# Bioassay Guided Fractionation of Marine *Streptomyces* sp. GMY01 and Antiplasmodial Assay using Microscopic and Flow Cytometry Method

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| Info Article | ABSTRACT |
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| | Marine-derived *Streptomyces* sp. GMY01 is a potential actinobacteria which have anticancer activities. In previous studies, several anticancer agents exhibited antiplasmodial activity. This study aimed to evaluate antiplasmodial activity of GMY01 extract using bioassay guided fractionation. Crude extract of ethyl acetate was obtained from supernatant of 11 days fermentation in starch nitrate medium. Crude extract was fractionated using *n*-hexane and methanol solvent. Re-fractionation was applied using flash chromatography and column chromatography. Antiplasmodial assay was performed on *Plasmodium falciparum* FCR3 and *P. falciparum* 3D7 by microscopic method using thin blood smear + Giemsa stain, and was confirmed by flow cytometry method using SYBR Green I stain. Toxicity assay was performed on Vero cells line (normal cells). Main constituent of active fraction was analyzed using LCMS/MS. The result of the study showed that ethyl acetate-methanol fraction has high antiplasmodial activity ($IC_{50}$=3.96μg/mL) with very low toxicity on Vero cells ($IC_{50}$=30,072μg/mL). Bioassay guided fractionation resulted F4.7 which has the highest *Plasmodium* inhibition (94.3% at 5μg/mL). Main constituent analysis showed $C_{10}H_{13}NO$ (163.09971 Da) and $C_{11}H_{15}NO_{13}$ (209.10519 Da) as two mayor compounds in F4.7. This study guided the isolation and structure elucidation of potential compound in further research.<br>**Key words:** Actinomycetes, drug discovery, malaria, antiplasmodial assay, SYBR Green I |

## INTRODUCTION

*Streptomyces* as the main sources of new bioactive compounds is used for producing two-thirds of all currently available antibiotics. It has a very large genome size, between 6.2 and 12.7 Mb, and 5% of its genome is devoted to the synthesis of secondary metabolites (Undabarrena *et al*., 2017; Weber *et al*., 2015). In the past ten years, the discovery of new compounds from marine bacteria, especially from the *Streptomyces* genus, has led to the discovery of new anticancer (Nguyen *et al*., 2020; Dhaneesha *et al*., 2019). In the previous study, we discovered the marine bacterium *Streptomyces* sp. GMY01 (7.9Mbp) which has very high potential for therapeutic agent especially as anticancer (Herdini *et al*., 2016) . The GMY01 extract shows anticancer activities on T47D and MCF7 breast cancer cell lines (Farida *et al*., 2007; Werdyani *et al*., 2017).

Malaria is a critical disease in humans caused by *Plasmodium* majority *Plasmodium falciparum* parasite infection transmitted by the female *Anopheles* mosquito bite (Vega-Rodríguez *et al.*, 2015). The emergence of drug-resistant *Plasmodium* drives the efforts of the expert to find and develop new drugs. The discovery of new antimalarial agents from marine actinobacteria is tremendously limited because 72% of antiplasmodial natural products are still plant-sourced (Tajuddeen & Van Heerden, 2019). New antimalarial drugs could be developed from anticancer compounds. Previous studies have shown that antiplasmodium has a synergetic effect similar to that of anticancer compounds (Crespo-Ortiz & Wei, 2012; Das, 2015). In another study, an anticancer compound inhibited P.*falciparum* and *P. berghei* (Sumanadasa *et al.*, 2012).

Based on the synergy properties of anticancer and antiplasmodium compounds, we predict that *Streptomyces* sp. GMY01 also has potential as an antiplasmodium. In this research, we evaluated antiplasmodial activity of *Streptomyces* sp. GMY01 using bioassay guided fractionation. For antiplasmodial assay, we used microscopic method using thin blood smear with Giemsa stain (Hall & Fauci, 2009) and flow cytometry method using SYBR Green I nucleic acid dye (Rebelo *et al.*, 2013). To predict active compounds on potential fraction, we conducted main constituent analysis using Liquid Chromatography–Mass Spectrometry/Mass Spectrometry (LCMS/MS).

