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The presence of endophytic actinobacteria in mangosteen peel (*Garcinia mangostana*) and its antioxidant activity

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Abstract. *Larasati F, Batubara I, Lestari Y. 2020. The presence of endophytic actinobacteria in mangosteen peel (*Garcinia mangostana *L.) and its antioxidant activity. Biodiversitas 21: 1488-1497.* Mangosteen (*Garcinia mangostana* L.) is a family member of Clusiaceae which is rich in secondary metabolite compounds that can function as antioxidants. Besides being produced by its host plant, the bioactive compounds can also be produced by endophytic actinobacteria. The purpose of this study was to explore the presence of endophytic actinobacteria from mangosteen peel and determine its antioxidant activity. The actinobacteria were isolated, purified, morphologically characterized, molecularly identified, extracted with ethyl acetate and tested for antioxidant properties. The antioxidant activity was assayed using DPPH (2.2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) methods. The components of extracts were separated by Thin Layer Chromatography (TLC) and bioautography was done to determine the antioxidant bands. As a result, five isolates of endophytic actinobacteria in mangosteen peel showed to have difference in aerial mycelium color, substrate mycelium color, and types of spore chains. Based on 16S rRNA gene analysis, AGM3.2 isolate showed similarity with *Streptomyces griseochromogenes* ATCC 14511 (T) 99.06%. AGM3.1 had similarity with *Streptomyces osmaniensis* OU-63 (T) 98.35%. Meanwhile, AGM2.3 were similar to *Streptomyces xanthophaeus* NBRC B-5414 (T) 99.82%, AGM2.2 had similarity with *Streptomyces xanthophaeus* NBRC B-5414 (T) 98.95%. In addition, AGM2.1 has homology with *Streptomyces goshikiensis* NBRC 12868 (T) 99.52%. Using both DPPH and ABTS, supernatant of AGM2.1 showed the highest antioxidant activity indicated by 36.96 and 98.80 inhibition, respectively. Antioxidant capacity of ethyl acetate extract of AGM2.1 was 22.22 µg AEAC/mg extract (DPPH) and 20.34 µg AEAC/mg extract (ABTS). Meanwhile, ethyl acetate extract of mangosteen peel had antioxidant capacity by 21.17 µg AEAC/mg extract (DPPH) and 18.75 µg AEAC/mg extract (ABTS). Antioxidant bioautographic analysis of mangosteen peel ethyl acetate extract was compared with alpha mangosteen standard. The results showed that alpha mangosteen presence in the mangosteen extract with the same Rf value of 0.64 with standard. Meanwhile, actinobacterial ethyl acetate extract from AGM3.1, AGM2.3, AGM2.2, AGM2.1 each have the same Rf value with the alpha mangosteen standard. However, the spot for alpha mangosteen had dark red color, while spots of the four actinobacterial isolates showed to have blue color indicating different antioxidant compounds. The blue spot indicates the flavone, flavanone, flavonol, and isoflavone. These compounds include a subgroup of flavonoid compounds. Ethyl acetate extract AGM3.2 does not have spot compounds with the same Rf value as the alpha mangosteen standard. Study clearly shows that endophytic actinobacteria from mangosteen peel have potency as antioxidant.

Keywords: Antioxidant, characterization, endophytic actinobacteria, *Garcinia mangostana*

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) belongs to family of Clusiaceae, which is widely found in Asia and Africa (Chattopadhyay and Kumar 2006). Previous phytochemical studies reported that mangosteen contains secondary metabolites including xanthone, flavonoid, and phenolic (Ibrahim et al. 2018). These bioactive compounds closely link to antioxidant properties. In Asian countries, mangosteen has been used in traditional medicines for treatment of various diseases such as skin infection, dysentery, diarrhea, and cholerae (Muhsinin et al. 2016). In fact, secondary metabolites are not only synthesized in plants, but also in endophytic microbes living in plant tissues. Endophytic microbes are microbes that live in plant tissue without causing symptoms of disease in the host. Endophytic microbes such as bacteria, fungi, and actinobacteria are known to be able to associate in plant tissue. Endophytes associated with mangosteen have been reported, such as *Botryodiplodea theobromae* isolated from peel of *Garcinia xanthochymus* and *Fusarium* sp. isolated from peel of *Garcinia gummigutta*, in which these microbes potentially as antioxidative properties (Prakash et al. 2013).

