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Research Article

IN VITRO POTENCY AND TOXICITY OF *STREPTOMYCES* SP. FERMENTATION PRODUCT AS AN ANTIMALARIAL THERAPY AGAINST *PLASMODIUM FALCIPARUM*

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ABSTRACT

Objective: This research aims to study the activity of a *Streptomyces* sp. fermentation product as an antimalarial modality in HepG2 cells.

Methods: The effects of the product against *Plasmodium falciparum* 3D7 were examined using an *in vitro* technique parasite. The potency of the *Streptomyces* sp. fermentation product was examined by determining the half maximal inhibitory concentration (IC_{50}), and the mechanism was studied using transmission electron microscopy (TEM). Toxicity tests were also conducted.

Results: The *Streptomyces* sp. fermentation product had an IC_{50} of 0.001 µg/ml against the parasite, versus values of 0.054 and 0.022 µg/ml for quinidine and prodigiosin, respectively. TEM revealed no formation of hemozoin. The *Streptomyces* sp. fermentation product was non-toxic in HepG2 cells based on its cytotoxicity concentration 50% of 1.380 µg/ml.

Conclusion: The *Streptomyces* sp. fermentation product has potential as a potent and non-toxic antimalarial therapy.

Keywords: Antimalarial, *Streptomyces* sp. Fermentation Product, Half maximal inhibitory concentration, Transmission electron microscopy, Toxicity.

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INTRODUCTION

Malaria remains a global health problem, particularly in tropical areas [1]. According to Indonesia's Ministry of Health, the incidence of malaria declined between 2011 and 2015 from 1.75 per population to 0.85 per population [2]. However, the emergence of antimalarial drug resistance has threatened the goal of eradicating malaria [3]. The resistance of *Plasmodium falciparum* to artemisinin has been detected in five countries in the Greater Mekong Subregion [4].

Some drugs used to treat malaria are obtained from plants, such as quinine and artemisinin. Quinine was initially isolated from the bark of the *Cinchona* tree [4]. Artemisinin was developed from the Chinese herb *Artemisia annua* (qinghao) [5]. Other plant-based substances with potent antimalarial effects include the alcoholic extract of the root bark of *Uapaca nitida* (Euphorbiaceae) in Tanzania and extracts from *Hernandia voyroni* (Hernandiaceae) in Madagascar [6]. Research in Iran found that extracts from *Buxus hyrcana*, *Erodium oxyrrhnchum*, *Glycyrrhiza glabra*, and *Ferula oopoda* had significant antimalarial activity [7].

Studies have also identified potent antimalarial effects for the fermentation products of bacteria. Among them, Baba *et al*. described the antimalarial activity of the fermentation product of the endophytic bacterium *Streptomyces* SUK10 [8]. Boonlarppradab *et al*. revealed that the fermentation products of *Saccharopolyspor*a A and B had antimalarial activity with half maximal inhibitory concentration (IC_{50}) of 4.1 and 3.9 µM, respectively [9].

Meanwhile, *Streptomyces* spp. has been widely studied for their antibiotic, antifungal, and anticancer activities [10]. Among these, *S. griseous* was the first species to be used in the industrial production of antibiotics [11]. In addition, research on the antimalarial effects of *Streptomyces* spp. has also been conducted. Isaka *et al*. reported that *Streptomyces spectabilis* isolated from soil samples in Thailand exhibited antimalarial activity against *P. falciparum* K1 [12]. *Streptomyces* spp. can produce various secondary metabolites [13,14]. For example, *Streptomyces* spp. from the ocean can produce the secondary metabolite prodigiosin, which has confirmed antimalarial effects [15,16].

