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MORPHOLOGICAL CHARACTERIZATION AND ANTIOXIDANT ACTIVITY OF ACTINOBACTERIA FROM *Xylocarpus granatum* GROWING IN MANGROVE HABITAT

KARAKTERISASI MORFOLOGI DAN AKTIVITAS ANTIOKSIDAN AKTINOBAKTERI DARI Xylocarpus granatum YANG TUMBUH DI HABITAT MANGROVE

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ABSTRACT

Xylocarpus granatum produces various bioactive compounds with diverse biological activities, one of them as an antioxidant. Endophytic actinobacteria can also produce bioactive compounds. This study aimed to explore the existence of the endophytic actinobacteria of X. Granatum and determine its antioxidant activity. The research method begins with the isolation of endophytic actinobacteria from fruits, seeds, and leaves using selective media Humic Acid (HV). Furthermore, characterizing the obtained isolates using four different media, namely YSA, ISP2, ISP3, and ISP4, measured antioxidant activity for actinobacterial supernatants and actinobacterial ethyl acetate extracts using the DPPH method. The results showed that from X. granatum, 15 isolates of endophytic actinobacteria were obtained from fruit, seeds, and leaf. Endophytic actinobacteria isolates from X. granatum showed different colors of aerial mycelium, substrate mycelium, and spore chain type. XGF11, XGF12, and XGF4 isolates produced high antioxidant activity of the supernatant; 57.30%, 49.77%, and 41.90% inhibition, respectively. The antioxidant capacity of XGF12 ethyl acetate extract was 501.60 µg AEAC/mg extract and 465.47 µg AEAC/mg extract for XGF11 ethyl acetate extract. DNA extraction and 16S rRNA amplification from endophytic actinobacteria X. granatum according to Presto Mini gDNA Bacteria Kit Protocol. Based on 16S rDNA gene analysis, isolates XGF12 and XGF11 showed 99.16% and 99.42% similarities with Streptomyces xylanilyticus, respectively. Meanwhile, XGF4 showed to have 99.82% similarities with Brevibacterium sediminis.

Keywords: *antioxidant, biodiversity, endophyte, ethyl acetate extract,16S rRNA*

ABSTRAK

Xylocarpus granatum menghasilkan berbagai senyawa bioaktif dengan aktivitas biologis yang beragam, salah satunya sebagai antioksidan. Actinobacteria endofit juga dapat menghasilkan senyawa bioaktif. Penelitian ini bertujuan untuk mengetahui keberadaan aktinobakteri endofit X. granatum dan mengetahui aktivitas antioksidannya. Metode penelitian diawali dengan melakukan isolasi aktinobakteri endofit dari buah, biji, dan daun menggunakan media selektif Humic Acid (HV). Selanjutnya dilakukan karakterisasi isolat yang diperoleh menggunakan empat media berbeda yaitu YSA, ISP2, ISP3, dan ISP4, mengukur aktivitas antioksidan supernatan aktinobakteri dan ekstrak etil asetat aktinobakteri menggunakan metode DPPH. Ekstraksi DNA dan amplifikasi gen 16S rDNA dilakukan sesuai protokol Presto Mini gDNA Bacteria Kit. Hasil penelitian menunjukkan bahwa dari X. granatum diperoleh 15 isolat actinobacteria endofit dari buah, biji, dan daun. Isolat aktinobakteri endofit dari X. granatum menunjukkan perbedaan warna pada miselium aerial, miselium substrat, dan penataan rantai spora. Supernatan dari isolat XGF11, XGF12, dan XGF4 menghasilkan aktivitas antioksidan yang tinggi, secara berurutan 57,30%, 49,77%, dan 41,90% penghambatan. Kapasitas antioksidan ekstrak etil asetat XGF12 adalah 501,60 g AEAC/mg ekstrak dan 465,47 g AEAC/mg ekstrak untuk ekstrak etil asetat XGF11. Berdasarkan analisis gen 16S rDNA, isolat XGF12 menunjukkan kemiripan 99.16% dan XGF11 menunjukkan kemiripan 99.42% dengan Streptomyces xylanilyticus, sedangkan isolat XGF4 menunjukkan kemiripan 99.82% dengan Brevibacterium sediminis.

Kata kunci: *antioksidan, biodiversitas, endofit, ekstrak etil asetat, 16S rRNA*

INTRODUCTION

Xylocarpus granatum is a mangrove plant from the *Meliaceae* family whose environment is in extreme conditions. *X. granatum* contains limonoids used by coastal communities as traditional medicine [1]. This plant can produce tyrosinase inhibitors, has antioxidant activity, and specific secondary metabolites. Most coastal communities use the fruit as a traditional powder for skincare and traditional medicine. *X. granatum* is known to produce alkaloids, triterpenoids

(limonoids), steroids, and phenolics. Limonoid compounds are reported as the most dominant compounds in this plant and have various biological activities [2].

