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Short Communication: In vitro antimicrobial and antimalarial screening of a crude extract of *Streptomyces* sp. AB8 isolated from Lapindo Mud Volcano Area, Sidoarjo, Indonesia

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Abstract. *Arifiyanto A, Setyaningrum E, Nukmal N, Aeny TN. 2021. Short Communication: In vitro antimicrobial and antimalarial screening of a crude extract of* Streptomyces *sp. AB8 isolated from Lapindo Mud Volcano Area, Sidoarjo, Indonesia. Biodiversitas 22: 2817-2823. Streptomyces* is a potential bacterial genus that has been investigated extensively as a source of natural microbial compounds. Its potential metabolites have been widely developed for pharmaceutical, pathogen control, and other applications in agriculture. This study aimed to determine the ability of the *Streptomyces* sp AB8 crude extract in inhibiting *Plasmodium* and pathogenic microbes. *Streptomyces* was cultured on Gause synthetic media for 10 days. The fermented broth culture media has dissolved in a 1:1 mixture of ethyl acetate and methanol. Biochemical characterization of this isolate has carried out using the standard methods. In-vitro antimalarial activity assay was performed using a chloroquine-sensitive *Plasmodium falciparum* strain 3D7. Fresh type O-positive human erythrocytes were suspended at 4 percent hematocrit in a complete medium to maintain culture. The inhibitory concentration (IC_{50}) was determined using probit analysis. The results showed the extract of *Streptomyces* sp. AB8 contains phenolic and alkaloids. *Streptomyces* sp. AB8 extract can inhibit *Dickeya zeae* N-Unila 5, *Dickeya zeae* N-Unila 10, *Aspergillus* sp IK3, and *Escherichia coli* growth. The results also showed that the IC_{50} value of extract against *P. falciparum* 3D7 was 17.56 ug/mL. Further research was needed to determine the types of purified bioactive compounds and their bioactivity.

Keywords: Alkaloids, antimalarial, antimicrobial, phenolic, *Streptomyces*

INTRODUCTION

Malaria is a disease caused by the *Plasmodium* parasite, is one of the deadliest diseases in tropical countries (Hay et al. 2004). It was transmitted by the bite of a female *Anopheles* sp. infected with *Plasmodium.* Malaria in humans was caused by five different *Plasmodium* species, i.e., *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* (Singh et al. 2013).

Unfortunately, the *Plasmodium* sp. parasite was resistant to antimalarial drugs such as chloroquine, amodiaquine, mefloquine, and artemisinin, according to many studies (Cui et al. 2015). Malaria research was actively being conducted to combat parasite resistance. The analysis of metabolites from different plants has been performed (Alkandahri et al. 2019; Fatmawaty et al. 2017; Okokon et al. 2017; Orabuezea et al. 2020; Zeleke et al. 2017), as well as metabolites from microorganisms to combat multi-drug resistant *Plasmodium.* Metabolites derived from microbes were considered to be more desirable due to their shorter life cycles.

Streptomyces, a Gram-positive bacteria, is a member of the actinobacteria phylum which is known to have antimicrobial, anticancer (El-Naggar and El-Ewasy 2017), anti-inflammatory (Gomathi and Gothandam 2019), and antioxidant (Li et al. 2018). Several *Streptomyces*, such as *Streptomyces hygroscopicus* subsp. *hygroscopicus* (Fitri et al. 2019; Nugraha et al. 2020), *Streptomyces* SUK10 (Zin et al. 2017), and *Streptomyces spectabilis* BCC 4785 (Isaka et al. 2002) have been studied for their antimalarial activity. *Streptomyces* sp. AB8 was obtained from the Lapindo mud volcano (Arifiyanto et al. 2020), has not been investigated for its antimalarial activity. This study aimed to conduct an initial screening of the antimicrobial and antimalarial activity of *Streptomyces* sp. AB8.