The alkaloid prodigiosin is a member of the prodiginine family, which has been reported to have antibacterial, antifungal, antimalarial, and antiprotozoal activity [17]. Papireddy *et al.* studied prodigiosin, undecylprodigiosin, and metacycloprodiginin, finding that all three had potent antimalarial activity, with IC_{50} of 8.0, 7.7, and 1.7 nM, respectively [18]. Lazaro *et al*. reported that heptyl prodigiosin displayed antimalarial activity *in vitro* [19]. Rahul *et al*. observed that prodigiosin inhibited the formation of *Trypanosoma brucei gambiense*, with an IC_{50} of 0.158±0.019 g/ml, and exerted antimalarial activity with an IC_{50} of 1.1±5.9 pg/ml *in vitro* [20]. The spectrum of the antifungal activity of prodigiosin is believed to be comprehensive, including species of *Candida*, *Aspergillus*, *Penicillium*, *Saccharomyces*, *Cryptococcus*, and *Histoplasma* [21]. Prodigiosin reportedly has a mechanism of action similar to quinine, namely, inhibiting the formation of hemozoin, which can be examined through transmission electron microscopy (TEM). TEM is useful in analyzing nearly all cellular components, including the cytoskeleton, membrane systems, and organelles [22,23].

Meanwhile, a compound or extract should be tested for toxicity to assess its potential harm to living things. Most drugs are metabolized in the liver, making HepG2 cells a suitable model to examine their metabolism. Therefore, this study used HepG2 cells to assess the efficacy, mechanism of action, and potential toxicity of a *Streptomyces* sp. fermentation product against the malaria-causing parasite *P. falciparum*.

METHODS

Parasite

This study used the *P. falciparum* 3D7 strain originating from the University of Tokyo that is propagated regularly at the Eijkman Institute

for Molecular Biology. *P. falciparum* cells were thawed from liquid nitrogen using NaCl and 20% RPMI complete medium. *P. falciparum* 3D7 cells were propagated in 10% RPMI complete medium, with medium replacement every 2 days. Parasitemia was assessed using thin blood smears with Giemsa staining. Cultures of *P. falciparum* 3D7 that caused 2% parasitemia were used to determine IC_{50}.

Drug

The fermentation product of *Streptomyces* spp. was obtained from LIPI. As controls, quinidine and prodigiosin were obtained from the Laboratory of Pharmacology, University of Indonesia.

Viability testing

Viability testing was performed using a 96-well plate, with cultures of *P. falciparum* at a volume of 180 ml included in each well. A 20-ml stock sample of the *Streptomyces* sp. fermentation product was added to well #12 and then homogenized. A sample was taken from this well, introduced into well #11, and homogenized, and this process was repeated until well #4 were reached. The dilution method was also performed for the quinidine and prodigiosin stocks. The plate was incubated in a candle jar at 37 °C for 48 h. Parasitemia was assessed by making a thin blood smear stained with 10% Giemsa solution and counted under a microscope. A curve of IC_{50} values was then created using the results between the test concentration and the percentage of growing parasites.

TEM

P. falciparum 3D7 was incubated with the fermentation products of *Streptomyces* spp. at the IC_{50} concentration, and untreated controls were incubated in a candle jar at 37 °C for 48 h. The samples were then diluted with a solution of 2.5% glutaraldehyde (in 0.1 M cacodylate buffer + 3% sucrose) and incubated with shaking for 48 h at 4 °C. The samples were then washed with 0.1 M cacodylate buffer + 3% sucrose 3 times. The obtained pellets were incubated in a solution of 2% osmium tetraoxide + 2.5% $K_3Fe(CN)_6$ and washed 3 times with 0.1 M cacodylate buffer + 3% sucrose. The pellets were then dissolved in 10%, 30%, 50%, 70%, 95%, or 100% ethanol. Pellets were then incubated with pure propylene oxide at room temperature for 1 h and centrifuged.

Pellets were then incubated with Spurr's resin (1:1) at room temperature for 30 min and then centrifuged, after which half of the supernatant was incubated for 30 min at room temperature and centrifuged. The supernatant was then removed, and the pellet was incubated in Spurr's resin overnight under vacuum. Samples were transferred to new tubes. Spurr's pure resin was introduced into each sample, which was incubated under vacuum at 70°C overnight. Parasite cells were stained using uranium salts.

