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In vitro Test for Inhibition of *Plasmodium falciparum* 3D7 Parasites using *Streptomyces hygroscopicus* subsp. *hygroscopicus* Strain i18, Isolated from a Pineapple Farm in Lampung

Endah Setyaningrum[1], Achmad Arifiyanto[1]*, Nismah Nukmal[1], Titik Nur Aeny[2], Meishy Handerlin Putri[1] and Ulin Ni'mah Setiawati[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Lampung, Indonesia.
[2]Department of Plant Protection, Faculty of Agriculture, Universitas Lampung. Jl. Prof. Soemantri Brojonegoro No 1, Gedong Meneng, Rajabasa, Bandarlampung 35144, Lampung, Indonesia.

Abstract

Increasing natural ingredient awareness and utilization has created increased demand for sources of natural medicinal ingredients, including sources of compound used to treat malaria. *Streptomyces* is a genus of prokaryote well recognized for its production of antibiotics and other pharmaceutically useful compound. This study aimed to assess the ability of unpurified fermentation metabolites to inhibit *Plasmodium* parasites. A strain of bacteria identified as *Streptomyces hygroscopicus* subsp. *hygroscopicus* strain i18 were isolated from pineapple fields in Lampung province, and was cultured and fermented on liquid synthetic Gause medium for 10 days. The supernatant was separated from the cells and extracted with ethyl acetate-methanol (1:1). *Plasmodium falciparum* 3D7 was used for antiplasmodial testing. Metabolites were tested qualitatively using a phytochemical approach. Saponins and triterpenoids were found to be present in the extract. Parasite inhibition as measured using probit analysis and yielded an IC_{50} value of 11.07 g.m/L. These findings suggest further examinations of this extract (e.g. assessment of off target effects) are warranted.

Keywords: antiparasitic, actinomycetes, antimalarial, parasite, microbes

***Correspondence:** achmad.arifiyanto@fmipa.unila.ac.id; +62-721 704625

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INTRODUCTION

Malaria remains a significant medical challenge, particularly in tropical countries[1]. *Plasmodium* parasites are responsible for malaria, which is transmitted by the bite of female *Anopheles* mosquitoes[2]. Malaria is often found in tropical coastal and rainforest habitats. It is endemic to Lampung province with a high rate of incidence[3,4]. Therefore, better resources for malaria control are needed.

Malaria control using a variety of natural ingredients are now widely pursued[5], due to increasing resistance to synthetic antimalarial drugs. Exploration for antimalarial natural products has not been limited to plants. Animals and microbes have also been assessed[6]. *Streptomyces* is one of many microbial genera that has long been known for its anti-parasitic and anti-pathogenic metabolites, including antimalarials[7].

Streptomyces are Gram-positive bacteria found scattered across various habitats. These bacteria are capable of controlling pathogen populations and producing biosurfactants[8]. A previous study succeeded in isolating the bacteria *Streptomyces hygroscopicus* subsp. *hygroscopicus* i18[9]. These bacteria were shown to inhibit the growth of pathogens that caused root and stem rot, namely *Dickeya zeae*.

Plasmodium development is inhibited by the same bacterial strains, according to previous reports[10]. Determining the lethal doses of distilled extracts has been the subject of many antiparasitic studies. Distillation-based purifications are expensive and time-consuming[11]. It is important to know whether a crude extract obtained from *Streptomyces hygroscopicus* subsp. *hygroscopicus* can inhibit *Plasmodium* parasites without purification. This study thus aimed to learn more about *Plasmodium* inhibition by unpurified *Streptomyces hygroscopicus* subsp. *hygroscopicus* strain i18 fermentation extract.

MATERIALS AND METHODS

Cell culture and fermentation

Bacteria were cultured in liquid Gause media (LGM) with an adjusted pH of 7.3[12] . LGM media consists of soluble starch 20.0 g, KNO_3 1.0 g, NaCl 0.5 g, $MgSO_4$ x 7 H_2O 0.5 g, K_2HPO_4 0.5 g, $FeSO_4$ x 7 H_2O 10.0 mg, dissolved in 1000 mL distilled water. Culture supernatant for extraction was prepared by scaling up cultures. Initially, a 10 mL test tube of LGM was inoculated with a single loop inoculum. After 24 h incubation, the 10 mL starter culture was used to inoculate 90 mL of LGM. This culture was incubated at room temperature for 10 days. Supernatant was obtained by centrifuging the culture for 30 min at 6000 rpm. Crude supernatant was filtered through filter paper and extracted with a 1:1 methanol-ethyl acetate solvent mixture [13]. The solvent was then evaporated using a rotary evaporator, yielding a paste that was lyophilized to form a powder.

