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Rhizosphere actinobacteria isolated from *Pometia pinnata* and its antimicrobial activity

ISRA JANATININGRUM[1,♥], AMELIA ZAHRA[1], FITRIYANTI[1], VIVI ANGGIA[1,2]

[1]Program Study of Pharmacy, Faculty of Health Sciences, Universitas Islam Negeri Syarif Hidayatullah Jakarta. Jl. Ibnu Taimia IV, South Tangerang 15419, Banten, Indonesia. Tel.: +62-217-4716718, ♥email: isra.janatiningrum@uinjkt.ac.id

[2]Program Study of Pharmacist Profession, Faculty of Health Sciences, Universitas Islam Negeri Syarif Hidayatullah Jakarta. Jl. Ibnu Taimia IV, South Tangerang 15419, Banten, Indonesia

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Abstract. *Janatiningrum I, Zahra A, Fitriyanti, Anggia V. 2024. Rhizosphere actinobacteria isolated from* Pometia pinnata *and its antimicrobial activity. Biodiversitas 25: 1007-1014.* Actinobacteria are currently the largest antibiotic-producing bacteria due to their extreme environments such as soil, roots and rhizosphere. The diversity of rhizosphere actinobacteria is affected by plant exudates. *Pometia pinnata* is a plant that has long been used for traditional medicine in Indonesia. This study was aimed at isolating and evaluating isolate and evaluate the actinobacteria rhizosphere of *P. pinnata*, to screen their antibacterial and antifungal properties. Isolation of actinobacteria was done by using serial dilution method and grown on media. About 34 actinobacteria were successfully isolated from the rhizosphere of *P. pinnata* and 50% of them showed index antimicrobial activity for *Staphylococcus aureus* ATCC 6538 (0.52), *Streptococcus pneumoniae* ATCC 1705 (2.87), and *Escherichia coli* ATCC 25922 (1.28). Several microbial isolated microbes (20.8%) were found capable in inhibiting fungal growth. The highest antibacterial activity against all bacteria was shown by RM12 isolate. The aqueous fraction of RM12 isolate demonstrated a significant inhibitory activity against *S. aureus* ATCC 6538 and *E. coli* ATCC 25922 at concentrations of 250 ppm, 500 ppm and 1000 ppm. TLC bioautography showed that aqueous fraction RM 12 do not belong to the polyphenol or phenol compound group. The RM12 was characterized by using 16S ribosomal RNA sequence found to belong to actinobacteria genera to Str*eptomyces bungoensis*.

Keywords: 16S rRNA, actinobacteria, antibacterial, antifungal, diversity

INTRODUCTION

Soil contains important organic-inorganic substances and harbors a diverse group of bacteria, fungi and protozoa. The microbial community develops as a result of the abundance of nutrients exudated from plant roots which supports microbial growth. Root exudates are components generated by plant roots and released into the surrounding plant rhizosphere. The composition of the exudate produced by plant roots varies depending on the type of plant, growth phase and physical factors such as pH, type of soil, humidity, and temperature. The presence of root exudates in plant rhizosphere contributes to the beneficial interaction between microorganisms and plants. Rhizosphere soil in the surrounding plant roots, provides a habitat for microbial population contributed by the presence of plant exudates (Ambarwati et al. 2019; Imchen et al. 2019; Janatiningrum and Lestari 2022; Wulandari et al. 2020).

Actinobacteria bacterial group is one of the largest populations of microorganisms which presents various and extensive habitats on Earth. These bacteria are characterized as Gram-positive bacteria, are widely present in soil and aqueous habitats, colonizing plants with filamentous structures, and almost 90% of actinobacterial genera have been isolated from soil due to organic-inorganic contents. The rhizosphere soil possesses more actinobacteria than soil without a root system, because it contains a diverse chemical composition of exudate (Imchen et al. 2019; Janatiningrum and Lestari 2022). The compounds in root exudates consist of primary and secondary metabolites. Primary metabolites are simple organic compounds that plants and surrounding microorganisms need, such as carbohydrates, organic acids, and amino acids. Primary metabolites are secreted in larger quantities than secondary metabolites. Secondary metabolite compounds produced by root exudates for example flavonoids, alkaloids, phenols, glucosinolates, auxins, etc (Vives-peris et al. 2019).

