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Inhibition of secondary metabolite extract of *Streptomyces sp*. on *Plasmodium falciparum* in vitro: A Study of soil sediment of Papua's Hamadi mangrove forest

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ABSTRACT

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Background: Reports about the resistance of antimalarial drugs demand exploration and development of new drugs. Indonesia's vast maritime area and abundance of mangrove forests have the potential for the development and discovery of active anti-cancer substances, antibiotics and antiplasmodial. Studies on the potential of new drugs from this sector are still limited.

Objective: The purpose of this study was to see the potential of *Actinomycetes* genus *Streptomyces sp.* as an antiplasmodial in the Hamadi Coast of Papua.

Methods: Retrieval of mangrove soil sediments to isolate *Streptomyces* was located in the Hamadi-Jayapura mangrove forest. Selective isolation was conducted by SCA (Starch Casein Agar) selective media. Identification of *Streptomyces sp* was examined microscopically through observation of colony morphology and gram staining. The results of isolation of *Streptomyces* were then fermented on FM3 media until finally secondary metabolite extract of *Streptomyces* was obtained. The secondary metabolite extract of *Streptomyces* was tested for its inhibition on the development of *Plasmodium falciparum*.

Result: Analysis of inhibition of the secondary metabolite extract of *Streptomyces sp* on the development of *Plasmodium falciparum* showed good results because the extract of *Streptomyces sp* with a concentration of 100 µg/mL could suppress average growth values of *Plasmodium falciparum* to only 0.66% with an average inhibitory value of 95.20%. This value followed levels of extract concentration by the lowest concentration of 0.01 µg/mL, the average value of *Plasmodium falciparum* growth which increased to 10.89% with an average inhibition value of 20.83%. IC_{50} analysis of *Plasmodium falciparum* in culture for 48 hours was 0.12 µg/mL, and this value was very good because a test of substance has very good inhibitory activity when the IC_{50} value is ≤ 10 µg/mL.

Conclusion: *Streptomyces*' secondary metabolite extract from mangrove sediments showed a very good ability to inhibit the growth of *Plasmodium falciparum* so that it could have potential as a source of antiplasmodial.

Latar Belakang: *Laporan adanya resistensi obat-obatan antimalaria menuntut eksplorasi dan pengembangan obat baru. Kawasan maratim Indonesia yang luas dan kelimpahan habitat hutan mangrove berpotensi untuk pengembangan dan penemuan zat aktif anti kanker, antibiotik, antiplasmodium. Informasi dan penelitian potensi obat baru dari sektor ini masih sangat terbatas dilakukan penelitian.*

Tujuan: *Tujuan penelitian untuk melihat potensi Actinomycetes genus Streptomyces sp sebagai antiplasmodia di Pantai Hamadi Papua.*
Metode: *Pengambilan sedimen tanah mangrove untuk mengisolasi Streptomyces berlokasi di hutan mangrove Hamadi-Jayapura. Isolasi selektif dilakukan dengan media selektif SCA (Starch Casein Agar). Identifikasi Streptomyces sp dilakukan dengan cara mikroskopis melalui pengamatan morfologi koloni dan pengecatan gram. Hasil isolasi Streptomyces selanjutnya difermentasi pada media FM3 hingga pada akhirnya didapatkan ekstrak metabolit sekunder Streptomyces. Ekstrak metabolit sekunder Streptomyces diuji daya hambatnya terhadap perkembangan Plasmodiun falciparum.*
Hasil: *Analisa daya hambat ekstrak metabolit sekunder Streptomyces sp terhadap perkembangan Plasmodium falciparum menunjukan hasil yang baik karena ekstrak Streptomycessp dengan konsentrasi 100 µg/mL dapat menekan nilai rata-rata pertumbuhan Plasmodium falciparum menjadi hanya 0,66% dengan nilai rata-rata penghambatan yaitu 95,20%. Nilai ini mengikuti tingkatan konsentrasi ekstrak yang diberikan hingga pada konsentrasi terendah yaitu 0,01 µg/mL, nilai rata-rata pertumbuhan Plasmodium falciparum menjadi meningkat yaitu 10,89% dengan nilai rata-rata penghambatan yaitu 20,83%. Analisis* IC_{50} *Plasmodium falciparum pada kultur selama 48 jam yaitu 0,12µg/mL dan nilai ini termasuk sangat baik karena suatu zat uji memiliki aktivitas daya hambat sangatbaik bila nilai* $IC_{50} \leq 10$ *µg/mL.*
Kesimpulan: *Ekstrak metabolit sekunder Streptomyces dari sedimen kawasan mangrove menunjukan kemampuan daya hambat pertumbuhan Plasmodium falciparum sangat baik sehingga berpotensi sebagai sumber antiplasmodium.*