Toxicity test

HepG2 cell cultures were taken from an −80 °C freezer and thawed. The cell suspensions were then introduced into 15-ml Falcon tubes; Dulbecco's Modified Eagle's medium (DMEM) complete medium (DMEM + 1% penicillin-streptomycin + 10% fetal bovine serum) was added, and the tubes were centrifuged at 500 rpm for 10 min. For each tube, the supernatant was discarded, complete medium was added, and the 20-ml sample was placed in 75-cm^2 culture flasks. The cultures were incubated at 37°C in a 5% CO_2 atmosphere, and the medium was replaced every 2–3 days. Cells were harvested after approaching confluence. Cells were washed with PBS and incubated in 3 ml of 0.25% trypsin in the incubator for 15 min. Then, complete medium was added, and cells were centrifuged at 500 rpm for 10 min. The supernatant was discarded, 1 ml of complete medium was added, and the number of cells was counted using a hemocytometer. HepG2 cells were cultured in 24-well plates at a density of 1×10^5 cells per well. After incubation for 24 h, cells were exposed to various concentrations of the *Streptomyces* sp. fermentation product and incubated for 24 h. The cells were subsequently washed with PBS and incubated in 0.25% trypsin for 15 min, after which cells were incubated in complete medium. The cell suspension was then centrifuged at 500 rpm for 10 min. The supernatant was discarded, and cells were incubated in 1 ml of PBS. Next, 10 ml of the cell suspension was added to 10 ml of Trypan blue and then homogenized. Then, 10 ml of this mixture was placed in a hemocytometer chamber, and cell viability was calculated. This method was also performed for quinidine. The results of cell viability were used to determine the cytotoxicity concentration 50% (CC_{50}).

RESULTS

Cell Viability

The inhibitory effects of the *Streptomyces* sp. fermentation product on the growth of *P. falciparum* 3D7 were examined in comparison to those of quinidine and prodigiosin. The IC_{50} of the fermentation product of *Streptomyces* spp. was 0.001 µg/ml (Table 1 and Fig. 1).

Conversely, the IC_{50} for quinidine was 0.054 µg/ml (Table 2 and Fig. 2).

In addition, the IC_{50} for prodigiosin was 0.022 µg/ml (Table 3 and Fig. 3).

TEM analysis of the fermentation product of *Streptomyces* spp.

The morphology of parasites was examined using TEM at ×8000 (Fig. 4). In the absence of treatment, the parasite grew normally with firm borders and healthy organelles, and hemozoin was produced.

Table 1: Inhibitory effects of the *Streptomyces* sp. fermentation product on *P. falciparum* 3D7 growth

Concentration (µg/ml)	Parasitemia (%)	Parasite growth (%)
No treatment	3.1	100.0
10^{-3}	1.7	54.3
10^{-2}	1.3	40.4
10^{-1}	0.7	21.3
1	1.1	34.0
10	0.9	29.8
10^2	0.5	16.0
10^3	0.0	0.0
10^4	0.0	0.0
10^5	0.0	0.0

P. falciparum: Plasmodium falciparum

Table 2: Growth of *P. falciparum* 3D7 following exposure to quinidine

Concentration (µg/ml)	Parasitemia (%)	Parasite growth (%)
No treatment	2.38	100.0
5×10^{-5}	2.13	89.5
5×10^{-4}	2.62	110.3
5×10^{-3}	1.92	80.6
5×10^{-2}	0.59	24.8
5×10^{-1}	0.64	26.9
5	0.47	19.8
50	0.16	6.5
5×10^2	0.00	0.0
5×10^3	0.00	0.0

P. falciparum: Plasmodium falciparum

Table 3: Growth of *P. falciparum* 3D7 following exposure to prodigiosin

Concentration (µg/ml)	Parasitemia (%)	Parasite growth (%)
No treatment	4.69	100.0
10^{-7}	6.34	135.3
10^{-6}	5.87	125.3
10^{-5}	5.48	116.9
10^{-4}	5.04	107.5
10^{-3}	5.22	111.4
10^{-2}	3.29	70.2
10^{-1}	0.59	12.5
1	0.00	0.0
10	0.00	0.0