X. granatum fruit peel can inhibit the tyrosinase enzyme system with an IC_{50} value of 784.87 µg mL^{-1} (monophenolase) and 1176.66 µg mL^{-1} (diphenolase) at a maximum concentration of 2000 µg mL^{-1} [3]. In addition, Zamani [4] also reported the ability of *X. granatum* seeds as a tyrosinase inhibitor and antioxidant agent with an IC_{50} value of monophenolase of 323.11 µg mL^{-1} and IC_{50} value diphenolase of 1926.03 µg mL^{-1}.

Endophytic microbes can also produce secondary metabolites similar to those produced by their host plants. Endophytic microbes have been proven to be a source of natural bioactive compounds, one of which is antioxidants. The benefits of endophytic microbes can be used as an alternative to producing various bioactive compounds from their hosts. One of the endophytic microbes that have the potential to produce bioactive compounds is actinobacteria. Actinobacteria can live as endophytes that can produce the same bioactive compounds as their host. Actinobacteria are known as microbes rich in secondary metabolites with a vast life span, one of which is found in plant tissues [5]. In addition, groups of endophytic microbes such as actinobacteria have been known as producers of bioactive compounds used in industrial development, such as in the pharmaceutical field. These actinobacteria represent the most important group of microbes due to their economic value and play an essential role in biotechnology [6].

Actinobacteria have long been known as endophytic microbes that can produce secondary metabolites with various biological activities such as antioxidants. Antioxidants are molecules that can stabilize free radicals before they attack cells. The mechanism of action of antioxidants is to complement the lack of electrons and inhibit the chain reaction of the formation of free radicals. Mangrove ecosystems show potential natural resources such as new actinobacteria due to their high species diversity. In addition, mangroves can form a unique environment because they are influenced by tidal, muddy soil due to intermittent flooding. This ecosystem is very rich in organic matter, nitrogen, and sulfur content to be used by microorganisms [7].

Endophytic actinobacteria in mangroves are an excellent source for producing bioactive compounds due to their different environmental conditions from the land. Previous research has been conducted by Ariansyah et al. [8] and succeeded in finding several endophytic actinobacteria *X. granatum* from the rhizosphere, leaves, and fruit that have indications as antioxidants. However, information regarding the molecular identity and morphological diversity of actinobacteria from the fruit, seed, and leaf of *X. granatum* has not been reported, so it is essential to study the endophytic actinobacteria *X. granatum* as a source of germplasm for antioxidants.

METHODS

Isolation of endophytic actinobacteria *Xylocarpus granatum* was collected from Torosiaje village, Popayato districts, Gorontalo, North Sulawesi. Samples used came from the fruits, seeds, and leaves. Surface sterilization of samples was carried out using the method of Coombs and Franco [9]. Samples were cut to a size of 1 cm x 1 cm, then soaked in 70% ethyl alcohol for 1 minute, 1% NaOCI for 5 minutes, 70% ethyl alcohol for 1 minute, and rinsed using sterile distilled water three times. The sample was then ground aseptically using a mortar until smooth. A total of 0.1 mL of sample suspension was inoculated into HV media (*humic acid*) and incubated for 14 days. Colonies of endophytic actinobacteria were purified using International Streptomyces Project (ISP) 2 media.

Morphological characterization of endophytic actinobacteria. The pure isolates were morphologically characterized based on pigmentation, substrate mycelium color, and aerial mycelium color on four different media, namely ISP2, ISP3, ISP4, and YSA. Then, microscopic characterization was carried out on the arrangement of the spore chains using a light microscope at 400x [10].

Screening antioxidant activity of endophytic actinobacteria. Endophytic actinobacteria were subcultured using solid ISP4 media and then incubated at room temperature for ten days. After that, the endophytic actinobacteria colonies were taken with a sterile straw with a diameter of 0.5 cm, and it inoculated into 30 mL of ISP2 media as much as one circle. The culture was incubated at room temperature for ten days using a rotary shaker at 110 rpm. Next, the culture was centrifuged at 3000 rpm for 15 minutes at 4°C. Supernatant and cell biomass were separated. The cell biomass was dried in the oven at 60°C, and the supernatant was used for screening for antioxidant compounds. A total of 1000 µl supernatant was put into a cuvette then reacted with 1000 µl of 0.125 mM *2,2-diphenyl-1-pikrilhidrazil* (DPPH) solution for antioxidant screening using the DPPH method [11]. The reaction mixture was homogenized and incubated in a dark room for 30 minutes. The absorbance of the suspension can be measured at a

