RESEARCH ARTICLE

Antimalarial potency and cytotoxicity studies of secondary metabolites from fractions 38K, 38T, and 56 *Streptomyces hygroscopicus* subsp. *hygroscopicus in vitro*

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Recent research has revealed that *Plasmodium falciparum* (*P. falciparum*) possesses an Artemisinin-based Combination Therapy (ACT) resistance mechanism. To address this issue, new antimalarial chemicals are required. *In vitro*, *in vivo*, and *in silico* tests have been conducted on metabolite extracts of *Streptomyces hygroscopicus* subsp. *hygroscopicus* (*S. hygroscopicus*) and showed antimalarial effects through various mechanisms. The aim of this research was to determine the effectiveness and cytotoxicity of active fractions 38K, 38T, and 56 of *S. hygroscopicus* secondary metabolites as antimalarial. Fractionation procedure was performed using flash column chromatography BUCHI Reveleris® PREP Purification System. Effectiveness test was conducted on *Plasmodium berghei* (*P. berghei*) culture by parasite inhibition counts. The cytotoxicity effect of *S. hygroscopicus* was observed utilizing the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide Assay (MTT Assay) on Michigan Cancer Foundation-7 (MCF-7) breast cancer cell culture. Inhibition Concentration 50% (IC_{50}) and Cytotoxicity Concentration 50% (CC_{50}) was obtained from probit analysis. *S. hygroscopicus* fractions 38K, 38T, and 56 showed antimalarial activity against *P. berghei*, with fraction 38T having the strongest activity as antimalarial from selectivity index. Fraction 38T had the IC_{50} with the value of 11.66 µg/mL which included in the promising activity classification of IC_{50}. Almost all asexual stages of parasite morphology were damaged by the fractions. MCF-7, a cell line used to study antimalarial cytotoxicity, was unaffected by any of the three fractions due to its CC_{50} exceeding 50 µg/mL. *S. hygroscopicus* fractions 38K, 38T, and 56 of the metabolite extracts contain non-toxic compounds that can damage the morphology of intra-erythrocytic stage and inhibit the growth of *P. berghei in vitro*.

Keywords: antimalarial; effectivity; cytotoxicity; *Streptomyces hygroscopicus; Plasmodium berghei*.

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Introduction

Malaria is a parasitic infection spread by the bite of *Plasmodium* infected female *Anopheles*. *Plasmodium* species that can infect humans include *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. In 2019, malaria posed a threat more than half of the world's population by causing 229 million morbidities and 409,000 deaths [1]. Malaria is most widespread in isolated areas with little economic development. Indonesia is a developing country with a tropical

climate that is perfect for high malaria infection transmission rates [2].

Artemisinin-based Combination Therapy (ACT) have become the fundamental therapy for malaria. However, research in the Greater Mekong sub-region of Southeast Asia has shown that *P. falciparum* has a mechanism of resistance to ACT. The discovery of new antimalarial compounds is a solution to overcome this problem [3]. To address this issue, *Actinomycetes* have been extensively researched as biological control agents for pathogens in recent years. *Actinomycetes* are the most abundant source of bioactive metabolites with antibacterial, antifungal, anticancer, antioxidant, antiparasitic, and anti-inflammatory properties [4]. *Actinomycetes* are gram-positive, filamentous, spore-forming bacteria. *Streptomyces sp* is a species from the *Actinomycetes* class that is capable of producing bioactive metabolite compounds and has potential as an antimalarial [5]. This fact is supported by the results of a recent study which showed that *Streptomyces hygroscopicus* subsp. *hygroscopicus* (*S. hygroscopicus*) has activity as an antimalarial agent [6].

In vitro, in vivo, and *in silico* studies have been conducted on metabolite extracts of *S. hygroscopicus* and showed antimalarial effects through various mechanisms. Extracts of *S. hygroscopicus* contain eponemisin analogs that are able to inhibit the function of the Ubiquitin Proteasome System (UPS) resulting in a decrease in the initiation of protein translation and protein degradation that causes failure of cell stress response and ends in cell death [7]. Liquid chromatography–mass spectrometry (LC–MS) identification showed that *S. hygroscopicus* contain the compound 6,7-dinitro-2-[1,2,4] triazole-4-yl-benzo [de] isoquinoline-1,3-dione which is an isoquinoline derivative. This compound was carried out a reverse molecular docking study and showed activity as an antimalarial with its ability to bind strongly to the adenylosuccinate synthetase protein [8].