P. falciparum: Plasmodium falciparum

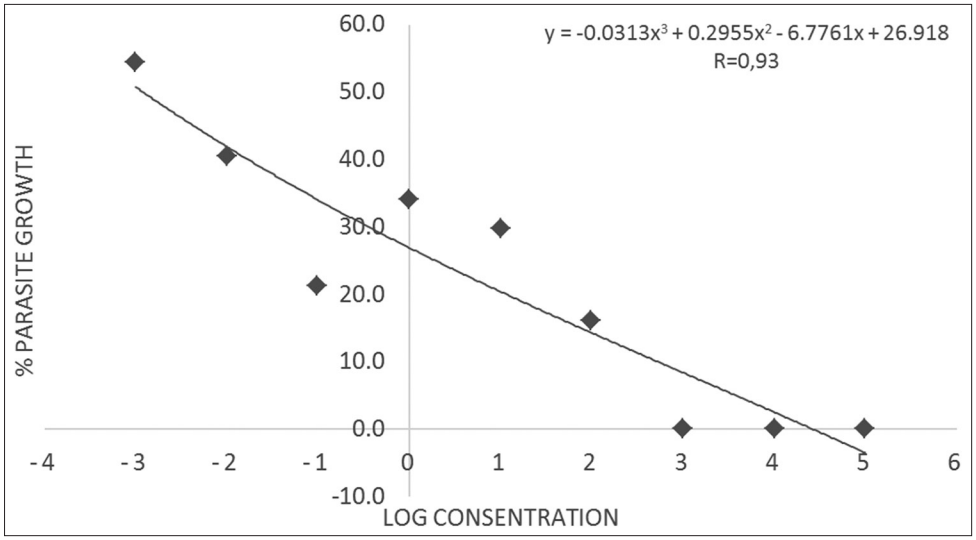

Fig. 1: The growth curve of *Plasmodium falciparum* 3D7 following exposure to the *Streptomyces* sp. fermentation product

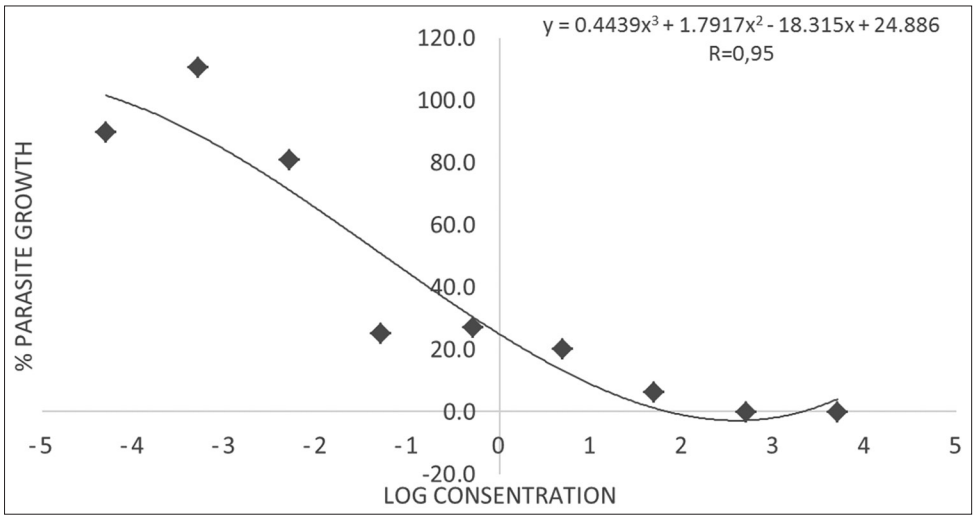

Fig. 2: The growth curve of *Plasmodium falciparum* 3D7 following exposure to quinidine

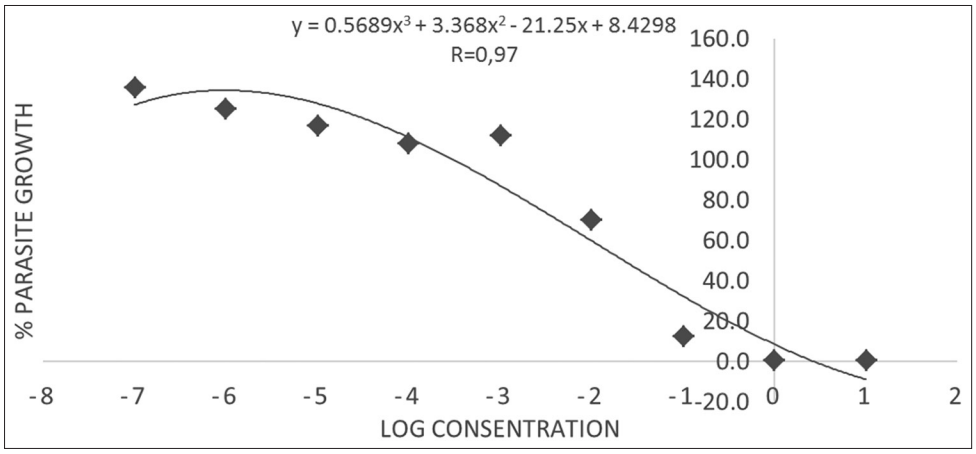

Fig. 3: The growth curve of *Plasmodium falciparum* 3D7 following exposure to prodigiosin