wavelength of 517 nm. The percentage of DPPH resistance is measured using the formula :

$$\%\text{Inhibition} = [1 - (A_S - A_C) : (A_0 - A_C)] \times 100\%$$

As = Sample absorbance
Ac = Control absorbance
A0 = Blank absorbance

Extraction of antioxidant substance from the supernatant of endophytic actinobacteria. The supernatant was extracted using ethyl acetate in a ratio of 1:1. The supernatant and ethyl acetate layer was separated using a separating funnel, and the ethyl acetate layer was concentrated using a rotary evaporator at 45 ºC. Extract yield was determined.

Determination of antioxidant capacity of ethyl acetate extract. Antioxidant capacity was expressed in *ascorbic acid equivalent antioxidant capacity* (AEAC). Determination of antioxidant capacity was carried out after determining the standard curve of ascorbic acid (vitamin C). The absorbance measurement results obtained are entered into the line equation of the standard curve obtained so that the results obtained can show the equivalence of antioxidant in the sample with ascorbic acid [11].

Statistical analysis. DPPH inhibitory activity by supernatant actinobacteria endophytic *X. granatum* and linear regression analysis to determine the average antioxidant capacity of each sample were repeated three times with Microsoft Excel. Data analysis was carried out statistically using one-way analysis of variance (ANOVA).

DNA extraction and amplification of 16S rDNA. The process of isolating genomic DNA from actinobacteria isolates was carried out according to the *Presto Mini gDNA Bacteria Kit Protocol*. The concentration and purification of the genomic DNA can be measured using the Nanodrop Maestro-Nano Pro Spectrophotometer (Maestrogen). The actinobacteria genomic DNA was amplified using the *Polymerase Chain Reaction* (PCR) method. This PCR method used 16S rDNA gene primers that are specific for actinobacteria, namely 16Sact1114 R (5'-GAGTTGACCCCGGCRGT-3') and 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') [12]. DNA amplification used PCR conditions with pre-denaturation steps at 94°C for 5 minutes, denaturation at 92°C for 1 minute, primer attachment at 53°C for 30 seconds, elongation at 72°C for 30 seconds, and post elongation at 72°C for 3 minutes. This step is carried out for up to 30 cycles, with the primer attachment temperature being increased to 55°C.

DNA sequencing and phylogenetic analysis. The amplification of the actinobacteria 16S rDNA gene then determined the nucleotide base sequence. The 16S rDNA sequence homology was searched using BLAST on GenBank using the Eztaxon program, which can be accessed on the *EzBioCloud.net* website. Phylogenetic tree construction was carried out using the *neighbor-joining* (NJT) method using MEGA software with 1000 times bootstrap [13].

RESULTS AND DISCUSSION

Endophytic actinobacteria of *Xylocarpus granatum*. Fifteen isolates of endophytic actinobacteria *X. granatum* were isolated with different growth times (Table 1). This study used samples from the village of Torosiaje, Gorontalo, North Sulawesi. According to Baderan et al. [14], this village has a coastal area that strongly supports the growth of various mangrove species, such as *X. granatum.* Coastal conditions have a substrate in the form of mud consisting of sand and clay as a habitat for mangrove growth.

There were 15 isolates of endophytic actinobacteria that were successfully isolated from the fruit, seed, and leaf of *X. granatum*. These isolates were obtained with different growing times using humic acid (HV) media. This media is selective for growing actinobacteria and contains nutrients that are very supportive for the growth of actinobacteria in greater numbers and inhibiting the growth of fast-growing bacteria [15].

Table 1. The difference in growing time for the endophytic actinobacteria *X. granatum* on humic acid media

Source	Code of Isolate	Growing time (Week)
Fruit	XGF_2	3
	XGF_4	4
	XGF_5	
	XGF_6	3
	XGF_7	
	XGF_{11}	7
	XGF_{12}	
Seeds	XGS_1	17
	XGS_3	8
	XGS_4	
	XGS_5	9
	XGS_6	
	XGS_9	
Leaves	XGL_2	10
	XGL_3	

In addition, HV media contains humic acid, which actinobacteria can use as a source of nutrients for their growth. The different growth

times of these isolates were probably due to the ability of each isolate to use the nutrients contained in HV media, the ability of various isolates to grow and adapt to new environments, and differences in plant tissue structure.

