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Antiplasmodial activity and genome mining study of marine-derived *Streptomyces* sp. GMY01

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ABSTRACT

High resistance to chloroquine in most malaria-endemic area in the world leads to the need for new antimalaria drugs. Marine bacterium *Streptomyces* is the source for potential new antimalarial molecules. This research aimed to investigate antiplasmodial activity of marine-derived of *Streptomyces* sp. GMY01 and to identify potential active compounds using genome mining study. *In vitro* antiplasmodial activity assays using flow cytometry method showed that the ethyl acetate extract of this bacterium had high antiplasmodial activity (IC_{50} value of 1.183 µg/mL) on *Plasmodium falciparum* FCR3. Genome mining analysis of whole-genome sequences using antiSMASH 6.0 beta version revealed that *Streptomyces* sp. GMY01 had 28 biosynthetic gene clusters (BGCs), including the genes encoding polyketide synthase, non-ribosomal peptide synthetase, terpene, lanthipeptide, bacteriocin, butyrolactone, ectoin, siderophore, and others. The known BGCs were predicted to be involved in the production of known compounds from gene clusters ranged from 5 to 100% similarity. Ongoing purification and elucidation of the structures will allow identification of the active compounds produced by marine-derived *Streptomyces* sp. GMY01.

ABSTRAK

Tingginya tingkat resistensi terhadap klorokuin di hampir semua daerah endemik malaria di dunia mendorong perlunya obat antimalaria baru. Bakteri laut *Streptomyces* merupakan sumber molekul antimalaria baru yang potensial. Penelitian ini bertujuan untuk mengkaji aktivitas antiplasmodial bakteri asal laut *Streptomyces* sp. GMY01 dan mengidentifikasi senyawa aktif potensial menggunakan studi *genome mining*. Uji antiplasmodial *in vitro* dengan metode *flow cytometry* menunjukkan ekstrak etil asetat bakteri ini memiliki aktivitas antiplasmodial tinggi (IC_{50} = 1,183 µg/mL) pada *Plasmodium falciparum* FCR3. Analisis *genome mining* sekuen genom menyeluruh menggunakan antiSMASH 6.0 versi beta menunjukkan bahwa *Streptomyces* sp. GMY01 memiliki 28 gen kluster biosintesis yang mengkode poliketida sintase, non-ribosomal peptide sintetase, terpene, lanthipeptide, bacteriocin, butirolactone, ectoin, siderophore, dan lainnya. Gen kluster biosintesis yang telah diketahui diprediksi berperan dalam produksi senyawa-senyawa yang telah diketahui dengan similaritas 5 – 100%. Purifikasi senyawa dan elusidasi struktur sedang dilakukan akan mengidentifikasi senyawa aktif yang dihasilkan bakteri asal laut *Streptomyces* sp. GMY01.

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INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium* that is transmitted through the bite of a female *Anopheles* mosquito.[1] Malaria remains a major health problem worldwide due to resistance to currently available antimalarial drugs especially chloroquine. The drugs resistance caused malarial treatment failure in almost all malaria endemic areas.[2] Therefore, the World Health Organization has recommended artemisinin-based combination therapies (ACT) as first-line drugs to replace sulfadoxine–pyrimethamine and chloroquine in the treatment of malaria. However, recent studies have reported cases of artemisinin resistance to *P. falciparum* in Southeast Asia.[3,4] The emergence and spread of drug-resistant *Plasmodium* species possibly trigger the re-emergence of malaria outbreaks, thereby encouraging efforts to discover and develop new drugs.[5,6]

Previous studies revealed new antiplasmodial compounds isolated from *Streptomyces* bacteria.[7,8] *Streptomyces* bacteria have been the main source of commercial drug and antibiotic candidates for decades.[9,10] *Streptomyces* has provided bioactive molecules for more than 10,000 of the 18,000 known bacterial bioactive compounds. *Streptomyces* have a linear chromosome with an extremely large genome size (between 6.2 and 12.7 Mb); approximately 5% of the genome is responsible for the synthesis of secondary metabolites.[11]