Conversely, when the parasite was exposed to the fermentation product of *Streptomyces* spp., hemozoin was not produced.

Toxicity of the fermentation product of *Streptomyces* spp. in HepG2 cells was evaluated.

The toxicity of the fermentation product of *Streptomyces* spp. in HepG2 cells was evaluated using Trypan blue staining. Then, a CC_{50} curve for the fermentation product of *Streptomyces* spp. relative to the untreated controls was created. The toxicity of quinidine was similarly examined. At concentrations ≤100 pg/ml, the *Streptomyces* sp. fermentation

product did not induce significant cytotoxicity (Table 4). Following exposure to higher concentrations, the CC_{50} of the fermentation product was calculated as 1.380 µg/ml (Fig. 5).

Conversely, quinidine at a concentration of 200 µg/ml was completely cytotoxic to HepG2 cells, whereas cell viability exceeded 50% for smaller concentrations (Table 5). After calculating the cell viability curve, the CC_{50} was determined as 160 pg/ml (Fig. 6).

DISCUSSION

Natural products have long been investigated in drug discovery, included antimalarial drugs. 45% of clinically used antimalarial drugs were derived from natural products [24]. An example is the first antimalarial drug, quinine, which was isolated from the bark of the *Cinchona* spp. (Rubiaceae) [6]. In 1972, a group of Chinese researchers isolated the antimalarial drug artemisinin from the traditional Chinese herb *A. annua* (Asteraceae) [25]. In addition, *Actinomycetes* bacteria also produce a wide range of bioactive compounds, some of which have antimalarial effects [24].

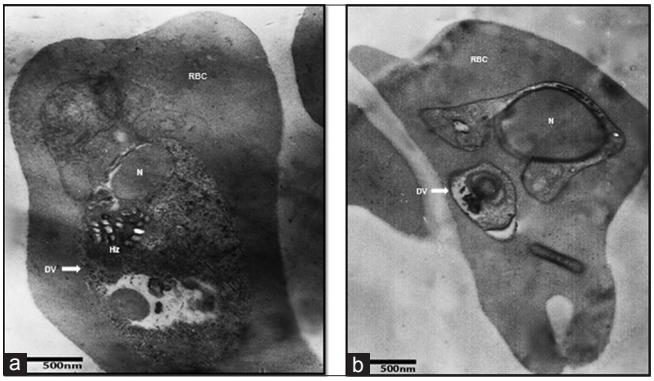

Fig. 4: Morphology of *Plasmodium falciparum* 3D7 assessed through transmission electron microscopy at ×8000 magnification. (a) In the absence of treatment, hemozoin was produced. (b) Hemozoin was not produced when the parasite was exposed to the fermentation product of *Streptomyces* spp.

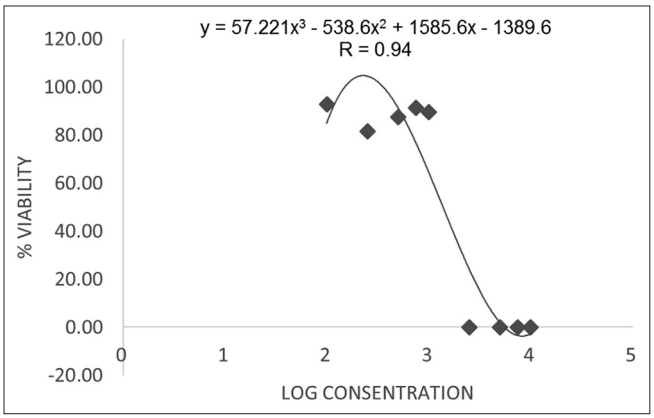

Fig. 5: The cell viability curve following exposure to the *Streptomyces* sp. fermentation product

