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Determination of Antioxidant and Antibacterial Compound of *Streptomyces* sp. Sae4034 and Its Inhibitory Mechanism on *Staphylococcus Aureus*

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Abstract
The genus Streptomyces is known as producers of bioactive compounds that have antibacterial and antioxidant properties. *Streptomyces* sp. SAE4034 was known to produce antibacterial compounds that can inhibit and kill pathogenic bacteria, including *Staphylococcus aureus*, but the group of antibacterial compounds and their inhibitory mechanisms were not yet known. The ability of *Streptomyces* sp. SAE4034 in producing antioxidants is unknown yet. This research aimed to determine the classification of antibacterial compounds produced by *Streptomyces* sp. SAE4034, its potency in producing antioxidant compounds, and to observe the inhibitory mechanisms of antibacterial compounds produced by *Streptomyces* sp. SAE4034 against *S. aureus* using cell leakage method. The results of this research showed that *Streptomyces* sp. SAE4034 produce compounds in the groups of alkaloid, terpenoid, flavonoid, and polyphenol. Compounds with Rf values 0.40 and 0.47 which includes into polyphenol and 0.72 which includes into alkaloid group has the ability to inhibit the growth of *S. aureus*. The MIC value was 256 µg/mL. *Streptomyces* sp. SAE4034 has a very strong antioxidant activity that can scavenge DPPH free radical with IC_{50} value of 32,14 ppm and has an antioxidant enzyme activity that is superoxide dismutase amount of 63,10 U/mL The inhibition mechanism of Streptomyces sp. SAE4034 crude extract against *S. aureus* was through leaking of the cell wall or cell membrane resulting in the release of nucleic acid and protein of *S. aureus* cells and the highest cell leakage was at 2 doses of MIC.

Key Words: *Antibacteria, Antioxidant, Cell leakage, Streptomyces sp. SAE4034*

INTRODUCTION

Streptomyces is known as bacteria that produce bioactive compounds (Hasani *et al*., 2014). More than 7000 bioactive compounds used clinically were the result of Streptomyces, including antibacteria and antioxidant drugs (Tan *et al*., 2017). *Streptomyces* sp. SAE4034 is a collection of isolates owned by the Microbiology Laboratory, Faculty of Biology, Jenderal Sudirman University. This isolate was isolated from the rhizosphere mud of the *Rhizosphora apiculata* mangrove in Segara Anakan, Cilacap. *Streptomyces* sp. SAE4034 is known to produce antibacterial compounds that can inhibit and kill pathogenic bacteria, including *Staphylococcus aureus* (Ryandini *et al*., 2018). However, the group of antibacteria compounds and the mechanism of their inhibition against *S. aureus* are not yet known.

S. aureus is a Gram-positive bacterium found in the human body, especially on the skin and nasal cavity. *S. aureus* can also cause various infectious diseases such as endocarditis, pneumonia, nosocomial, and food poisoning in animals and humans and it is commensal or pathogenic (Gnanamani *et al*., 2017). The cell wall structure of *S. aureus* consists of peptidoglycan as the main component, which is not an effective barrier and is not protected by the outer layer. These conditions may weaken the cell wall or cell membrane so that antibacterial compounds can easily lyse cells. When a cell undergoes lysis, cell contents such as proteins or nucleic acids will come out of the cell. Cell leakage tests can predict bacterial inhibition by antibacterial compounds (Singh *et al*., 2014).

The potency of *Streptomyces* sp. SAE4034 in producing antioxidants is not yet known. Antioxidants are compounds that can inhibit oxidative reactions caused by the attachment of free radicals that can cause serious diseases such as heart disease, cancer, premature aging, and others (Tan *et al*., 2017). The methods used in this study to test the antioxidant activity are the 2,2 diphenyl-1-picrylhydrazyl (DPPH) analysis method and Superoxide Dismutase (SOD) enzyme activity method. The DPPH method can determine the value of the inhibitory concentration$_{50}$ (IC_{50}) of antioxidant compounds. IC_{50} is a value obtained from the concentration of antioxidant compounds that can reduce or eliminate 50% of free radical activity (Ikhlas, 2013). SOD is an antioxidant enzyme that can counteract free radicals (Rahman *et al*., 2012).

This research aimed to determine the classification of antibacterial compounds produced by *Streptomyces* sp. SAE4034, its potency in

producing antioxidant compounds, and to observe the inhibitory mechanisms of antibacterial compounds produced against *S. aureus* using cell leakage method

MATERIAL AND METHOD

The materials used include isolates of *Streptomyces* sp. SAE4034 and *S. aureus* obtained from Microbiology Laboratory collection, media: Muller Hinton Agar (MHA, Merck), Starch Casein Nitrate Agar (SCNA, g/L: soluble starch 10, casein 0,3, KNO_3 2, $MgSO_4.7H_2O$ 0,05, K_2HPO_4 2, NaCl 2, $CaCO_3$ 0,02, $FeSO_4.7H_2O$ 0,01, Agar 18, distilled water up to 1000mL), Starch Casein Nitrate Broth (SCNB, similar to SCNA without agar), Nutrient Agar (NA, Oxoid), Nutrient Broth (NB, Oxoid), chemicals and reagents: alcohol 70%, ethanol 96%, Lugol's iodine, spiritus, crystal violet, safranin, H_2O_2, Tetramethyl-para-phenylenediamine-dihydrochloride, methanol, chloramphenicol, pure vitamin C, 2.2-Diphenyl-1-picrylhydrazyl (DPPH), Superdioxide Dismutase (SOD) Kit, phosphate buffer pH 7, tween 80, toluene, ethyl acetate, acetic acid, silica gel GF_{254}, Whatman paper no 1, Dragendroff's reagent, vanillin-sulfuric acid reagent, citroborate acid, and 6 mm paper disc.