The potential biotechnology of *Streptomyces* species can be revealed through genome mining techniques, where biosynthetic gene clusters (BGCs) can be identified and can be used to predict the chemical core structures of molecules.[10] *Streptomyces* contain the largest number of BGCs, such as the gene encoding polyketide synthetase (PKS), non-ribosomal peptide synthetases (NRPSs), or PKS–NRPS hybrids.[11] Over the last 10 years, several new active compounds from *Streptomyces* have been revealed through genome mining approaches[12,13]

In the previously study, *Streptomyces* sp. GMY01 from the marine sediment in Krakal Beach, Yogyakarta, Indonesia was successfully isolated and identified.[14] The biological assay of GMY01 extract exhibited anticancer activity against the breast cancer cell lines MCF7 and T47D.[15,16] However, the active compounds detected from GMY01 have not been identified and their antiplasmodial activity have not been investigated. This study aimed to investigate antiplasmodial activity of *Streptomyces* sp. GMY01 extract and to identify of potential active compound using genome mining study.

MATERIALS AND METHODS

Biological material

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. Human *Plasmodium falciparum* FCR3 was obtained from the Eijkman Institute for Molecular Biology, Jakarta, Indonesia.

Fermentation and extraction

Streptomyces sp. GMY01 was maintained in ISP-2 agar medium (Difco, Sparks, USA). Then, *Streptomyces* sp. GMY01 was cultured at 28 °C with 180 rpm agitation for 3 days in a 250 mL Erlenmeyer flask containing 100 mL tryptic soy broth (Difco, Sparks, USA) as the seed medium. Then, the cells were transferred into four 1000 mL flasks containing 500 mL starch nitrate broth (SNB) as the production medium and incubated for 11 days at 28 °C with 180 rpm agitation in a shaking incubator. The SNB medium contained 0.5 g NaCl, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 20 g starch in 1000 mL distilled water. Secondary metabolites were obtained by separating the cell biomass from the liquid using refrigerated centrifugation at 4137 × g at 4 °C for 15 min. The supernatant was extracted two times with an equal volume of ethyl acetate and evaporated to obtain

the crude extract. The all chemical reagents and solvents were purchased from Merck KGaA, Darmstadt, Germany.

Antiplasmodial assay using flow cytometry

Plasmodium falciparum FCR-3 was cultured in accordance with the previous method with minor modifications.[17] The cultures were maintained in 2% fresh human erythrocytes (red blood cells, RBCs) (O+, male) suspended in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Thermo Fisher Scientific, USA) containing 10% human serum (O^+, male) and 500 µg gentamicin (Indofarma, Bekasi, Indonesia) per liter. The flasks were incubated at 37 °C under a gas mixture of 5% O_2 and 5% CO_2. Every 3–4 days, the infected erythrocytes were transferred to a fresh complete medium with uninfected erythrocytes. The stock culture was synchronized with 5% sorbitol as described by Lambros and Vanderberg.[18] For the antiplasmodial assay, the extract was prepared by adding 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) (w/v) at various concentrations (0.25 – 8 µg/mL). The *Plasmodium* growth inhibition assay was performed in a total volume of 200 µL using 96-well microplates (Iwaki), with each plate containing a 100 µL extract solution and 100 µL *Plasmodium* inoculum at the parasitemia level of 5% in a 5% CO_2 incubator (CellXpert C170i, Eppendorf AG, Hamburg, Germany) at 37 °C for 3 × 24 h of incubation. *Plasmodium* growth was observed by making thin blood film preparations with Giemsa stain (Merck, Germany) for microscopic observation (Nikon YS100 biological microscope, Japan). Flow cytometry analysis was carried out by SYBR Green I stain in accordance with the work of Rebelo with minor modifications.[19] The extract solution and chloroquine as a control at various concentrations were used as the treatments. For each measurement, 200 µL *Plasmodium* culture (approximately 1.8×10^{11} RBCs) was centrifuged to separate the cells from the medium. Ten microliters of cells were stained with 1 µL DNA-specific dye SYBR Green I (1× the concentration) (Invitrogen, Carlsbad, USA) and 10 µL CD235a antibody (eBioscience, San Diego, USA). After 10 min of incubation in the dark, the stained sample was immediately analyzed by flow cytometry using a 535/45 nm bandpass filter in front of the detector (BD FACS Calibur flow cytometer, USA). The results of flow cytometric analysis were analyzed using CellQuest software. The RBCs in uninfected and infected samples were detected by their characteristic forward and side scatter properties. Staining with the anti-glycophorin CD235 antibody was used to establish that all events detected represented RBCs. The percent Plasmodium inhibition was obtained by formula:

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

A: SYBR Green I fluorescens intensity in control (RPMI medium)
B: SYBR Green I fluorescens intensity in treatment

The half maximal inhibitory concentration (IC_{50}) values of the extracts were obtained using regression analysis of $\log_{10}$ concentrations of the extract against percent *Plasmodium* inhibition. This analysis was performed using Graph Pad Prism 9.

The protocol of the study was approved by the Medical and Health Research Ethic Committee, the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta (ref. no. KE/FK/0279/EC/2019).

Genome mining analysis

The whole-genome sequence of *Streptomyces* sp. GMY01 was determined by next-generation sequencing platforms using 454 pyrosequencing technology (454 GS FLX) and HiSeq1000 (Illumina) as published in a previous study.15 In this study, the identification gene clusters involved in the biosynthesis of secondary metabolites and predictions of the core structures produced were conducted by using antiSMASH 6.0 beta version,20 which is available at http://antismash.secondarymetabolites.org.

Accession number(s): This whole-genome shotgun project has been deposited at Data Bank of Japan/European Nucleotide Archive/GenBank under the accession number JABBNA000000000. The version described in this paper is version JABBNA010000000.

RESULTS

From the supernatant of 10 L of 11-days old cultured in liquid SNB medium, 792 mg crude ethyl acetate extract was obtained from the supernatant. The antiplasmodial assay using flow cytometry analysis showed that the crude extract of GMY01 inhibited *P. falciparum* FCR3 growth. The IC_{50} value of the crude ethyl acetate from the supernatant was 1.183 µg/mL whereas IC_{50} value of chloroquine was 1.820 µg/mL (FIGURE 1). The low IC_{50} of the GMY01 extract indicates its potential as an antiplasmodial.

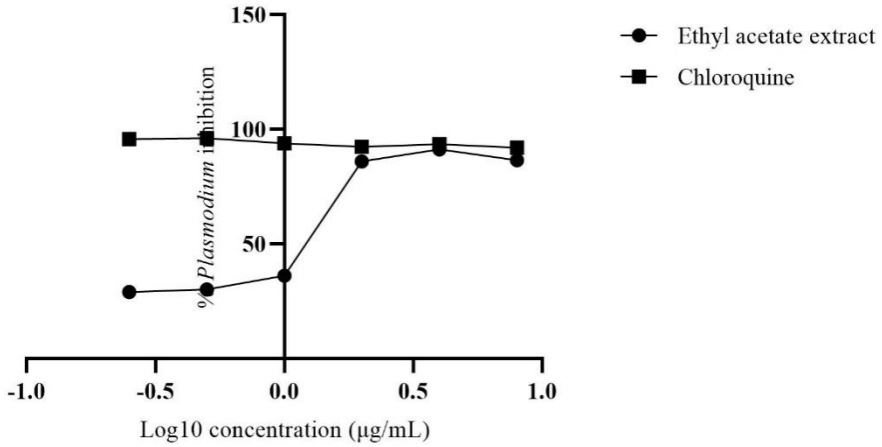

FIGURE 1. *Plasmodium falciparum* FCR3 inhibition by ethyl acetate extract of *Streptomyces* sp. GMY01 and chloroquine at various concentrations

Fluorescens intensity of SYBR Green I show the number of *Plasmodium* parasite that infected RBC. The intensity of the SYBR Green I fluorescence was calculated based on the gated percent value of the total events in the upper right region. the upper right was the positive region of the SYBR green I antibody (infected RBC) and was the positive region of the CD235a antibody (total RBC). The results of flow cytometry analysis in FIGURE 2 shows that extract treatment (2 µg/mL) (FIGURE 2.a) has low fluorescens intensity of SYBR Green I compared with the controls (infected RBC without drug) (FIGURE 2.c), and it was similar with chloroquine treatment (2 µg/mL) as a commercial antimalarial (FIGURE 2.b). FIGURE 4.d shows very low fluorescens intensity of SYBR Green I in uninfected RBC (uninfected RBC). This indicated that SYBR Green I was selective stain for genetic material (DNA) of *Plasmodium*.