MATERIALS AND METHODS

Culture and fermentation

Streptomyces sp. strain AB8 was rejuvenated on yeast starch agar (YSA) medium, Yeast Starch Agar medium consisted of 2.0 g yeast extract (Oxoid™), 10.0 g soluble starch (Sigma-Aldrich), 15.0 g of agar powder (Sigma-Aldrich), and 1000 mL distilled water adjusted to a pH 7.3. Bacteria were cultured at room temperature for 1–5 days. The bacterial stock was cultured on a slanting agar medium. According to Arifiyanto et al. (2021) and Ezeonu

and Ejikeme (2016), Bacteria were characterized by several biochemical tests.

Gause's medium consists of soluble starch, KNO_3, NaCl, $MgSO_4.7H_2O$, K_2HPO_4, $FeSO_4.7H_2O$, and distilled water was used as the fermentation medium (Lin et al. 2012). One loop of bacterial inoculum was added to 100 mL of Gause's medium. The fermentation was carried out at a speed of 150 rpm in a shaking incubator at room temperature. Bacterial growth was observed for 10 days and measured using a spectrophotometer at 600 nm wavelength. A free-cell supernatant was separated from cell biomass using centrifugation at a speed of 6000 rpm, for 10 minutes. The supernatant was filtered through filter paper and extracted using a 1:1 methanol-ethyl acetate. A rotary evaporator was used to evaporate the solvent at 40-50°C for 40 minutes to obtain concentrate extract and was subsequently lyophilized into powder (Setyaningrum et al. 2021).

Antimicrobial activity

Fourteen day-olds of *Aspergillus* IK3 in a test tube were added with sterile distilled water parasite, then one percent molasses and 0.5 mL Tween 80 were added to the test tube, and homogenized using a vortex mixer. Conidia's suspension separated, then fungal spore cell density was adjusted to 10^8 cells/mL using a hemocytometer. It was observed under a microscope with 400x magnification (Agustina et al. 2019; Rosa et al. 2020).

Some swabs of bacterial suspension ages 18-24 hours were taken and then put into a test tube containing 5 mL of sterile 0.9% NaCl so that the turbidity was comparable to that of 0.5 McFarland suspension (Darmawan et al. 2017; Putri et al. 2021).

E. coli and *Dickeya zeae* were grown on Mueller Hinton Agar (MHA) at a 10^8 CFU/mL density in a Petri dish. One loop of *Streptomyces* sp. AB8 aged 24-hour was inoculated on media and challenged in the center of MHA media that has been inoculated with bacteria. An antifungal test was conducted on Potato Dextrose Agar media against *Aspergillus* IK3. Clear zones formation was observed after 24 hours of incubation at room temperature (Sumardi et al. 2020).

In vitro antimalarial assay

The in vitro antimalarial activity test was carried out using chloroquine-sensitive *Plasmodium falciparum* strain 3D7. As much as 1 mg of the extract was dissolved in 100 µL of DMSO (concentration 10.000 µg/mL) as a stock solution. The stock solution was diluted serially. Synchronous parasites (ring stage) with about 1% parasitemia were used in this test. Fresh type O-positive human erythrocytes were suspended at 4 percent hematocrit in a complete medium to maintain culture (Baniecki et al. 2007). Using a high-throughput liquid handler, parasites were supplemented into a 96-well plate. Two microliters of the test solution with various concentrations were added to the well. The final concentrations of the extract were 100, 10, 1, 0.1, and 0.01 µg/ mL. The gas mixture (O_2 5%, CO_2 5%, N_2 90%) was applied to the test plate that has been inserted into the chamber. The chamber containing the test plate was incubated for 48 hours at 37°C. The culture was then harvested and a thin blood layer was made with 20% Giemsa staining.

Data were analyzed by counting the number of infected erythrocytes per 1000 normal erythrocytes to determine the growth percentage of *Plasmodium* and inhibitory activity of the extract against *Plasmodium*. A blood smear was observed under a microscope. The growth percentage was calculated using the following formula;

$$\% \text{ growth} = \% \text{ parasitemia} - D0 \quad (1)$$

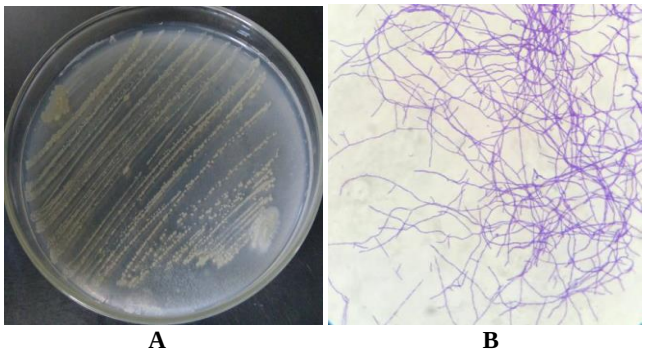
Where: D0 is the initial hour of growth (%)