INTRODUCTION

Almost half of the world's population currently lives in malaria-endemic areas as clinical cases of malaria in 2017 was estimated in a number of 219 million people and caused 435,000 deaths. Meanwhile, the drugs available for treatments of the malaria are quickly decreased in their effectiveness due to the rapid development of *Plasmodium sp* resistance. For example, artemisinin combination therapy as a recommended malaria treatment in endemic countries shows an alarming spread of resistance in various regions in Southeast Asia. Considering options for malaria treatments, it is necessary to find new drugs for future malaria therapy.[1]

Malaria disease is caused by protozoan genus *Plasmodium* that transmits through bites of female *Anopheles sp* mosquito. Five species can cause malaria, for example, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi*. Two types of *Plasmodium* that can cause outbreaks in Asian and African countries are *Plasmodium falciparum* and *Plasmodium vivax* that are the most virulent of all species.[2]

The control and treatment of malaria can be resisted by the rapid development of *Plasmodium sp* resistance and *Plasmodium knowlesi* infections that are steadily increasing. These urge scientists to discover new anti-malaria drugs with new targets and mechanisms. One of the possible agents as a new source of malaria drug is *Actinomycetes*, bacteria that are widely distributed in terrestrial and aquatic ecosystems.[3] These bacteria provide bioactive metabolism with antibacterial, antifungal, anticancer, anti-oxidant, anti-malaria, and anti-inflammatory activities.[4] *Actinomycetes* are gram-positive bacteria, filaments, and some of the secondary metabolites.[5] *Actinomycetes* groups comprise of 97 new species, representing 9 genera and representing 27 rare *Actinomycetes* families , with the largest number of new isolates from *Pseudonocardiaceae*, *Demequinaceae*, *Micromonosporaceae*, and *Nocardioidaceae* families. Besides, there are 167 new bioactive species provided by 58 different rare Actinomycetes species representing 24 genera. Most of the compositions released by the *Actinomycetes* have antibacterial, antifungal, antiparasitic, anticancer or antimalarial activity.[6] The highest amount of natural secondary metabolite products are extracted from the genera: *Nocardiopsis*, *Micromonospora*, *Salinispora*, and *Pseudonocardia*. The genus Micromonospora is revealed as the richest source of bioactive natural products that are chemically diverse and specific.[7] Genus *Streptomyces* Sub-order Streptomycineae has important roles in producing bioactive compounds of secondary metabolites that have potential as anticancer,

antifungal, antibacterial, enzyme inhibitors and antimalarial.[8,9]

The mangrove forest is a very productive ecosystem to protect coastline areas located in tidal zones in the tropics and subtropics. Mangrove ecosystems are diverse habitats for flora and fauna of marine, freshwater and terrestrial species.[10] Indonesia is an archipelago country with long coastlines with the potential of mangrove forests having large sedimentation that can produce Actinobacteria as a new antibiotic, anticancer and antimalarial species.[11] Actinobacteria are significant components of microbial populations in most soils including mangrove areas.[10] This study looks at the potential of the Actinobacteria genus *Streptomyces sp* from the mangrove forest, especially sediments of Hamadi Beach in Jayapura as a barrier to the growth of *Plasmodium falciparum*.