Morphological diversity of endophytic actinobacteria *X. granatum*. A total of 15 isolates of endophytic actinobacteria *X. granatum* were successfully purified on ISP2 media. Isolates grown on four different types of media produced different colors of aerial mycelia and substrates, different surface textures, colony shapes, and arrangement of spore chains. This can be used as an initial parameter for morphological characterization because from these results, the differences produced by each isolate (Table 2). Observation on isolates aged 14 days showed various aerial mycelia colorations, primarily pure white, cream, traffic white, and silk gray. The results of this observation are the same as those obtained by Ariansyah et al. [8]; they succeeded in finding the endophytic actinobacteria *X. granatum* with aerial mycelia characteristics of white and bright yellow. In addition, the isolates obtained produced the mycelium color of the substrate in the growth medium. However, the mycelium color of the resulting substrate is mostly the same.

In addition to different growing times, isolates also had various aerial mycelia color morphology and substrate, colony shape, and spore chain arrangement. The morphological diversity can be seen clearly after growing on four different media YSA, ISP2, ISP3, and ISP4. It happens because the composition of each of these media triggers the growth of different actinobacteria colonies. The results showed that most of the aerial mycelia and substrate colors were almost identical, but the resulting growth directions differed. The difference in mycelium color is due to the different responses of each isolate in using the carbon and nutrient source in each medium [16].

Most actinobacteria growth was excellent in ISP4 media because ISP4 contained starch, calcium carbonate, and ammonium sulfate, which supported the growth of actinobacteria, especially the *Streptomyces* group [17]. In addition, the composition of ISP4 contains more minerals, and the carbon source used is more complex than YSA, ISP2, and ISP3 media. The results obtained are supported by previous research by Malek et al. [18], which showed that ISP4 was the most effective medium in growing actinobacteria from mangrove forests in Pahang with an isolated recovery of 31.7%.

Table 2. Color diversity of the actinobacteria endophytic mycelium *X. granatum* on different media

Code	Aerial mycelium color				Substrate mycelium color			
	YSA	ISP 2	ISP 3	ISP 4	YSA	ISP 2	ISP 3	ISP 4
XGF_2	Traffic white	Traffic white	Traffic white	Pure white	Oyster white	Ivory	Traffic white	Cream
XGF_4	Papyrus white	Agate grey	Agate grey	Traffic white	Signal yellow	Zinc yellow	Signal yellow	Sand yellow
XGF_5	Traffic white	Traffic white	Pure white	Traffic white	Traffic white	Lemon yellow	Silk grey	Silk grey
XGF_6	Cream	Papyrus white	Cream	Cream	Lemon yellow	Ivory	Cream	Cream
XGF_7	Traffic white	Traffic white	Pure white	Cream	Beige	Ivory	Pebble grey	Golden yellow
XGF_{11}	Pure white	Pure white	Traffic white	Pure white	Silk grey	Ivory	Silk grey	Pebble grey
XGF_{12}	Traffic white	Pure white	Traffic white	Traffic white	Pebble grey	Ivory	Silk grey	Telegrey
XGS_1	Dusty grey	Dusty grey	Dusty grey	Pebble grey	Olive brown	Olive yellow	Golden yellow	Ochre brown
XGS_3	Traffic white	Signal grey	Traffic white	Traffic white	Ivory	Ivory	Sand yellow	Oyster white
XGS_5	Oyster white	Papyrus white	Oyster white	Pure white	Silk grey	Golden yellow	Silk grey	Cream
XGS_6	Oyster white	Papyrus white	Oyster white	Pure white	Silk grey	Saffron yellow	Silk grey	Cream
XGS_9	Oyster white	Papyrus white	Oyster white	Pure white	Silk grey	Golden yellow	Silk grey	Cream
XGL_2	-	-	-	Pure white	-	-	-	Pebble grey
XGL_3	-	-	-	Pure white	-	-	-	Silk grey

Note: (-) = Does not produce mycelium

The different mycelium color is caused by the composition of the media used, where each different composition in the media can trigger the growth of different isolates. In addition, other factors that can affect the color of the mycelium are the temperature and pH used. The color of the mycelium produced was very diverse. The similarity of the color aerial mycelium and the substrate obtained did not guarantee that the isolates were from the same group. According to Retnowati et al. [17], the characteristics of actinobacteria colonies are not slimy and adhere very tightly to the surface of the medium. The texture of actinobacteria colonies is like powder, and sometimes there is a texture like cotton. Actinobacteria colonies can be distinguished from other bacteria groups by observing the shape of the colonies on the solid media used for growth [19]. In addition, the colony shape of each isolate also varied. Endophytic actinobacteria colonies were medium in size with irregular edges, slight in length with a texture like cotton, and many other shapes (Figure 1).