The inhibition ability is expressed as a percentage using the formula below:

$$\text{The percentage of inhibition} = 100\ \% - ((Xu - Xk) \times 100\%) \quad (2)$$

Where:
Xu = % growth on the test solution
Xk = % growth on the negative control

The two formulas above were used to determine the concentration of extract to inhibit the growth of parasites by 50%. The lethal concentration 50 (LC_{50}) was estimated using probit analysis based on data of the inhibition percentage.



A **B**

Figure 1. A. Colonies of *Streptomyces* AB8 on Yeast Starch Agar; and B. The Gram-stained colony observed under microscope with 1000x magnification

RESULTS AND DISCUSSION

Antimicrobial activity of *Streptomyces* extract

Streptomyces sp. AB8 bacteria were isolated from rhizosphere soil in the Lapindo mud volcano field, Sidoarjo. Previous research showed that *Streptomyces* sp. AB8 could inhibit the growth of pathogenic bacteria (*Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 25922) (Arifiyanto et al. 2020). *Dickeya zeae* as a causative agent of stem and root rot in agricultural and plantation commodities (Aeny et al. 2020) was also inhibited by *Streptomyces* sp. AB8 (Table 1). The results suggested that crop damage caused by *D. zeae* could be controlled by the extract of *Streptomyces* sp. AB8 to overcome economic losses. These bacteria could also inhibit the growth of *Aspergillus* IK3, a cockroach pathogen, and *Escherichia coli*, which was isolated from wastewater around the campus of the University of Lampung.

Streptomyces is a Gram-positive bacteria that belong to the order *Actinobacteriales* with branched mycelium and chain-shaped. These bacteria could grow in a variety of environments, such as rhizosphere, marine debris, weathered leaf litter, plant endophytes, and insect symbionts (Mathew et al. 2020). The initial screening revealed that *Streptomyces* sp. AB8 could utilize glucose, fructose, cellulose, and lactose, galactose, sucrose, chitin, mannan, and amylum (Table 2) as its carbon source. As a decomposer group of microbes, *Streptomyces* has the ability to degrade complex molecules such as cellulose and mannan (Sasongko et al. 2015). Chitin is the main component of fungal cell walls. *Streptomyces* ability to decompose chitin potentially used to control mold. (Okay et al. 2013).

Biochemical characterization of *Streptomyces* sp. AB8

The development of new drug candidates has been driven by the resistance of pathogenic microbes to antibiotics and the risk to human health they pose. Microbial metabolites are increasingly being used to treat harmful bacteria. *Streptomyces*, a Gram-positive bacteria, have been shown to restrict the growth of various harmful bacteria. This bacterial community was thought to be in the middle of the bacteria-mold spectrum producing superior secondary metabolites such as triterpenoids, flavonoids, and alkaloids, and antibiotics. Gram-negative bacteria and pathogenic fungi were a challenge for humans health (Al-Ansari et al. 2019). Some of them were responsible for infection in humans. Pathogenic bacteria such as *Enterobacter aerogenes* and *Proteus mirabilis* were confirmed to be inhibited by *Streptomyces radiopugnans*, *Streptomyces atacamensis*, *Streptomyces fenghuangensis*, *Streptomyces verrucosisporus*, and *Streptomyces mangrove* isolates (Al-Ansari et al. 2019). In humans, *Aspergillus* genera cause diseases such as localized infections, deadly illnesses, allergic responses, and inhaled conidia (Mousavi et al. 2016). Production of lyase, chitinase, protease, and cellulase enzymes by *Streptomyces* is suspected of supporting antifungal activity. These enzymes can damage the cell wall of the mold, which was rich in components that were easily degraded by these enzymes. Therefore, *Streptomyces* spp could be used as antifungals (de Lima Procópio et al. 2012).

