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Identification and Antibacterial Activity of Actinomycetes Isolated From Medicinal Plant *Andrographis paniculata* Rhizosphere Soil

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Abstract

Objectives: Research related to the characterization of the secondary metabolites of actinomycetes rhizosphere medicinal plants, as well as the molecular identification of actinomycetes as an antibacterial compound producer was carried out on actinomycete isolates obtained from the rhizosphere soil of medicinal plants in South Sulawesi. The purpose of this study was to isolate actinomycetes from *Andrographis paniculata* rhizosphere and to determine their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Finally, actinomycetes that produce antibacterial compounds were identified as well.

Materials and Methods: Two isolates of actinomycetes were obtained from *A. paniculata* named SM-4 and SM-6, which were fermented in the starch nitrate broth (SNB) medium for 14 days. The supernatant and biomass of the fermentation yield were separated, and then the supernatant was extracted using ethyl acetate. Next, the antibacterial activity test was conducted by the diffusion method using a paper-disk. Eventually, the identification of actinomycetes SM-4 and SM-6 was based on the sequence analysis of the 16S rRNA gene using universal primers 27f and 1492r.

Results: The antibacterial activity of ethyl acetate extracts could inhibit the bacteria test to 2 mg concentration. Based on phylogenetic trees, actinomycetes SM-4 and actinomycetes SM-6 had the highest similarity with *Streptomyces griseorubiginosus* and *Streptomyces phaeopurpureus*, as well as *Streptomyces purfeofuscus* and *Kitasatospora azatica*, respectively.

Conclusions: In general, actinomycetes SM-4 isolated from rhizosphere *A. paniculata* had the highest similarity with *Streptomyces griseorubiginosus* and *Streptomyces phaeopurpureus* with a similarity level of 97%. In addition, SM 6 isolated from the rhizosphere of *A.s paniculata* demonstrated the highest similarity with *Streptomyces purfeofuscus* and *K. azatica* with similarity levels of 97%. Finally, the thin layer chromatography-bioautography test revealed that the ethyl acetate extract from actinomycetes SM-4 had antibacterial activity at Rf 0.14 and 0.4 and was assumed to be a class of terpenoid compounds.

Keywords: *Actinomycetes*, Antibacterial, 16S rRNA gene, *Andrographis paniculate*, Rhizosphere

Introduction

Increased antimicrobial therapy and the discovery of the new members of the antimicrobial group, through synthetic chemical screening or fermentation, spurred the development of the production of antimicrobial compounds over the past decade. On the other hand, the problem of antimicrobial resistance also increases by increasing its use (1). Multi-drug resistance (MDR) is a serious problem because treatment options can be extremely limited so that more effective treatment strategies or new antibiotics are necessary in this regard (2).

This triggers an exploration of the potential for natural resources in an effort to overcome this resistance problem.

According to (3), actinomycete is a microbial group that produces the most bioactive antibiotic compounds (70%), fungi (20%), and bacteria (10%). In addition, this microbial group is highly important in the environment because it is capable of biotransformation and metabolic processes in an extremely wide range (4). Actinobacteria are the main microbes in soil micro-ecosystems (5). Further, actinomycetes are the best source of secondary metabolites, and most of these compounds originate from single genus *Streptomyces*, the species of which are widely distributed in marine and terrestrial habitats (6).

Actinomycetes populations in rhizosphere soils are close to 40% of the total soil microflora. Furthermore, proteobacteria and actinobacteria are the dominant populations found in the rhizosphere of various types of plant species (7,8). Microorganisms are abundant in the rhizosphere because plant roots secrete exudates containing organic materials such as amino acids, sugars, and organic acids which are nutrients for microorganisms. Moreover, the composition and pattern of root exudates, plant species, and soil types affect the activity and population of rhizosphere microbes (9).

Khamna et al reported that there are 445 actinomycetes isolates from 16 rhizosphere soils of medicinal plants. Nearly 89% of isolates are of genus *Streptomyces* and 11% of them include other genera (10). Rante et al

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used microbes as a source of bioactive compounds, especially *Actinomycetes* from the *Orthosiphon stamineus* rhizosphere as the producer of antibacterial compounds against multidrug-resistant bacteria (11).