The tools used include autoclave (Hirayama, HL-36Ae), Laminar Air Flow (Labconco), microwave (Menumaster), Ultra Violet Cabinet (Camag), refrigerator (LG), temperature centrifuge machine (Hitachi Himzc CR7 Type), light microscope and stereo microscope (Olympus), spectrophotometer UV-Vis (Shimadzu UV Mini 1240), oven (Memmert), incubator (Memmert), analytical balance (Ohause, EO 2140), micropipette (Socorex) and tip, ose needle, glass wares (Duran or Pyrex), microwell plate (Biologyx), and TLC tools kit (Merck).

The research was conducted with survey method. The research parameters observed were inhibition zone diameter from antibacterial activity of *Streptomyces* sp. SAE4034 extract against *S. aureus*, Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values. Cell leakage due to the inhibitory mechanism of *S. aureus* by *Streptomyces* sp. SAE4034 was based on the Optical Density (OD) value and the classification of compounds contained in *Streptomyces* sp. SAE4034 extracts using the TLC based on the Retention factor (Rf) value and spot characteristics in the phytochemical screening method. The IC_{50} value of antioxidant was measured using DPPH method and SOD enzyme activity value.

Reculture isolate of *Streptomyces* sp. SAE4034 and *S. aureus*

Streptomyces sp. SAE4034 isolate was taken as much as one ose and streaked continuously on SCNA medium, and incubated at room temperature for at least 5 days. One ose of *S. aureus* isolate was taken and streaked continuously on slant NA medium in a test tube then incubated at room temperature for 24 hours.

Colony characterization of *Streptomyces* sp. SAE4034

Streptomyces sp. SAE4034 isolate was taken out as much as 1 ose, and quadrant streak was performed on SCNA plate medium and incubated at room temperature for > 7 days. Morphological observations were carried out using a stereomicroscope. The colony morphology observed included substrate and aerial mycelium, colony shape, colony size, colony surface, mycelium color, and pigmentation.

Gram staining of hypha (Cappucino & Sherman, 2014)

Streptomyces sp. SAE4034 isolate was taken out as much as 1 ose and put on object glass that had been dropped with distilled water. The isolate then fixed over bunsen fire 2-3 times. Crystal violet reagent as a basic dye was dropped on the isolate and left for 1 minute, object glass then washed with flowing distilled water and dried. Lugol's iodine reagent as a mordant dye was dropped on the isolate and allowed to stand for 1 minute, object glass then washed with flowing distilled water and dried. Ethanol reagent 96% as the bleaching agent was dropped repeatedly on the isolate until the last drop of ethanol that falls is clear, object glass then washed with flowing distilled water and dried. Safranin reagent as a comparison dye was dropped on the isolate and allowed to stand for 45 seconds, object-glass is then washed with flowing distilled water and dried. The preparation was observed using a light microscope with 1000x magnification. The positive interpretation of *Streptomyces* sp. SAE4034 is a purple cell indicating that it is a Gram-positive bacterium.

Aerial hypha characterization

Streptomyces sp. SAE4034 isolate was taken out as much as 1 ose and continuous streak was performed on SCNA medium in a Petri dish. The culture was incubated at room temperature for > 7 days. Aerial hypha characteristic observations include hyphal shape, spore chain, and branches are carried out using a light microscope.

Catalase assay of *Streptomyces* sp. SAE4034

Streptomyces sp. SAE4034 isolate was taken out as much as 1 ose and put on the object-glass using ose needle. H_2O_2 reagent was dropped on the bacterial isolates. The positive interpretation of *Streptomyces* sp. SAE4034 was characterized by the presence of gas bubbles above the isolate.

Oxidase assay of *Streptomyces* sp. SAE4034

Streptomyces sp. SAE4034 isolate was taken as much as 1 ose and put on filter paper above object glass using an ose needle. Tetramethyl- para-phenhylenediamine-dihydrochloride reagent was dropped on the bacterial isolates. The positive interpretation was characterized by the presence of dark-blue color in the colony.

Extract production of *Streptomyces* sp. SAE4034

Pure culture of *Streptomyces* sp. SAE4034 isolate was taken 1 ose and carried out tightly continuous streak on SCNA medium, then incubated for 7 days at room temperature. Isolate that grows was then made plugs using the sterile tip and cultivated in 200 mL of SCNB medium and incubated for 28 days at room temperature. On day 28th, culture isolate was filtered using Whatman No.1 filter paper, to separate mycelium. The mycelium was dried and measured its dry weight using an analytical balance. The filtrate was then extracted using ethyl acetate solutions by shaking for 40 minutes until separated between the media and the organic solvent. The organic solvent containing extract was separated and then evaporated at 70ºC for 2 hours.