There are several pathways of antimicrobial mechanisms of *Streptomyces* compounds. In general, antimicrobial mechanisms are the interactions of pathogenic microorganisms' biochemical, genetic, and cellular structures. (Chevrette et al. 2019). Metabolites of *Streptomyces* spp may cause the inhibition of DNA division. Disorders of RNA synthesis affect the ability of pathogens to produce proteins that play an important role in the cellular structure of membranes, functional enzymes, and the cell walls of pathogenic microbes. They were also inhibited pathogenic metabolism and structural breakdown (de Lima Procópio et al. 2012).

Alkaloids and phenols were detected in synthetic Gause broth after the fermentation process (Table 2). Alkaloids are present in a variety of plants and animals, including microbes. Alkaloids extracted from biological compounds had long been used to treat a range of symptoms (Zhou et al. 2013), such as cancer, painkillers, high blood pressure, nervous system damage, Parkinson's disease, and antimalarials were among them. Meanwhile, indole alkaloids isolated from *Streptomyces* sp. CT37 has been demonstrated to inhibit *Candida albicans* ATCC 10231 at low concentrations (Fang et al. 2020).

Table 1. The diameter of the growth inhibition by *Streptomyces* sp. AB8

Microbes	Colony diameter ($\bar{x}$ mm)	Clear zone ($\bar{x}$ mm)
Escherichia coli	0.56	0.40
Dickeya zeae N-Unila 5	1.60	0.70
Dickeya zeae N-Unila 10	1.50	0.50
Aspergillus sp IK3	0.20	0.10

Table 2. Biochemical characters of *Streptomyces* sp. AB8

Test	Result	Test	Result
Glucose	+	Phenol	+
Fructose	+	Alkaloids	+
Cellulose	+	Flavonoids	-
Lactose	+	Saponin	-
Galactose	+	Triterpenoids	-
Sucrose	+	Anthraquinone glycosides	-
Chitinase	+	Tannin	-
Mannanase	+	Catalase	+
Lipase	-	Indole	+
Amylase	+	Protease	+