Actinomycetes can also be isolated from the rhizosphere of the *Andrographis paniculata* (Acanthaceae) plant. It is an annual herb that grows abundantly in Southeast Asia including India, Sri Lanka, Java, Pakistan, Indonesia, and Malaysia. Further, this herb is efficacious for treating the gastrointestinal tract and upper respiratory infections, fever, herbs, sore throat, hepatitis, and a variety of other chronic and infectious diseases (12). Recently, medicinal plants have come to the forefront of bioprospecting in search for new sources of actinomycetes (13). In this study, actinomycetes were isolated from the rhizosphere of *A. paniculata* and then were tested for their antibacterial activity and identified using the 16S rRNA gene sequence.

Materials and Methods

Preparation of Isolate Actinomycetes

Actinomycete isolates from rhizosphere soil samples were taken from several medicinal plants in the Makassar City region of South Sulawesi Province and then regrown on the media starch nitrate agar (SNA) based on the protocol of (11).

Antagonistic Tests

The antimicrobial activity was determined by growing all actinomycetes isolates on SNA media. Then, the isolates were incubated for 7 days and cut into a small agar block using s stainless steel cylinder. Next, test bacteria were inoculated in petri dishes in the nutrient agar (NA) medium. Then, agar blocks transferred to the NA medium contained test bacteria. The inhibition zone was observed after incubation at 37°C for 24 hours. Each isolate was observed to inhibit test bacteria which were characterized by the formation of clear zones around the isolates (11).

Fermentation and Extraction

The active isolates were pre-cultured in 500 mL Erlenmeyer flasks containing 100 mL of the SNA liquid medium and incubated at room temperature for 3 days. The prekultur (starter) was transferred into an Erlenmeyer 500 mL containing 100 mL of the same medium. In addition, fermentation was carried out at room temperature for 14 days at a shaking rate of 150 rpm. Then, microbial growth media were filtered to separate biomass and fermentation liquid. Next, the fermented liquid was extracted 2 times with ethylacetate (1:1 v/v) in a separating funnel for 20 minutes. Finally, the obtained extract was evaporated and then stored in the desiccator for further use in the next test.

Antibacterial Activity

The antibacterial activity was determined by the bioassay method based on the method of Badji et al and Pandey on test bacteria. Furthermore, ethyl acetate extract 10 mg/mL was dissolved in organic solvents. Then, 10 µL of the extract were put on a disc paper (6 mm in diameter). After the evaporation of all solvents, the paper discs were placed on the surface of the media which had been inoculated with test bacteria by the streak method. All plates were then incubated for 24 hours at 37°C. Eventually, antibacterial activity was observed by the presence of a bacterial growth inhibition zone around the disc paper (14,15)

Molecular Characterization of Isolates of Actinomycetes

DNA Preparation

DNA isolation was done using the method by Song et al. The 7-day-old liquid culture of actinomycetes was put into a 1 mL sterile microtube and then centrifuged at 13 000 rpm for 5 minutes, and finally, the supernatant was discarded. The pellets were washed with Tris EDTA (TE) 400 µL, centrifuged at 13 000 rpm for 5 minutes, resuspended with 400 µL buffer SET (75 mM NaCl, 25 mM EDTA, and 20 mM Tris HCl), and a pH of 7.5 and then 50 µL of lysozyme (10 mg/mL) was added as well. Next, the pellets were incubated in a water bath at 37°C for 1 hour and homogenized by inversion every 15 minutes. Moreover, 50 µL of SDS 10%, protease 20 µL, and RNA-ase 50 µL were added and then incubated with a 65°C water bath for 2 hours and inverted every 0.5 hours. After 1 hour of incubation, 167 µL of NaCl 5 M was added to pellets. After completion of incubation, 400 µL of chloroform cold was added, incubated at room temperature for 0.5 hours, and inverted every 10 minutes, and finally, centrifuged at 13 000 rpm for 10 minutes. Additionally, the aqueous phase was transferred to a new tube, then isopropanol or 2-propanol 1x was added to the volume of the supernatant, carefully homogenized, and stored at -20 °C (overnight) or -80 °C (1 hour). After reaching the room temperature, it was centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed, 100 µL of ethanol 70% cold was added and homogenized, then ethanol was removed and the pellet was dried and dissolved/resuspended with TE 25 µL. Finally, the purity of the DNA solution was checked using a spectrophotometer at λ260 and λ280 nm, and the amount of DNA was measured at λ260 nm (16).