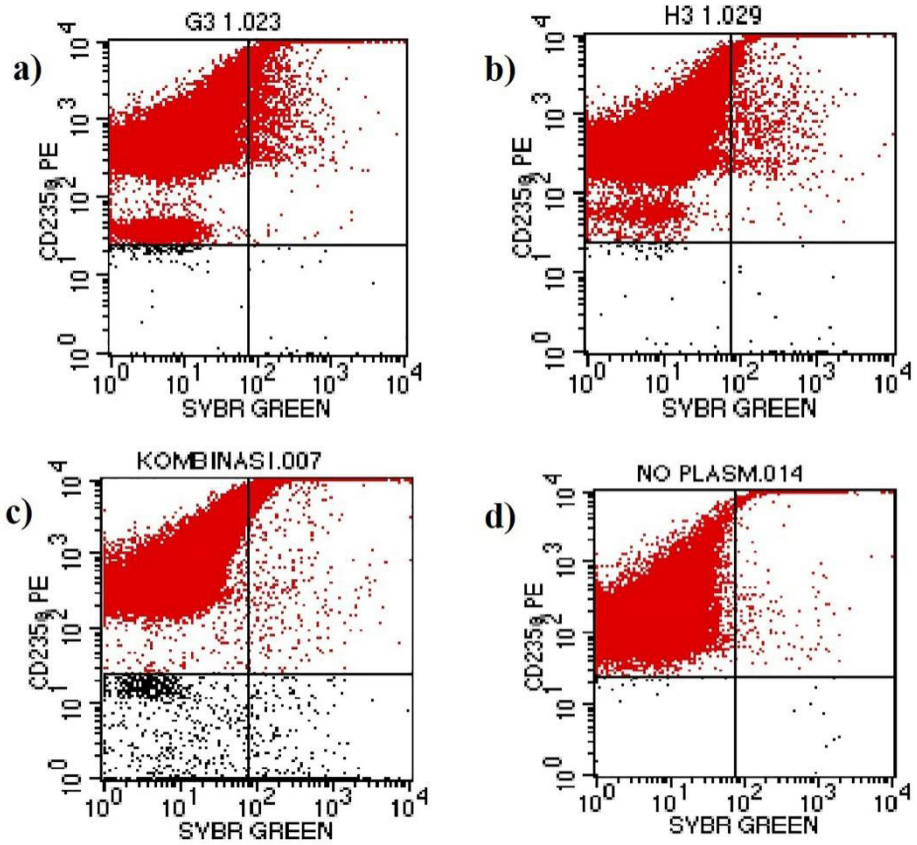

FIGURE 2. Flow cytometry analysis using SYBR Green I stain for *Plasmodium* DNA intensity and CD235a for red blood cells (RBC) intensity. *Plasmodium falciparum* FCR-3 growth on 2 μg/mL ethyl acetate extract of *Streptomyces* sp. GMY01 (a), 2 μg/mL chloroquine (b), the positive control (infected RBC without drug) (c), the negative control (uninfected RBC) (d).

The genome mining analysis of the whole-genome sequence of GMY01 using antiSMASH 6.0 revealed the 28 regions consisting of 23 known regions and 5 unknown regions, which encoding NRPS modules, PKS, terpene, ectoin, lanthipeptide, siderophore, butyrolactone, and others (TABLE 1). Genes encoding NRPS and PKS types were dominant in the BGC analysis. The BGCs were predicted to be involved in the production of putative secondary metabolite such as geosmin, ectoine and albaflavenone with 100% deduced amino acid sequence similarities; microansamycin, deimino-antipain, venezuelin, mirubactin, scabichelin, hopene, abyssomicins M-X and spore pigments with ≥ 50% similarity; and lysocin, herboxidiene, stenothricin, saframycin A/B, streptobactin, S56-p1, grincamycin, scleric acid, isorenieratene, saframycin A, vazabitide A and informatipeptine with ≤ 50% similarity.