## MATERIAL AND METHODS
### Biological materials

*Streptomyces* sp. strain GMY01 was isolated from a marine sediment sample collected from Krakal Beach (8°8'44"S 110°35'59"E), Gunungkidul, Yogyakarta Province, Indonesia (Farida *et al.*, 2007). *Streptomyces* sp. GMY01 was deposited at the Indonesian Culture Collection (WDCM 769), Indonesian Institute of Sciences (LIPI) as InaCC A147 and NITE Biological Research Center (NBRC, WDCM 825) Japan with registration number NBRC 110111. The nucleotide sequence of the 16S rRNA gene was assigned to GenBank accession no. MN922642. *Plasmodium falciparum* FCR3 and *P. falciparum* 3D7 was obtained from the Eijkman Institute, Jakarta, Indonesia. Vero (ATCC-CCL81) cell line were obtained from the Faculty of Medicine, Health Public and Nursing, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia. *In vitro* assays for antiplasmodial activity used human blood from adult male donors and was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, UGM (KE/FK/0279/EC/2019).

### Fermentation and extraction

GMY01 bacteria were maintained in International *Streptomyces* Project-2 (ISP-2) agar medium (Difco, Sparks, USA). GMY01 was cultured at 28°C with 180rpm agitation for 3 days in a 250mL Erlenmeyer flask which contains 100 mL of tryptic soy broth (Difco, Sparks, USA) as the seed medium. For fermentation, the cell culture was transferred into four 1000mL flasks which contains 500mL of starch nitrate broth (SNB) medium and was incubated for 11 days at 28°C with 180 rpm agitation in a shaking incubator (Ghanem *et al.*, 2000). Secondary metabolites were obtained by separating the cell biomass from the liquid using refrigerated centrifugation at 4137 × *g* at 4°C for 15min (Farida *et al.*, 2007). The supernatant was extracted twice with an equal volume of ethyl acetate and evaporated using evaporator machine (Buchi, Switzerland) to obtain the crude extract. The crude ethyl acetate extract was dissolved in methanol and fractionated using an equal volume of *n*-hexane to separate the polar and nonpolar fractions.

### Fractionations

The active fraction was re-fractionated using flash chromatography (Reveleris™, Buchi, Switzerland) and a C-18 column cartridge with water-acetonitrile as the mobile phase to obtain separated fractions. The ethyl acetate-methanol fraction was dissolved in methanol combined with celite (Merck, Germany), with a fraction: celite ratio of 1: 3 (w/w), and dried. Flash chromatography was performed on the water ethyl acetate-methanol fraction based on the procedure manual for dry loading samples. All targeted fractions were weighed and evaluated for antiplasmodial assays. The second re-fractionated was performed with column chromatography method using Si 60 (40-63μm) (LichoPrep, Merck) with 30 of column length and 1cm of column diameter and methanol: chloroform (4:1) as eluent.

### Antiplasmodial assay

The human parasite *P. falciparum* was cultivated using the Trager and Jensen method with minor modifications (Trager & Jensen, 2005). *Plasmodium* was maintained in 2% human erythrocytes (red blood cells, RBCs) (O+, male) and

suspended in RPMI 1640 (Gibco, Thermo Fisher Scientific, USA) including 10% human serum (O+, male) and 500 μg of gentamicin (Indofarma, Bekasi, Indonesia) per liter. Flask cultures were incubated in a $CO_2$ incubator with 5% $CO_2$ at 37°C. Before it was used in treatments, the culture was synchronized with 5% sorbitol (Lambros & Vanderberg, 1979) (Mustofa *et al*., 2007). The sample was prepared by adding 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) (w/v) at various concentrations. The *Plasmodium* growth inhibition assay was performed in a total volume of 200 μL using 96-well microplates. Each microplate which contains 100μL of extract solution and 100 μL of *Plasmodium* inoculum at the parasitemia level of 5% was placed in a 5% $CO_2$ incubator (CellXpert C170i, Eppendorf AG, Hamburg, Germany) at 37°C for 3×24h. All treatments were performed in triplicate. The *Plasmodium* growth was investigated by making thin blood film preparations with Giemsa stain and further observed using a microscope (Nikon, Japan). The parasitemia was calculated from a minimum of 1,000 RBCs.