The metabolite extract of *S. hygroscopicus* also contains Tryptanthrin which is a weak alkaloid group compound as an antimalarial. *In silico* analysis shows that Tryptanthrin has reversal chloroquine resistant properties allowing *S. hygroscopicus* to be a potential antimalarial candidate with a dual mechanism strategy when used in combination with Chloroquine. In previous study, it was explained that active fraction numbers 41 and 44 of the secondary metabolite extract of *S. hygroscopicus* were able to inhibit the *Plasmodium berghei* (*P. berghei*) density [9]. Other active fraction numbers, particularly 15 and 16, also shown antimalarial activity by inhibiting 50% of the growth of *P. falciparum* strain 3D7 parasites and were non-toxic to human cells [10]. *In vitro* studies showed that extracts of *S. hygroscopicus* metabolites caused damage to parasite morphology, decreased the number of infected erythrocytes and decreased *P. falciparum* 3D7 DNA [6]. *In vitro* studies showed that Fractions 14 and 36K of the metabolite extract of *S. hygroscopicus* subsp. *hygroscopicus* contain non-toxic compounds that can damage the morphology and inhibit the growth of *P. berghei* [11]. Ariel has conducted extraction, fractionation, and identification methods of secondary metabolites from the fermentation of *S. hygroscopicus*. The results showed that there were 13 points on the Thin Layer Chromatography (TLC) results of fractionation I and 14 points on the TLC of fractionation II. These points demonstrated the content of compounds of the Monoterpenes, Triterpenes, Steroids, Saponins, Kumarin, Scopoletin, and Alkaloids groups in the secondary metabolite fraction [12]. Currently, only fractions 14, 15, 16, 36K, 41, and 44 have been profiled and tested for effectiveness as antimalarials [9]. There are still a number of active *S. hygroscopicus* fractions that may have antimalarial effects. In previous research which examined the content of compounds in the secondary metabolite fraction of *S. hygroscopicus* by chromatography in UV light, the fractions 38K with an Rf value of 0.24 showed dark gray spots. When compared with the literature, it was stated that the compound *contained* coumarin, scopoletin, or alkaloids [12].

Meanwhile, fractions 38T with an Rf value of 0.24 showed blue spots and it was known as saponin. Fractions 56 with Rf value 0.42 showed bright blue spots known *as* monoterpenes, triterpenes, and Steroids. Each fraction obtained from fractionation II.

Due to the limited research on the antimalarial effects and toxicity of each fraction, this study aimed to determine the effectiveness and toxicity of active fraction numbers 38K, 38T, and 56 of *S. hygroscopicus* secondary metabolites as antimalarial.

Materials and Methods

This study used a true experimental research design. *S. hygroscopicus* bacteria were fermented and extracted by previous researchers to obtain secondary metabolites. *S. hygroscopicus* was obtained from LIPI Microbial Collection Cibinong and sub-cultured in the Laboratory of Microbiology, Faculty of Medicine, Universitas Brawijaya (Malang, Indonesia). The active metabolites were then fractionated and obtained fraction numbers 38T, 38K, and 56. Each fraction was tested for effectiveness and cytotoxicity. For efficacy test, fractions 38T, 38K, and 56 were evaluated against *P. berghei in vitro* at doses of 0.25, 1.25, 6.25, 31.25, and 156.25 µg/mL, respectively. The degree of parasitemia from microscopic observation was used as the test parameter. In the cytotoxicity test, fractions 38T, 38K, and 56 each divided into five concentrations of 0.25, 2.5, 25, 250, and 2,500 µg/mL and tested against Michigan Cancer Foundation-7 (MCF-7) cell line using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) Assay. The results of the toxicity test in the form of percentage of viable cells were used to determine the Cytotoxicity Concentration 50 (CC_{50}) as the test parameter. This study was in accordance with the Helsinski Declaration and approved by the Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia (Approval No. 13/EC/KEPK/01/2022).

Fractionation of *Streptomyces hygroscopicus* subsp. *hygroscopicus* secondary metabolite

The fractionation process was performed using Sepacore® Flash Chromatography and Reveleris® PREP Purification System Chromatography with gradient ethyl acetate and n-hexane solvents and was done by PT BUCHI Indonesia (BSD City, Tangerang, Indonesia). The results were validated with TLC using ethyl acetate : n-hexane eluent combination decided by previous research as the combination of eluent volume and reagent type of spraying in TLC procedure as independent variable to analyze the compounds in the metabolite. Spot characteristics appeared at TLC plate such as retardation factor (Rf) and color of spot after spraying process as dependent variable. This spot characteristics was matched with the compounds group database.

Preparation of mice donor and *Plasmodium berghei* thawing

Male albino Balb/c strain mice with 6–8 weeks of age and an average body weight of 20–25 grams obtained from Laboratory of Parasitology, Faculty of Medicine, Universitas Brawijaya (Malang, Indonesia) were used as mice donors. *Plasmodium berghei* was also obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Brawijaya. Frozen erythrocyte pellets infected by *P. berghei* were thawed and centrifuged at 2,000 rpm for 5 minutes. The pellets were then washed twice using Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) and diluted appropriately for donor mice. Each experimental animal received an intraperitoneal injection of 200 µL *P. berghei* infected erythrocyte solution. Observation of the degree of parasitemia in mice was carried out every 2 days using blood smears obtained from the tails of mice with the assumption that there was a twofold increase in parasitemia in

blood smears. After the degree of parasitemia reached at least 5%, the parasite culture was taken completely from the blood through the heart of the mice through cardiac puncture after the mice were euthanized using chloroform. Histopaque was used to isolate the red blood cells from the leukocyte- and monocyte-infected mice. Then the infected red blood cells were cultured in 24 well plates using 20% Fetal Bovine Serum (FBS), 0.1% gelatin in RPMI for 48 hours in a CO_2 incubator at 32°C and a hematocrit of 2.5%.