Some of the isolates obtained could produce pigment on the ISP2 medium. The pigment consists of various colors, such as lemon yellow for isolates XGS_1, XGF_{11} and XGF_{12}. Isolate XGF_2, XGS_6, XGF_7, and XGS_3 have ivory color pigmentation; light ivory color pigmentation is produced by isolates XGS_5, XGS_9, and XGF_4 (Figure 2).

Figure 1. Colony form of endophytic actinobacteria *X. granatum* using a stereo microscope with a magnification of 40x

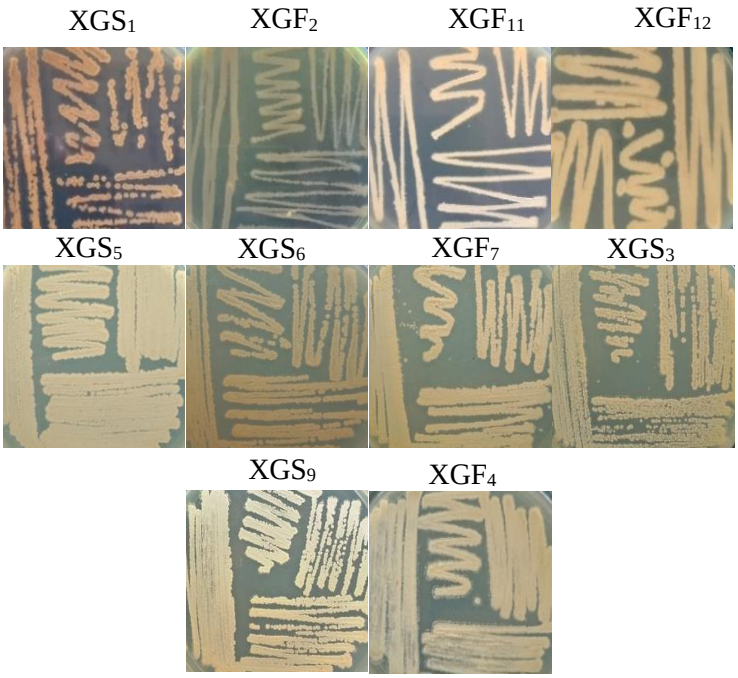

Figure 2. The pigment produced by actinobacteria isolates on ISP2 media

Microscopic observation for spore chain arrangement showed that isolates XGS6, XGF6, XGL_2, XGF_{11}, XGF_5, XGF_4, XGF_2, XGL_3, XGS_9, XGF_7, and XGS_4 had the spore chain type rectiflexibiles. XGS_3 had retinaculiaperti spore chain type, then XGF_{12} and XGS_5 had spiral spore chain type. Rectiflexibiles spore chain type is a type of straight spore chain, and retinaculiaperti is a spore form with hooks (Figure 3).

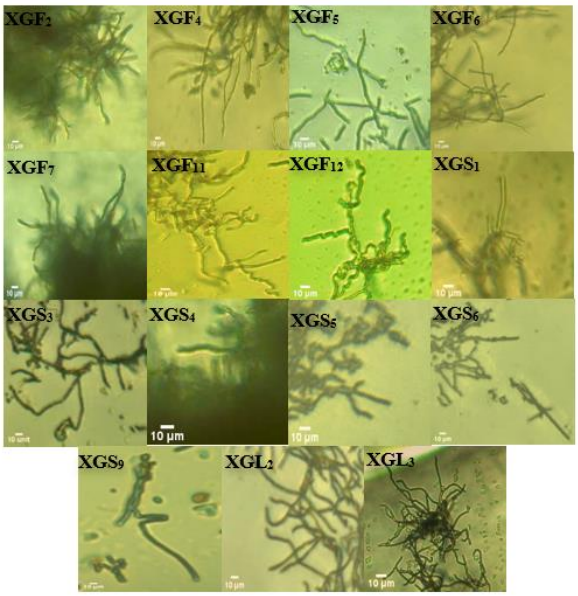

Figure 3. The spore chain type of endophytic actinobacteria *X. granatum* aged 14 days in ISP2 media was observed through a light microscope with 400x magnification

Antioxidant activity of supernatant. The results showed that all the tested isolates have antioxidant activity. This is evidenced by the fact that all isolates could produce percent inhibition, where percent inhibition was the ability of the bioactive compounds produced by each isolate to reduce DPPH free radicals. XGF_{11} isolates produced the highest percentage of inhibition at 57.30% (Table 3). However, compared with ascorbic acid, which was used as a positive control, the rate of inhibition of ascorbic acid was still higher. The percentage of inhibition produced by each isolate was very diverse. This may be due to the different mechanisms of the bioactive compounds produced by each isolate in reducing DPPH free radicals.