Several studies have been carried out regarding actinobacteria from rhizospheric soil that have potential as antimicrobials. Antibacterial compounds produced by rhizosphere actinobacteria can inhibit Gram-positive and Gram-negative bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Arifiyanto et al. 2020; Fatmawati et al. 2018; Retnowati et al. 2018). In addition to its ability to inhibit pathogenic bacteria, rhizospheric actinobacteria is also studied to have antifungal activity. The most common antifungal activity found in actinobacteria is inhibiting *Fusarium* genus (Elshafie et al. 2023; Mariastuti et al. 2018; Zhang et al. 2021). Actinobacteria are also well-known for their benefits in producing antibiotics for the pharmaceutical industry. About 70% of the antibiotics are produced by genus *Streptomyces*, and 16% by strains that belong to genera of actinobacteria (Shrestha et al. 2021). Nevertheless, the rate of producing new compounds from

terrestrial actinobacteria has moderately decreased, where the rate of isolation of known compounds have significantly increased.

In recent years, antimicrobial resistance is rapidly increasing on a global scale and is spreading from one country to another faster than previously thought. Antibiotic resistance can be the cause of prolonged illness, disability and death. There are several pathogens that are on WHO's priority list that urgently need for new antibiotics. These pathogens include *S. pneumoniae*, *E. coli*, *S. aureus* and *C. albicans* (WHO 2017). Therefore, the study of new natural antibiotic sources which is able to overcome the resistance is compulsory to be conducted.

Plants not only provide nutrients for microorganisms but several plant species also contain unique antimicrobial metabolites in their exudates (Olanrewaju and Babalola 2019). Indonesia has plentiful types of plants are used as medicinal plants. *Pometia pinnata*, often known as matoa is a plant originating from West Papua, Indonesia which is spread across several regions islands in Indonesia. *Pometia pinnata* is known as ornamental plants that can cure several diseases such as fever, diarrhea, flu, and diabetes (Pakaya et al. 2021; Sidoretno 2022). Moreover, *P. pinnata* has been reported to have potential as antimicrobials agent (Irawan and Sirait 2020), due to potential secondary metabolite content of rhizosphere actinobacteria Actinobacteria isolated from *P. pinnata* rhizosphere has never been reported before as an antimicrobial agent. As a consequence, the search for potential actinobacteria in producing bioactive compounds is necessary to be performed. Bacterial resistance continues to increase, causing the search for new sources of antibiotics continue at this time. There are many unexplored actinobacteria with novel bioactive compounds, including antibiotics. Rhizosphere actinobacteria of *P. pinnata* is one source for new antibiotics. So, this study aims to isolate the rhizosphere actinobacteria *P. pinnata* which has potential as antimicrobial agent which can be developed as a source of new antibiotics.

MATERIAL AND METHODS

Samples collection

Sampling was carried out in November 2022 in Pekanbaru, Riau Province. Soil samples were collected from around the *P. pinnata* roots, about 1-10 cm from the soil surface to the tip of the plant roots.

Isolation and purification of rhizospheric actinobacteria

The soil sample was dried at 60°C for 2 hours, and then 1 g of the dried soil sample was dissolved into 9 mL of 0.9% NaCl. It is then diluted in the concentration of 10^{-5}. About 100 µL of the sample was cultured by spread plate method on HVA media containing of (Na_2HPO_4 (0.5 g), $MgSO_4.7H_2O$ (0.05 g), KCl (1.71 g), $CaCO_3$ (0.02 g), $FeSO_4.7H_2O$ (0.01 g), agar (18 g), distilled water (1 L)). Furthermore, the media was incubated for 7 days at 25-28°C (Janatiningrum and Lestari 2022).

Actinobacterial colonies were purified by using ISP4 media involving streak plate method. The growth isolated were then inoculated using a toothpick by scratching into 4 quadrants on ISP4 agar media consisting of ($MnCl_2.7H_2O$ (0.1g), $ZnSO_4.7H_2O$ (0.1g), $FeSO_4.7H_2O$ 0.1g K_2HPO_4 (1g), $MgSO_4.7H_2O$ (1g), NaCl (1g), $(NH_4)_2SO_4$ (2g), $CaCO_3$ (2g), $FeSO_4.7H_2O$ (1 g), agar (18 g)). The bacteria cultured were then incubated for 7 days at 25-27°C (Hayakawa and Nonomura 1987).