Antibacterial activity test of *Streptomyces* sp. SAE4034 extract against *S. aureus* using diffusion method (Kirby Bauer method

One ose *S. ureus* was inoculated into NB medium then incubated at 37 ºC for 24 hours. Amount 1 mL of pathogenic bacterial culture was put into a Petri dish and then 20 mL of MHA medium was added by pour plate. Disc paper with 6 mm diameter was dropped with extract of *Streptomyces* sp. SAE4034 was placed on MHA medium. For positive control, disc paper with 6 mm diameter is dripped with antibiotic chloramphenicol. For negative control, disc paper with 6 mm diameter was dropped with 20 µL of aquadest and placed on the MHA medium. Petri dishes are then incubated at 37ºC for 24 hours, observed and calculated the inhibition zone formed by the formula:

$$D(mm) = \frac{D1+D2}{2}$$

Details:
D= average diameter of inhibition zone
D1= vertical diameter of inhibition zone
D2= horizontal diameter of inhibition zone

Determination of Minimum Inhibitory Concentration (MIC) value of *Streptomyces* sp. SAE4034 extract toward *S.aureus*

Determination of MIC value was carried out using NB as much as 0.1 mL of microwell plate then each well are added with *Streptomyces* sp. SAE4034 extract which had been dissolved in aquadest with concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/mL. Total of 0.1 mL of *S. aureus* culture was added to each well. For positive control, it was done by adding 0.1 mL of NB medium then added with chloramphenicol antibiotics, then added with 0.1 mL of the *S. aureus* culture. Meanwhile, for negative control, this was done by adding 0.1 mL of *S. aureus* culture to 0.1mL NB medium. Microwell plate that has been filled with the test solution, positive control, and negative control were incubated at 37ºC for 24 hours. The well containing lowest concentration with clear suspension shows that the related concentration becomes the minimum inhibitory concentration of the *Streptomyces* sp. SAE4034 extract antibacterial activity.

Determination of Minimum Bactericidal Concentration (MBC) value of *Streptomyces* sp. SAE4034

Determination of the MBC value was carried out by taking 1 ose of test solution used in the MIC test and then streaking it on MHA media prepared in Petri dish, then incubated at 37ºC for 24 hours. The parameter used was the presence or absence of bacterial growth on the agar media, indicated by the presence or absence of white spots on the agar media.

Characterization of compounds from *Streptomyces* sp. SAE4034 extract using thin layer chromatography (TLC) method

Silica plate (silica gel GF254) 10 cm x 20 cm in size that has been cut into appropriate size (1,0 cm x 10 cm) was prepared and activated in an oven at 150ºC for 10 minutes. Extract of *Streptomyces* sp. SAE4034, as much as 10 µL was spotted on the plate as an initial marker of the stationary phase. The plates were then immersed in eluent solution (mobile phase) with a mixture of toluene: ethyl acetate: methanol (10:0.2:3). After the compound was eluted, the plate was dried at 80ºC for 2 hours. The plate is observed under UV light with 366 nm wavelength and measured the Rf value formed by the following formula:

$$Rf\ value = \frac{As}{Ao}$$

Details:
As = Distance traveled by the sample
Ao = Distance traveled by the solvent

Bioactive compound analysis using phytochemical screening method

A phytochemical test was used to identify spots on the chromatographic plate based on the type of compounds. The resulting group of compounds can be alkaloids, terpenoid, flavonoids, polyphenols, or polyketide. The identification of the polyketide compound was carried out by observing the spots at the 366 nm wavelength. The identification of alkaloid compound groups was carried out by detecting the eluted spots by spraying the plates using Dragendroff's reagent and then heating them in an oven at 100°C for 5 minutes. The presence of alkaloids is indicated by the formation of orange or orange-yellow spots. The identification of terpenoid compound was carried out by detecting the eluted spots by spraying it on the plate using a vanillin-sulfuric acid reagent, then heating it in an oven at 100°C for 10 minutes. The presence of terpenoids was indicated by the formation of black-purple spots.

The identification of flavonoid compound was carried out by detecting the eluted spots by spraying on the plate using citroborate reagent, then heating it in an oven at 100°C for 5 minutes. The presence of flavonoids was indicated by the formation of yellow, green, or purplish-blue spots, which can be observed under 366 nm UV light. The identification of polyphenols compound is carried out by detecting spots eluted by spraying on a plate with $FeCl_3$ reagent, the chromatogram plate that has been sprayed is heated in an oven for 5 minutes at a temperature of 100ºC.

Bioautography assay

Bioautography test aims as a method of active compounds detection that has antibacterial activity. Amount 5-6 mL culture of *S. aureus* in NB medium was sprayed on the TLC plate surface evenly. The TLC plate was then incubated in a petri dish that had been given wet cotton to create a humid atmosphere for 1 x 24 hours at 37ºC. The TLC plate that had been sprayed with *S. aureus* was then sprayed with TTC (Tetrazolium Chloride) 2% and then re-incubated for 1 x 24 hours at 37ºC. Observations were made by looking at the inhibition zone formed by interpreting creamy spots in areas that were not overgrown with bacteria against purple background. The evaluation of antibacterial activity is expressed as presence of inhibition zones and the Rf values from previous analytical TLC to reflect the activity.

