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Antiplasmodial Activity of the Low Molecular Weight Compounds from *Streptomyces* sp. GMR22

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ABSTRACT

Low molecular weight (LMW) antiplasmodial compounds isolated from bacteria, particularly *Streptomyces* have not been widely reported. This study aimed to identify LMW compounds from *Streptomyces* sp. GMR22 as antiplasmodial. Isolation of the LMW compounds from the supernatant of fermentation culture using solvent of *n*-hexane:ethylacetate (EtOAc) (85:15v/v)and identified using gas chromatography-mass spectrometry (GC-MS). Antiplasmodial assay of *n*-hexane:EtOAc extract was carried out *in vitro* against *P. falciparum* (3D7). Parasitemia percentage obtained through microscopic observations and 50% inhibitory concentration (IC_{50}) obtained through probit analysis. The confirmatory antiplasmodial test was done by flow cytometry using SYBR Green I for *Plasmodium* DNA and anti-human CD235a for erythrocyte. The LMW compounds were investigated using SwissADME for drug-likeness. *n*-Hexane:EtOAc extract contained 21 LMW compounds from alcohol, hydrocarbon, ester, aromatic/diester, diester, fatty acid, and triester classes, which possessed moderate antiplasmodial activity with an IC_{50} value of 38.61 ± 19.06 µg/mL. Confirmation by flow cytometry analysis showed that the extract at 50 µg/mL exhibited antiplasmodial activity based on a decreased *Plasmodium* DNA intensity as compared to the control group. The result of drug-likeness screening obtained that 3 LMW compounds were drug-likeness, namely phenylethyl alcohol, ethyl citrate, and di-n-butyl phthalate. *Streptomyces* sp. GMR22 produced LMW compounds as antiplasmodial, and further study was needed to reinvestigate and to identify antiplasmodial active compounds.

Keywords: Low molecular weight compounds, *Streptomyces*, antiplasmodial, antimalaria

INTRODUCTION

Molecular weight compound is one of the considerations for the successful discovery of drug compound candidates. This consideration emerged after High Throughput Screening (HTS) era, where many compounds have high molecular weights. The higher molecular weights cause compounds tend to be lead-like rather than drug-like. According to the Rule of 5 (Ro5), drug-like compounds have low molecular weights of less than 500g/mol. Low molecular weight is one of the keys of drug-likeness for the discovery of oral bioavailability candidates (Lipinski, 2004).

Low molecular weight (LMW) compound is one of the products of secondary metabolites produced by microbes, particularly soil-dwelling bacteria (Tyc *et al*., 2017). *Streptomyces*, the soil-dwelling bacteria, are a well-known source for the

production of secondary metabolites, including LMW compounds. The LMW compounds of less than 500g/mol or 500 Da are commonly volatile (Schmidt *et al*., 2015), and known to possess biological activities (de Lima Procópio *et al*., 2012; Wu *et al*., 2015; Xing *et al*., 2018).

Streptomyces sp. GMR22 is one of the soil-dwelling bacteria isolated from Cajuput rhizospheric soil at Wanagama I Forest, Yogyakarta, Indonesia (Nurjasmi *et al*., 2009). This microbe is known to produce LMW compounds which possessed biological activity (Sukmawati *et al*., 2018), and predicted to encode biosynthetic pathways of terpenes (Herdini *et al*., 2017). The LMW compounds could be extracted and isolated using an organic solvent mixture. One of the LMW compounds was known as terpene (Jiang *et al*., 2016).

Previous studies revealed that monoterpenes and sesquiterpenes isolated from essential oils exhibited antiplasmodial activity *in vitro* against the W2 and K1 strain of *P. falciparum* (Boyom *et al*., 2011; Durant *et al*., 2014; Mota *et al*., 2012). Moreover, triterpenes also exhibited *in vitro* antiplasmodial activity (Isaka *et al*., 2010). The antiplasmodial compounds isolated from bacteria, particularly *Streptomyces* have not been widely reported. Therefore, *Streptomyces* sp. GMR22 is considered to produce LMW compounds, including terpenes as antiplasmodial.