Antimalaria effectivity assay

The antimalarial effectivity was determined by counting the percentage of parasite inhibition. Groups of *P. berghei* cultures were exposed to the active metabolites' fractions at the concentrations of 0.05, 1.25, 6.25, 31.25, and 156.25 mg/L. A positive control was exposed to 2 µg Artemisinin, whereas negative control was exposed to 2 µg 1% DMSO. The culture then incubated for 48 hours at 32°C with 5% CO_2. Next, the culture was taken and centrifuged. The pellet from the culture was used to make blood smears on an object glass. Giemsa staining was performed before observation on a microscope with a magnification of 1,000x for 1,000 erythrocytes. The percentage of parasite inhibition was obtained based on the following formula [13].

$$\%\ Parasite\ inhibition = 100\% - \left(\frac{Xs}{Xc}\right) \times 100\%$$

where *Xs*, the degree of parasitemia in the sample group, was the percent average growth of parasites treated with select doses. *Xc*, the degree of parasitemia in the control group, was the percent average growth of parasites treated with 1% DMSO. The degree of parasitemia was calculated based on the total infected erythrocytes in 5,000 observed erythrocytes. The percentage of parasitemia degree was obtained from the following formula [14]:

$$\%\ Percentage\ of\ Parasitemia = \frac{\sum \text{IRBC}}{\sum \text{Total RBC}} \times 100\%$$

where IRBC was the infected red blood cell. The Inhibitory Concentration 50 (IC_{50}) is the concentration of *S. hygroscpicus* extract that can inhibit the growth of 50% of *P. berghei* culture and was obtained through SPSS (IBM, Armonk, New York, USA) probit analysis.

Human breast cancer cell culture *(*MCF-7*)*

MCF-7 cell was obtained from the American Type Culture Collection (HTB-22-ATCC) (Bioresource Center, Manassas, VA, USA). MCF-7 was cultured using RPMI 1640 medium with added 10% FBS, 1% Penicillin-Streptomycin, and 1.5% $NaCHO_3$. MCF-7 cells which stored in cryotube were centrifuged and incubated at 37°C for 24 hours. Incubation was carried out until a sufficient number of cells with a density of 70-80% covered the flask. MCF-7 cells in the culture flask were observed under an inverted microscope. The subculture was done when the cells were attached and reached 80-90% confluent. Cells were ready to be harvested when cells were > 80% confluent.

Cytotoxicity test using MTT assay

MCF-7 cultured cells were diluted to a final cell concentration of $8x10^3$ cells/100 µL. Cells were then transferred into each well of a 96-well microplate as much as 100 µL per well. Microplates were then incubated in an incubator at 37°C and 5% CO_2 for 24 hours. Secondary metabolite extracts of *S. hygroscopicus* with 5 concentration series (0.05, 0.5, 5, 50, 500 mg/L) were introduced into the wells containing 100 µL of cells in each with three repetitions. The microplate was then homogenized and incubated for 48 hours. MTT assay kit (BIOTIUM Inc., Hayward, GA, USA) was applied following the manufacturer's instructions. 100 µL of MTT solution was added to each well and incubated for 3 hours at 37°C in 5% CO_2 incubator until purple crystals formed. Cell conditions were examined with an inverted microscope. Living cells would react with MTT reagent and form purple color. If formazan had clearly formed, 100 µL of 10% SDS stopper reagent was added. The absorbance of formazan was read using a Zenix-320 microplate reader at the wavelength of 630

nm. The percentage of viability towards MCF-7 cells was calculated with the following equation [15].

$$\% \text{ Viability} = \frac{\text{OD Sample}}{\text{OD Control}} \times 100\%$$

Data obtained from the MTT assay results were used to calculate the CC_{50} value using SPSS probit analysis.

Statistical Analysis

Data in this study were analyzed using the SPSS version 25 (IBM, Armonk, New York, USA). One way ANOVA test, Tukey posthoc test, Pearson correlation test, and Linear regression test were performed with a *P* value less than 0.05 as significant difference.

Results and discussion

Fractionation of *Streptomyces hygroscopicus* subsp. *hygroscopicus* secondary metabolite

During the fractionation process, various weights of fractions were generated in each tube resulting 47 fractions from fractionation I (Sepacore® Flash Chromatography) and 60 fractions from fractionation stage II (Reveleris® PREP Purification System Chromatography). Fractionation was performed using column chromatography with hexane and ethyl acetate solvents to simplify the extract samples containing various components. In a previous study, fractionation II showed spots in fractions 38K and 56 when exposed to UV light at 254 and 366 nm with retardation factors of 0.24 and 0.42, respectively [12]. These spots were considered as monoterpenes, triterpenes, and steroid compounds because they showed gray or purple fluorescence at UV 254 nm and bright blue fluorescence at UV 366 nm. In fraction 38T, when sprayed with p-Anisaldehyde - sulfuric acid, the extract of saponin metabolites was obtained. In the *in vitro* research of Fitri *et al*, it was said that antimalarial activity had been identified in the fractionation of secondary metabolite extracts from *S. hygroscopicus* bacteria through the mechanism of inhibition of mitochondrial enzymes characterized by a decrease in *Plasmodium falciparum* Lactate Dehydrogenase (PfLDH) concentration [6]. Additionally, Nugraha *et al* demonstrated through an *in silico* approach that isoquinoline derivatives contained in the metabolites of *S. hygroscopicus* had antimalarial activity through the interaction between isoquinoline derivatives with several proteins such as adenylosuccinate synthetase, falcipain 2, glucose-6-phosphate isomerase, glycerol kinase, enoyl-acyl carrier protein reductase, and dihydrofolate reductase-thymidylate synthase [8]. The potential of metabolites derived from secondary metabolite extracts of *S. hygroscopicus* needs further development as an effective antimalarial agent. Fractionation aims to simplify extract samples that contain various components. Fraction numbers 38K, 38T, and 56 were selected because they contain secondary metabolites that have antimalarial activity [12]