Antioxidant activity testing with the DPPH method

The extract of *Streptomyces* sp. SAE4034 0.01 g dissolved in methanol until it reaches 200 ppm concentration. The dissolved extract was then made into the concentration of 10, 20, 30, and 40 µg/mL. An amount of 2 mL for each concentration was taken and placed in a different test tube. Each concentration of the solution was added with 2 mL of 0.1 mM DPPH solution, then homogenized. Ascorbic acid (vitamin C) as a standard comparison solution was dissolved in 200 ppm. Concentrations of 10, 20, 30, and 40 µg/mL were made, and 2 mL was taken for each concentration and placed in different test tubes. Each solution's concentration was added with 2 mL of 0.1 mM DPPH solution as a control, then homogenized. The sample solution and comparison solution that had been added with DPPH was then incubated at room temperature for 30 minutes in a dark room. The positive interpretation of DPPH inhibition was characterized by a change in color of the solution from the original purple to yellow. DPPH solution was used as a control solution and methanol was used as a blank solution. The absorbance of sample solution, comparison solution, control solution, and blank solution were measured by a spectrophotometer at 517 nm wavelength. The DPPH absorption inhibition presentation was calculated by the formula (Rao and Rao, 2013):

$$\text{DPPH inhibition} = \frac{Control\ OD - sample\ OD}{Control\ OD} \text{ x } 100\%$$

Details:
DPPH Inhibition (%)
Control OD: control absorbance
Sample OD: sample absorbance

Sample solution and comparison solution then calculated the minimum inhibition value to inhibit DPPH radical or the half inhibitory concentration (IC_{50}) using the equation (50 = ax + b) from the linear regression curve. According to Molyneux (2004) the antioxidant properties based on the IC50 value can be stated as follows:

Table 1. IC Value of Antioxidant Activity

IC_{50} value	Antioxidant properties
<50 ppm	Very strong
50 ppm-100 ppm	Strong
100 ppm-150 ppm	Moderate
150 ppm-200 ppm	Weak

Measurement of SOD enzyme activity

SOD enzyme activity test was carried out with Superoxide Dismutase Assay Kit from Randox laboratories. Measurement begins with the preparation of sample solution, standard solution, and blank solution. The sample solution used 20 µL *Streptomyces* sp. SAE4034 extract added 1.000 µL of SOD buffer solution and 100 µL of xanthine oxidase solution in the sample tube. The standard solution used 20 µL standard solution added with 1000 µL SOD buffer solution and 100 µL xanthine oxidase solution in a standard tube. The blank solution uses 1.000 µL of SOD buffer solution added with 100 µL of xanthine oxidase. Three solutions were homogenized and measured at 520 nm wavelength using a UV-Vis spectrophotometer. SOD enzyme activity was measured using the formula (Randox laboratories, 2009):

$$\text{SOD activity (U/mL)} = \frac{As}{AO} \text{ x Ks}$$

Details:
As= the absorbance value of the sample solution
Ao= the absorbance value of standard solution
Ks= standard concentration (30.65) mU/L

Analysis of protein and nucleic acid leakage of S. aureus bacterial cells

Pathogenic bacterial suspension of *S. aureus* was cultured in NB media for 24 hours at room temperature, then 10 mL is taken, added with 0.5 mL of tween 80 in Falcon tube. The test bacteria solution then cold centrifuged at 3500 rpm for 20 minutes. The filtrate was removed and pellets in the tube were washed with 0.1% phosphate buffer pH 7,0 for 2 times, then suspended in 10 mL of pH 7.0 phosphate buffer. Furthermore, the extract of *Streptomyces* sp. SAE4034 was added with the concentration of 1 MIC (256 µg/mL), 2 MIC (2x256 µg/mL) and

control, incubated in a shaker incubator at 37ºC for 24 hours. The suspension was centrifuged at 3500 rpm for 15 minutes. The supernatant was taken and its absorbance measured at 260 nm and 280 nm wavelength using UV-Vis spectrophotometer. The 280 nm wavelength was used to determine nitrogen from cell proteins, while the 260 nm wavelength was used to determine nitrogen from cell nucleic acids.

RESULT AND DISCUSSION

The confirmative test result of *Streptomyces* sp. SAE4034 isolate showed that the macromorphological characteristics of the isolate that was 14 days old and grown on the SCNA medium was circular-shaped colonies, 2-4 mm in colony diameter, powdery colony surfaces, white-colored mycelium substrates, cream-colored aerial mycelium, and pigments was not diffused. Characteristics of micromorphology isolates that was 20 days old that had a spiral and fragmenting branches shape, long chained spores and branched with 20-50 spores, fragmented spore chains into cocoids. *Streptomyces* sp. SAE4034 isolate were Gram positive, positive catalase, and positive oxidase. The results of this characterization are in accordance with Ryandini *et al*. (2018).

Antibacterial compounds are produced from *Streptomyces* sp. SAE4034 which grown in SCNB medium for 28 days at room temperature. According to Wardana *et al*. (2017), at 28 days old of incubation there was no biomass increase in isolate but the inhibition ability to bacteria was increase. According to Tyas *et al*. (2021), liquid culture of *Streptomyces* sp. SAE4034 with 28 days old has entered the stationary phase. Secondary metabolite compounds such as antioxidants and antibacteria are produced by bacteria in the stationary phase. According to Pratiwi *et al*. (2018), secondary metabolites are products resulting from the secondary metabolism of microorganisms. Secondary metabolite products are required for microorganisms as emergency nutrients to survive in the stationer phase. Liquid culture of *Streptomyces* sp. SAE4034 was then filtered to separate mycelium biomass and fermented liquid. The yield obtained in this research was 32.182 %.