MATERIAL AND METHODS

Streptomyces sp. GMR22 isolate

Streptomyces sp. GMR22 was isolated from Cajuput rhizospheric soil at Wanagama I Forest, Yogyakarta, Indonesia, as obtained from the previous study (Nurjasmi *et al*., 2009). GMR22 isolate has been deposited at Indonesian Culture Collection (InaCC A148), Research Center for Biology, Indonesian Institute of Sciences, and NITE Biological Research Center (NBRC), Japan (NBRC 110112). GMR22 16S RNA sequence has been submitted in the National Center for Biotechnology Information (NCBI), accession code MN922646.

Fermentation, extraction, and isolation of LMW compounds

GMR22 isolate was inoculated on International Streptomyces Project-2 (ISP-2) medium (Alimuddin *et al*., 2010). The spore culture was inoculated into 50mL tryptic soy broth (TSB) and incubated at 28 °C for 3 days on a rotary shaker (150 rpm) as seed culture. Then, it was inoculated into 450 mL starch nitrate broth (SNB) for metabolites production (soluble starch 20.0 g, NaCl 0.5 g, KNO_3 1.0 g, $K_2HPO_4.3H_2O$ 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, per liter) and incubated at 28 °C for 11 days on a rotary shaker (150 rpm). The supernatant was collected by centrifugation at 5000 rpm for 10 minutes then filtered by Whatman No.1 filtrate paper. The extraction method was carried out using an organic solvent of *n*-hexane:EtOAc (85:15v/v) mixture, according to Jiang *et al*. (2016) obtained a solid brown extract.

Identification of LMW compounds and drug-likeness screening

n-Hexane:EtOAc extract was dissolved in 200 µL *n*-hexane then analyzed using GC-MS (Jiang *et al*., 2016). In this study, GC-MS (Shimadzu QP-2010S) used RTX-5 capillary column (30m x 0.25mm x 0.25µm), positive ionization 70eV mode, carrier gas = Helium at 0.5mL min^{-1} flow rate, column pressure = 13.7 kPa, linear velocity = 25.9 cm sec^{-1}, total flow = 28.0mL min^{-1}, injector temperature = 300°C splitless mode. Oven temperature started at 70 °C (for 8 minutes), then raised 5 °C min^{-1} to 300 °C (for 26 minutes). Mass spectra were used in scan mode ranging from 28-600m/z. The resulted mass spectra were analyzed by comparing with commercial mass spectra (NIST12, NIST62, WILEY229). The identified compounds were investigated for drug-likeness using the SwissADME web tool, which was accessed through https://www.swissadme.ch (Daina *et al*., 2017).

Plasmodium culture and *in vitro* antiplasmodial assay

The 3D7 strain of *P. falciparum* was obtained from the Eijkman Institute, Jakarta, Indonesia. *In vitro* antiplasmodial assay was carried out using donor blood type O^+. The *Plasmodium* was cultured *in vitro* continuously, according to Trager and Jenson (1978), incubated in an incubator at 37°C with 5% CO_2. The trophozoite-phase of *Plasmodium* was synchronized by the addition of 5% D-sorbitol every 48h, as reported by Lambros and Vanderberg (1979) and Mustofa *et al*. (2007).

One milligram *n*-hexane:EtOAc extract was dissolved in 10µL DMSO stock solution (100,000µg/mL). The extract was diluted using RPMI medium to obtain final tested concentration of 0.78, 6.25, 25, 50, 100, 200µg/mL. One hundred microlitre RPMI medium (negative control) and tested concentration of extract, respectively, were plated in 96-well microplate and followed by the addition of 100µL *Plasmodium* culture

(trophozoite-phase at 0.5-1% parasitemia, 1% hematocrit) resulting from the synchronization. The antiplasmodial assay was carried out with triplicate, incubated for 72h in an incubator at 37°C with 5% CO_2. Parasitemia was observed by making thin blood films stained by 10% Giemsa stain. Microscopic observation of the parasitemia was done using light microscopy (Nikon) at 1000x magnification in 1000 observed erythrocytes. The percentage of *Plasmodium* growth inhibition was obtained by counting the parasitemia on the control group multiplied by 100% (Jenett-Siems *et al*., 1999).