Morphology characterization of actinobacteria

Actinobacteria were characterized macroscopically and microscopically. The macroscopic was carried out by observing 7-day-old actinobacterial isolates in ISP 4 medium, which was incubated at 28-30°C. Observations included aerial mycelium color, substrate mycelium color and pigment soluble using the RAL color chart. Microscopic morphology was carried out by observation using a microscope with 10x40 magnification (Optika microscopes Italy). On microscopic observation, actinobacterial isolates observed in the arrangement of their spores (Shirling and Gottlieb 1966).

Primary screening of antimicrobial assay

Antibacterial test

Antibacterial activity was done by measuring diameter zone and index of inhibition of rhizosphere actinobacteria isolates against *S. aureus* ATCC 6538, *S. pneumoniae* ATCC 1705, and *E. coli* ATCC 25922. The antibacterial test was carried out using the agar plug method in which the density of pathogenic bacteria was in the exponential phase (106-108). 1% of pathogenic bacteria was put into 100 mL of Mueller Hinton agar medium at 40°C then homogenized and poured into a petri dish. Actinobacterial isolates on ISP 4 medium (7 days old, 28-30°C) were cut into 6 mm diameters. The actinobacterial isolate pieces are then placed on Mueller Hinton agar which has been inoculated with pathogenic bacteria. After that it was incubated at 37°C for 24 hours. Three replicates were performed for each actinobacterial isolate. The zone of inhibition of bacteria was observed in case of antibacterial compounds produced by the actinobacteria (Janatiningrum et al. 2022).

Antifungal test

Antifungal activity test was done using *Candida albicans* ATCC 10231. Actinobacteria isolates on ISP 4 medium (7 days old, 28-30°C) were divided into a diameter of 6 cm and placed on Potato dextrose agar which was cultured with *C. albicans* ATCC 10231 and then incubated at 37°C for 24 hours. After incubation, diameter of inhibition was calculated to show antifungal activity. Determination of antifungal activity was performed in duplo replicates for each actinobacterial isolate (Janatiningrum et al. 2022).

The clear zone index is calculated using this formula:

$$\text{The clear zone index} = \frac{\text{average diameter of clear zone} - \text{average diameter of isolate}}{\text{average diameter of isolate}}$$

Identification base on 16S rRNA gene of rhizospheric actinobacteria

Actinobacteria which have the highest antibacterial activity were identified by sequncing the 16S rRNA gene. The spores and mycelium of bacteria endophytes were collected in 1.5 mL microtube and extracted using Presto Mini gDNA Bacteria Kit according to the protocol. The concentration and purity of the DNA genome were quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

Total genomic DNA was amplified using the Polymerase Chain Reaction (PCR) method with 16S specific primers for bacteria 1492R 5'-GGTTACCTTGTTACGACTT-3' and 27F 5'-AGAGTTTGATCCTGGCTCAG-3' (Bruce et al. 1992). The PCR condition was as follows: pre-denaturation (94°C, 5 minutes), annealing (55°C, 30 seconds), elongation (72°C, 30 seconds), post elongation (72°C, 3 minutes), followed by a 30 cycles amplification. The amplification results were visualized using gel electrophoresis with EtBr dyes on a UV transilluminator.

The amplified product was sent to the sequencing services company (Genetica sciences). The sequencing results are processed with Seqtrace software. Then the sequences are compared and identified by Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI). This step aims to find regions of similarity between sequences. Afterward, the phylogenetic tree was constructed using MEGA 7 software with a neighbor-joining method approach.

Secondary metabolite production and extraction

Fermentation test was carried out to extract secondary metabolites from selected actinobacteria culture. Selected actinobacterial isolates were fermented using the shaker method. Actinobacterial isolates were cultured on ISP 4 medium (7 days old, 28-30°C). A starter solution was done by taking 3 pieces of actinobacterial isolate culture (diameter 6 mm) at 100 mL ISP-2 medium. After that, 1% starter culture mixture into 2 liters ISP-2 medium, then it was fermented for 7 days in a shaker incubator with a speed of 120 rpm. About 2 liters of fermented culture was obtained. Then, the fermented culture was centrifuged at 4000 rpm for 5 minutes. The supernatant was separated and collected from the biomass (Gebreyohannes et al. 2013; Nugraha et al. 2020).