Morphology of parasite

Changes in parasite morphology after administration of the 38K, 38T, and 56 fractions of *S. hygroscopicus* were detected in blood smears of erythrocytes infected with *P. berghei* and cultured for 48 hours under a light microscope. At the concentration of 0.25 g/mL, fractions 38K was observed to have ruptured schizonts surrounded by merozoites (A1), whereas a ring-form image was found in distinct erythrocytes but at the same concentration (A2). Then, at the concentration of 1.25 µg/mL, an image of immature schizonts (B1) and mature schizonts containing several merozoites (B2) were displayed. At the concentration of 6.25 µg/mL, several trophozoites in crisis form (C1) and mature trophozoites with irregular cytoplasm (C2) were seen. At the concentration of 31.25 µg/mL, trophozoites with cytoplasmic damage causing irregular cytoplasmic shape (D1) and trophozoites in crisis (D2) were observed. At the concentration 156.25 µg/mL, several trophozoites in crisis form dominated the image

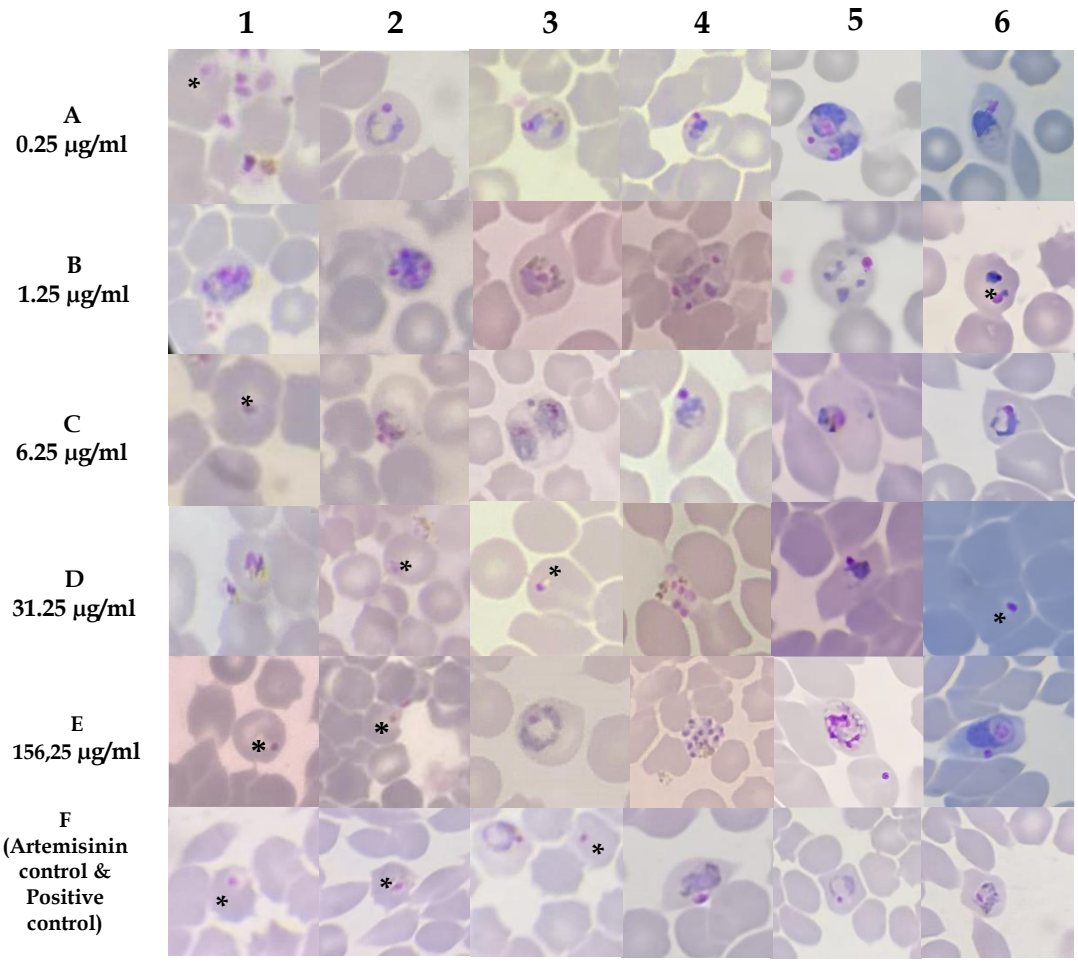


Figure 1. Morphological changes of *Plasmodium berghei* after 48 h incubation with fractions 38K, 38T, and 56 of *S. hygroscopicus* subsp. *Hygroscopicus* metabolite extracts. **A:** 0.25 µg/mL. **B:** 1.25 µg/mL. **C:** 6.25 µg/mL. **D:** 31.25 µg/mL. **E:** 156.25 µg/mL. **F1-F3:** Artemisinin control. **F4-F6:** Positive control.