The highest antioxidant activity was created by isolates derived from fruit. Compared with other plant organs, more fruit endophytes were obtained which have antioxidant activity. The results support the traditional usage of this plant as an ingredient for treating skin. According to Batubara et al. [20], *X. granatum* is used by coastal communities as an ingredient to make traditional medicine, produce tyrosinase inhibitors, and be used as an antioxidant.

Table 3. Inhibitory activity of DPPH supernatant actinobacteria endophytic *Xylocarpus granatum*

Isolate code	Average DPPH radical inhibition (%)
XGF_2	26.93 ±0.25^{e}
XGF_4	41.90 ±0.26^{h}
XGF_5	41.83 ±0.35^{h}
XGF_6	6.80 ±0.78^{a}
XGF_7	32.57 ±1.46^{f}
XGF_{11}	57.30 ±0.17^{j}
XGF_{12}	49.77 ±0.29^{i}
XGS_1	31.87 ±0.25^{f}
XGS_3	34.47 ±0.06^{g}
XGS_4	30.03 ±0.12^{e}
XGS_5	9.13 ±0.12^{b}
XGS_6	19.70 ±0.36^{c}
XGS_9	41.77 ±1.00^{h}
XGL_2	26.53 ±0.51^{h}
XGL_3	6.43 ±0.75^{a}
Ascorbic acid (100 ppm)	98.57 ±0.35^{k}

Numbers followed by the same letter notation are not significantly different at the 95% test level (Tukey's test)

The seeds of this fruit are used as a traditional powder for skincare [21]. Hendrawan et al. [22] say leaves of *X. granatum* contain phenolic compounds that function as antioxidants. The phenolic compounds can be used as antioxidants because they can prevent new free radicals by converting them into stable molecules.

XGF_{11} produced the highest inhibition of DPPH scavenging activity among all isolates (Table 3). A color change from purple to yellowish in the sample after being reacted with DPPH occurs because the compound donates a hydrogen atom to the DPPH radical to reduce it to a more stable form of DPPH-H *(1,1-diphenyl-2-pikrilhidrazil).* Testing antioxidant activity using the DPPH method is one of the most common and relatively rapid methods to determine a sample's antioxidant activity. Antioxidant activity can be measured through the effects of antioxidants in controlling the oxidation process. The DPPH method was used to examine the activity of antioxidant compounds in inhibiting the oxidation process (2,2-*diphenyl-1-pikrihildrazil*) [23].

The working principle of this DPPH method is the ability of antioxidant compounds to stabilize free radical compounds through electron transfer mechanisms or hydrogen donors. In general, the DPPH method is used to test antioxidant activity in vitro. This method has several advantages compared to other methods; DPPH method only requires fewer samples during the analysis process; the procedure is more superficial, sensitive, and realtively fast. However, the DPPH method must be carried out very carefully because it is susceptible, easily degraded by light, oxygen, and pH [24]. In this test, ascorbic acid (Vitamin C) was

used as a positive control because ascorbic acid is a secondary antioxidant that reduces free radicals and prevents chain oxidation reactions. In addition to the DPPH method, the determination of antioxidant activity can also be carried out using the ABTS method (*2.2-azinobis-3-ethylbenzatiazolin-6-sulphonic acid*). The ABTS solution is blue-green, but if it's reacted with a sample containing antioxidant compounds, it will be reduced to become more stable so that the solution becomes colorless. The working principle of this ABTS radical is the ability of antioxidant compounds to stabilize free radical compounds by donating proton radicals. The advantage of this ABTS method is that it can be used at different pH levels, so it is instrumental in measuring the antioxidant activity of samples extracted using acid solvents, faster reaction times, and the specific absorbance produced. However, this method has weaknesses, such as not describing the body's defense system against free radicals so that the ABTS method is only used as a comparison method.

The antioxidant capacity of ethyl acetate extract. The potential actinobacteria supernatant was extracted using semipolar ethyl acetate solvent. The ethyl acetate solvent was intended to make the bioactive compounds' components varied, both polar and nonpolar groups. Potential isolates used to determine antioxidant capacity were XGF_{11}, XGF_{12}, and XGF_4, with various yield percentages. XGF_{11} has a yield value of 0.33%, XGF_{12} is 0.40%, and XGF_4 has the smallest yield value of 0.23%.