Measurement of DNA Concentration and Purity

DNA purity and concentration were determined using a spectrophotometer at wavelengths of 260 nm and 280 nm. The purity value is obtained from the ratio of optical density (OD) values at wavelengths of 260 nm and 280 nm (OD260/OD 280). The comparison value between 1.8 and 2.0 shows high DNA purity. However, comparisons below 1,8 indicate the contamination of compounds with large molecular weights such as proteins. Eventually, a comparison value above 20 represents the contamination of compounds with small molecular weights such as RNA (17,18). DNA concentration values were determined using the following formula = OD 260 x dilution factor x 50.

Amplification of 16S rDNA Sequence

16S rDNA sequences were amplified using the polymerase chain reaction (PCR) method with 27 m polymerase and primary DNA tags (5'AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'GGTTACCTTGTTACGACTT-3'). The thermal conditions of the cycle were regulated as the denaturation of target DNA at 98°C for 3 minutes, followed by 30 cycles at 94°C for 1 minute, primary annealing at 54°C for 1 minute, and primary extension at 72°C for 5 minutes. At the end of the cycle, the mixing reaction was set at 72°C for 5 minutes and then cooled at 4°C. Then, PCR amplification was detected using the agarose electrophoresis gel and visualized with UV fluorescence after being painted with ethidium bromide. The obtained PCR products were then sequenced, and sequencing results were compared with genomes from bank databases using NCBI BLAST (www.ncbi.nih.gov/).

Phylogenic Analysis

Sequencing similarity was obtained from sequencing 16S rRNA genes from strain 20/BLP using BLAST (NCBI). The data sequencing of 16S rRNA gene isolates 20/BLP and data using the 16S rRNA gene from reference strains obtained from International databases (NCBI: National Center for Biotechnology Information) were then prepared with a Program File Editor. In addition, the 16S rRNA sequences of the selected isolates were matched or aligned using the CLUSTAL X program (19) to nucleotide sequences having similarities from bank data. Then, the results of subsequent alignments were used to produce phylogeny trees using the PHYLIP program (20). The result is the value of nucleotide similarities over 16S rRNA between isolates SM-4 and SM-6 and the reference strain.

TLC-Bioautography

The ethyl acetate extract from isolate SM-4 was bottled on TLC plates developed with mobile phase n-hexane: ethyl acetate (6: 0.5). The spots were detected by TLC and visualized under the UV light of λ254 and λ3675 nm. TLC-bioautography testing was done by attaching the TLC plate (which has sterilized with a UV light for 1 hour) on the media inoculated with test bacteria. Furthermore, it was incubated at 4°C for 4 hours to stop the growth process of test microbes and allow time for the diffusion of active compounds to grow, and finally, re-incubated at 37°C for 24 hours. The spots that indicated the presence of inhibition zones (marked by the formation of clear zones around the spots) were expressed as active spots.

Results and Discussion

This research was conducted by preparing the results of SM-4 and SM-6 isolates from the rhizosphere of Sambiloto which were obtained using a new SNA medium scratched on a petri dish and incubated at 37°C. The results of preparation showed the appearance of the same colony as the original colony.

Based on Figure 1, isolates SM-4 and SM-6 have fairly good growth in SNA media with the color of the aerial mycelium for isolate SM-4 with grayish-white and brownish substrate mycelium, and SM-6 isolates with grayish-white air mycelium and the black substrate mycelium. This is in accordance with the finding of research conducted by Dhanasekaran and Jiang, demonstrating that substrate mycelium from actinomycetes can provide colors such as white or almost colorless to yellow, blue, purple, brown, red, pink, orange, green, black, or other colors (21). However, Locci and Sharples found that the characteristics of other aerial mycelia from Streptomyces are pigments which can have colors from white or gray to yellow, orange, lavender, blue, and green, thus they are often referred to as "color wheel" (22).