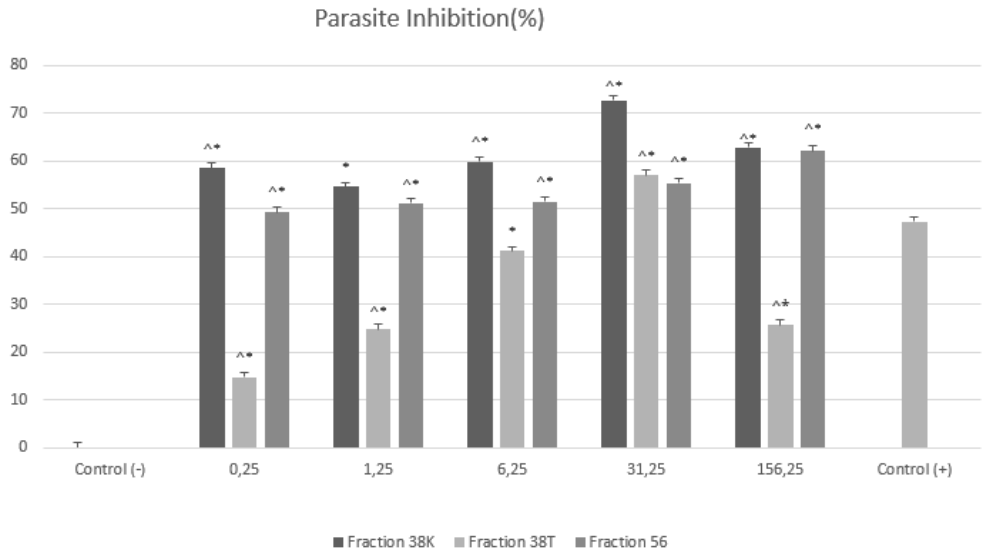
(E1,2). In the 38T fraction, at the concentration of 0.25 µg/mL, the 38K fraction showed a ring-form stages (A3), whereas the same concentration in other erythrocytes revealed a mature schizont image with multiple merozoites (A4). An image of immature schizonts was then discovered (B3 and B4) at the dosage of 1.25 g/mL. Both the ring form (C4) and immature schizonts (C3) were observed at the concentration of 6.25 µg/mL. The observations at the concentration of 31.25 g/mL were dominated by trophozoites in the form of a crisis (D3) and by ruptured schizonts surrounded by merozoites (D4), whereas at the concentration of 156.25 µg/mL, several ring-form (E3) as well as ruptured schizont surround by merozoites (E4) were obtained. At the concentration of 0.25 µg/mL, fraction 56 revealed the presence of immature schizonts (A5 and A6). Then, at the dose of 1.25 µg/mL, a ring form stages were observed (B5), while the observation results were dominated by a trophozoite stages in the shape of a crisis (B6). A ring form (C5) and trophozoites in crisis form (C6) were produced at the concentration of 6.25 µg/mL. A trophozoite stage with cytoplasmic damage was produced at the dose of 31.25 µg/mL, resulting a ring form in crises form (D5) and a trophozoite stages in crisis form (D6). At the concentration of 156.25 µg/mL, immature schizont were displayed (E5 and E6). The observation in Artemisinin control were dominated by crisis form (F1-F3). Whereas in

positive control, a trophozoite with irregular cytoplasm (E4) and *ring-form* (F4-F6) were seen (Figure 1). The results showed that all three fractions interfered with parasite stage development, which suggested that the antimalarial activity of the fractions was the result of synergistic interactions of the various components contained in the fractions. These results were similar to previous studies that extracts of *S. hygroscopicus* metabolites could damage parasite morphology. The schizonts and trophozoites failed to develop and appeared morphologically damaged with loss of cytoplasmic content and a picnotic nucleus called crisis formed. The crisis form is characterized by loss of cytoplasm, the nucleus is attracted to the edge of the parasite cytoplasm, and the chromatin is thick, dense, and dark [16].

***Plasmodium berghei* growth inhibition**

The parasite inhibition effects of the intervention groups were observed using light microscopy to determine the percentage of parasite-infected erythrocytes. The degree of parasitemia and the inhibitory effects of *S. hygroscopicus* fractions 38K, 38T, and 56 were significantly ($P < 0.05$) (Figure 2A). Compared to the negative control, each treatment group had a significant decrease in parasite-infected cells. The negative control used in this study which did not receive any intervention had a parasitemia percentage value of 4.75±0.14% and had zero percent parasite inhibition. The positive control used 0.01 mM Artemisinin with a parasitemia percentage value of 2.35±0.19% and a parasite inhibition percentage value of 50.61±4.10%. Fraction 38K showed the greatest percentage inhibition at the concentration of 31.25 µg/mL with a parasite inhibition percentage value of 73.37 ± 1.49%, followed by the concentration of 156.25 µg/mL with a parasite inhibition percentage value of 62.71 ± 2.31%. Both concentrations were higher than the percentage of positive control inhibition. The one-way ANOVA test showed significant differences among the intervention groups with the P value less than 0.05. Unfortunately, the relationship between fraction concentration and degree of parasitemia was not significant with P value = 0.656 and r = 0.274 (Pearson correlation test), indicating no correlation. R^2 value of 0.463 from linear regression analysis revealed that treatment administration was responsible for 46.3% of parasitemia inhibition. Fraction 38T showed the greatest percentage of inhibition at the concentration of 31.25 µg/mL with a parasite inhibition percentage value of 56.99 ± 2.37%, which was higher than the positive control percentage. The percentage of inhibition was followed by the concentration of 6.25 µg/mL, which showed a percentage value of parasite inhibition of 41.09 ± 0.43%, but the percentage was still low compared to the positive control group. After treatment, a one-way ANOVA test of the degree of parasitemia inhibition revealed significant differences among the intervention groups ($P < 0.05$). The relationship between fraction concentration and degree of parasitemia was significant with *P* value = 0.000 and r = 0.510 (Pearson correlation test), indicating a positive correlation. R^2 value of 0.260 from linear regression analysis revealed that treatment administration was responsible for 26% of parasitemia inhibition. Fraction 56 showed the greatest percentage inhibition at the concentration of 156.25 µg/mL with a parasite inhibition percentage value of 62.83 ± 0.69%. The percentage of inhibition was followed by concentration of 31.25 µg/mL with a parasite inhibition value of 55.26 ± 0.35% followed by concentrations of 6.25 µg/mL and 1.25 µg/mL which respectively showed parasite inhibition percentage values of 51.38 ± 1.03% and 51.13 ± 1.72%. After treatment, a one-way ANOVA test of the degree of parasitemia inhibition revealed significant differences among the intervention groups ($P < 0.05$). The relationship between fraction concentration and degree of parasitemia was significant with *P* value = 0.000 and r = 0.902 (Pearson correlation test), indicating a positive correlation. R^2 value of 0.813 from linear regression analysis revealed that treatment administration was responsible for 81.3% of parasitemia inhibition.