Antioxidant capacity is expressed in *ascorbic acid equivalent antioxidant capacity* (AEAC) [11]. The results showed that the highest antioxidant capacity was produced by XGF_{12} isolate which was 501.60 µg AEAC/mg extract (Figure 4). Based on the perspective isolates, XGF_4, XGF_{11}, and, XGF_{12} the antioxidant capacity was determined. The unit used to determine this antioxidant capacity is the Ascorbic Acid Equivalent Antioxidant Capacity (AEAC). Before the measurement, the antioxidant capacity, the supernatant of each isolate was extracted using ethyl acetate. XGF_{12} produced the highest yield extract. The yield obtained shows the number of components of bioactive compounds in the extract that can be extracted by ethyl acetate solvent. If the yield is small and the activity is high, it cannot be used for commercial purposes. The antioxidant capacity results showed that the ethyl acetate extract of XGF_{12} isolate had the highest capacity (Figure 4). The results obtained are different from the results obtained when determining the antioxidant activity of the supernatant, where the highest antioxidant activity was produced by XGF_{11} (Table 3). This might happen because the sample has not undergone the extraction process when determining the antioxidant activity. However, when determining the antioxidant capacity, the samples used were extracted using ethyl acetate as solvent by the maceration method. In this extraction stage, the compounds present in the sample are separated based on their polarity.

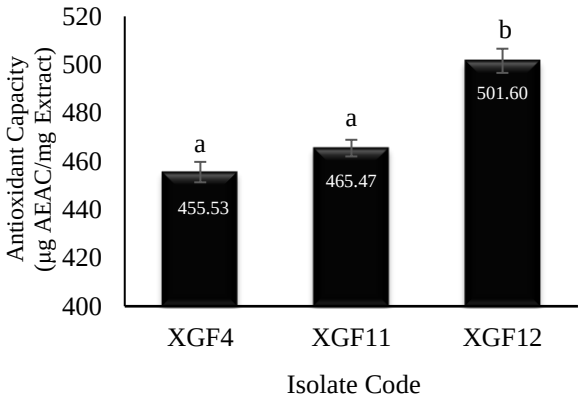

Figure 4. Antioxidant capacity of actinobacteria ethyl acetate extract. The numbers followed by the same letter in the same test method showed no significant difference based on the Tukey test $P<0.05$

Molecular identity of endophytic Actinobacteria *X. granatum* based on 16S rRNA. The potential isolates were further tested using a molecular approach based on the 16S rRNA gene. Based on the results of BLAST analysis, XGF_{11} and XGF_{12}. The amplification results showed that XGF_4, XGF_{11}, and XGF_{12} have a base size of ±1080 bp. Actinobacteria isolates were identified as similar to the homologous strains (comparison) in the *EzBioCloud* database (Table 4). XGF_4 had the highest similarity of 99.82% with *Brevibacterium sediminis* FXJ8.269. Meanwhile, XGF_{11} and SR2-123 had the similarity with *Streptomyces xylanilyticus* by 99.42% and 99.16%, respectively.

The close relationship with *Streptomyces* based on molecular characterization is also supported by the morphological characteristics produced when observing morphological diversity: the rapid growth of hyphae and aerial mycelium formation. According to Procipio et al. [25], hyphae that grow fast and form aerial mycelium are characteristics of the *Streptomyces* group. The *Streptomyces* group is the largest group of actinobacteria, about 77% of the total actinobacteria. In addition, *Streptomyces* is widely used in the health sector because it can produce various secondary metabolites. Several previous studies stated that *Streptomyces* is a group of actinobacteria that can produce secondary metabolites, one of which was found by Zhang et al. [26], who discovered new species, *Streptomyces*

sp. MS1/7 with the potential to produce excellent antioxidant activity because it contains the phenolic compound *2-Allyloxyphenol*.

Streptomyces mangrovisoli MUSC 149 and *Nocardiopsis alba GME22* were known to have potential as an antioxidant. These two isolates were from the *Streptomyces* and *non-Streptomyces* groups used as a comparison. Based on the phylogenetic tree, the kinship of isolates XGF_4, XGF_{11}, and XGF_{12} were far separated from the *S. mangrovisoli MUSC 149* and *Nocardiopsis alba GME22* (Figure 5).

The phylogenetic tree results showed that XGF_{11} and XGF_{12} isolates were in the same group and had a very close relationship with *S. xylanilyticus*. In contrast, XGF_4 had a very close relationship with *B. sediminis.* Previous research has found that *S. xylanilyticus* SR2-123 comes from the soil. This isolate has enzyme activity as β-Galactosidase and β-Glucosidase and other enzyme activities [27]. Other research conducted by Chen et al. [28] found that isolates *B, sediminis* sp. was isolated from deep-sea sediments. The isolate was found to have naphthol-AS-BI-phosphohydrolase, acid phosphatase, and oxidase activities.