Antioxidants have a very good defense mechanism especially in humans against pathologies associated with free radical attacks. Free radicals or Reactive Oxygen Species (ROS) are chemicals substances that can be molecules or atoms that do not have electron pairs in the outer layers of their molecules, this causes ROS to be very reactive (Borek 2001). Antioxidant compounds are known to have the ability to delay or prevent chain oxidation reactions caused by free radicals. Antioxidative compounds serve as reducing agents capable to neutralize toxic ROS before they cause cellular damages (Wolf 2005). Antioxidant activity can be determined by assay using the

DPPH and ABTS methods. The DPPH (2,2´diphenyl picrylhydrazyl) and ABTS (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) methods are among the most widely used methods because of their simple measurement, short assay time (Mishra and Bisht 2012). The activity of antioxidant compounds in inhibiting DPPH and ABTS radicals can be observed from the color changes measured at 517 nm wavelength for the DPPH method (Molyneux 2004) and wavelength 734 nm for the ABTS method (Shalaby and Shanab 2013).

Actinobacteria that have antioxidant abilities have been reported previously. For instance, *Streptomyces hydrogenans* live in *Aloe vera*, reported showing antioxidant activity as represented by IC_{50} 5.58 ± 0.26 µg/mL (Nafis et al. 2018). In addition, *Streptomyces malaysiense* sp. nov. isolated from mangrove soil showed inhibitory activity of 27.24 ± 1.91% and 27.87 ± 2.19% assessed by DPPH and ABTS assays, respectively (Ser et al. 2016). The presence of actinobacteria in mangosteen peel still rarely reported. Therefore, the purpose of this study was to explore the endophytic actinobacteria diversity in mangosteen peel, and determine its antioxidant activity.

MATERIALS AND METHODS

Sample collection

Mangosteen fruits (*Garcinia mangostana* L.) were collected from Kampung Cengal, in Leuwiliang, Bogor, West Java. The sample was sorted to ensure that mangosteen had no defects.

Isolation and purification of endophytic actinobacteria

The selected mangosteen was washed using sterile distilled water. Surface-sterilization of the fruits was carried out at gradual levels, according to method of Radji et al. (2011). Subsequently, sample was soaked in alcohol 70% for 1 min, then soaked in NaOCl 1% for 5 min, alcohol 70% for 1 min, ultimately rinsed with sterile distilled water for 3 sec. Sterilized peel samples are separated from the fruit flesh, thinly sliced, and weighed as much of 5 g. A total of 0.1 mL crushed peel suspension was then plated on Humic Acid Vitamin B (HV) medium containing cycloheximide and nalidixic acid and Incubated for 14-30 days at room temperature (±28°C). Actinobacteria colonies that grew on the agar medium were purified on International *Streptomyces* Project (ISP) 4 medium was added with nystatin at a concentration of 100.0000 IU/mL.

Morphological characterization of endophytic actinobacteria

The 14 days old colonies were macroscopically observed for substrate mycelium, aerial mycelium color, pigmentation using microscope stereo (BESTCOPE BS3060B), while microscopic observation was used to study spore chain, observed under light microscope (Olympus CX21F51) at magnification of 400x.

Molecular identification of endophytic actinobacteria

The spores and mycelia of endophytic actinobacteria grown in ISP4 medium for 14 days were collected and transferred into 1.5 mL Eppendorf tube. The extraction of these samples was performed using *Geneaid Presto Mini gDNA Bacteria Kit* in accordance with standard protocol. Concentration and purity of the DNA was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). The 16S rRNA gene was amplified using specific primers for actinobacteria 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Bruce et al. 1992) and 16Sact1114R (5GAGTTGACCCCGGCRGT-3') (Martina et al. 2008). PCR mixture (50 µL) contained 25 µL MyTaqTM Extract-PCR Kit, 5 µL primer 27F (100 pmol), 5 µL primer 16Sact1114R (100 pmol), 4 µL template (100 ng/µL), and 11 µL nuclease-free water. PCR condition was performed in pre-denaturation at 94°C for 5 min, 15 cycles of denaturation (denaturation at 94°C for 1 min, annealing at 53°C for 30 sec, elongation at 72°C for 30 sec, post-elongation at 72°C for 3 min), and followed by the second 15 cycles performed at 55°C. The resulting PCR products were electrophoretically analyzed for 20 min at 70 V, then soaked in ethidium bromide (EtBr) for 15 min, and visualized under UV transilluminator. For identifying actinobacteria, the sequence of 16S rRNA gene was compared with sequence database in Genbank using EzBioCloud web. Furthermore, the sequences that were compared were constructed by phylogenetic trees with MEGA X software, bootstrap analysis 1000x repetitions using the neighbor-joining method (Tamura et al. 2018). Outgroups used *Nocardiopsis gilva* (YIM 90087).