Confirmatory antiplasmodial test by flow cytometry

Confirmatory antiplasmodial test of *n*-hexane:EtOAc extract was analyzed using flow cytometry. *Plasmodium* DNA was stained using SYBR Green I (Invitrogen, USA) (Dery *et al*., 2015), and CD235a (anti-human, eBioscience, USA) as a marker for erythrocyte. The confirmatory test was carried out independently in the same culture manner as the previous antiplasmodial assay. The final concentration of the tested extract was 50µg/mL, and DMSO (0.1% v/v) as the extracting solvent was used as a control group. The test was performed in duplicate with *Plasmodium* uninfected-erythrocyte was used as a comparison.

Two hundred microlitre of each treatment was centrifuged at 7000rpm for 10min. Then, each of 50µL erythrocyte pellet was transferred into a microtube. Afterward, 2µL of CD235a and 2µL of SYBR Green I (1000x final concentration) were added consecutively then it was incubated in the dark at room temperature for 30min. The pellet was washed once by adding 1mL of PBS and centrifuged at 2000rpm for 5min. Pellet was then collected and dissolved in 400µL flow cytometry buffer for subsequent analysis using a flow cytometer (BD FACSCanto™ II).

Ethical clearance

In vitro antiplasmodial assay used donor blood from an adult male with blood type O^+, and approved by the Medical and Health Research Ethics Committee (MHREC) of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (KE/FK/0869/EC/2019). The ethics committee was recognized by The Forum for Ethical Review Committees in Asia and the Western Pacific (FERCAP).

Statistical analysis

The result of *in vitro* antiplasmodial assay was presented as mean ± standard error of the mean (SEM) of parasitemia percentage and growth inhibition percentage. One-way ANOVA followed by the LSD post hoc statistical test was done using SPSS 24 software (IBM Corp., USA). The antiplasmodial activity was defined by 50% inhibitory concentration (IC_{50}) obtained through probit analysis (95% confidence interval) using SPSS 24 software (IBM Corp., USA).

RESULTS AND DISCUSSION

Identification of LMW compounds and drug-likeness screening

The detected compounds profile from *n*-hexane:EtOAc extract after analysed using GC-MS is presented on the chromatogram (Figure 1). Twenty one compounds were identified as alcohol, hydrocarbon, ester, aromatic/diester, diester, fatty acid, and triester classes, which have molecular weights of less than 500g/mol (Figure 1). Meanwhile, 4 silicates have molecular weights of more than 500g/mol (Table I). The result of drug-likeness screening using SwissADME based on the Ro5 showed that 3 compounds were drug-likeness, 16 compounds were non-drug-likeness, and 2 compounds could not be predicted. The classified drug-likeness compounds are phenylethyl alcohol, ethyl citrate, and di-n-butyl phthalate (DBP).

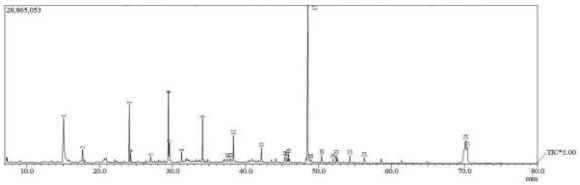

Figure 1. GC-MS chromatogram of *n*-hexane:EtOAc extract

The main component in the extract was di-n-octyl phthalate (DOP), an aromatic/diester class that had the highest area proportion (30.77%). Other main components were phenylethyl alcohol (13.06%) and 2-butyl-1-hexadecanol (11.41%) from alcohol class, followed by 1-tetradecene (7.87%), tridecane (6.05%), and (E)-3-octadecene (5.21%) from hydrocarbon class. Meanwhile, other compounds were below 5%. Additionally, this study found that the extract contained silicate anion tetramer, which is commonly nanoparticle composite.

Table I. Identification of the LMW compounds from *n*-hexane:EtOAc extract and drug-likeness screening for potential compound candidates.