TABLE 1. Genome mining analysis of the *Streptomyces* sp. GMY01 genome using antiSMASH 6 beta version

Region	Biosynthesis Gene Cluster (BGC) Type	Most similar known cluster	Similarity (%)	Length (bp)	Origin Organism
4.1	NRPS	No matches found	-	-	-
13.1	NRPS, Lanthipeptide class ii	Lysocin	9	71,777	*Lysobacter* sp. RH2180-5
21.1	Type 3 PKS (T3PKS)	Herboxidiene	6	159,472	*Streptomyces chromofuscus*
21.2	NRPS, NAPAA	Stenothricin	13	54,895	*S. roseosporus*
23.1	NRPS	Saframycin A/B	12	62,804	*S. lavendulae*
24.1	Terpene	Geosmin	100	2,181	*S. coelicolor*
24.2	RiPP like, NRPS	Streptobactin	47	26,478	*Streptomyces* sp. ATCC 700974
24.3	Type 1 PKS (T1PKS), NRPS-like, NRPS, Butyrolactone, REE-containing	Microansamycin	67	69,750	*Micromonospora* sp. HK160111
24.4	Siderophore	No matches found	-	-	-
25.1	Ectoin	Ectoine	100	3,366	*S. anulatus*
26.1	NRPS	Deimino–antipain	66	15,484	*S. albulus*
27.1	Lanthipeptide class iv	Venezuelin	50	5,339	*S. venezuelae.*
27.2	RiPP-like	No matches found	-	-	-
27.3	NRPS, other	S56-p1	17	67,922	*Streptomyces* sp. SoC090715LN-17
28.1	NRPS	Mirubactin	50	27,717	*Actinosynnema mirum*
29.1	T1PKS, NRPS	Scabichelin	90	30,020	*S. scabies*
30.1	Lanthipeptide class iv	No matches found	-	-	-
36.1	Terpene	Albaflavenone	100	2,468	*S. coelicolor* A3(2)
38.1	Siderophore	Grincamycin	5	41,909	*S. lusitanus*
42.1	Butyrolactone	Scleric acid	23	18,478	*S. sclerotialus*
45.1	Terpene	Isorenieratene	15	9,777	*S. argillaceus*
48.1	NRPS	Saframycin A	12	62,804	*S. lavendulae*
49.1	Terpene	Hopene	53	13,757	*S. coelicolor* A3(2)
53.1	T1PKS, hgIE-KS	Vazabitide A	17	40,455	*S.* sp. SANK 60404
53.2	Bacteriocin, Lanthi-peptide	Informatipeptin	8	14,866	*S. viridochromogenes* DSM 40736
55.1	Butyrolactone	No matches found	-	-	
60.1	T1PKS	Abyssomicins M-X	56	70,308	*Streptomyces* sp. LC-6-2
63.1	Type 2 PKS (T2PKS)	Spore pigment	83	11118	*S. avermitilis*

NRPS: Non-ribosomal peptide synthetase; PKS: Polyketide synthase; NAPAA: non-alpha poly-amino acids; RiPP: ribosomally synthesised and post-translationally modified peptide

DISCUSSION

Streptomyces sp. GMY01 displayed strong activity against *P. falciparum* FCR-3 using flow cytometry analysis. SYBR Green I could be applied for antiplasmodial assays because the dye specifically colors *Plasmodium* DNA.[19]

High fluorescens intensity of SYBR Green I indicate high levels of parasitemia. Conversely, low SYBR Green I fluorescens intensity indicate *Plasmodium* inhibition.

The IC_{50} values of the antiplasmodials in this study were almost the same as those in other research, such as the active compounds of *Streptomyces* BCC26924 with IC_{50} values against *P. falciparum* K1 between 0.24 and >10 µg/mL.[8] Isaka[7] revealed that the metacycloprodigiosin compounds, namely, bafilomycin A and spectinabilin from *Streptomyces spectabilis* BCC 4785, obtained 0.005–7.8 µg/mL IC_{50} against *P. falciparum* K1.