The active metabolites were extracted using the maceration method. First, a fermented supernatant was soaked with ethyl acetate (1:1). The supernatant mixture and ethyl acetate solvent were placed in an incubator shaker for 2 hours at 120 rpm. After incubation, the ethyl acetate phase was evaporated using a rotary evaporator and the supernatant was dried using freeze-dry. The concentrated crude extract was then redissolved with 1% DMSO solvent (Kurnianto et al. 2020).

Secondary screening of Actinobacterial extract

Antimicrobial assay of actinobacterial extract was evaluated using the disc diffusion method against pathogenic microbes. After adjusting the turbidity to 0.5 MC Farland standard, bacteria strains were suspended and swabbed on agar media. About 20 µL of each actinobacterial extract (1000 ppm, 500 ppm, 250 ppm, 125 ppm and 62.5 ppm) was impregnated into paper disc (6 mm) and introduced onto upper layer of inoculated agar plates. The plates were incubated at 37°C for 24 hours. Antimicrobial activity of actinobacteria extract was compared with chloramphenicol as positive control and DMSO as negative control. Diameter of inhibition zone was measured to determine antimicrobial activity (Apsari et al. 2019).

Fraction compounds analysis by Thin Layer Chromatography (TLC)

The analysis of fractions of selected rhizosphere actinobacteria extracts was performed on silica gel Thin Layer Chromatography (TLC) plates (silica gel GF254, Merck). Aqueous fraction was transferred on TLC plate and developed in CH2Cl2:MeOH (3:1) whereas ethyl fraction was eluted using Hex: EA 1:2. Separated spots were visualized under 254 nm and 366 nm ultraviolet wavelength followed by spraying with spray reagent 5% FeCl3 (Praptiwi et al. 2019b).

RESULTS AND DISCUSSION

The rhizosphere environment has an redundant diversity of microorganisms and has its uniqueness. The rhizosphere is in direct contact with plant roots and is actively enriched by the complex mixture of nutrients provided by the plant, in a process known as rhizodeposition (Ling et al. 2022). Each rhizosphere environment attracts a unique microbial community according to its plant species (Essarioui et al. 2017). The microorganisms present in the rhizosphere depend on soil type, host species, host plant genotype, and root system architecture (Saleem et al. 2018). Most microorganisms that live in the rhizosphere environment have the potential to inhibit the growth of bacteria and other fungi (Oberhofer et al. 2019). These microorganisms include actinobacteria which are very abundant around the rhizosphere environment (Li et al. 2020). *Pometia pinnata* is a plant that is used empirically by Indonesian people to treat various infectious diseases, and based on research, the *P. pinnata* plant has potential as an antimicrobial. The bioactive compounds produced by *P. pinnata* are inseparable from their interactions with microbes that live in their rhizosphere environment.

Then, the 34 isolates underwent morphological characterization, which can be seen in Table 1. According to Shirling and Gottlieb (1966) ISP-4 media is one of the standard media for observing the morphology of actinobacterial growth. Based on morphological characterization, 27 isolates produced aerial mycelium and 7 isolates did not form aerial mycelium. The morphological diversity of actinobacteria is illustrated mainly by the substrate mycelium, which leads to the formation of diverse spore structures. Mature spores show a variety of colors such as grey, white, yellow or pink and others. Only

2 isolates that could produce pigments, RM13 and RM14. RM13 isolates produced orange-brown pigments and RM14 produced brown-beige colors after 21 days of growing on ISP-4 media (Figure 1). The appearance of pigments can be influenced by their environment, the ability of spores to survive under unfavorable conditions in some *Streptomyces* can produce pigments (Janatiningrum et al. 2018). Table 1 below shows the diversity of morphological characteristics of actinobacteria isolated from the rhizosphere *P. pinnata.*

Microscopic morphology was performed using a light microscope with 10x40 magnification (Figure 2). Based on the results of microscopic observations, almost all of the isolates of actinobacteria have a spiral arrangement of spores. The isolates were identified based on the Journal of Systematic Bacteriology, according to Shirling and Gottlieb 1966. Actinobacterial isolates of *P. pinnata* rhizosphere have mycelium that characterizes the genus *Streptomyces sp.* Table 2 below shows the ability of rhizospheric actinobacteria to inhibit the test pathogen.