Qualitative metabolites screening

Secondary metabolites content was determined by analyzing samples of the powdered extract. Testing was performed using a previously described qualitative approach[14]. Phenols, alkaloids, flavonoids, saponins, triterpenoids, anthraquinone glycosides, and tannins were prominent among identified compounds.

Infrared Spectroscopy

Infrared (IR) spectra of the crude extract were obtained using an Agilent Cary 630 spectrometer. Infrared spectra with wave numbers ranging from 400 to 4000 cm^{-1} were obtained. Resolution was reported in two sizes per wave number.

In vitro antiplasmodial testing procedure

Antimalarial activity testing was performed *in vitro*[15]. This study used *Plasmodium falciparum* strain 3D7, which chloroquine-sensitive. One mg of sample extract was diluted in 100 mL of DMSO (stock solution, concentration 1.000 mg/mL). Serial dilution were made from the stock solution. In this study, synchronous parasites (ring stage) approximating 1% parasitemia were used. Fresh type O-positive human erythrocytes were used to maintain cultures, which were suspended at 4 percent hematocrit in complete medium[16]. Parasites were added to 96-well plates using a high-throughput liquid handler. Two microliters of various dilutions of extract were then added. Final extract concentrations were 100, 10, 1, 0.1, and 0.01 μg/mL. Plates were incubated under controlled gas condition (O_2 5%, CO_2 5%, N_2 90%). Plates were incubated for 48 h at 37°C, then erythrocyte slides prepared using 20% Giemsa staining.

Data were analyzed by counting the number of infected erythrocytes per 1000 total

erythrocytes microscopy. These data were used to calculate growth and inhibition percentages. The following formula was used to determine the growth percentage:

$$\% \text{ growth} = \% \text{ parasitemia} - D_0 \quad ...(1)$$

Where D_0 represents initial 1% parasitemia prior to incubation (%)

Inhibition is expressed as a percentage, and calculated using the formula below;

$$\text{Percent inhibition} = 100\ \% - ((Xu/Xk) \times 100\% \quad ...(2)$$

Description

Xu = % growth in the presence of extract

Xk = % growth in the negative absence of extract

Extract concentration that inhibited parasite growth by up to 50% (EC_{50}), was calculated using the two formulae above. The inhibitory concentration (IC_{50}) was determined using probit analysis and inhibition percentage results.

RESULTS

Streptomyces is genus of soil bacteria known to produce numerous secondary metabolites with useful human applications. Saponin and triterpenoid class compounds are produced by *Streptomyces hygroscopicus* subsp. *hygroscopicus* i18. Table 1 presents the findings of our secondary metabolite survey.

Table 1. Screening of metabolites compound results

Test	Result
Phenols	-
Alkaloids	-
Flavonoids	-
Saponins	+
Triterpenoids	+
Anthraquinone glycosides	+
Tannins	-

Saponin and triterpene extracts from *Streptomyces hygroscopicus* subsp. *hygroscopicus* strain i18 inhibited *Plasmodium* parasite cells growth by up to 81.11 percent at 100 µg/mL. Growth inhibition was achieved at concentrations of 10 µg/mL (57.63%, 1µg/mL (31.78%), 0.1 µg/mL (23.82%), and 0.01 µg/mL (8.91%). Fig. 1 illustrates this data in a logarithmic format. Inhibition of the growth of *Plasmodium* parasite cells was dose dependent. The probit data found that a treatment dosage of 11.07 µg/mL could suppress population growth by 50% (Fig. 2).