Note: + : positive reaction; - : negative reaction

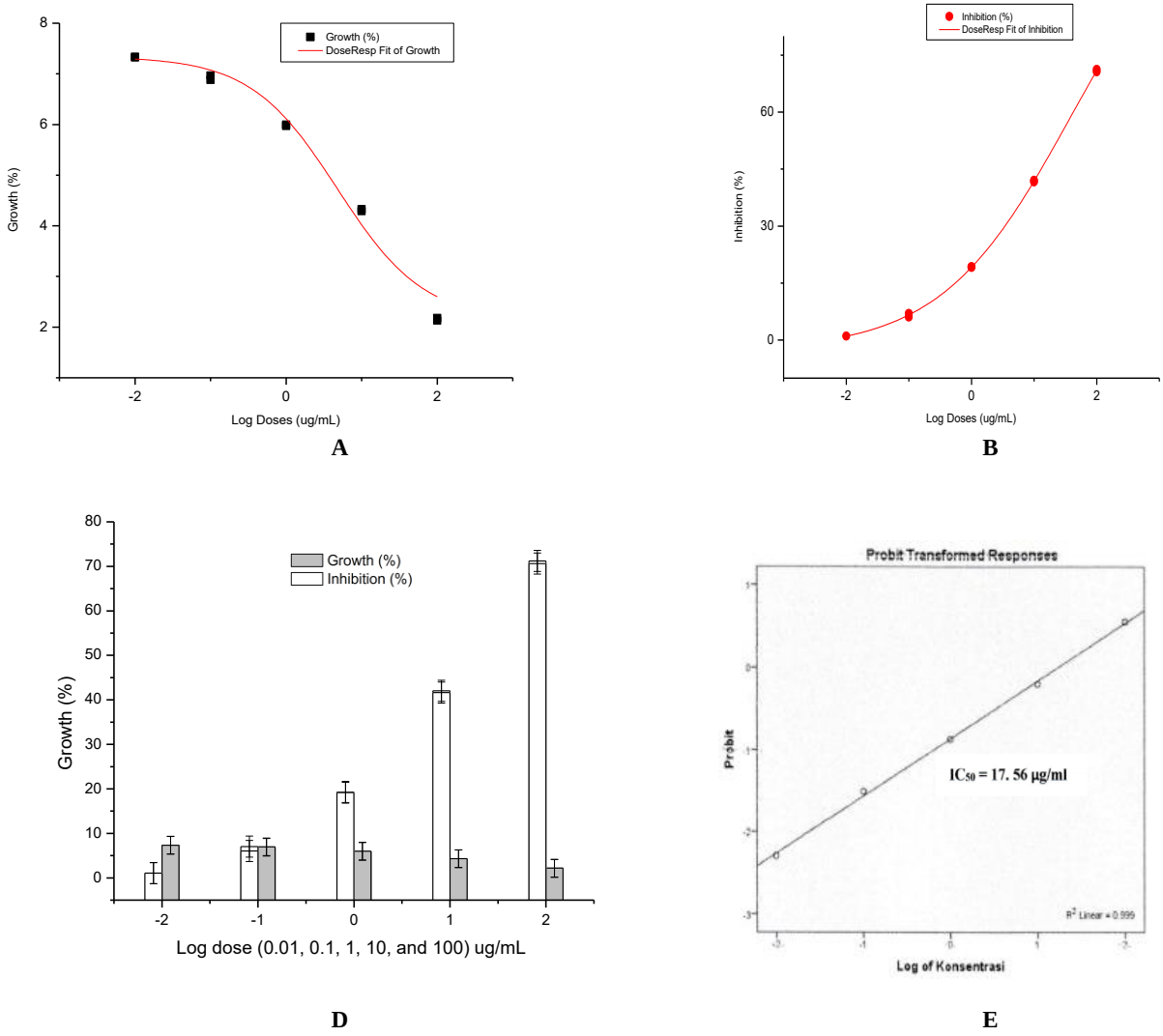


Figure 2. The effect of *Streptomyces* extracts on growth (A), growth inhibition (B), the ratio of growth to inhibition (C), the IC_{50} value of extract against *Plasmodium* parasites (D)

Alkaloids, which have a wide structural variation, are distinguished by the presence of a specific nitrogen atom. The majority of alkaloids have only one nitrogen atom, but others had up to five. This nitrogen was discovered in the form of a primary amine (RNH2), secondary amine (R2NH), or tertiary amine (R3NH) (R3NH) (Cushnie et al. 2014). Many alkaloids contain oxygen in addition to carbon, hydrogen, and nitrogen (Cushnie et al. 2014). Figure 3 showed that there was H bonded NH at an absorbance range of 3070-3350 cm^{-1}. This was presumed as a sign of the presence of alkaloids. The acetate and benzoic groups occurred at 1073.23 cm^{-1} and above, followed by double bond -CH=CH- (cis) at 650-750 cm^{-1}.

Phenols also present in the liquid media after the fermentation process (Table 2). It was confirmed by the emergence of spectroscopy bands around 990 until 1060 cm^{-1}. The secondary cyclic alcohols are found at these spectra. Phenols donate hydrogen to react with reactive nitrogen and oxygen species as an antioxidant (Pereira et al. 2009). Phenols also have a variety of biochemical and pharmacological properties such as antiviral, anticancer, antimalarial, and anti-inflammatory activities.

Antimalarial activity

Streptomyces extract at a concentration of 100 µg/mL was able to inhibit parasite growth up to 70.92%, with an average parasite growth rate of 2.15%. Parasitic growth was suppressed at a rate of 4.31 % with an inhibitory percentage of 41.84 percent at a concentration of 10 µg/mL. Extract concentration of 1 µg/mL resulted in parasite growth of 5.96% with an inhibition percentage of 19.23%. While a concentration of 0.1 µg/mL resulted in inhibition of 6.55% with parasite growth of 6.93 and a concentration of 0.01 µg/mL resulted in inhibition of 1% with growth of 7.33%. Overall, the treatment resulted in lower growth compared to the control. The increasing extract concentration was linear with inhibition, and vice versa, parasite growth increased with decreasing concentration (Figure 2). The inhibitory concentration to suppress a half parasites population (IC_{50}) was achieved at 17.56 µg/mL.