Confirmation of the antiplasmodial assay was carried out by flow cytometer analysis using SYBR Green I stain (Rebelo *et al*., 2013) with minor modifications. The extract concentration for treatment was 0.25–8μg/mL. For each measurement, 200μL *Plasmodium* culture (approximately $1.8 \times 10^{11}$ RBCs) was centrifuged at 7000rpm for 10min to separate the cells from the medium. Ten microliters of RBC were stained with 2μL DNA-specific dye SYBR Green I (500× the final concentration) (Invitrogen, Carlsbad, USA) and 2μL CD235a antibody (eBioscience, San Diego, USA). After 30min of incubation in the dark, the stained sample was washed once by 1mL of PBS and centrifuged at 2000rpm for 5min. The RBC pellet was collected and dissolved in 400μL flow cytometry buffer (BD) and immediately analyzed by flow cytometry using a 535/45nm bandpass filter in front of the detector (BD FACSCanto™ II, USA). The results of flow cytometric analysis were quantitatively analyzed using BD FACSDiva 8.0.2 software. Staining with the anti-glycophorin CD235 antibody was used to establish that all detected events represented RBCs.

The percent *Plasmodium* inhibition was obtained by formula:

$$\% \ inhibition = \frac{A - B}{A} \ x \ 100$$

A: The parasitemia or SYBR Green I fluorescens intensity in control (RPMI medium); B: The

parasitemia or SYBR Green I fluorescens intensity in treatment

The quantitative data of antiplasmodial assay on microscopic method and flow cytometry method were presented as mean ± standard deviation (SD) of parasitemia percentage and growth inhibition percentage. One-way ANOVA followed by Dunnett's multiple comparison test for analysis of treatment and two-way ANOVA for analysis of interaction between two methods was done by using GraphPad Prism 8.4.3 software. The half maximal inhibitory concentration ($IC_{50}$) values of the extracts or fraction were determined by nonlinear regression analysis of $\log_{10}$ concentrations of the extract versus percent *Plasmodium* inhibition which uses GraphPad Prism 8.4.3 software. The interaction between two methods was analyzed by two-way ANOVA.

**Toxicity assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) assay was employed to evaluate cytotoxicity of the extract on Vero cells as normal cells (Hansen *et al*., 1989; Hansen *et al*., 1989). Vero cells were grown in Dulbecco's Modified Eagle Medium–high glucose (Gibco, Thermo Scientific, USA) which were supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Scientific, USA). The cells were seeded into a sterile flat bottom 96-well microplate (Iwaki) at a density of $5 \times 10^3$ cells/well and allowed to adhere overnight at a total volume of 100μL in a humidified incubator (5% $CO_2$, 37°C). One hundred microliters of extract solution (0–1000μg/mL in 0.1% DMSO) was added to the cells and incubated for 24h before performing the MTT assay. Then, 100μL of 5mg/mL MTT solution was added to each well, were incubated at 37°C in a $CO_2$ incubator for 4h and was added by 100uL sodium dodecyl sulphate (SDS) to stop reaction. The amount of formazan product was determined spectrophotometrically at 595nm using a microplate reader (Bio-Rad, California, USA). All treatments were performed in triplicate. Percent cell viability was calculated using the formula:

$$\% \ inhibition = \frac{(A - B)}{A} \ x \ 100$$

A: the mean absorbance of the control wells; B: the mean absorbance of the treated wells

The $IC_{50}$ values were determined by nonlinear regression analysis between the $\log_{10}$ concentrations of the extract versus percent cell inhibition using GraphPad Prism 8.4.3 software.