Fermentation was carried out on pure isolates aged 7 days in order to obtain secondary metabolites from actinomycetes isolates. Isolate fermentation was conducted using starch nitrate broth (SNB) liquid media, and then they were incubated for 11 days and shaken occasionally. According to Mulyadi and Sulistyani, at the age of 14 days, actinomycetes have generally entered the stationary phase, which is the microbial phase producing secondary metabolites including the pigment giving air into the isolate (23). After 14 days, there was a color change in the SNB medium, which was initially clear turned brownish with a mass of mycelia visible on the walls of the fermentation container. Color changes indicate that isolates have produced secondary metabolites, especially pigments while air mycelium attached to the glass wall is a sign of the presence of *Actinomycetes*.

In this study, fermentation was carried out using the growth media of SNB. A good growth medium is a medium that is able to provide carbon sources and other minerals needed for growth and activity (24). This study used SNB media because of its carbon and mineral content. The

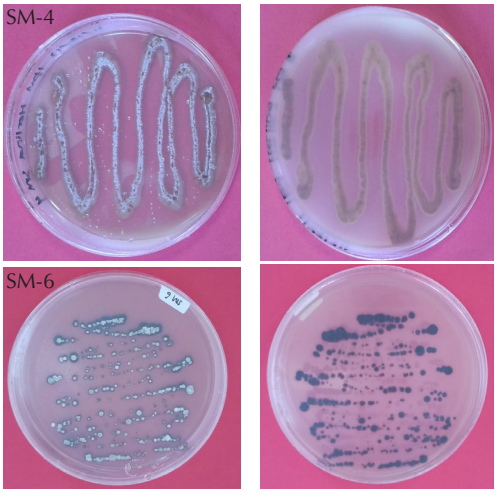

Figure 1. Isolates of Actinomycetes SM-4 and SM-6 Grown on SNA Medium and Incubated 10 Days. *Note.* A: Front view; B: Rear view.

carbon source of SNB media comes from the soluble starch which contains a diverse amount of C from the starch and glycerol. Further, inorganic nitrogen sources come from KNO3, and minerals are derived from magnesium, sodium, iron, and potassium which are compositions of SNB media (25). After day 14, fermentation is filtered so that it is separated from biomass and supernatant. The supernatant was extracted using ethyl acetate (1:1) which was repeated twice. Ethyl acetate is used because it is a semi-polar solvent and has low toxicity while not solving in water. In addition, ethyl acetate is often used as a solvent because able to solve more secondary metabolites which can provide antibacterial activity. The extraction results were then evaporated until obtaining thick extracts. The obtained extract was then tested for antibacterial activity by the diffusion method using a round disk paper with a diameter of 6 mm and a thickness of 0.5 mm. The diffusion method has several advantages such as easiness of doing and the ability to identify the sensitivity of various types of microbes to antibacterial at certain concentrations. The test results are provided in Table 1.

The antibacterial activity of isolates SM-4 and SM-6 showed a diameter inhibition against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) bacteria at the levels of 2 mg and 4 mg which can be categorized as broad-spectrum antibacterial level because they inhibit gram-positive and -negative bacteria.

SM-4 isolates with 2 mg levels had moderate antibacterial activity (5-10 mm) against *S. aureus* and *E. coli* bacteria while they had strong antibacterial activity (10-20 mm) against *S. aureus* and *E. coli* at 4 mg levels. On the other hand, SM-6 isolates with 2 mg levels had moderate antibacterial activity (5-10 mm) against *S. aureus* and *E. coli* bacteria whereas, at 4 mg levels, they had strong antibacterial activity (10-20 mm) against *S. aureus* and *E. coli*.

The inhibition activity of SM-4 isolates was greater than that of SM-6 isolates, this was clearly observed at 4 mg levels (Table 1) which was characterized by the formation of the inhibition zone diameter of isolate SM-4, which was greater than that of SM-6 isolates by differences in the cell wall structure between gram-positive and gram-negative bacteria.