Screening was carried out on *C. albicans*, *S. pneumoniae, S. aureus*, and *E. coli*. According to WHO, these test microbes are priority microbes that need to be searched for new sources of antibiotics, because these microbes are resistant to many antibiotics (WHO 2017). Based on the screening results for antimicrobial activity of *P. pinnata* rhizosphere actinobacteria, 17 isolates (50%) had antibacterial activity and 7 isolates (20.58%) had antifungal activity, which was indicated by the formation of an inhibition zone around the isolates (Figure 3). Research conducted by Apsari et al. (2019) found that 95% inhibition of *E. coli* by rhizosphere actinobacterial isolates originating from corn roots in East Nusa Tenggara, Indonesia. This study showed that actinobacterial from *P. pinnata* rhizosphere can inhibit bacteria *E. coli* 23.52%, *S. pneumoniae* 38.23%, and *S. aureus* 38.23%, which inhibits. Many isolates were able to inhibit all of the pathogenic bacteria tested at once, such as isolates RM 12 RM 31, RM 40, RM 45, RM 47. The actinobacterial isolate that had the greatest inhibitory activity was RM12 isolate, which was able to inhibit *S. aureus*, *E. coli* and *S. pneumoniae* with an average inhibition zone index of 0.52 mm, 2.86 mm, 1.27 mm (Table 2). Furthermore, the RM 12 isolate was used as the selected isolate to identify the 16S rRNA gene and secondary metabolite compounds were extracted.

Isolate RM12 shows 100% similarity with *Streptomyces bungoensis* based on the 16S rRNA gene analysis (Table 3). This is in accordance with morphological observations which show that RM 12 has similarities with the genus *Streptomyces*. *Streptomyces bungoensis* (BF26) which comes from several soils in Egypt is able to inhibit the fungi *Alternaria sesame*, *Fusarium oxysporum* and *Rhizoctonia solani* (Elshamy et al. 2022). Malisorn et al. 2020 obtained isolates RM 54 and RM 56 which are actinobacteria isolated from the rhizosphere soil of the plant *Albizia odoratissima* which show similarities to *S. bungoensis* and can inhibit *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633.

Phylogenetic tree shows how living things evolved from a common ancestor. Phylogenetic trees show which species are more related to each other through evolution, and which are less related. Its start from a root that shows evolutionary ancestry (Kapli et al. 2020). These roots then form a phylogenetic tree trunk that shows the lineage of that ancestor. Tree construction phylogenetics shows that strains RM 12 is on the same line and in the closest line of relationship with *S. bungoensis.* Based on its relationship, RM 12 is still closely related to *Streptomyces rubrogriseus* (Figure 4), which is a genus of actinobacteria that is well known for producing the antibiotic Streptomycin (Kusaka et al. 1968).

Table 1. Characteristics morphology of rhizosphere actinobacteria *P. pinnata* day-7. Color observations are according to the RAL chart

Code isolates	Colors of aerial mycelium	Colors of substrate mycelium	Pigmentation
RM1	Pure white	Broom yellow	-
RM2	Light pink	Antique pink	-
RM3	Pure white	Broom yellow	-
RM4	Platinum grey	Honey yellow	-
RM5	Peable grey	Ivory	-
RM6	-	Luminous orange	-
RM7	Cream	Broom yellow	-
RM9	-	Pastel orange	-
RM10	Silk grey	Silk grey	-
RM11	Silk grey	Silk grey	-
RM12	Pure white	Traffic yellow	-
RM13	Light ivory	Light ivory	Orange brown*
RM14	Telegrey 4	Orange brown	Brown beige*
RM15	Pure white	Ivory	-
RM16	Dahlia yellow	Saffron yellow	-
RM17	Light ivory	Oyster white	-
RM18	Pure white	Rape yellow	-
RM24	Oyster white	Telegrey 4	-
RM25	Red orange	Pastel orange	-
RM26	Pastel violet	Zink yellow	-
RM28	-	Luminous yellow	-
RM29	-	Sulphur yellow	
RM31	Red lilac	Traffic yellow	-
RM34	Signal white	Saffron yellow	-
RM35	Traffic white	Light pink	-
RM36	Deep orange	Luminous orange	-
RM38	-	Sun yellow	-
RM40	-	Rape yellow	-
RM43	Platinum grey	Traffic yellow	-
RM44	Traffic white	Cream	-
RM45	Light ivory	Oyster white	-
RM46	Beige brown	Pure white	-
RM47	-	Telemagenta	-