Table I. Bioactivities of *Streptomyces* sp. GMY01 fraction using liquid fractionations

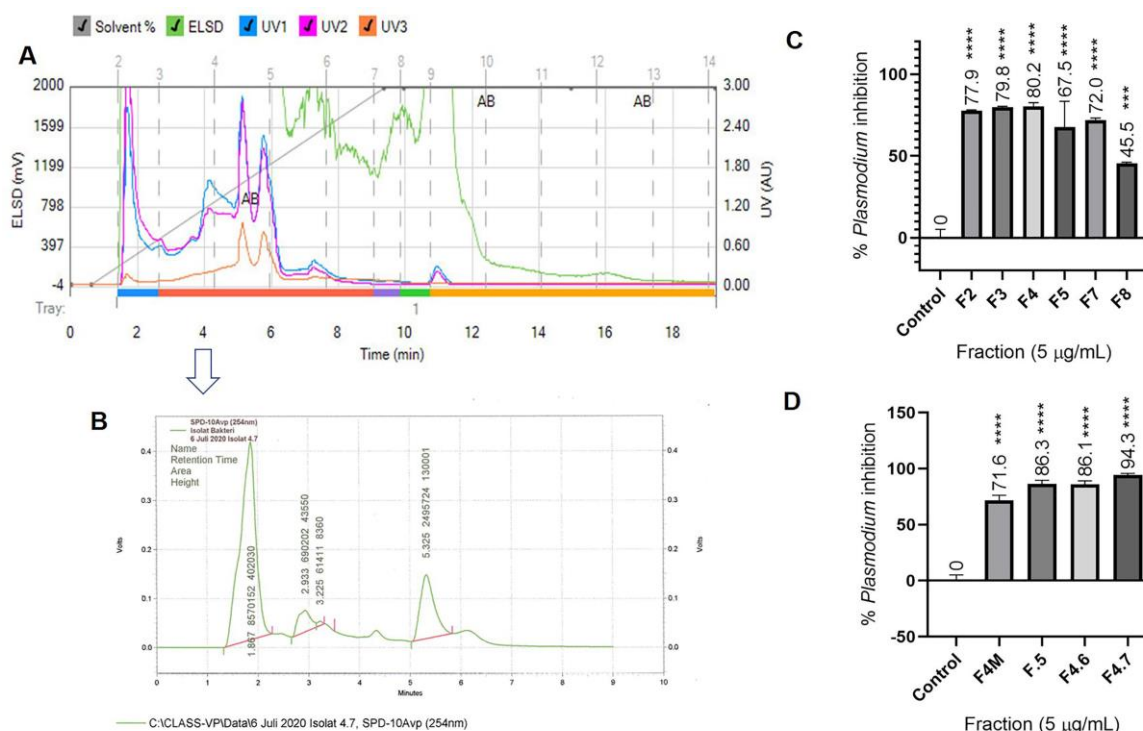| Fractions | IC$_{50}$ *P. falciparum* FCR3 (µg/mL) | IC$_{50}$ Vero (µg/mL) | Selectivity index (SI) |
|---|---|---|---|
| *n*-hexane-ethyl acetate | Not active | 68.45 | - |
| *n*-hexane free ethyl acetate | 3.83 | 865.3 | 225.9 |
| *n*-hexane-ethyl acetate-methanol | Not active | 57.29 | - |
| Ethyl acetate-methanol | 3.96 | 30,072 | 7,593.94 |
| Ethyl acetate residual | 6.24 | 52.85 | 8.47 |



Figure 1. Flash chromatography of ethyl acetate-methanol extract using Reveleris™ using C18 column and eluent water: acetonitrile (A), HPLC profile of fraction 4.7 from column chromatography using Lichoprep Si 60 (40–63 µm) (B), antiplasmodial assay on *P. falciparum* 3D7 of active fraction from flash chromatography (C) and from column chromatography (D). Value are expressed as mean ± SD (***$p<0.001$, ****$p<0.0001$).