The bandwidth at 3212.96 cm^{-1} observed by FTIR analysis (Fig 3) is postulated to be linked to intermolecular H bonds. A =C-H bond, which can be found in any organic compounds was observed at 3060.61 cm^{-1} in the spectrum. Carboxylate groups were present at 1424.23 cm^{-1} (CH2). At 990-1060 cm^{-1}, a secondary cyclic alcohol appeared. At

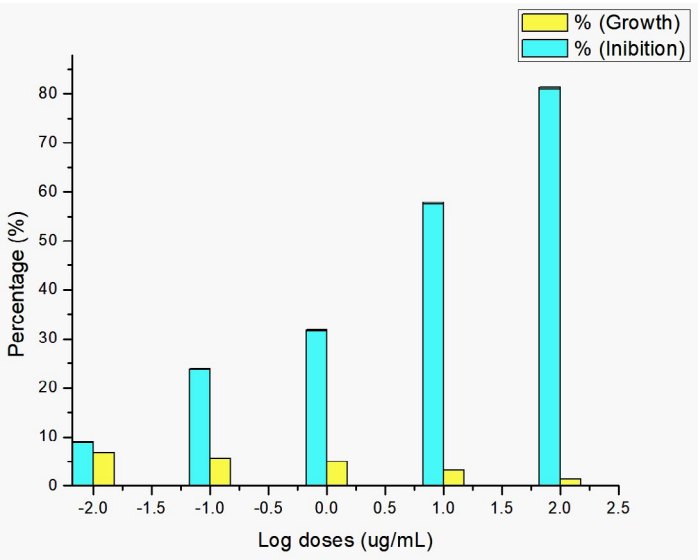

Fig. 1. The effect of giving bacterial extract on the growth of *Plasmodium* parasite.

approximately 905-915 and 985-995, an alkene (-CH=CH2) was discovered in the bandwidth. Strain on the C-O bond is indicated by the absorbance between 400 and 1250 cm^{-1}.

DISCUSSIONS

Streptomyces bacteria are considered to be probiotic bacteria that offer a variety of health benefits. Member of this genus have long been known to produce antibiotics[17]

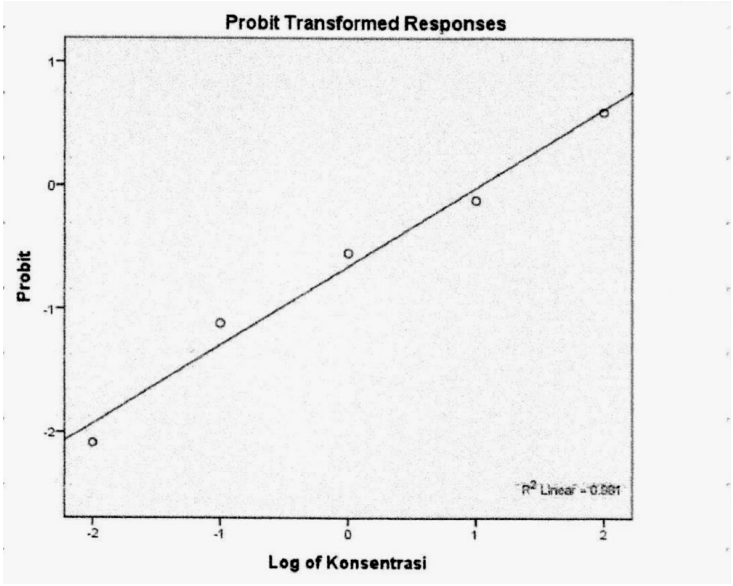

Fig. 2. Probit analysis of the inhibition of bacterial extract on the *Plasmodium* parasite.