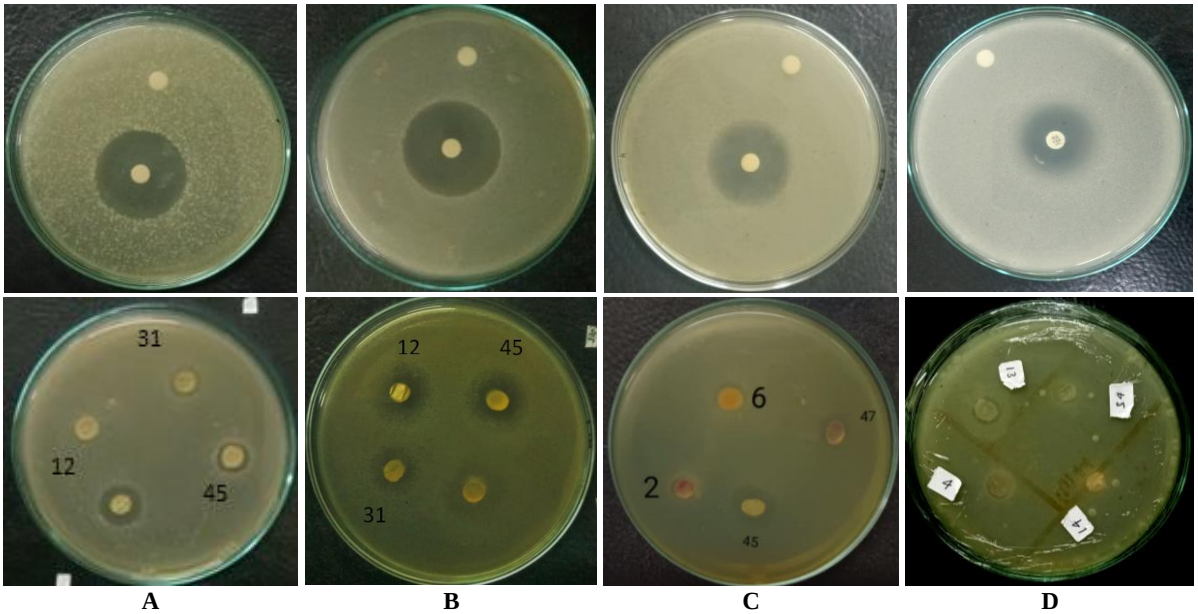
Note: Characterization use RAL Color Chart, *: Pigmentation age 21 days

A **B** **C** **D** **E**

Figure 1. Macroscopic morphology of rhizosphere actinobacteria *Pometia pinnata.* A. RM 3, B. RM 6, C. RM 12, D. RM 45, E. RM 47

A **B** **C** **D**

Figure 2. Microscopic morphology of rhizosphere actinobacteria *Pometia pinnata* (4x10 magnification).A. RM12,B. RM14,C. RM31,D. RM45 at 10 x 40 magnification. The red mark shows the form of arrangement actinobacterial spores



A **B** **C** **D**

Figure 3. Inhibition of antibacterial activity isolates actinobacteria rhizosphere *Pometia pinnata.* A. *E.coli*, B. *S. aureus*, C. *S. pneumoniae*, D. *C. albicans.* Positive and negative control (*above*), isolate of actinobacteria (*below*)

Table 4. Antibacterial activity of aqueous and ethyl acetate extracts of actinobacterial isolates of *Pometia pinnata* rhizosphere RM12

Samples/ Concentration (ppm)	Clear zone index					
	E. coli		*S. pneumonia*		*S. aureus*	
	Aqueous fraction	Ethyl fraction	Aqueous fraction	Ethyl fraction	Aqueous fraction	Ethyl fraction
Control (+)	3.58	3.66	3.16	2.99	3.16	2.99
Control (-)	0.0	0.0	0	0	0	0
1000	1.08	0.0	0	0	0.67	0
500	0.83	0.0	0	0	0.42	0
250	0.46	0.0	0	0	0.33	0
125	0.0	0.0	0	0	0	0
62.5	0.0	0.0	0	0	0.0	0