Determination of the actinobacteria supernatant antioxidant activity

Isolate of endophytic actinobacteria was subcultured in ISP4 medium for 10 days at room temperature (±28°C). The isolate was collected by 9 mm-sterile plastic straw, then planted in 30 mL ISP2 liquid medium. Afterward, the isolate was incubated at room temperature in a rotary shaker at 100 rpm. After 10 days of incubation, the liquid culture was centrifuged to separate supernatant and biomass using centrifuge (HERMILE Labortechnik GmbH type Z326 k) at speed of 6000 x g and 4°C for 15 minutes. The cell biomass was dried at 60°C for 5 h. Meanwhile, the supernatant antioxidant activity test followed the procedure described by Batubara et al. (2009). Antioxidant activity can be determined by assays using the DPPH and ABTS methods. The DPPH (2.2-diphenyl-1-picrylhydrazyl) and ABTS (2.2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) methods are among the most widely used methods because of their simple measurement, short assay time. Based on the method DPPH assay was performed through reacting 100 µL supernatant with 100 µL DPPH solution, while ABTS assay was carried out by reacting 20 µL supernatant with 180 µL ABTS solution. Each experiment was measured at triplicates. Reaction mixture was homogenized and incubated in darkroom for 30 min. Furthermore, absorbance was detected using ELISA reader at wave-length of 517 nm (for DPPH assay) and 734 nm (for ABTS assay). The antioxidant activity is reported by % inhibition.

Extraction of bioactive compounds in endophytic actinobacteria

The isolate was collected by 9 mm-sterile plastic straw, then planted in 500 mL ISP2 liquid medium. Afterward, the isolate was incubated at room temperature in a rotary shaker at 100 rpm. After 10 days of incubation, the liquid culture was centrifuged to separate supernatant and biomass using centrifuge (HERMILE Labortechnik GmbH type Z326 k) at speed of 6000 x g and 4°C for 15 minutes. Bioactive compounds in endophytic actinobacteria were extracted using procedure reported by Lertcanawanichakul et al. (2015). Supernatant (500 mL) was added with ethyl acetate at 1:1 (v/v) in a separatory funnel, then shaken for 30 min. The layers formed were separated, between supernatant and solvent. The resulting supernatant was re-extracted using ethyl acetate with similar procedure. Finally, top layer (ethyl acetate fraction) was separated, and evaporated using rotary evaporator at 40°C. The extract was stored at 4°C for further analysis.

Extraction of bioactive compounds in mangosteen peel

Mangosteen fruits were washed and peeled. The peel was thin-sliced, dried at 60°C for 24 h (Palakawong et al. 2010), then pulverized using an electric blender. The peel was macerated by soaking it (100 g) in 500 mL ethyl acetate at room temperature (±28°C) for 2×24 h. Macerated sample was filtered through a filter paper to collect the solid fraction. Simplicia powder is re-macerated using ethyl acetate until clear extract was formed (at least 3 times). The extract was concentrated using low pressure at 40°C in a rotary evaporator. The viscous extract was stored at 4°C for subsequent analysis.