The inhibition test result of the extract from *Streptomyces* sp. SAE4034 using Kirby Bauer method (Table 2 and Figure 1) showed that *Streptomyces* sp. SAE4034 extract can inhibit the growth of *S. aureus* characterized by the presence of a clear zone around the disc paper. Disc paper that has been dripped with extract with a concentration of 256 µg/mL produces an average diameter of the inhibition zone of 15 mm In this test uses a positive control in the form of chloramphenicol with a concentration of 10 mg/mL. It produces an inhibition zone diameter of 25.5 mm. Inhibitory activity of *Streptomyces* sp. SAE4034 extract against *S. aureus* was categorized in the moderate category (Nedialkova & Naidenova, 2005). The difference in the inhibition zone diameter formed in the extract and the positive control was thought to be due to the presence of different concentrations between them. The positive control was chloramphenicol which was a broad spectrum antibiotic, while the crude extract still contained a mixture of metabolites, so that the inhibition zone formed by chloramphenicol was wider than the inhibition zone formed by the crude extract.The inhibitory ability of *Streptomyces* sp. SAE4034 extract against *S. aureus* was observed for more than 48 hours and still showed consistent inhibitory results it can be said that the extract has stable antibacterial abilities (Ryandini *et al*., 2018).

Table 2. Inhibition Zone Diameter Result of Antibacterial Activity from *Streptomyces* sp. SAE4034 against *S. Aureus*

No	Sample	Diameter (mm)	Category
1	*Streptomyces* sp. SAE4034 extract	15	Moderate
2	Positive control	25.5	Strong
3	Negative control	0	-

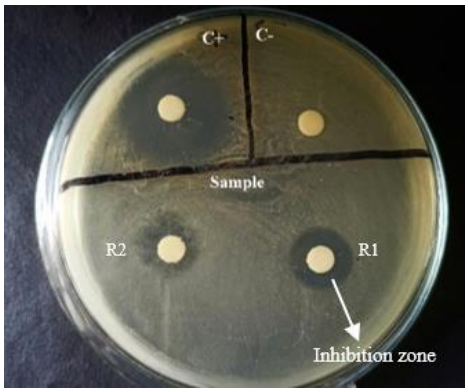

Figure 1. Inhibition Zone Diameter of Antibacterial Activity from *Streptomyces* sp. SAE4034 against *S. aureus*
Details: Control positive (C+); Control Negative (C-); Sample of SAE4034 extract repetition 1 (R1); Sample of *Streptomyces* sp. SAE4034 extract repetition 2 (R2)

The MIC test on *Streptomyces* sp. SAE4034 extract was carried out with microdilution method using 96-microwell plate. The MIC test was carried out to determine the minimum concentration of the extract that could inhibit the growth of bacteria. The results of the MIC test *Streptomyces* sp. SAE4034 extract against *S. aureus* (Figure 2) showed that the extract was able to inhibit the growth of *S. aureus* at a minimum inhibitory concentration of 256 µg/mL which was categorized as a strong inhibitor (Taechowisan *et al*., 2017).

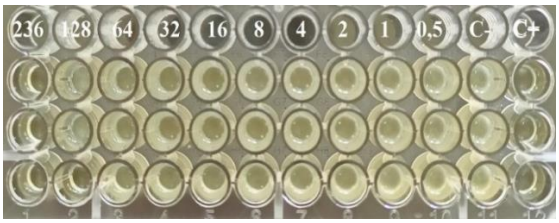

Figure 2. The MIC Test Result of *Streptomyces* sp. SAE4034 Extract Against *S. aureus*

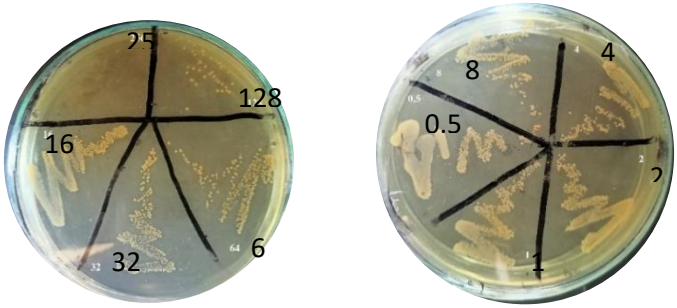

Figure 3. The MBC Test Result of *Streptomyces* sp. SAE4034 Against *S. aureus*
Details: Concentration of 256 µg/mL (no bacterial growth); Concentration of 128, 64, 32, 16, 8, 4, 2, 1, 0.5 µg/mL (bacterial growth performed)

The results obtained from the MIC test are then used for MBC testing. The results of the MBC test (Figure 3) showed that *Streptomyces* sp. SAE4034 extract can kill or bactericidal against *S. aureus* at a concentration of 256 µg/mL. According to Saraswati (2011), the MBC value can be seen from the presence or absence of *S. aureus* colonies growing on the media.