The selectivity index (SI) was calculated from the ratio of toxicity on normal cells to antiplasmodial activity (Valdés *et al*., 2010)

**LCMS/MS**

Mass spectrometry analysis was performed on a Xevo G2-XS QTof mass spectrometer (Waters MS Technologies, Milford, USA) (Zhang *et al*., 2019). Electrospray ionization was adopted. The scan range was from 100 to 1200m/z. The capillary and cone voltages were set at 0.8kV and 30kV, respectively, and positive electron spray mode was adopted. The desolvation gas was set to 1000 L/h at a temperature of 500°C, the cone gas was set to 50L/h, and the source temperature was set to 120°C. Ultra-performance liquid chromatography (UPLC) analysis was performed using a Waters Acquity Ultra Performance LC system. Chromatographic separation was carried out on an ACQUITY UPLC HSS T3 column (100mm ×2.1mm, 1.7urn) at a column temperature of 40°C. The mobile phase consisted of solvent A (0.1% formic acid in water, v/v) and solvent B (0.1% formic acid in acetonitrile), with gradient polarity (A:B) of

95:0.5 to 0.5:95. The flow rate was set at 0.3mL/min. The column and auto-sampler were maintained at 40°C and 20°C, respectively. The injection volume was 1uL. The data acquisition and processing were performed using UNIFI. The parameter used was retention time (RT) in the range of 1–16min.

## RESULT AND DISCUSSION

From the 10 L supernatant of 11-days old GMY01 cultured in liquid SNB medium, 792mg crude ethyl acetate extract was obtained. Fractionation of the crude ethyl acetate produced 322mg of *n*-hexane fraction, 37mg of ethyl acetate–methanol–*n*-hexane fraction, 378mg of ethyl acetate–methanol fraction, and 7mg of insoluble fraction. The antiplasmodial activity and toxicity on normal cells of fractions (Table I). *N*-hexane fraction has no effect on *Plasmodium* and has high toxicity on normal cells. *N*-hexane free ethyl acetate, ethyl acetate-methanol and ethyl acetate residual have similar activities on *Plasmodium* but own different toxicity on normal cells. The antiplasmodium activity criteria in *P. falciparum* from extracts of natural product are categorized as inactive if they have $IC_{50}$>100µg/mL while the extracts with $IC_{50}$<100µg/mL are classified as follows: low active if SI<4, partially active if SI 4-10 and active SI>10 (Valdés *et al.*, 2010). *N*-hexane free ethyl acetate, ethyl acetate-methanol and ethyl acetate residual have SI>10 but ethyl acetate-methanol fraction has the highest SI (7,593.94). This fraction selected for further fractionations.

Flash chromatography result on ethyl acetate-methanol fractionation (Figure 1.A). From 2-6min of running time shows high peak detected on 254, 280 and 366nm of UV wavelength. On bioassay of ten fractions shows that F2, F3, F4, and F7 have high antiplasmodial activities (>70%). All treatment except F8 have significant difference than control ($p$<0.0001) (Figure 1.C). Among all fractions, F4 has the highest antiplasmodial activity and has been selected for further fractionations. In further separation procedures by manual column chromatography using silica 60, we obtained ten fractions. From bioassay on *Plasmodium*, F4.5, F4.6 and F4.7 have high antiplasmodial activities (>80%) and have significant difference than control ($p$<0.0001) (Figure 1.D). Other fractions which have no effect on *Plasmodium* were not displayed on this report. F4.7 resulted high antiplasmodial activity (94.3%) at 5µg/mL of concentration. The purity analysis using HPLC (C18

column, water: acetonitrile (1:1), isocratic) showed that fraction 4.7 has single mayor compounds and two minor compounds (Figure 1.B).

The antiplasmodial activity of active F4.7 using microscopic methods was confirmed with flow cytometry method. On microscopic analysis, infected RBC on RPMI control medium show schizont stage *P. falciparum* 3D7 similar with DMSO 0.1% as sample solution (Figure 2.B1-B2). The *Plasmodium* still on ring stage like stage on initial assay (0h) (Figure 2.B3). This indicated that *Plasmodium* growth inhibited by 25µg/mL of F4.7 fraction treatment. The same results were shown in other studies using 100ng/mL of eurycomanone with an incubation time of 72h which produced 90% inhibition with *Plasmodium* which was discovered to be dominant in the ring and throphozoites stage (Sholikhah *et al.*, 2016). It indicates that based on microscopic assay, F4.7 has high inhibition on *Plasmodium*. This fact was supported by flow cytometry analysis using SYBR Green I stain.