METHODS

This study is experimental quantitative research. It was conducted at the Microbiology Laboratory and Parasitology Laboratory of the Papua Biomedical Research and Development Center. This study had obtained a Research Ethics Permission of the Health Research Ethics Commission of the Ministry of Health Litbankes Agency Letter Number LB.02.01/5.2/KAE.010/2016 concerning Exemption of Ethical Approval (Exempted). The samples in this study were *Streptomyces* ° isolated from *Actinomycetes* isolated from mangrove forest soil sediments and blood specimens infected with a single *Plasmodium falciparum* (+++). Mangrove forest sediment sampling was conducted in the mangrove area of Hamadi Beach in the rhizosphere (around the root system) by a thickness of sediment of 1-5 cm. Then it was then put into a 50 ml sterile plastic with storage in a laboratory at 4°C.[12,13] The samples previously were wet, but then they were dried until the water content vanished. After drying, the sediment in the oven then weighed 1 gram. The sediment was dissolved in Ringer's solution (1/4 strength) with a series of dilutions made of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Furthermore, pre-heated treatment was organized by heating in a 50°C water of bath to kill bacteria or fungi to stimulate the growth of *Actinomycetes*. Selective isolation of *Streptomyces sp* used Starch Casein agar Sterile agar (Composition per liter: 15.0 g agar, Soluble starch 10.0 g, K_2HPO_4. 2.0 g, KNO_3 2.0 g, NaCl 2.0 g, Casein 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4 \cdot 7H_2O$ 0.01 g). Inoculated 0.1 mL of a suspension sample from each dilution were put into the starch casein media, and then the surface of the plate was incubated at 27°C for two weeks. Growing colonies were isolated by using toothpicks and inoculated into nutrient media, and then they were incubated at 27°C for approximately two weeks. Observing shapes of the colony and its morphology was examined by using a 1000x magnification microscope. Further identification was conducted by gram staining, and then the morphology was observed by using a 1000 x magnification microscope.[12] Next, metabolite fermentation was conducted by using sterile FM3 media (Soluble starch 20 g, soy powder 15 g, yeast extract 5 g, peptone 2 g, $CaCO_4$ 4 g, sea salts 18 g in 1 L aquades pH 7.2-7.4).14 Then isolates of *Streptomyces* obtained were inoculated in the media and incubated at 37°C for 7 days.[15,16] Identification of the secondary metabolites was organized by using Thin Layer Chromatography (TLC) method with chloroform: methanol eluent solvents in various combination combinations from polar to non-polar.[2] Testing the inhibition of secondary metabolite extract of *Streptomyces sp* on *Plasmodium falciparum*, *Streptomyces* extract of 10 mg/10 mL was dissolved with DMSO and then diluted with aquadest 1 mL to obtain a concentration of 1000 µg/mL. The crude dose series of *Streptomyces* extract was made into 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, and 0.01 µg/mL, diluted by using RPMI media + 0.5% albumax. A pipette as much as 20 µL series above dose was put into the wells. The plate was ready to be tested on *Plasmodium falciparum*.[1,17]

Data analysis

The data were statistically analyzed by using probit analysis to determine the concentration

of inhibitory of *Plasmodium falciparum* 50% of *Streptomycetes sp* extract.

RESULTS

Streptomyces isolates in this study were obtained from mangrove forest sediments, and it was grown into Starch Casein Agar (SCA) media as shown in Figure 1.

Characterization of colony shapes and morphological identification through observation under a microscope at 1000x magnification could be seen in Figure 2. Observation of the morphological shape of *Streptomyces sp* can be recognized from the shape of the white colony. In the colonies found aerial mycelium and substrate mycelium. colony media aroma smells like soil.[18,19]

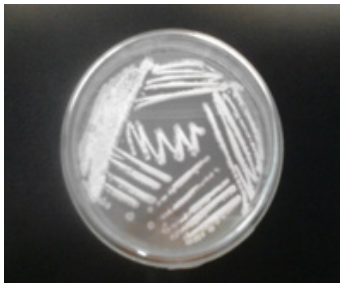

Figure 1. Morphological appearance of *Actinomycetes* genus colonies on SCA media.

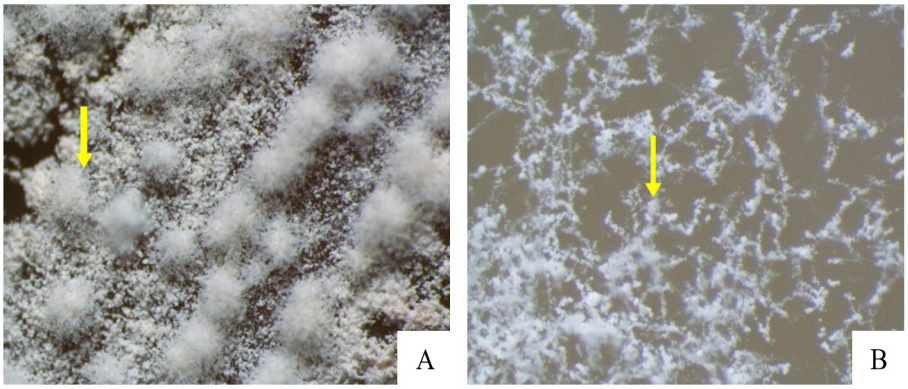

Figure 2. Microscopic identification of *Streptomyces sp* isolates with 1000 times magnification: A) aerial mycelium appearance B) Substrate mycelium appearance