Peak	RT (min)[a]	Compound	MW (g/mol)[b]	Area (%)	Class[c]	Drug-likeness (Ro5)[d]					
						Lipophilicity	Size	TPSA	Solubility	Saturation	Flexibility
1	15.076	Phenylethyl alcohol	122	13.06	Alcohol	v	v	v	v	v	v
2	17.668	1-Dodecene	168	1.99	Hydrocarbon	x	v	v	v	v	v
3	24.084	1-Tridecene	182	6.05	Hydrocarbon	x	v	v	v	v	v
4	24.274	Tridecane	184	0.98	Hydrocarbon	x	v	v	v	v	v
5	27.002	4-Methyl-undecane	170	0.79	Hydrocarbon	x	v	v	v	v	v
6	29.438	1-Tetradecene	196	7.87	Hydrocarbon	x	v	v	v	v	v
7	29.578	4,8-Dimethyl-tridecane	212	1.94	Hydrocarbon	x	v	v	v	v	v
8	31.247	Ethyl citrate	276	1.32	Ester	v	v	v	v	v	v
9	34.123	(E)-3-Octadecene	252	5.21	Hydrocarbon	x	v	v	v	v	v
10	37.560	(Z)-3-Methyl-2-decene	154	0.78	Hydrocarbon	x	v	v	v	v	v
11	37.932	Di-n-butyl phthalate	278	0.94	Aromatic/ diester	v	v	v	v	v	v
12	38.344	1-Nonadecene	266	3.26	Hydrocarbon	x	v	v	v	v	v
13	42.185	(E)-3-Eicosene	280	1.81	Hydrocarbon	x	v	v	v	v	v
14	45.395	Oleamide	281	1.02	Fatty acid	x	v	v	v	v	v
15	45.702	1-Dococene	308	0.67	Hydrocarbon	x	v	v	v	v	v
16	45.875	Dioctyl-adipate	370	0.84	Diester	x	v	v	v	v	v
17	48.555	Di-n-octyl-phthalate	390	30.77	Aromatic/ diester	x	v	v	v	v	v
18	48.961	1-Heptacosanol	396	0.63	Alcohol	x	v	v	v	v	v
19	50.445	Silicate anion tetramer	888	1.19	Silicate	o	o	o	o	o	o
20	52.005	2-Methyl-1-hexadecanol	256	0.71	Alcohol	x	v	v	v	v	v
21	52.441	Silicate anion tetramer	888	0.81	Silicate	o	o	o	o	o	o
22	54.307	Silicate anion tetramer	888	0.73	Silicate	o	o	o	o	o	o
23	56.291	Silicate anion tetramer	888	0.91	Silicate	o	o	o	o	o	o
24	70.166	2-Butyl-1-hexadecanol	298	11.41	Alcohol	o	o	o	o	o	o
25	70.372	Glyceryl pentanoate	344	4.32	Triester	o	o	o	o	o	o

a: Retention time (minute); b: molecular weight; c: source: https://pubchem.ncbi.nlm.nih.gov; d: SwissADME prediction analysis based on Ro5 (Lipinski *et al.*, 2001); v: eligible to Ro5; x: not eligible to Ro5; o: could not be predicted

It hypothesized that *Streptomyces* sp. GMR22 might secrete enzymes and could lead to the synthesis of silicate such as *Actinobacter* sp., which has been reported by Singh *et al*. (2008).

Extraction using an organic solvent mixture aimed to obtain the LMW compounds, including the class of terpenes. However, the terpenes were not identified. The GC-MS used in this study was RTX-5 capillary column, which was different from Jiang *et al*. (2016). Yamada *et al*. (2015) reported that 2-methylisoborneol was only the trace that could be detected in liquid culture after analyzed using GC-MS. Therefore, the terpenes level in the extract might also be trace.

This study found that the classified drug-likeness compounds were phenylethyl alcohol, ethyl citrate, and DBP from alcohol, ester, and aromatic/diester classes, respectively. This elucidates that the compounds have physicochemical properties, which are associated with acceptable aqueous solubility and intestinal permeability. Therefore, the compounds are predicted to be suitable for oral bioavailability candidates (Daina *et al*., 2017; Lipinski, 2004).