Antioxidant activity test

The antioxidant capacity is carried out by the DPPH and ABTS method each performed at 3 repetitions. This antioxidant activity follows the procedure described by Batubara et al. (2009). For DPPH assay, ethyl acetate extract of actinobacteria and ethyl acetate extract mangosteen peel was made at concentration of each 500 µg/mL, while ascorbic acid as control was prepared at 5 concentrations. Each solution (100 µL) was reacted with 100 µL DPPH (75 mM in 100 mL ethanol). The mixture was then incubated in a dark room for 30 min, and the absorbance was measured using ELISA reader at 517 nm. Absorbance (y) and concentration (x) of the control was plotted to arrange linear regression. ABTS antioxidant assay was carried out based on the method of Re et al. (1999). For ABTS assay, 7 mM ABTS solution was reacted with 2.45 mM potassium peroxidisulphate ($K_2S_2O_8$), then incubated in a dark room for 14-16 h. The absorbance was measured at 734 nm. ABTS which is ready to be used as a radical had absorbance value of 0.68-0.70. The extract and control (each 20 µL) were reacted with 180 µL ABTS oxidized radicals. After 30 min of incubation, in a dark room, absorbance was determined at 734 nm. Antioxidant capacity (equivalent to ascorbic acid) was quantified using similar procedure mentioned in DPPH method.

Thin-layer chromatography (TLC) analysis

TLC experiment was carried out using plate silica gel 60 F_{254} as stationary phase (Merck). Standard and sample solution was plotted in TLC plate and left at room temperature till dry (Liza et al. 2017). TLC plate was placed in chromatography chamber previously saturated with eluent chloroform: methanol (9 : 1 v/v) for 30 min (Wijayanti et al. 2018). The chamber was closed until the mobile phase reached upper limit, then the plate was released and dried. Subsequently, eluted plate was detected and analyzed for Rf using CAMAG Reposrostar 3 integrated with WinCATS software. Detection was performed using UV 254 nm and 366 nm.

Bioautography of antioxidant activity

TLC plate from previous stage was sprayed with DPPH 5 mM in methanol, then incubated in a dark room for 30 min. The yellowish bands that have antioxidant activity with a purple plate background as DPPH. The bands were compared with those in chromatogram previously detected in UV 254 nm and 366 nm for quantification of the Rf.

Statistical analysis

Linear regression for determination of antioxidant activity was calculated in Microsoft Excel. Data were statistically evaluated using one-way analysis of variance (ANOVA), while significant difference among means was verified using Tukey test at $P<0.05$.

RESULTS AND DISCUSSION

Diversity of endophytic actinobacteria in mangosteen peel

The results of this study were obtained as many as five endophytic actinobacterial isolates from mangosteen peel. The isolate criteria chosen for assay antioxidant activity were pure colonies, which were supported by observations of macroscopic and microscopic characteristics and molecular analysis results that showed the five isolates had similarities with *Streptomyces*. Morphological characteristics of the five colonies actinobacteria isolates have different colors of aerial mycelium and substrate mycelium when grown in various media (ISP2, ISP3, ISP4, and YSA). However, all isolates did not produce pigmentation in growth medium (Table 1).

In terms of macroscopic characteristics, morphology of the colonies seemed attached to medium surface, like cotton due to presence of aerial mycelium covering the surface of colonies (Figure 1).

Microscopically, all five isolates showed difference in type of spore chains. Based on method of Shirling and Gottlieb (1966) AGM3.2, AGM3.1, AGM2.3, AGM2.2 showed type spiral spore chain, while AGM2.1 showed *retinaculum apertum* (long spiral) spore chain (Figure 2). The results of microscopic observation reinforce the notion that five isolates actinobacteria demonstrated similar characteristics to *Streptomyces*.

Molecular identity based on 16S rRNA gene

Homology sequence analysis using EzBioCloud showed that the five isolates identified based on 16S rRNA had similarities with the genus *Streptomyces*, this was consistent with their morphological characterization (Table 2). AGM3.2 isolate showed similarity with *Streptomyces griseochromogenes* ATCC 14511 (T) 99.06%. AGM3.1 had similarity with *Streptomyces osmaniensis* OU-63 (T) 98.35%. Meanwhile, AGM2.3 were similar to *Streptomyces xanthophaeus* NBRC B-5414 (T) 99.82%, AGM2.2 had similarity with *Streptomyces xanthophaeus* NBRC B-5414 (T) 98.95%. In addition, AGM2.1 has homology with *Streptomyces goshikiensis* NBRC 12868 (T) 99.52%. The results of submitting DNA sequences obtained access numbers and strain isolates. AGM3.2 is species *S. griseochromogenes* strain AGM32 with access number MT197301. AGM3.1 is species *S. osmaniensis* strain AGM31 with access number MT197302. AGM2.3 is a *S. xanthophaeus* strain AGM23 with access number MT197303. AGM2.2 is a *S. xanthophaeus* strain AGM22 with access number MT197304 and AGM2.1 is a *S. goshikiensis* strain AGM21 with access number MT197305.