Table 4. Molecular identity of endophytic actinobacteria *X. granatum* compared with homologous strains based on the 16S rRNA gene sequence

Isolate code	Accession number	Species	Strain	Similarity (%)
XGF_4	KX356313	*Brevibacterium sediminis*	FXJ8.269(T)	99.82
	BCSJ01000023	*Brevibacterium epidermidis*	NBRC 14811(T)	99.08
XGF_{11}	LC128341	*Streptomyces xylanilyticus*	SR2-123(T)	99.42
	AY999720	*Streptomyces coerulescens*	ISP 5146(T)	98.54
XGF_{12}	LC128341	*Streptomyces xylanilyticus*	SR2-123(T)	99.16
	AY999720	*Streptomyces coerulescens*	ISP 5146(T)	98.20

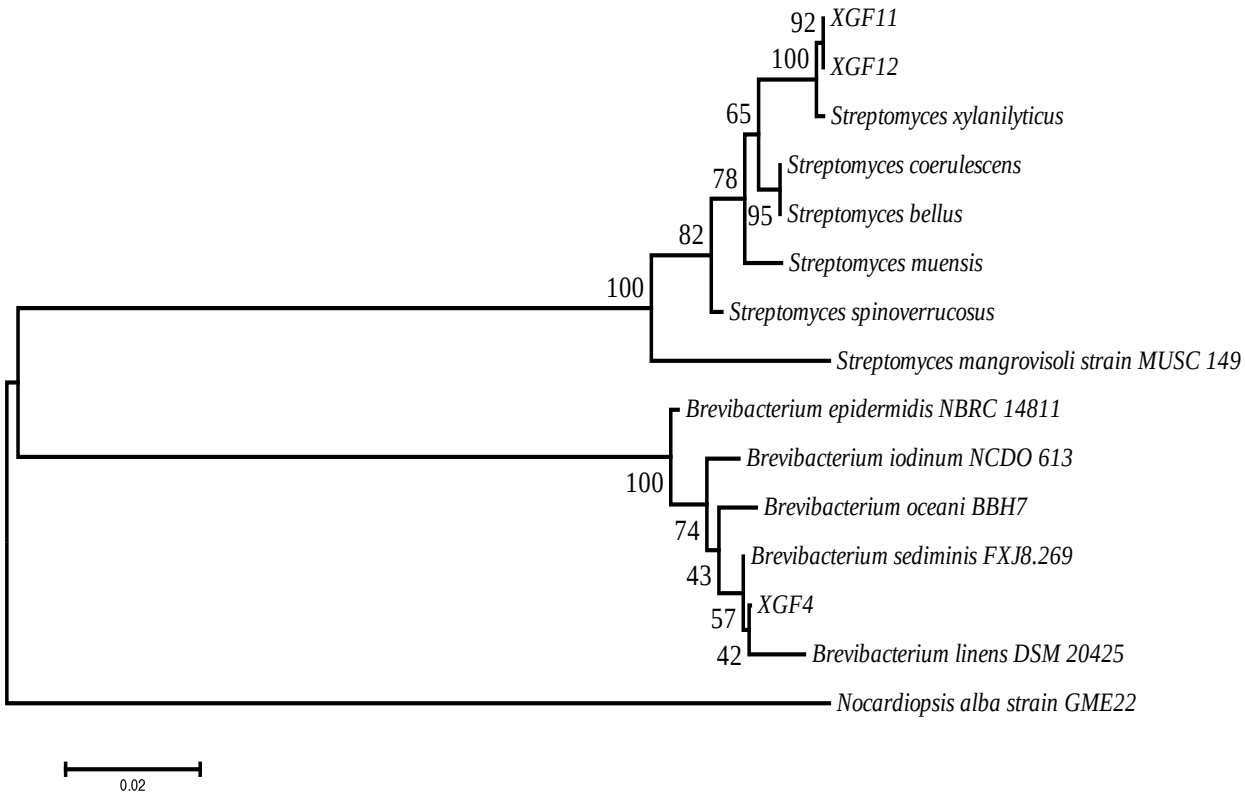

Figure 5. Phylogenetic tree of endophytic actinobacteria *X. granatum* based on 16S rRNA gene analysis

CONCLUSION

Endophytic actinobacteria were found in *X. granatum* as many as 15 isolates with different growth times and morphological diversity. All isolates could produce various antioxidant activity, which XGF11, XGF12, and XGF4 had 57.30%, 49.77%, and 41.90%, respectively. Based on the 16S rRNA gene analysis, XGF11 and XGF12 isolates had similarity with *S. xylanilyticus* SR-123, while XGF4 had similarity with *B. sediminis* FXJ8.269.

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