There was different fluorescens intensity of SYBR Green I on P2 area. P2 area represented the red blood cells (RBC) that infected by *Plasmodium* (positive SYBR Green I). The high intensity of SYBR Green I indicated the high *Plasmodium* DNA or the high parasitemia (Figure 2.A1-A2). The low intensity of SYBR Green I was detected on 25µg/mL F4.7 treatment (Figure 2.A3). Based on parasitemia value, there was difference between microscopic and flow cytometry assay (Figure 2.C). On microscopic assay, schizont stage having multi nuclei was counted as one parasite. Meanwhile, on flow cytometry assay, intensity of SYBR Green I depends on number of *Plasmodium* DNA on RBC. Multi nuclei stage on *Plasmodium* infected RBC detected as high intensity. Percentage of inhibition on microscopic assay was higher than flow cytometry assay (Figure 2.D). Nevertheless, both in microscopic and flow cytometry assay, 25µg/mL of F4.7 treatment has higher inhibition than RPMI and DMSO 0.1%.

Statistical analysis showed that there was significant interaction of parasitemia percentage ($p$<0.0001) and *Plasmodium* inhibition percentage ($p$=0.0003) between microscopic and flow cytometry method (Table II). The two methods have their own objectives, advantages, and limitations. Thin blood films are preferred to examine the morphology of parasites and to determine species (Bejon *et al.*, 2006) and the staging of *Plasmodium* (Sholikhah *et al.*, 2016).
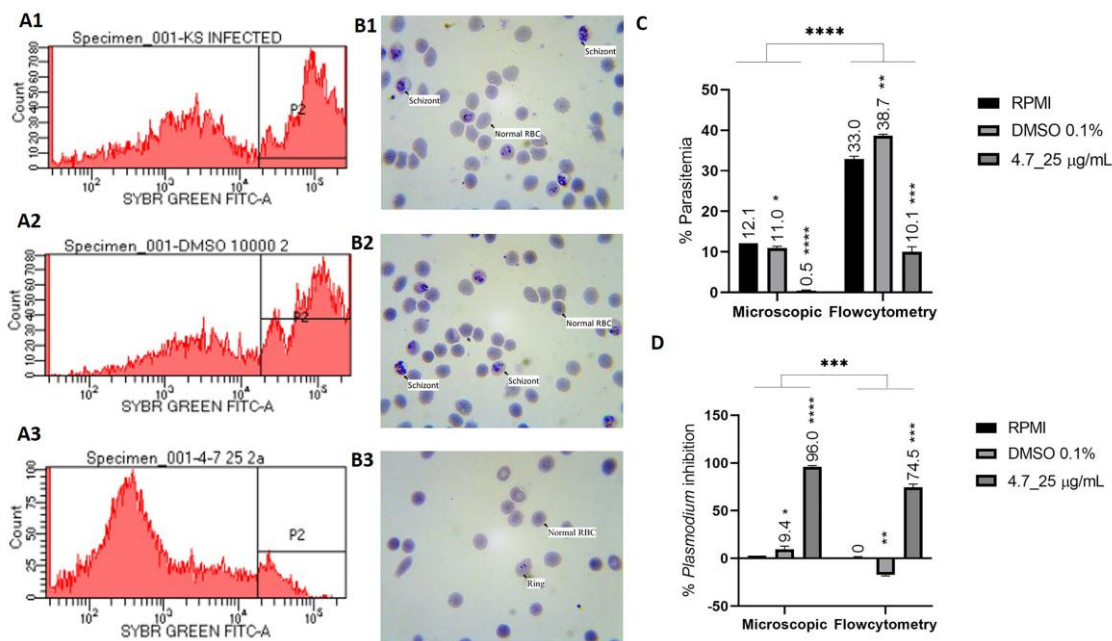
Figure 2. Fluorescens intensity of SYBR Green I on flow cytometry assay (A), parasitemia of *Plasmodium falciparum* 3D7 on microscopic assay (B). 1: Infected red blood cells (RBC) on RPMI medium (control), 2: Infected RBC on RPMI medium+ 0.1% DMSO, 3: Infected RBC on 25 µg/mL of F4.7 GMY01. Percentage of parasitemia (C) and percentage of *Plasmodium* inhibition (D) on antiplasmodial activities of F4.7 using microscopic and flow cytometry method. Value are expressed as mean ± SD (* $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Table II. Statistical analysis of interaction between two methods on antiplasmodial assay

