, acetonitrile eluent was used in analyzing the extract fraction of *S.hygrosocpicus* in detecting peaks of secondary metabolite compounds in the analyzed fraction [17]. In addition, acetonitrile is an eluent used in HPLC analytic [6], showing an initial peak at fraction 16 which is a DMSO solvent at RT 4-10 minutes. When DMSO was running by HPLC using an acetonitrile eluent with a gradient concentration, DMSO was detected at vulnerable RT 0-10 minute [18]. HPLC usually using for tested the purity of active fraction with mobile phase consisting of water – acetonitrile gradient at the flow rate 1ml/min [14]. HPLC analysis is a methods for detecting the concentration of important active compound [19]. In this present study, we recorded chromatogram of *Streptomyces* compare with standard area as showed in the following result.

Cytotoxicity Analysis using WST-8 Assay

The viability of colorectal cancer *DLD-1* cell line after being treated with ethyl acetate extract fraction of *Streptomyces hygroscopicus* subsp. *Hygroscopicus* for 48 hours at the highest dose (80 ppm) was 45.6485%, while at 9 other dose variations, the percentage of cell line viability of colorectal cancer *DLD-1* was still more than 92.9738%. These results indicated that the fraction of *Streptomyces hygroscopicus* subsp. *Hygroscopicus* needs a very high dose to be able to inhibit the proliferation process by almost 50%. The study showed that the cytotoxicity effect of fraction 15 against colorectal cancer *DLD-1* cell line occurred only at a dose of 80 ppm. This cytotoxic effect arised because of the presence of secondary metabolites contained in the ethyl acetate fraction of *Streptomyces hygroscopicus* subsp. *Hygroscopicus*. One of them is eponemycin that acts as a proteasome inhibitor with α,β-epoxyketone as an active group [20]. The inhibition of protein degradation through 20S proteasome activity by eponemycin causes inhibition of cell proliferation due to the decreased initiation of protein translation [21]. The failure of protein degradation results in cell stress response characterized by increased *Reactive Oxygen Species* (ROS) which causes inhibition of proliferation [22]. Overall the dose lower than 80 ppm is safe for the treatment of fractions *of S. hygroscopicus* metabolite (Figure 5).

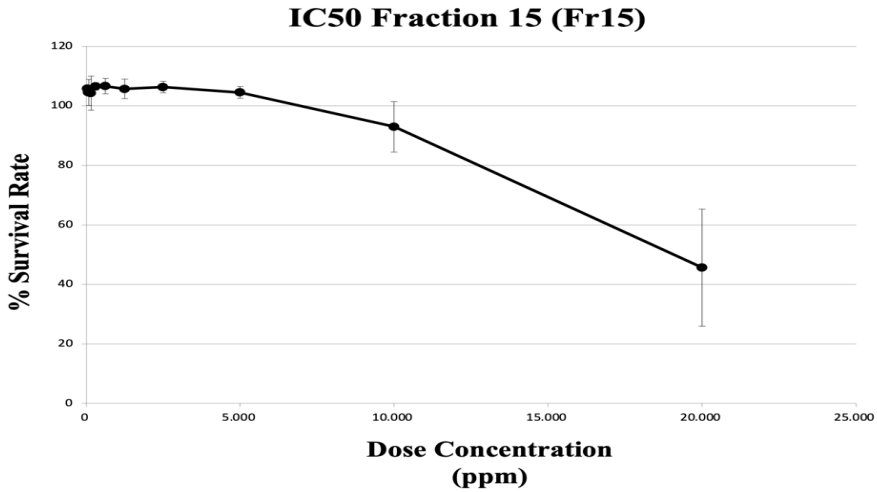


Figure 5. *WST-8 test after exposing for 48 hours*
Showed only at a dose of 80 ppm of fraction 15 of *Streptomyces hygroscopicus* Subsp. Hygroscopicus gave inhibitory affect more than 50% against *the DLD-1 cell line*.

CONCLUSIONS

Based on the above results, fraction 15 and 16 of ethyl acetate extract of *S. hygroscopicus* was able to inhibit more than 50% growth of *P. falciparum* 3D7 *in vitro* by analyzing lactate dehydrogenase (LDH) and *Plasmodium* L-malate:quinone oxidoreductase (*Pf*MQO) enzymes in mitochondrial membrane. It was also proven that the two fractions were non-toxic to human cells in the cytotoxicity test.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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