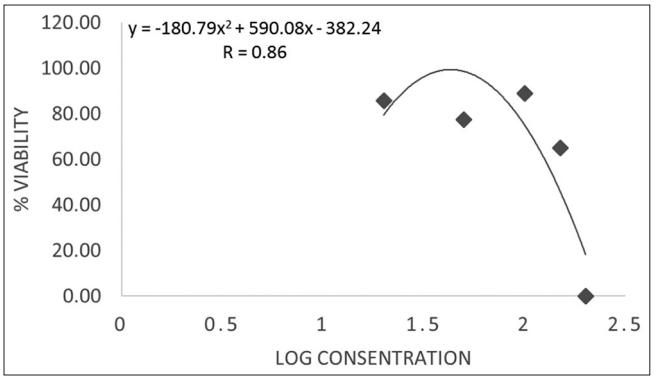

Fig. 6: Cell viability curve following quinidine exposure

In prior research using *S. spectabilis* BCC 4785 isolated from soil samples, the metabolite metacycloprodigiosin was found to have antimalarial activity. Prodigiosin is a secondary metabolite produced by *Streptomyces* and *Serratia* spp. The *Streptomyces* sp. fermentation product supposedly contains prodigiosin, a secondary metabolite, and alkaloid [26]; thus, it might have the same mechanism of action as the alkaloids quinine and quinidine. In another study, Lazaro *et al*. found that heptyl prodigiosin has activity similar to quinine against sensitive *Plasmodium* 3D7 [19].

In a previous study, Intaraudom *et al*., using a crude extract of *Streptomyces* sp. BCC27095 isolated from soil samples in Thailand, reported an IC_{50} of 2.72 µg/ml against a multidrug-resistant *P. falciparum* K-1 strain [27]. Another study using crude extracts of *Streptomyces* sp. BCC26924 reported an IC_{50} of 0.77 µg/ml against the same strain [28]. Meanwhile, Rakotondraibe *et al*. recorded IC_{50} of 2.5–10 µg/ml for extracts of *Streptomyces* spp. against *P. falciparum* Dd2 strains [24]. These results illustrate that the fermentation product of *Streptomyces* spp. has good antimalarial activity. However, it must be noted that this study used drug-resistant strains, whereas prior research used drug-resistant *Plasmodium* spp.

In this study, the fermentation product of *Streptomyces* spp. appeared to have stronger antimalarial activity than the control agents, possibly because it contains several secondary metabolites with antimalarial effects. Meanwhile, the controls were only single agents. Therefore, further research is needed to more closely identify the antimalarial agents.

P. falciparum degrades hemoglobin to acquire nutrients for growth. This results in increased heme levels, which are toxic to the parasite. To overcome this toxicity, the parasite polymerizes heme into hemozoin, which is nontoxic. In this study, it appears that hemozoin was not formed following exposure to the *Streptomyces* sp. fermentation product, resulting in heme accumulation and death of the parasite.

Table 4: Cell viability following exposure to the fermentation product of *Streptomyces* spp.

Concentration (µg/ml)	% viability
No treatment	100
100	92.90
250	81.42
500	87.43
750	91.26
1000	89.62
2500	0.00
5000	0.00
7500	0.00
10,000	0.00

Table 5: Cell viability following quinidine exposure

Concentration (µg/ml)	% viability
No treatment	100.00
20	85.56
50	77.22
100	88.89
150	65.00
200	0.00

Herbs are used as medicines in developing countries based on the presence of secondary metabolites [29]. Bacteria such as *Streptomyces* spp. also produce secondary metabolites, and thus, toxicology testing is needed to ensure the safe use of fermentation products. The current analysis using HepG2 cells indicated that the fermentation product should be nontoxic in comparison to the controls.

CONCLUSION

The fermentation product of *Streptomyces* sp. exhibited potency as an antimalarial agent, with an IC_{50} value of 0.001 µg/ml. The CC_{50} of the fermentation product in HepG2 cells was 1380 µg/ml. The test results indicate that the *Streptomyces* sp. fermentation product exerts strong antimalarial effects without causing toxicity, although additional research is needed to verify these findings.

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