Table 2. Antimicrobial activity of rhizospheric actinobacteria

Isolates code	Clear zone index			
	E. coli	*S. pneumonia*	*S. aureus*	*C. albicans*
Control (+)	5.65	3.08	2.7	2.3
Control (-)	0	0	0	0
RM1	0.23	0	0.88	0
RM2	0	0.15	0.23	0
RM3	0	0.18	0.12	0
RM4	0	0	0.09	0.13
RM5	0	0	0.10	0
RM6	0	0	0.12	0
RM7	0	0.08	0	0
RM12	1.28	2.87	0.52	0
RM13	0.13	0.43	0	0.07
RM14	0	0	0	0.14
RM15	0	0	0	0.14
RM16	0	0	0	0.20
RM24	0	0	0.08	0
RM26	0	0	0	0.18
RM28	0	0	0	0.17
RM31	0.48	1.13	0.19	0
RM36	0	0.04	0	0
RM40	0.25	0.15	0.69	0
RM43	0	0.42	0	0
RM44	0.11	0.41	0	0
RM45	1.22	1.56	1.68	0
RM46	0	0.92	0.31	0
RM47	0.04	0.43	0,64	0

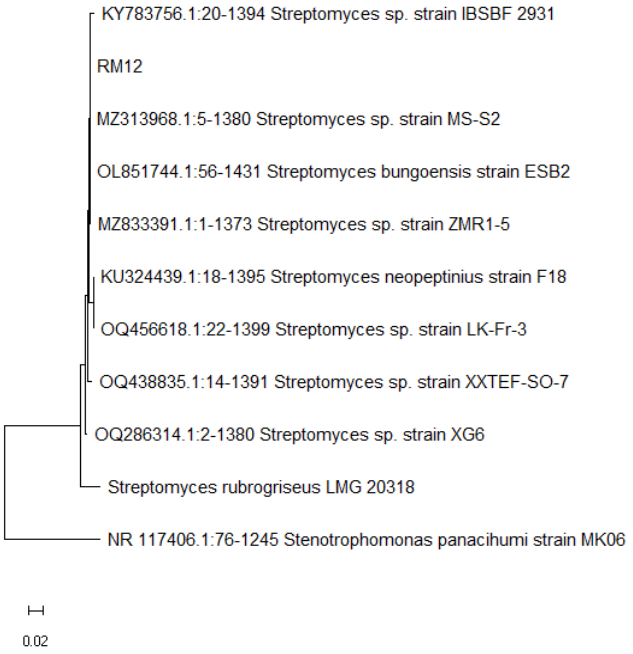


Figure 4. Phylogenetic tree construction 16S rRNA gene of RM12 actinobacteria rhizosphere isolate using neighbor joining approach

Table 3. Similarity of 16S rRNA gene isolate actinobacteria *Pometia pinnata* rhizosphere RM12 compared to gene database in NCBI GenBank

Species	Strain	Similarity (%)	Accession number
Streptomyces bungoensis	ESB2	100	NR_174270.1
Streptomyces sp.	MS-S2	100	MZ313968.1
Streptomyces sp.	ZMR1-5	99.71	MZ833391.1
Streptomyces neopeptinius	F18	100	KU324439.1

Actinobacterial isolates of *P. pinnata* rhizosphere which have been screened were selected based on the largest inhibition zone and are broad spectrum isolates. The selected isolate was RM12 isolate. This isolate was inhibited *S. aureus* ATCC 6538 (0.52), *S. pneumonia* ATCC 1705 (2.86) and *E. coli* ATCC 25922 (1.27). Actinobacterial inhibitory ability against pathogenic bacteria indicated by the magnitude of the inhibition Zone Index (Izh) for each isolate. Based on the size of the inhibition zone index, it is divided into 3 groups; low (Izh <0.4), medium (Izh 0.4 - < 6) and high Izh ≥6 (Retnowati et al. 2017). Based on this category, isolate RM12 has medium antibacterial activity against *S. aureus* and high against *S. pneumonia* and *E. coli.*

The inhibitory activity of actinobacterial isolates against Gram-negative and Gram-positive bacteria is influenced by differences in the type of bioactive compound produced by each isolate, structure and bacterial cell wall compositions, population size, and incubation time. Research conducted by Retnowati et al. (2017) regarding isolation and activity tests actinobacterial antibacterial from the mangrove rhizosphere in the Torosiaje Gorontalo mangrove forest obtained an inhibition zone index for *E. coli* (0.86) and *S. aureus* (5.2). The results of the antibacterial activity test can be seen in Table 4 that the RM12 aqueous fraction can inhibit *E. coli* and *S. aureus* at 250 ppm.