The previous studies revealed that phenylethyl alcohol possessed antifungal activity (Xing *et al*., 2018), whereas DBP was reported to possess antimicrobial activity (Roy *et al*., 2006). Meanwhile, ethyl citrate was the first report in this study. This finding showed that the classified drug-likeness compounds were considered to have other biological activities.

One of the classified non-drug-likeness compounds was DOP, which possessed biological activities. Fitriastuti *et al*. (2020) reported that ethanol extract fraction of temu mangga (*Curcuma mangga* Val.) contained DOP, which also identified using GC-MS. They reported that DOP possessed potency as an antimalaria compound. Moreover, DOP was reported to possess antimicrobial activity (Zothanpuia *et al*., 2018).

***In vitro* antiplasmodial assay**

The result of antiplasmodial assay of *n*-hexane:EtOAc extract (Figure 2). It showed that the parasitemia percentage was inversely correlated to the growth inhibition percentage.

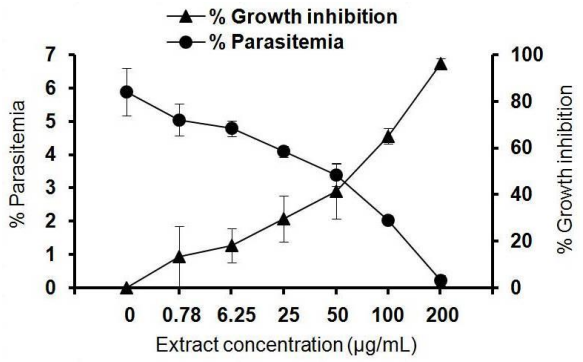

Figure 2. The correlation between parasitemia percentage and growth inhibition percentage of *P. falciparum* culture after treatment of extract concentration at 0, 0.78, 6.25, 25, 50, 100, 200µg/mL.

One-way ANOVA statistical test showed that the extract reduced the parasitemia percentage significantly as compared to the control group ($p < 0.00$). Likewise, the LSD post hoc test showed there was a significant difference between each extract treatment compared to the control group ($p<0.05$). The extract activity as antiplasmodial obtained an IC_{50} value of 38.61±19.06µg/mL. This shows that the extract exhibited moderate antiplasmodial activity with IC_{50} below 50µg/mL as reported by Kaharudin *et al*. (2020).

The compound's content of the extract is believed to be active antiplasmodial compounds. DOP, one of the main compounds, was reported being potential as antimalaria (Fitriastuti *et al*., 2020). They reported that the antimalaria test was carried out by a heme polymerization inhibition method, and the result obtained an IC_{50} value of 1.479µg/mL. Although phthalates are commonly encountered as plasticizers or pollutants from industrial wastes (Xu *et al*., 2020), other studies revealed that phthalate derivatives could be biologically produced by either *Streptomyces* (Mangamuri *et al*., 2016; Roy *et al*., 2006; Zothanpuia *et al*., 2018) or filamentous fungi through shikimic acid pathway (Tian *et al*., 2016).

In this study, the use of donor blood type O^+ aimed to successfully facilitate the *in vitro* cultivation of *Plasmodium* parasites. Theron *et al*. (2018) reported that *P. falciparum* preferred to invade donor blood type O^+ rather type A^+, B^+, and AB^+ for an *in vitro* culture. Thereby, the donor blood type O^+ became an advantage for the *in vitro* cultivation of *P. falciparum*.

Confirmatory antiplasmodial test by flow cytometry

The confirmatory antiplasmodial test results and fluorescence intensity of *Plasmodium* DNA and detected erythrocyte are also presented (Figure 3).

Area of SYBR Green I and CD235a, which stained the *Plasmodium* DNA and erythrocyte, respectively (Figure 3). Q1 indicates the area of SYBR Green I negative and CD235a positive. Q2 indicates the area of *P. falciparum* infected-erythrocyte and SYBR Green I positive. Q3 indicates the area of SYBR Green I negative, but CD235a positive for erythrocyte debris. Q4 indicates the area of *P. falciparum* uninfected-erythrocyte and CD235a positive. The fluorescence intensity of SYBR Green I represents the DNA of *Plasmodium* infected-erythrocyte, and the fluorescence intensity of CD235a represents the detected erythrocyte. The % total erythrocyte represents the detected erythrocyte count. The % erythrocyte (uninfected) represents the *P. falciparum* uninfected-erythrocyte count, whereas the % parasitemia (infected) represents *P. falciparum* infected-erythrocyte count. The % inhibition is the *P. falciparum* growth inhibition percentage after the treatment compared to the control.