The results of bioactive compounds separation in the *Streptomyces* sp. SAE4034 extract was carried out by the TLC method. The stationary phase used in this study was silica gel GF_{254} and the mobile phase used was toluene: acetic ethyl: methanol (10:0.2:3). The results of TLC method obtained seven spots of compounds on the plate with Rf value of 0.27, 0.33, 0.36, 0.40, 0.47, 0.72, and 0.86 (Figure 4). Based on Ryandini *et al*. (2018), separation of bioactive compounds in the *Streptomyces* sp. SAE4034 extract using chloroform: ethyl acetate: acetic acid (3:3:1) mobile phase obtained 4 spots of the compound on the plate with Rf values of 0.36, 0.45, 0.54, and 0.6. The difference in the visible of Rf value could be due to the difference in the eluent used as the mobile phase. According to Wulandari (2011), the factors that cause the difference in Rf value includes the sample preparation before TLC testing, the characteristics and size of the plate used, the volume and composition of the eluent as a mobile phase, equilibrium conditions, moisture on the plate, temperature in chamber, and sample size.

The TLC plate was then identified the group of compounds using a phytochemical test (Figure 5). The compound spots with a Rf value of 0.86 were identified as a group of terpenoid compounds with purple-black interpretation after being sprayed using vanillin sulfuric acid reagent and heated in an oven at 100ºC for 10 minutes. The compound spots with Rf values of 0.36, 0.40, and 0.47 were identified as a group of polyphenolic compounds with the interpretation of blue-black spots after being sprayed using $FeCl_3$ reagent and heated in an oven at 100ºC for 5 minutes. Phenolic compounds when sprayed using $FeCl_3$ reagent will give a purple, blue or black color interpretation (Harborne, 1987). The color change reaction in polyphenolic compounds occurs due to the formation of Fe^{3+} ion complexes with -OH groups of phenolic compounds (Rohadi, 2016).

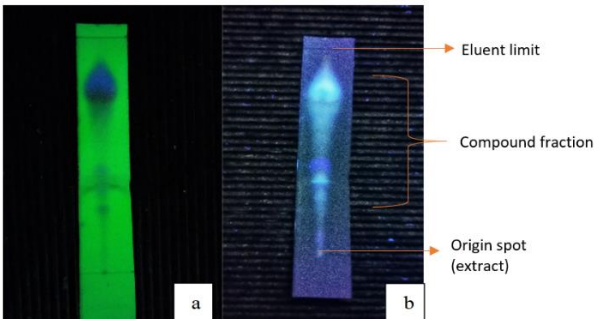

Figure 4. The Results of Compound Separation from *Streptomyces* sp. SAE4034 Extract using TLC Method
Details: TLC visualization result at λ 254 nm (a); TLC visualization result at λ 366 nm (b)

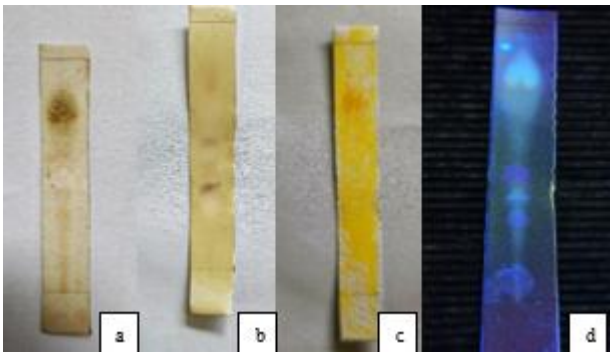

Figure 5. Results of Compound Identification from *Streptomyces* sp. SAE4034 Extract using Phytochemical Test
Details: Terpenoid compound using vanillin sulphuric acid reagent (a); polyphenolic compound FeCl3 reagent (b); alkaloid compound using Dragendroff reagent (c); Flavonoid compound using citroborate reagent in UV visualization λ 366 nm (d)

The spots of compounds with Rf values of 0.27 and 0.33 were identified as a group of flavonoid compounds with a purplish-blue interpretation which were observed using a UV transilluminator with a wavelength of 366 nm after being sprayed using citroborate reagent and heated in an oven at 100ºC for 5 minutes. The color change reaction in flavonoid compounds is caused by H_3BO_3 which can form chelate complexes with ortho dihydroxy and hydroxy carbonyl in flavonoids. Flavonoid compounds are indicated by the presence of yellow, green, or purplish-blue spots visible at 366 nm UV light (Wagner & Baldt, 1996). The compound spots with a Rf value of 0.72 were identified as a group of alkaloid compounds with the interpretation of yellowish orange spots after being sprayed using Dragendroff's reagent and heated in an oven at 100ºC for 5 minutes. Alkaloids are indicated by the presence of red-orange or yellowish-orange spots on a yellow background with the detection of Dragendroff's reagent (Wagner & Bladt, 1996). According to Begum *et al* (2017), the results of phytochemical screening of a compound may vary depending on the eluent used during the TLC test.