The identification was followed by gram staining which showed branched and purple (gram-positive) mycelium, and this was that characterized members of the genus *Streptomyces sp*, as shown in Figure 3.[12]

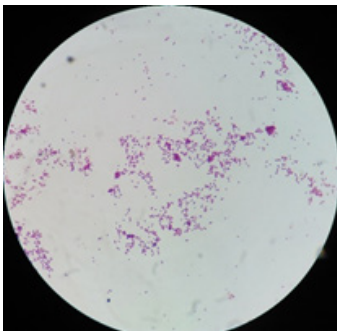

Figure 3. Identification of *Streptomyces sp* isolates by microscopic gram stain with 1000 times magnification.

The *Streptomyces sp* isolates were fermented by using FM3 media and incubated for two weeks at room temperature and at the speed of 200 rpm. The fermented solution was evaporated on a hotplate until it was dry, and then it was dissolved by using methanol to obtain a slightly turbid yellowish final extract as shown in Figure 4.

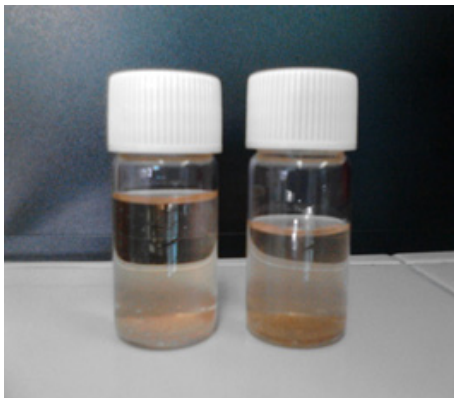

Figure 4. The methanol extract of secondary metabolite of *Streptomyces sp.*

The bioactive compounds contained in the extract of *Streptomyces sp* were identified by using Thin Layer Chromatography (TLC), where the extract sample was bottled on the TLC plate and then dipped into a chamber containing an eluent (methanol: chloroform) which has been firstly allowed to saturate. Results of the identification of secondary metabolite by using the TLC can be seen in Table 1. This qualitative observation showed presence of yellow and orange stains on ratios of polarity to methanol and chloroform solvents.

Table 1. Results of retention degrees (Rf) observation of TLC plates for ethyl acetate extracts of secondary metabolites of *Streptomyces* by using methanol and chloroform solvents

MeOH : $CHCl_3$ Comparison	**Eluent distance (cm)**	**Stain distance (cm)**	**Rf value**	**Information**
9 : 1	6	0.3	0.05	Two blotches of an orange compound
8 : 2	6.2	5	0.8	One spot of light yellow stain
7 : 3	6.5	2.9	0.44	One spot of orange stain
6 : 4	-	-	-	-
5 : 5	-	-	-	-
4 : 6	-	-	-	-
3 : 7	6	3	0.5	One blotch of an orange compound
2 : 8	-	-	-	-
1 : 9	6	4.2	0.7	One yellow stain

This study used *Plasmodium falciparum* taken from patients with stage 3 who were positive malaria (+++) meaning that there were 1 - 10 parasites per 1 microscopic field of view in thick blood smears. At this stage, the parasites were in a condition that was quite effective for infecting erythocytes. The breeding process of *Plasmodium falciparum* in the RPMI media was

conducted continuously by changing the media every 24 hours, and the sampling of thick blood smears was made to calculate the growth of the parasites.

Table 2 shows the results of microscopic observation of *Plasmodium falciparum* density. The density of the parasite (Parasitemia) was calculated based on observation of 40 fields of view (LP) microscopes or about 10,000 erythrocytes; therefore, the calculation was obtained:

$$\text{Parasitemia (\%)} = \frac{868}{10.000}\ x\ 100\ \% = 8{,}68\ \%$$

Table 2. Observation results of the *Plasmodium falciparum* parasitemia by microscopic examination of 1000 x thick blood smears

LP	Σ Parasite	LP	Σ Parasite	LP	Σ Parasite	LP	Σ Parasite
1	34	11	39	21	21	31	8
2	29	12	41	22	15	32	7
3	30	13	30	23	12	33	9
4	29	14	30	24	18	34	9
5	25	15	26	25	23	35	9
6	42	16	27	26	21	36	16
7	41	17	15	27	14	37	19
8	42	18	12	28	17	38	11
9	22	19	17	29	12	39	15
10	30	20	25	30	13	40	13