| Methods | RMPI | DMSO 0.1% | F4.7 (25µg/mL) | Mean | Diff * | *p* value ** |
|---|---|---|---|---|---|---|
| **% parasitemia** | | | | | | |
| Microscopic | 12.10±0.014 | 10.96±0.424 | 0.48±0.139 | 7.849 | -19.37 | <0.0001 |
| Flow cytometry | 32.95±0.636 | 38.65±0.354 | 10.05±1.202 | 27.22 | | |
| **% *Plasmodium* inhibition** | | | | | | |
| Microscopic | 5x10^{-5}±0.118 | 9.39±3.503 | 96.03±1.145 | 35.14 | 16.07 | 0.0003 |
| Flow cytometry | 0.00±1.923 | -17.29±1.068 | 74.50±3.43 | 19.071 | | |

Diff*: Difference between means; *p* value**: interaction between two methods.

Table III. Detected compounds of fraction 4.7 using LCMS/MS analysis

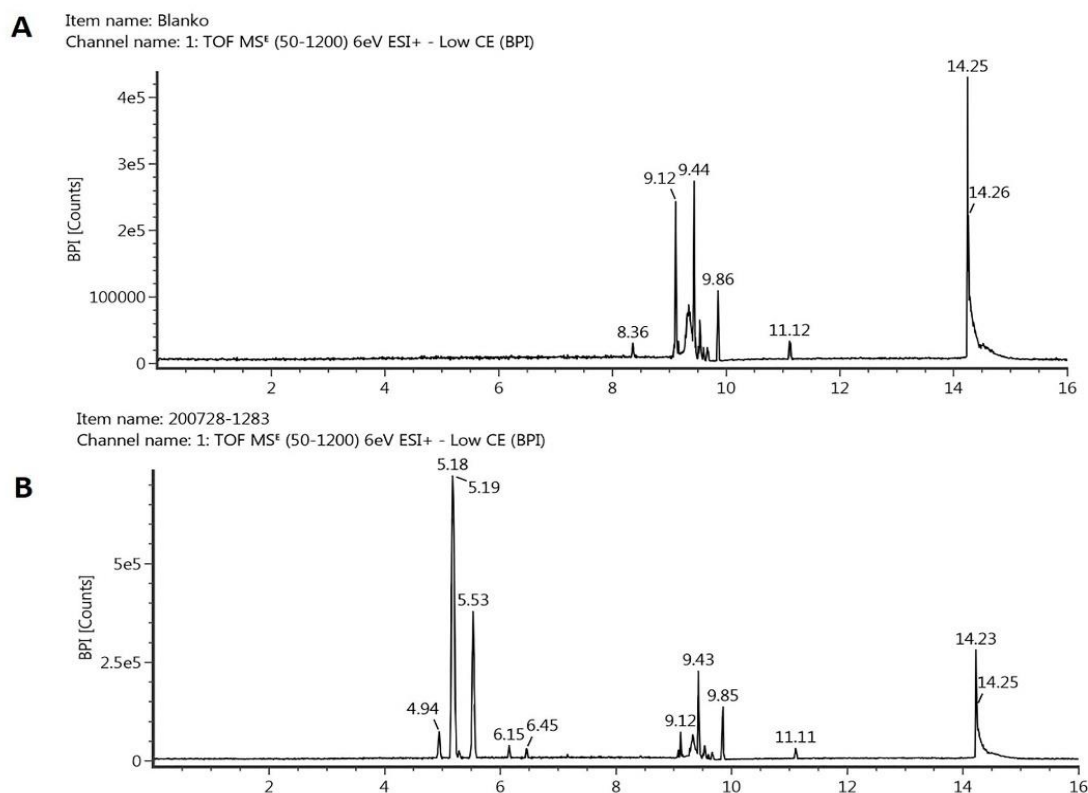| Compounds | Retention times (min) | Observed m/z | Neutral mass (Da) | Adducts | Mass error (mDa) |
|---|---|---|---|---|---|
| Ephedrine ($C_{10}H_{15}NO$) | 4.95 | 166.1220 | 165.11536 | +H | -0.6 |
| $C_{10}H_{13}NO$ | 5.19 | 164.1064 | 163.09971 | +H | -0.6 |
| $C_{11}H_{15}NO_3$ | 5.54 | 210.1120 | 209.10519 | +H | -0.4 |
| $C_{26}H_{22}O_4$ | 6.16 | 421.1391 | 398.15181 | +Na | -0.2 |
| $C_{23}H_{22}N_2O_7$ | 6.46 | 439.1494 | 438.14270 | +H | -0.5 |