The antiplasmodial activity produced by *Streptomyces* GMY01 was suggested to be related to its anticancer activity. The results of this study were similar to those of a previous research. The ethyl acetate extract of GMY01 had anticancer activity against human breast cancer cells, with IC_{50} values of 19 µg/mL in T47D cells and 7 µg/mL in MCF7 cells.[14] Other studies reported that the anticancer compound SB939 inhibit *P. falciparum* at all cycles in either the asexual or exoerythrocytic phase and cerebral malaria caused by *P. berghei.*[6] In addition, the antimalarial compound artemisinin and its analogs show anticancer activity and have synergistic effect with available anticancer drugs without increasing the toxicity to normal cells.[21,22] This result was consistent with the hypothesis that anticancer compounds could be antiplasmodial.

Whole-genome sequence analysis showed that *Streptomyces* sp. GMY01 is closely related to *Streptomyces albus* J1074.[15] Genome mining analysis using antiSMASH 6.0 showed that *Streptomyces* sp. GMY01 has a large group of NRPS- and PKS-encoded secondary metabolites. These results indicate that the marine origin of *Streptomyces* has high potential to be developed as a large source of active compound. However, compared with others marine *Streptomyces*, which has almost the same genome size, the BGCs in GMY01 are less than those in *Streptomyces* MP131-18.[23]

Biological activities of putative secondary metabolite based on genome mining study showed in TABLE 2. Most of the compounds predicted to be produced by *Streptomyces* sp. GMY01 has biological activities as antimicrobial, antibiotic, and antitumor - anticancer.

TABLE 2. Biological activities of putative secondary metabolites resulted by *Streptomyces* sp. GMY01 based on genome mining study

Most similar known cluster	Biological activity
Lysocin	Antibiotic [24]
Herboxidiene	Anti-cholesterol [25]
Stenothricin	Antibiotic [26]
Saframycin A/B	Antibiotic antitumor [27]
Geosmin	Earthy smelling substance [28]
Streptobactin	A new catechol-type siderophore[29]
Microansamycin	Antiproliferative [30]
Ectoine	Natural cell protectant [31]
Deimino–antipain	Peptidic protease inhibitor [32]
Venezuelin	unknown
S56-p1	unknown
Mirubactin	Siderophore [33]
Scabichelin	Siderophore [34]

Albaflavenone	Antibiotic [35]
Grincamycin	Antitumor – anticancer [36]
Scleric acid	Anti-Mycobacterium [37]
Isorenieratene	Antioxidant [38]
Saframycin A	Antitumor [39,40]
Hopene	unknown
Vazabitide A	Immunosuppressive effect [41]
Informatipeptin	unknown
Abyssomicins M-X	Antimicrobial, antitumor, anti-Mycobacteria, anti-HIV[42]
Spore pigment	unknown

In previous studies, it was known that antibiotic and anticancer compounds had the ability as antiplasmodial. Nocathiacins are a thiazolyl peptide group of antibiotics which have the ability to inhibit *P. falciparum in vitro*.[43] Tetracyclines, macrolides, quinolones, and rifampin also demonstrated *in vitro* activity against *P. falciparum* [44] An anticancer, novobiocin-ferrocene conjugates display moderate anticancer against the MDA-MB-231 breast cancer line and promising antiplasmodial against *P. falciparum* 3D7 strain.[45] In the other hand, several antiplasmodial also have activity as anticancer such as primaquine homodimers which have antiplasmodial activities on *P. berghei* and *P. falciparum* and anticancer activities on three cancer cell lines (MCF-7, HCT116, H 460).[46] Three classes of antimalarial drugs are well-established and experimental: artemisinin, synthetic peroxide and DHFR (dihydrofolate reductase) inhibitors show inhibition of proliferation in human cancer cells.[47]

This research has contributed to antiplasmodial discovery as candidates for antimalarial drugs from bacteria, especially *Streptomyces*. Bacteria that are known to produce anticancer and antimicrobial metabolites also have the potential to produce antiplasmodial. This study will accelerate the discovery of alternative antimalarial compounds.

CONCLUSION

Marine-derived bacteria *Streptomyces* sp. GMY01, previously known to have anticancer activity, also known to have high antiplasmodial activity. Genome mining analysis of the whole genome sequence shows that this bacterium produces putative compounds from secondary metabolites. These putative compounds are known to have some antimicrobial, antibiotic, anticancer and, antitumor activity. Further study would be conducted to isolate the active antiplasmodial compound from the *Streptomyces* sp. GMY01.

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