Alkaloids, terpenoids, flavonoids, and polyphenols compounds obtained from phytochemical screening are known to have the ability as antioxidant compounds. The mechanism of action of flavonoids is through the chelation of metal ions which can cause oxidation reactions such as Fe^{2+} and Cu^{2+} ions which can form the formation of free radicals. In addition, flavonoids can also donate hydrogen atoms to free radicals caused by ROS (Arnanda & Nuwarda, 2019). Polyphenols have a mechanism to scavenge free radicals by donating H atoms from the active OH group to free radicals (Papuc *et al*., 2017). Terpenoids are a type of lipophilic antioxidant that can inhibit lipid peroxidation, besides that the antioxidant mechanism of terpenoids is to fight reactive species such as superoxide and metal ion chelates Fe^{2+} and Cu^{2+} (Jafar *et al*., 2020). Alkaloids as antioxidant compounds have a mechanism of donating H atoms to free radicals (Sudirman, 2011).

The antibacterial ability of the compound group from *Streptomyces* sp. SAE4034 extract was detected by direct bioautography method. Direct bioautography was performed by inoculation of *S. aureus* directly on the TLC plate which was then sprayed with TTC 2% reagent for visualization. The dehydrogenase reaction of *S. aureus* inoculated on the plate will convert TTC into purple formazan which will give a purple color interpretation on the plate. Compounds on the plate that can inhibit the growth of *S. aureus* are indicated by the presence of an inhibition zone with the interpretation of cream-colored spots that appear on the plate with a purple background (Choma & Jesionek, 2015). Compounds that can inhibit growth of *S. aureus* based on the results of direct bioautography (Figure 6) showed that the polyphenolic compound with Rf value 0.40 and 0.47, and alkaloid compound with Rf value 0.72 can inhibit the growth of *S. aureus*. According to Rachmawati *et al*. (2011), the antibacterial mechanism of phenolic compounds at low concentrations is through cytoplasmic destruction which can cause nuclear leakage, while at high concentrations phenolic compounds can coagulate with cellular proteins. Bacteria that are in the cell division phase will be more easily inhibited by phenolic compounds. During the cell division phase, the phospholipid layer around the cell is in a very thin condition, so that phenolic compounds are easier to damage the contents of bacterial cells. According to Othman *et al*. (2019), The action mechanism of antibacterial from alkaloids compound is interfere the constituent component of peptidoglycan in bacterial cells, so that the cell wall layer is not completely formed and causing cell death.

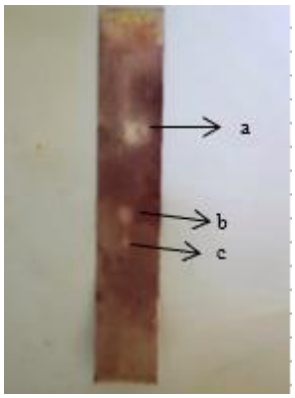

Figure 6. Inhibition of *Streptomyces* sp. SAE4034 Extract against *S. aureus* using Direct Bioautography Method
Details: alkaloid compound with Rf value 0.72 (a); polyphenolic compound with Rf value 0.47 (b); polyphenolic compound with Rf value 0.40 (c)

The antioxidant ability of *Streptomyces* sp. SAE4034 extract was tested for its activity in inhibiting free radicals through the DPPH method. *Streptomyces* sp. SAE4034 extract can inhibit the activity of DPPH free radicals 0.1 mM up to 58.60% at a concentration of 40 µg/mL (Table 3). In the research of Tan *et al*. (2018), showed that *Streptomyces* sp. MUM292 extract has DPPH free radical inhibitory activity amount of 35.98%. In another study Tan *et al*. (2019), showed that *Streptomyces* sp. MUM265 extract has DPPH free radical inhibitory activity amount of 42.33%. The difference in the results obtained can be caused by several factors such as differences in solvents, differences in extract concentrations, and the composition of compounds contained in the extract.

The minimum inhibition value to inhibit DPPH radical can be seen from the IC_{50} calculation results from *Streptomyces* sp. SAE4034 extract which is based on the regression curve equation y= 0.7553x + 26.367. The IC_{50} calculation results obtained a value amount of 32.14 ppm which indicates that the *Streptomyces* sp. SAE4034 extract has very strong antioxidant activity in inhibiting the DPPH free radicals. This is based on Molyneux (2004), that if the IC_{50} value of a compound is < 50 ppm, the antioxidant activity is categorized as very strong, 50-100 ppm is categorized as strong, 100-150 ppm is categorized as moderate, and 150-200 ppm is categorized as weak antioxidant activity.

The antioxidant activity of *Streptomyces* sp. SAE4034 extract was then compared with pure antioxidants such as ascorbic acid (vitamin C). The results of antioxidant activity on ascorbic acid (Table 4) showed that ascorbic acid could inhibit DPPH radical activity 0.1 mM up to 92.13%. The calculation result of the IC_{50} value calculated based on the regression curve equation y= 1.4093x + 38.6. The IC_{50} calculation results obtained a value of 8.09 ppm which indicates that ascorbic acid has a very strong antioxidant activity in inhibiting DPPH free radicals. Based on Matuszewska *et al*. (2018), ascorbic acid is a well-known antioxidant that is used as a standard of comparison in antioxidant activity.