A.



B. (1) (2) (3)

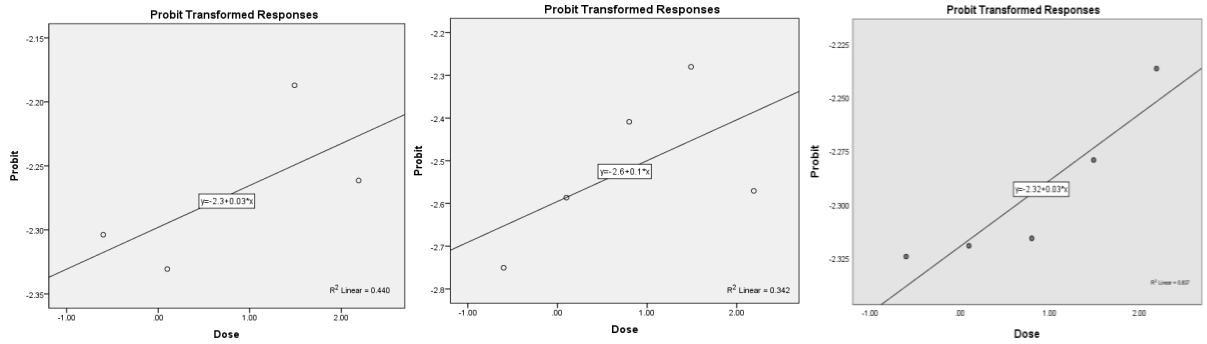


Figure 2. Antimalarial activity test of fractions 38K, 38T, and 56 of metabolite extract of *S. hygroscopicus* subsp. *Hygroscopicus* against *P. berghei* after 48 hours. **A.** Percentage growth inhibition of *P. berghei*. **B.** IC_{50} analysis of fractions 38K (1), 38T (2), and 56 (3) using linear regression analysis of log concentration and probit parasite inhibition. y = 0.03x - 2.3 with R^2 = 0.440 is the equation for fraction 38K with IC_{50} value 31.583 µg/mL which classified as moderate inhibition, while y = 0.1x - 2.6 with R^2 = 0.342 is the equation for fraction 38T with IC_{50} value 11.566 µg/mL classified as promising inhibition, while y = 0.03X - 2.32 with R^2 = 0.837 is the equation for fraction 56 with IC_{50} value 73.29 µg/mL classified as weak inhibition.

Inhibition Concentration 50 (IC_{50}) from fraction 38K, 38K, and 56

In this study, probit analysis of parasite inhibition was used to calculate IC_{50}, followed by linear regression analysis using SPSS. Probit analysis of fraction 38K resulted in the equation y = 0.03x - 2.3 with R^2 = 0.440. The IC_{50} for fraction 38K was calculated from this equation to be 31.583 µg/mL. While y = 0.1x - 2.6 with R^2 = 0.342 was used to calculate the IC_{50} of fraction 38T. IC_{50} for fraction 38T was calculated from this equation to be 11.566 µg/mL. For fraction 56, y = 0.03x - 2.32 was obtained with R^2 = 0.837. The IC_{50} for fraction 56 determined by this equation was at a concentration 73.29 µg/mL (Figure 2B). Parasite inhibition is the inhibitory effect caused by the treatment given. Inhibition Concentration 50 (IC_{50}) is the concentration of *S. hygroscopicus* that is able to cause 50% inhibition of parasites. Based on the results of this study, fractions 38K, 38T, and 56 of metabolite extracts of *S. hygroscopicus* can inhibit *P. berghei in vitro*. The results showed differences of parasite inhibition among the three fractions. In fraction 56, the results showed that the higher concentration of the fraction, the higher percentage of parasite inhibition that were obtained. However, in