Total= 868 *Plasmodium falciparum*

For testing, it was necessary to dilute the parasitic density from 8.68% to 0.8% by adding hematocrit red blood cell and RPMI media solution.The inhibitory test results at the incubation stage for 48 hours with various concentration of the secondary metabolite extract of *Streptomyces* were 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0 , 01 µg/mL. The negative control used only the solvent blank. The positive control used in this study was the active substance Quinin with the highest concentration variation of 100 µg/mL and the lowest concentration of 0.01 µg/mL (Table 3).

Table 3. Percentage of parasitemia and average inhibition of *Plasmodium falciparum* after given ethyl acetate extract secondary metabolite compounds of *Streptomyces sp* in vitro for 48 hours

Concentration (µg/mL)	Parasitemia (%)	Inhibition (%)
Control (-)	13.76	0
100 µg/mL	0.66	95.20
10 µg/mL	2.32	83.16
1 µg/mL	4.97	63.86
0,1 µg/mL	5.16	62.48
0,01 µg/mL	10.89	20.83

IC 50%= Concentrations that cause growth inhibition of *Plasmodium falciparum* by 50% = 0.12 µg / mL.

The inhibition test results in Table 3 showed that the extract of *Streptomyces sp* with a concentration of 100 µg/mL could suppress average growth values of the parasite to only 0.66% with an average inhibition value of 95.20%. This value followed the extract

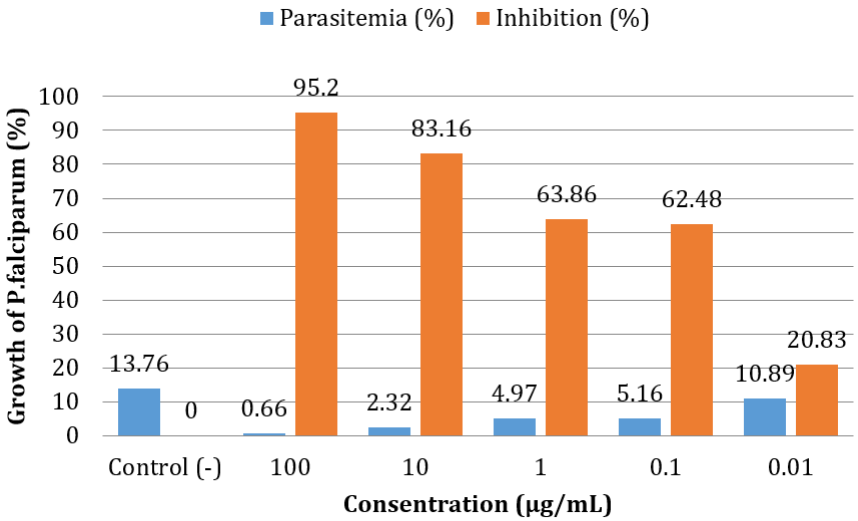

Figure 5. Inhibition of growth of *Plasmodium falciparum* incubated for 48 hours at various concentrations.

concentration level which is decreased in to the lowest concentration of 0.01 µg/mL, and the average growth value of the parasite increased into 10.89% with an average inhibitory value of 20.83%. Analysis of the relationship between the increased concentration and the amount of growth inhibitory activity of *Plasmodium falciparum* was examined by using Probit analysis. Therefore, the inhibition concentration for 50% inhibition (IC_{50}) for 48 hours could be calculated, and its result was 0.12 µg/mL.

DISCUSSIONS

Efforts made by researchers today is to find bioactive compounds by exploring nature to discover alternatives for new drugs. *Streptomyces sp* is the most dominant genus of *Actinomycetes* in producing potential bioactive compounds from secondary metabolites.[20] *Streptomyces sp* originating from mangrove sediments with specific and rich environmental condition is to support their sustainability. The formation of secondary metabolites in this species is also supported by changing conditions of salinity and pH of coastal waters.[21]

The results of isolation of *Streptomyces sp* on SCA (Starch Casein Agar) media showed white colony in filamentous forms morphologically, but physiologically it had bacterial character and specific soil-like odor; therefore, this genus is included in the Actinobacteria group.[18]