Molecular characterization and identification are based on the sequence analysis of the 16S rRNA gene using universal primers 27f and 1492r (26). The nucleotides from sequence 16S rRNA analysis from actinomycetes 20/BLP were used for BLAST (Basic Local Alignment Search Tool) through http://blast.ncbi.nlm.nih.gov/Blast.cgi to find out the kinship between the reference strains. The results of the analysis of the 16S rRNA gene with the reference strain are described as phylogenetic trees.

Table 1. Inhibition Zones of the Secondary Metabolites of Actinomycetes Isolates SM-4 and SM-6 Against *Staphylococcus aureus Escherichia coli bacteria*

Content Extract in Paper Disk	Extract	Inhibitory Zones (mm)	
		E. coli	*S. aureus*
2 mg	SM-4	9.23±1.0	8.9±1.23
4 mg		12.16±0.78	12.24±0.77
2 mg	SM-6	7.56±1.38	7.57±0.63
4 mg		10.05±0.4	10.32±1.34
Positive control	Amoxicillin	32.36±1.23	25.58±2.63

BLAST results showed that actinomycetes SM-4 isolated from the rhizosphere of Sambiloto medicinal plants had the highest similarity with *Streptomyces griseorubiginosus* and *Streptomyces phaeopurpureus* with a similarity level of 97%. Furthermore, SM-6 isolated from the rhizosphere of Sambiloto medicinal plants represented the highest similarity with *Streptomyces purfeofuscus* and *K. azatica* with a similarity level of 97%. Based on phylogenetic trees, SM-4 isolates had the closest relationship with *Streptomyces griseorubiginosus* and *Streptomyces phaeopurpureus* (Figure 2) and SM 6 isolates were closely related to *Streptomyces purfeofuscus* and *K. azatica* (Figure 3).

Streptomyces griseorubiginosus isolated from French soil was reported to produce antibiotic Cinerubins A and B (27) while Palaniyandi et al found that *Streptomyces phaeopurpureus* produces extracellular protease. Microbial proteinase has the ability to inhibit spore adhesion, and appressorium formation can be used to suppress the formation of infection by fungal pathogens (28). Streptomyces are the most important bacteria genus for the production of secondary metabolite. These soil bacteria are characterized by complex differentiation cycles. Moreover, they produce about two-thirds of all antibiotics such as clavulanic acid (29), neomycin (30), chloramphenicol (31), avermectin insecticide (32), chloramphenicol (33), insecticide avermectin (34), immunosuppressant tacrolimus (35), and strong antitumoral platenolides (36).

TLC-Bioautography

The results of the activity test demonstrated that the ethyl acetate extract obtained from actinomycetes SM-4 had a greater inhibition zone compared to SM-6 so that it was followed by bioautographic thin layer chromatography (TLC) testing analysis. TLC was used for separating chemical components based on the principle of adsorption and partitioning which is determined by the stationary phase (adsorption) and the mobile phase (eluent). The stationary and mobile phases included TLC GF254 and n-hexane: ethyl acetate (6: 0.5), respectively. The TLC plate was detected by UV 254 nm and 366 nm.

Additionally, the TLC plate was placed on the surface of the nutrient agar medium which was inoculated with *E. coli* and *S. aureus* bacteria. TLC plates were planted for 15-30 minutes aiming at enabling the active compound on the plate to diffuse on agar. After 15-30 minutes, the

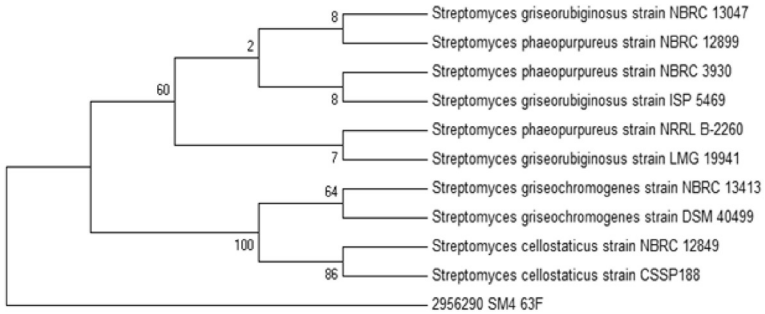

Figure 2. Phylogeny Trees Constructed Using the Neighbor-joining Tree Method Based on 16S rRNA Sequencing Showing the Phylogenetic Relationships of SM-4 Strains.