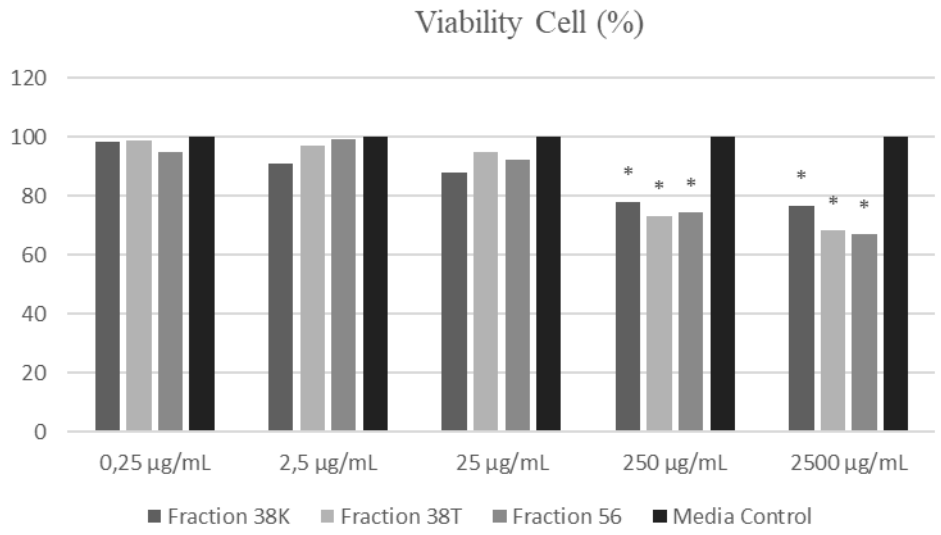


Figure 3. Diagram analysis of the viability cell of fractions 38K, 38T, and 56 of *S. hygroscopicus* subsp. *hygroscopicus* metabolite extracts. The result showed that all percentage concentrations of cell death were more than 50%. The result showed fractions 38T, 38K, and 56 with concentrations 250 µg/mL and 2,500 µg/mL were significant compared with control group. *: significant difference compared to the control group.

fractions 38K and 38T, the results showed that the best concentration in the percentage of parasite inhibition was the concentration of 31.25 µg/mL. Fraction 38K showed greater potential in inhibiting parasites than fractions 38T and 56, with the greatest inhibition up to 73.37% at the concentration of 31.25 µg/mL. This study showed that *S. hygroscopicus* had antimalarial properties against *Plasmodium sp*. Previous research on fractions 15 and 16 of *S. hygroscopicus* secondary metabolites showed more than 50% growth inhibition against *P. falciparum* using the lactate dehydrogenase (PfLDH) enzyme assay method *in vitro* [10]. IC_{50} in this study was determined through SPSS probit analysis using percentage inhibition data. IC_{50} means the concentration of fractions 38K, 38T, and 56 secondary metabolites of *S. hygroscopicus* that can inhibit the growth of *P. berghei* by 50%. In this study, the IC_{50} of fraction 38K was 31.583 µg/mL, while fraction 38T had an IC_{50} of 11.66 µg/mL, and fraction 56 had an IC_{50} of 73.29 µg/mL. The highest parasite inhibition percentages observed in fraction 38K did not match the IC_{50} value because the 38K fraction showed results that did not depend on concentration. Based on the literature, the anti-plasmodial activity of a compound can be classified as high activity (IC_{50} < 5 µg/mL), promising (5 µg/mL < IC_{50} < 15 µg/mL), moderate (15 µg/mL < IC_{50} < 50 µg/mL), and inactive (IC_{50} > 50 µg/mL) [18]. Fraction 38T showed promising activity, thus indicating that the fraction contained antimalarial compounds. Previous research obtained IC_{50} results from crude extract *Streptomyces sp*. AB8 against *P. falciparum* 3D7 of 17.56 µg/mL [17]. Another study stated that the IC_{50} of *S. hygroscopicus* strain i18 against *P. falciparum* using probit analysis was 11.07 µg/mL [18].

Cytotoxicity of fraction 38K, 38T, and 56

The cytotoxicity test was performed on MCF-7 cell line by MTT assay. Figure 3 showed the effects of *S. hygroscopicus* extract in fractions 38K, 38T, and 56 on the degree of MCF-7 cell viability. All concentrations of fractions 38K, 38T, and 56 had viability values higher than 50%. The Cytotoxic Concentration 50 (CC_{50}) value was determined by plotting the extract concentration on the X axis and the percentage of cell viability on the Y axis to obtain a concentration response curve. The CC_{50} values of fraction 38K, 38T, and 56 from secondary metabolite extract of *S.*