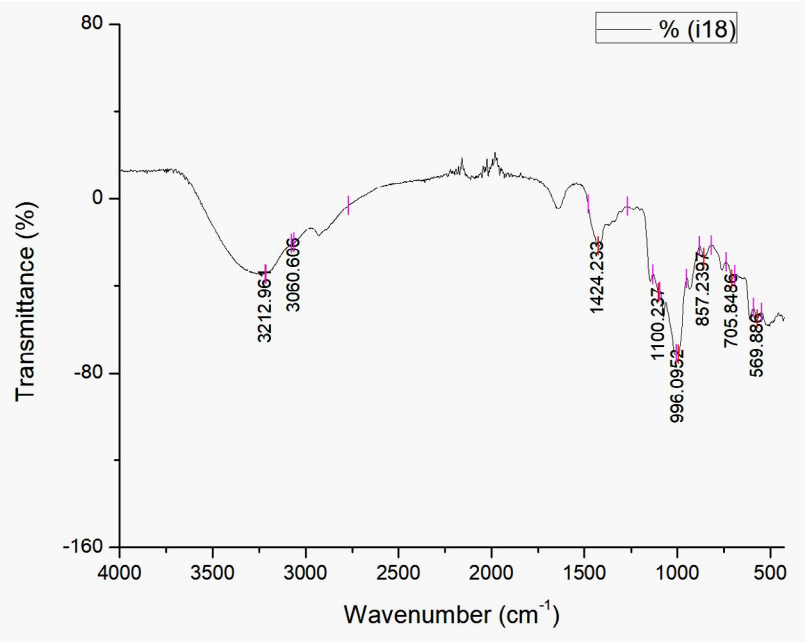

Fig. 3. Infrared spectroscopy using FTIR at biosurfactant produced by *Streptomyces hygroscopicus* subsp. *hygroscopicus* strain i18 fermentation extract

Antioxidants, pigment, and anticancer agents are also produced by these bacteria[18]. The i18 strain produces saponins and triterpenoids, according to preliminary screening results for secondary metabolites (Table 1). Saponins have been described as having biosurfactant[19]. Biosurfactants can lower water's surface tension, disrupting the conformation of a target microorganism's cell membranes[20]. Saponins can produce emulsifying foam which can disrupt the stability of the hydrophobic core of phospholipid bilayers, causing micelles to form. Saponins have been shown to inhibit growth of pathogenic bacteria[21].

Saponins and triterpenoids are closely related. Compounds in this group can be cyclic or non-cyclic, alcohol (OH), aldehyde, and carboxylic acid groups[22]. Spectroscopic analyses revealed the presence of a carboxylate group (Fig. 3). This carboxylate-containing isoform could be anti-diabetic from a physiological standpoint. Some natural pigments are triterpenoids[23]. Triterpenoids dissolve readily in polar solvents, including water, owing to their composition. Geraniums' triterpene content has also been reported to be antibacterial[24].

Since saponins in corporate glycosides in their structure[25], the existence of anthraquinone glycosides in our *Streptomyces* exctract was not unexpected. This compound is structurally related to anthracene, but has a carbonyl group on each of the two atoms on opposite sides of the central ring. The color of this pigment ranges from yellow to orange. When ammonia is applied to anthraquinones, the majority develop a characteristic color reaction in Bontrager reagent tests[26]. In our extract, the solution became reddish, while the anthrone and dianthrone remained golden. Carboxyl groups are also present in anthraquinones, which can be extracted using alkaline media. Anthrones and anthranols (which reduce glycosides) are made from anthraquinones[27].

The antimalarial compound gancidin was isolated from *Streptomyces* sp[7]. At doses of 6.25 and 3.125 µg/kg, it can inhibit nearly 80% of malarial parasites. In these experiment purified gancidin was used. Unpurified extract from strain i18, displayed an inhibitory concentration of 11.07 µg/mL. The toxicity of a candidate compound has no bearing on its antiplasmodial efficacy. We did, however, consider the toxicity of non-antimalarial compounds. Despite high toxicity toward *Plasmodium*, the extract could poison non-target cells[28]. Because this was a preliminary investigation, the compounds used in this analysis were not purified. Further testing would be needed in the future due to the demand for pure compound that could be used for mechanistic and *in vivo* testing. Effect on metabolism and cell damage could explain any toxicities in human usage.

CONCLUSIONS

Preliminary studies were carried out using crude extract fermented by *Streptomyces hygroscopicus* subsp. *hygroscopicus* strain i18, which contained of saponins, triterpenoids, and anthraquinone glycosides. This crude extract displayed the potential to inhibit the growth of *Plasmodium* parasites. Its half maximal inhibitory concentration (IC_{50}) towards *Plasmodium* parasites was 11.07 µg/mL. The results of this preliminary study need to be followed up with further research to establish physiological mechanisms and efficacy of pure compounds as antimalarial agents.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interests.

AUTHORS' CONTRIBUTION

ES and AA did the conceptualization, approach, study, tools, evaluation, and editing of experimental results. NN and TNA drafted the manuscript and reviewed it. MHP and UNS performed the experiments.

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DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Not applicable.

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