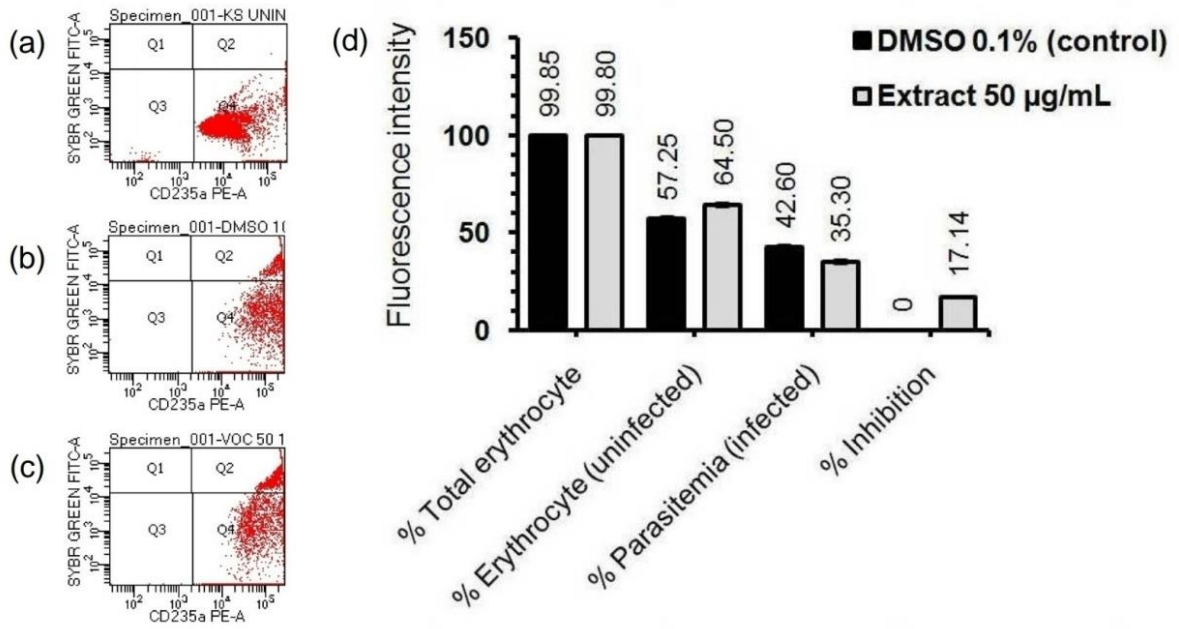

Figure 3. The flow cytometry analysis results of *Plasmodium* DNA and erythrocyte on the group of (a) *P. falciparum* uninfected-erythrocyte and *P. falciparum* culture after treatment of (b) DMSO and (c) extract 50 µg/mL, respectively, which showed a distinction percentage among groups based on (d) the fluorescence intensity of the detected erythrocyte (both total and uninfected), the percentage of parasitemia (the DNA of *P. falciparum* infected-erythrocyte), and the percentage of inhibition of *P. falciparum* growth

The detected erythrocyte intensity in both groups was equal. Thus, the total erythrocyte count was proportional. The control group showed uninfected-erythrocyte intensity lower than the treatment. This is in line with Kotepui *et al*. (2015). They reported that *P. falciparum* infection with high parasitemia showed a decreased total erythrocyte count as compared to *P. falciparum* infection with low parasitemia. The parasitemia percentage was found inversely correlated to the inhibition percentage on the flow cytometry. This is in line with the result of previous microscopic analysis. Hence, it shows that the extract exhibited potency as antiplasmodial.

CONCLUSION

Further research was needed to reinvestigate and to identify other antiplasmodial active compounds besides DOP resulted/generated from *Streptomyces* sp. GMR22.

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