Identification of secondary metabolite compounds in the methanol extract applied the thin layer chromatography (TLC) method using eluent solvents with a certain ratio. The TLC results were followed by observation under UV light. These observation results obtained yellow orange-yellow spots 22 by Rf value on polar solvents of 0.05-0.8 and Rf of non-polar solvents of 0.5-0.7. This showed that there is possibility of secondary metabolite that is soluble in water and organic solvents as potential candidates for active compounds with different types of solvents. A similar study by Bavya et al. (2011) and Bais et al. (2012) reported that the results of TLC extracted with chloroform was methanol (10:90 and 4: 1) eluents obtained with yellow stains with an Rf value of 0.6-0.9.[23,24]

The results of the inhibition of methanol extract of secondary metabolite of *Streptomyces sp* on the growth of *Plasmodium falciparum* in vitro can be seen in table 4. The concentration of methanol extract of secondary metabolite of *Streptomyces sp* with a concentration of 100 µg/mL could suppress the average growth value of *Plasmodium falciparum* by 0.66 % with an average inhibition value of 95.20%. The inhibitory value of secondary metabolite followed the increase in

the extract concentration dose, which decreased in to the lowest concentration dose of 0.01 µg/mL. The average value of *Plasmodium falciparum* growth increased into 10.89% with an average inhibition value of 20.83%. The negative control was also added in this test that showed an average parasitic growth rate of 13.76% with an average inhibitory value of 0%.

Another comparison included a positive control, an active substance Quinin at a concentration of 100 µg/mL and 0.01 µg/mL. The positive control at its highest and lowest concentration showed a growth value of *Plasmodium falciparum* of 0.86% and 6.42% respectively. Compared with the extract of *Streptomyces sp* with equal concentration, the growth value of *Plasmodium falciparum* in the highest and the lowest concentration were 0.66% and 10.84% respectively. Therefore, it could be concluded that the extract of *Streptomyces sp* has secondary metabolite compounds that can inhibit the development of *Plasmodium falciparum* with good effectiveness.

The results of IC_{50} analysis of *Plasmodium falciparum* in culture for 48 hours were 0.12 µg/mL. This IC_{50} value interpreted ability of the concentration of ethyl acetate extract of secondary metabolite compounds of *Streptomyces sp* in inhibiting the growth of *Plasmodium falciparum* in erythrocytes in vitro by 50%. The greater the effectiveness of the inhibition on the growth of *Plasmodium falciparum*, the smaller the IC_{50} value. The parameters in this particular test were a test of substance with the best activity by IC_{50} value ≤ 10 µg/ml. The activity is good if the IC_{50} value is between 10-50 µg/ml, and the activity is bad if the IC_{50} value is ≥ 50 µg/ml.[25] Study on the activity of secondary metabolite of *Streptomyces sp* is related to seaweed, obtaining IC_{50} value of 35.48 µg /mL.[26] Meanwhile, study on the inhibitory activity of secondary metabolite acetone extracts of *Streptomyces sp* originating from the soil showed IC_{50} value of 0.57 - 4.87 µg/mL.[4] There are differences in the inhibitory activity due to the types of organic solvent used for the extraction.[27]

Based on the IC_{50} results and comparisons of the IC_{50} value parameters, it could be inferred that the secondary metabolite compound of *Streptomyces sp* has the best inhibitory power. The mechanism of secondary metabolite of *Streptomyces sp* to inhibit the growth of *Plasmodium falciparum* is related to presence of chemical compounds that are active as inhibitors of proteases in Trypsin, Chymotrypsin and Proteinase K.[28] Proteases are needed by *Plasmodium* stage merozoites to breakdown and invase subsequent erythrocytes and to decrease hemoglobin by trophozoitin K. intraerythrocytic.[29] Other mechanism of the active substance are their cytotoxic characteristics such as quinone, trioxacarcin active substances, steroid compounds (ergosterols) and terpenoids found in the genus *Streptomyces*.[30,31]

Limitations of this study is that it only identified levels of the genus *Streptomyces sp* and its secondary metabolites limited to unfractionated methanol extracts. Further studies on the identification of chemical compounds are necessary.

CONCLUSION

Extracts of secondary metabolites of *Streptomyces sp* from sediments in the mangrove area of Hamadi Beach in Papua showed the ability to inhibit the growth of *Plasmodium falciparum* effectively; therefore, it has potential as a source of antiplasmodial.

CONFLICT OF INTEREST

There is no conflict of interest.

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