Figure 3. Chromatogram of blank (A) and F4.7 GMY01 (B) using LCMS/MS analysis

Microscopic method using thin blood smear with Giemsa stain is a standard method for observation (Hall & Fauci, 2009). Flow cytometry techniques are considered simple, fast and valid for monitoring *Plasmodium* growth (Fong & Wright, 2013). This method was most widely used in assays for *P. falciparum in vitro* and *in vivo* (Aguiar *et al.*, 2012). The concentration of antiplasmodial drug inhibition is considered to be more precisely determined by measuring the fluorescent DNA binding dye based on the fact that the host, red blood cells, lacks of DNA (Machado *et al.*, 2016).

Main constituent chromatogram of F4.7 by LCMS/MS analysis (Figure 3). Blank sample chromatogram (Figure 3.A) and F4.7 chromatogram (Figure 3.B). The peak after 8 min of retention times (RT) was shown as blank peak. There were five compounds detected on F4.7 at 4.9-6.4 min of RT (Table III).

The candidate identified compound with $C_{10}H_{13}NO$ formula at 5.19min of RT was mayor compound on F4.7. The second mayor compound ($C_{11}H_{15}NO_3$) was identified at 5.54 min of RT. The mayor compound in F4.7 predicted as isobutyranilide which has $C_{10}H_{13}NO$ formula and 163.099714 g/mol of exact mass (available on https://pubchem.ncbi.nlm.nih.gov/compound/Iso butyranilide, accessed on 9 November 2020). Based on bioassay study, this compound was inactive as anticancer assay. The second mayor compound was predicted as propoxur (2-isopropoxyphenyl-methylcarbamate) which has $C_{11}H_{15}NO_3$ formula and 209.105193 g/mol of exact mass. This compound was known to have activity as an insecticide (available on https://pubchem.ncbi.nlm.nih.gov/compound/49 44, accessed on 9 November 2020). Another compound was predicted as ephedrine which has $C_{10}H_{15}NO$ formula and 165.115364 g/mol of exact mass (available on https://pubchem.ncbi.nlm.nih.gov/compound/92 94, accessed 9 November 2020). Ephedrine is an alkaloid compound with potential bronchodilator and anti-hypotensive activity. Isolation and elucidation procedures of single compound are needed to conduct in further study. This effort

guided to obtain an active compound which is responsible as antiplasmodial compound.

## CONCLUSION

The fractionation method which uses liquid fractionation, flash chromatography and column chromatography can be used in screening antiplasmodial candidates from bacteria. Our result show ethyl acetate-methanol fraction has high antiplasmodial activity with very low toxicity on Vero cells. Bioassay guided fractionation resulted F4.7 as the highest *Plasmodium* inhibition and was confirmed by microscopic and flow cytometry assay. The main constituent analysis showed $C_{10}H_{13}NO$ (163.09971 Da) and $C_{11}H_{15}NO_{13}$ (209.10519 Da) two as mayor compound.

## ACKNOWLEDGEMENT

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