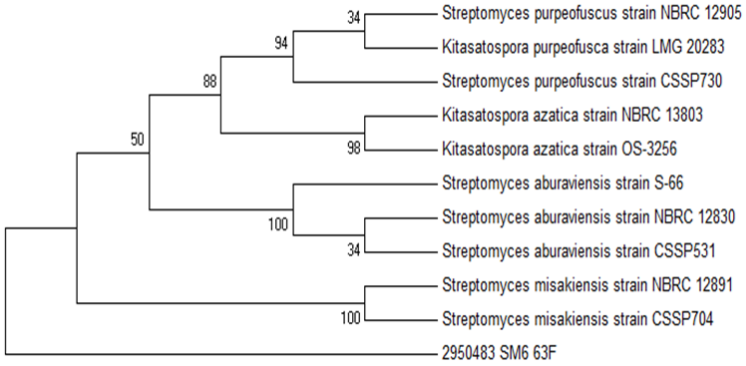

Figure 3. The Phylogeny Tree Constructed Using the Neighbor-Joining Tree Method Based on 16S rRNA Sequencing Indicating the Phylogenetic Relationship of SM-6 Strains

TLC plate was then removed from the surface of the medium. Antimicrobial compounds were diffused from the chromatogram plate into the media to inhibit bacterial growth after incubation for 1 X 24 hours marked by a clear zone on the surface of the former attached plate. The result of TLC-Bioautography test showed that the spot at Rf 0.14 and 0.4 had antibacterial activity (Figure 4).

Phytochemical screening (Figure 5) indicated that the spot which has antibacterial activity is thought to be a class of terpenoid compounds which are characterized by the discoloration of the spots in purple after spraying with anisaldehyde (36).

The mechanism of terpenoids as antibacterial compounds is assumed to involve the breakdown of the membrane by lipophilic components and has the main target, namely, the cytoplasmic membrane which refers to its hydrophobic nature (37).

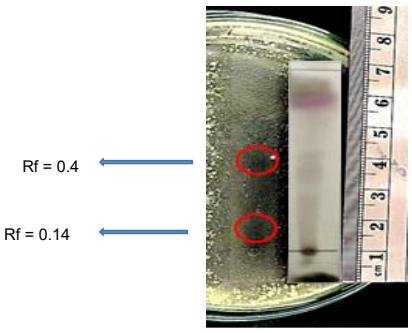

Figure 4. TLC-Bioautography Test Against *Staphylococcus aureus*. Note. TLC: Thin layer chromatography.

Conclusions

SM actinomycetes 4 isolated from the rhizosphere of Sambiloto medicinal plants had the highest similarity with *Streptomyces griseorubiginosus* and *Streptomyces phaeopurpureus* with a similarity level of 97%. In addition, SM 6 isolated from the rhizosphere of Sambiloto medicinal plants demonstrated the highest similarity with *Streptomyces purfeofuscus* and *K. azatica* with similarity levels of 97%.

Finally, the TLC-bioautography test revealed that the ethyl acetate extract from actinomycetes SM-4 had antibacterial activity at Rf 0.14 and 0.4 and thus was considered to be a class of terpenoid compounds.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

Not applicable.

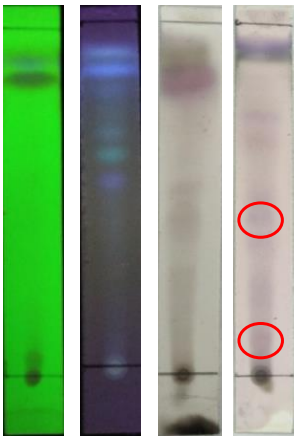

Figure 5. TLC Profile of Ethyl Acetate Extract From Actinomycetes SM-4 (A) UV 254 nm, (B) UV 366 nm, (C) H2SO4 10%, and (D) Anisaldehid. Note. TLC: Thin layer chromatography; UV: Ultraviolet.

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