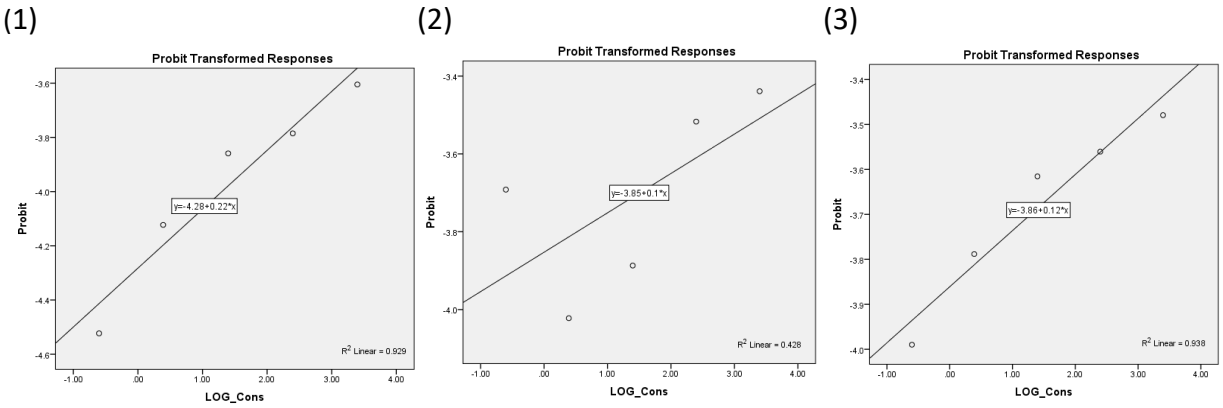


Figure 4. Cytotoxic concentration 50 analysis of fractions 38K, 38T, and 56 of metabolite extract of *S. hygroscopicus* subsp. *hygroscopicus* using linear regression analysis of log concentration and probit inhibition.

hygroscopicus were 427.868 µg/mL, 234.234 µg/mL, and 220.571 µg/mL, respectively. The result of the CC_{50} probit analysis of fraction 38K obtained the equation y = 0.22X - 4.28 with R^2 linear value of 0.929, whereas the CC_{50} probit analysis of fraction 38T obtained the equation y = 0.1X – 3.85 with R^2 linear value of 0.428, and y = 0.12X – 3.86 with R^2 linear value of 0.938 was used to calculate the CC_{50} of fraction 56 (Figure 4). Selectivity index (SI) is defined as the ratio of toxicity to human cells to the antimalarial activity of a compound. The selectivity index is determined by dividing the CC_{50} value by the IC_{50} value. From the results of this study, SI of fraction 38K was 13.547 µg/mL. Based on the classification of antiplasmodium activity, this value indicated active antiplasmodial activity because SI > 10. The SI of fraction 38T was 20.251 µg/mL, which showed active as antiplasmodial. Fraction 56 had the SI of 3,009 µg/mL, which indicated active marginally because SI < 4.

In the development of antimalarial compounds, it is necessary to conduct a cytotoxicity test to determine the safest and most harmless exposure dose for exposed cells. MCF-7 cell cultures were exposed to each fraction of *S. hygroscopicus* and the MTT Assay method was performed. The level of absorbance measured indicated the level of cell viability. According to the classification, if CC_{50} > 50 µg/mL, it is considered non-toxic, while CC_{50} < 50 µg/mL is considered toxic [16]. Fraction 38K, 38T, and 56 all had CC_{50} > 50 µg/mL, so the fractions were non-toxic to MCF-7 cells. This is similar to the results of previous studies of fractions 14 and 36K that showed non-toxic properties against MCF-7 cell culture [11]. Fractions 15 and 16 of *S. hygroscopicus* are known to have no toxic effects on DLD-1 cell line culture [10]. According to Valdes Classification, SI < 4 is considered marginally active, while SI 4-10 is considered partially active and SI > 10 is active [19]. Both fractions 38K and 38T had active antimalarial activity (SI > 10), indicating a favorable safety window between the effective concentration against the parasite and the toxic concentration to MCF-7 cells. However, the most active compound was 38T because the result of SI was higher than 38K.

This study demonstrated that the antimalarial compounds contained in fractions 38K, 38T, and 56 of *S. hygroscopicus* included coumarin, scopoletin, alkaloids, saponins, monoterpene, triterpene, and steroids. Those contents are similar to fractions 14 and 36K which we studied previously and showed a strong antimalarial effect by inhibiting the degree of parasitemia *in vitro* more effectively than Artemisin [11]. Steroids have hydrophobic properties so that they can facilitate active compounds into erythrocytes. The mechanism of action of steroids as antimalarials is to inhibit parasite

growth through inhibition of hemozoin formation (residual hemoglobin metabolism by parasites in the trophozoite phase) [20]. Saponins are able to synthesize biosurfactants so that they can reduce the surface tension of water and disrupt the conformation of *Plasmodium* cell membranes. Saponins can produce emulsifying foam that can destabilize the hydrophobic core of the phospholipid bilayer so that micelles are formed [18]. Triterpenoids and monoterped are included in the terpenoid group which is already known to have antimalarial activity. The structure of terpenoid compounds and their derivatives allows them to pass the erythrocyte membrane into cells through the lipid bilayer. These conditions result in inhibition of growth and invasion of *Plasmodium*. In addition, terpenoids are also able to disrupt the growth of *Plasmodium* by inhibiting protein synthesis [20]. Alkaloids are compounds that have been studied for their effects as antimalarials. In previous studies, alkaloid compounds were known to be able to inhibit the growth of *P. berghei* parasites through the mechanism of inhibiting the glycolysis pathway of *Plasmodium* enzymes, inhibiting heme detoxification, and hematin crystallization through interactions with heme and hematin [9]. Coumarin is known to have potential as an antimalarial. *In vitro* studies show that coumarin can inhibit the activity of important enzymes in the *Plasmodium* life cycle, such as topoisomerase enzyme and dihydrofolate reductase enzyme. Inhibition of these enzymes can interfere with the process of DNA replication and folic acid synthesis, which are important for *Plasmodium,* thus slowing down the growth and reproduction of the parasite. Scopoletin has high antioxidant effects that are beneficial in conditions of malaria infection. Malaria infection can cause increased oxidative stress in the body, and antioxidant compounds such as scopoletin can help protect host cells from oxidative damage caused by *Plasmodium* [21].

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