Table 3. Percentage Result of DPPH Inhibition by Antioxidant Compounds from *Streptomyces* sp. SAE4034 Extract

Control Absorbance	Concentration (µg/mL)	OD value (Average)	%DPPH Inhibition
0.500	10	0.322	35.600
	20	0.299	40.133
	30	0.267	46.667
	40	0.207	58.600

Table 4. Percentage Result of DPPH Inhibition by Antioxidant Compounds from Ascorbic acid

Control absorbance	Concentration (µg/mL)	OD value (Average)	%DPPH inhibition
0.500	10	0.248	50.333
	20	0.157	68.667
	30	0.079	84.200
	40	0.039	92.133

The activity of SOD enzyme was used to detect the ability of *Streptomyces* sp. SAE4034 in producing endogenous antioxidants capable to scavenging free radicals. The measurement result of SOD enzyme activity in the *Streptomyces* sp. SAE4034 extract (Table 5) shows that *Streptomyces* sp. SAE4034 is able to produce SOD enzyme with an activity of 63.10 U/mL. Based on the results of research by Tan *et al.* (2017), showed that *Streptomyces* sp. MUM212 extract has SOD enzyme activity amount of 37.47 U/mL and has the capability to scavenge superoxide anions which are free radicals produced from the xanthine-xanthine oxidase reaction. The SOD enzyme inhibits the xanthine-xanthine oxidase reaction by converting the superoxide anion radical into oxygen. Based on these results, *Streptomyces* sp. SAE4034 extract can be concluded to be able to scavenge superoxide anions. According to Tan *et al*. (2017), the SOD enzyme is one of the main enzymes that acts as an endogenous antioxidant. The SOD enzymes are able to scavenge free radicals, namely superoxide anions that cause oxidative stress in organisms. Kanth *et al*. (2011), added that SOD enzyme acts to catalyze the disproportion of superoxide anion (O_2^-) and Hydrogen (H^+) into hydrogen peroxide (H_2O_2) and oxygen (O_2) through a redox reaction.

Table. 5. Absorbance Value of SOD Enzyme Activity from *Streptomyces* sp. SAE4034 Extract

Solution	Absorbance value	SOD Enzyme Activity (U/mL)
Sample	0.245	
Standard	0.119	63.10
Blank	0	

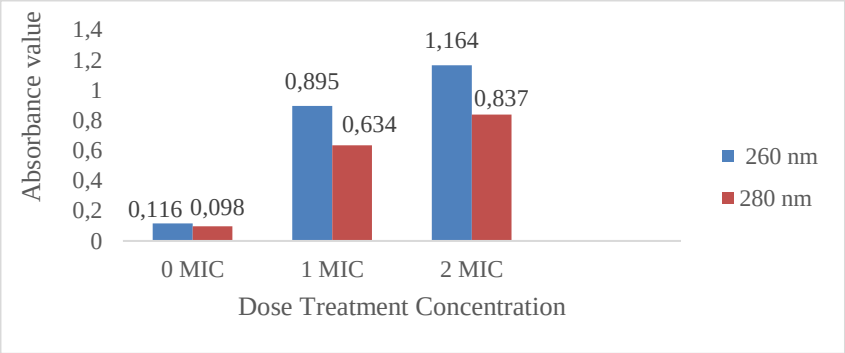

Figure 7. Absorbance Value Analysis of *S. aureus* Cell Leakage Test due to The Administration of *Streptomyces* sp. SAE4034 Extract

The results of cell leakage test (Figure 7) showed that the absorbance value at the 2 MIC dose treatment (2x256 µg/mL) showed higher results than the 0 MIC dose and 1 MIC dose treatment, this is in line with the statement of Nurkanto *et al*. (2010), in general, treatment with 2 MIC dose can cause more severe damage than treatment with 1 MIC dose. The absorbance value 260 nm wavelength tends to be higher than 280 nm wavelength in all treatments. This indicated that the contents of the cells which lysis due to leakage of *S. aureus* cells were more dominant in nucleic acids than proteins. According to Syarifuddin *et al*. (2018), the results of cell leakage were measured the absorbance values at 260 nm and 280 nm wavelength have different interpretations. The greater the absorbance value, the more the contents of the cell are lysis. The components of cell contents that leave the cell as measured at 260 nm are nucleic acids while at 280 nm are proteins.

According to Syarifuddin *et al*. (2018), one of the antibacterial mechanisms in inhibiting bacteria is antibacterial substances enter cells and attach to organelles so they interfere with cellular metabolic processes in bacteria. The membrane permeability of the bacterial cell becomes dysfunctional and can result in cell leakage and the release of cell components. The transcription and translation processes that occur in bacterial cells can be inhibited due to the intercalation of active compounds to DNA/RNA, this causes protein and nucleic acid leakage to occur.

CONCLUSION

Based on result and discussion, it can be concluded from this research that *Streptomyces* sp. SAE4034 produce compounds belonging to the group of alkaloid, terpenoid, flavonoid, and polyphenol. Compound with Rf value 0.40 and 0.47 which includes into polyphenol groups, and 0.72 which includes into alkaloid group has the ability to inhibit the growth of *S. aureus. Streptomyces* sp. SAE4034 has a very strong antioxidant activity that can scavenge DPPH free radical with IC_{50} value of 32.14 ppm and has an antioxidant enzyme activity that is superoxide dismutase amount of 63.10 U/mL. The inhibition mechanism of *Streptomyces* sp. SAE4034 extract was through cell leakage with release of nucleic acid and protein in *S. aureus* cells with the highest cell leakage at doses of 2 MIC.

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