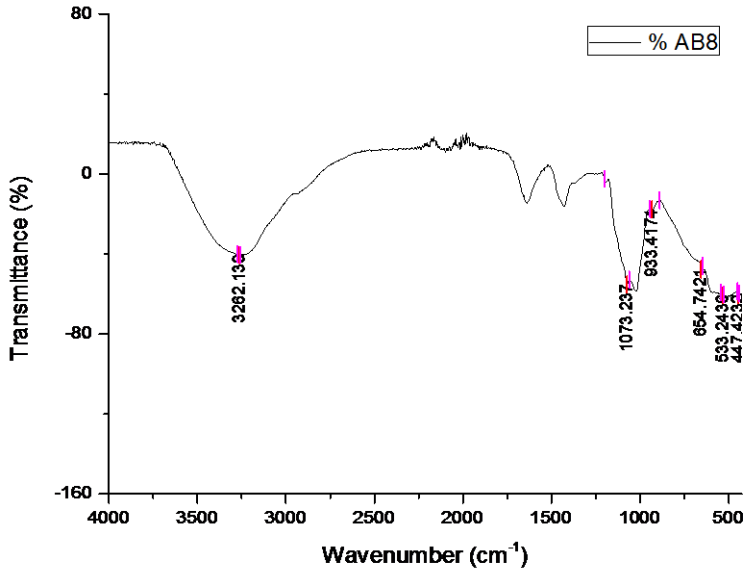


Figure 3. FT-IR spectra of crude extract produced by *Streptomyces* sp AB8

Several past studies have reported on the mechanism of alkaloids in preventing the growth of plasmodial and microbiological infections. Alkaloids inhibit nucleic acid synthesis by suppressing the enzyme dihydrofolate reductase in cell-free (Raimondi et al. 2019). It can prevent cell division by inhibiting the Z-ring formation in bacteria (Keffer et al. 2013). Another study by Godlewska-Żyłkiewicz et al. (2020) showed that alkaloids are sufficient to initiate bacterial homeostasis and disrupt the stability of the outer membrane and cytoplasmic (Cushnie et al. 2014). Alkaloids were also able to restrain fimbria-dependent biofilm formation in *E. coli* (Notarte et al. 2019).

Streptomyces cellulosae strain TES17 produced phenolic compounds with strong antioxidant properties and effective in damaging lung cancer cell lines (Rani et al. 2018). The antibacterial and antimalarial mechanism of phenolic compounds possibly due to the ability to inhibit respiratory electron transport systems, reduced outer membrane containing lipopolysaccharides, disrupts the structure of the cell wall, and the synthesis of cell wall becomes inefficient, affect the permeability, and disrupt the cell division protein FtsZ-ring (Aldulaimi et al. 2019).

The findings on *Streptomyces* extract's antimalarial activity were used as a starting point. Further study to determine its efficacy using an *ex vivo* or *in vitro* approach is needed (Sinha et al. 2017). Peter's Test or 4-day suppression test, histopathological observation, and cytotoxicity for non-cell-targeted are required (Kifle et al. 2020). A metabolite is categorized as antiplasmodial if this metabolite can suppress parasite growth by more than 30% compared to negative controls after completing a set of in vivo tests (Castro et al. 1996). In conclusion, the qualitative test approach was supported by infrared spectroscopic data indicating the presence of phenolic compounds and alkaloids from the bacterial metabolite *Streptomyces* AB8. The crude extract of *Streptomyces* sp AB8 can inhibit *Plasmodium*, with an IC_{50} value of 17.56 µg/mL. The extract of *Streptomyces* sp. AB8 could inhibit the growth of *Dickeya zeae* N-Unila 5, *Dickeya zeae* N-Unila 10, *Aspergillus* sp IK3, and *Escherichia coli*. These results on antimalarial and antimicrobial are preliminary results.

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