In this study, Aqueous extract and ethyl acetate extract from rhizosphere actinobacteria RM 12 were analyzed through TLC (Figure 5). TLC plat observation at 254 nm wavelength there are dark spots on a green background. Dark spots that detected at 250-260 nm wavelength might contain aromatic compounds such as aromatic amino acids and phenols (Harborne 1973). Figure 5 showed rhizosphere actinobacterial fraction contains several chemical compounds as indicated by several spots in each fraction and migration chemical compounds on the silica plate. 5% FeCl3 staining reagent to visualize spots of different colors. This will make it possible to show certain groups of chemical compounds such as phenols (Praptiwi et al. 2019a). In aqueous extract, one spots (R_f 0.74) were observed in 254 nm and 366 nm wavelength. After elavuate using $FeCl_3$ 5%. the aqueous fraction did not detect any phenolic or polyphenolic compounds. Differ with ethyl fraction, there are 4 spots (R_f 0.14; 0.2; 0.36; 0.5; 0.64) and detected there some phenolics compounds in R_f 0.36.

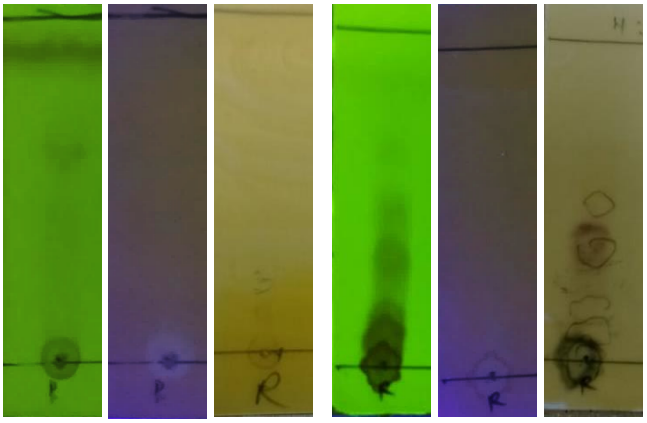


Figure 5. Thin layer chromatography bioautography of aqueous fraction (left) and ethyl acetate fraction (right). A. Visualization in 254 nnm. B. Visualization in 366 nm, and C. visualitation using $FeCl_3$ 5%

Meanwhile, the aqueous fraction has antibacterial activity. Based on this experiment, it can be concluded that the aqueous fraction that has antibacterial activity is not the phenol and polyphenol group. A class of compounds that have various antibacterial activities. Research by Cao et al. 2020 showed that actinobacteria isolated from the sea have antibacterial activity and it was identified that these compounds belong to the flavonoid group. *Streptomyces* sp. CRB46 was isolated from *Cyperus rotundus L.* rhizosphere in the Cemoro Sewu highland, Indonesia was detected to have antibacterial activity and this compound was included in the terpenoid group (Ambarwati et al. 2020). There are still many classes of antibacterial compounds produced by *Streptomyces* that have not been explored. Antibacterial compounds from Streptomyces RM 12 which can still be explored in the future for the development of antibacterial drugs.

In conclusion, a total of 34 actinobacterial isolates were isolated from the rhizosphere of *P. pinnata*, 27 actinobacteria isolates produced aerial mycelium and 2 isolates produced pigments. Morphological identification of *P. pinnata* rhizosphere actinobacteria having the most similarities with *Streptomyces*. There are 34 isolates, 50% had antibacterial activity against *S. aureus* ATCC 6538, *S. pneumonia* ATCC 1705 and *E. coli* ATCC 25922. A total 20.58% of actinobacteria isolates actively inhibited the growth of *C. albicans* ATCC 10231. RM 12 isolate was the highest activity in inhibiting microbial growth so that it was selected as the selected isolate for extraction. Based on the 16S rRNA gene, actinobacterial rhizosphere RM12 isolate has similarity with *Streptomyces bungoensis*. RM12 isolates aqueous fraction had antibacterial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 6538 at 250, 500, and 1000 ppm. TLC bioautography showed antibacterial compounds from rhizosphere actinobacteria RM 12 do not belong to the polyphenol or phenol compound group. Based on this research RM 12 isolates can be further developed as a source of microbial-based antibiotics drugs in the future.

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