Table 1. Morphological characteristics of endophytic actinobacteria isolated from mangosteen peel, grown in various media (ISP2, ISP3, ISP4, and YSA) for 14 days

Isolate code	Medium	Color of aerial mycelium	Color of substrate mycelium	Dissolved pigment
AGM3.2	ISP2	Oyster white	Ochre brown	-
	ISP3	Beige brown	Olive brown	-
	ISP4	Silk grey	Sand yellow	-
	YSA	Beige red	Pastel yellow	-
AGM3.1	ISP2	Graphite grey	Olive grey	-
	ISP3	Trafic grey	Stone grey	-
	ISP4	Basalt grey	Pale brown	-
	YSA	Cemnet grey	Grey white	-
AGM2.3	ISP2	Oyster white	Golden yellow	-
	ISP3	Pale brown	Brown beige	-
	ISP4	Pale brown	Olive brown	-
	YSA	Pale brown	Clay brown	-
AGM2.2	ISP2	Beige grey	Golden yellow	-
	ISP3	Beige grey	Light ivory	-
	ISP4	Oyster white	Silk grey	-
	YSA	Pale brown	Mahagony brown	-
AGM2.1	ISP2	Pure white	Olive brown	-
	ISP3	Oyster white	Sand yellow	-
	ISP4	Pure white	Golden yellow	-
	YSA	Pure white	Cream	-

Figure 1. Macroscopic morphology of 14 days-old endophytic actinobacteria colonies isolated from mangosteen peel, grown in various media (top to bottom): ISP 2, ISP3, ISP4 and YSA. Colonies were observed under stereomicroscope at magnification of 40x: A) AGM3.2, B) AGM3.1, C) AGM2.3, D) AGM2.2, E) AGM2.1

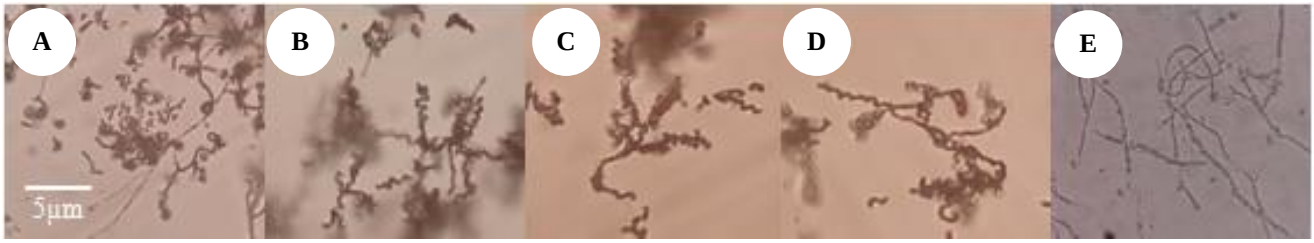

Figure 2. Microscopical morphology of 14 days-old endophytic actinobacteria isolated from mangosteen peel, observed under light microscope at magnification of 400x in ISP4 medium: A. AGM3.2, B. AGM3.1, C. AGM2.3, D. AGM2.2, E. AGM2.1

Table 2. The results of BLAST analysis on 16S rRNA gene endophytic actinobacteria in mangosteen peel according to 16S rRNA gene

Code	Species	Strain	Similarity (%)	Accession number
AGM3.2	*Streptomyces griseochromogenes*	ATCC 14511 (T)	99.06	CP016279
AGM3.1	*Streptomyces osmaniensis*	LGM 20094 (T)	98.35	FJ613126
AGM2.3	*Streptomyces xanthophaeus*	NRRL B-5414 (T)	99.82	JOFT01000080
AGM2.2	*Streptomyces xanthophaeus*	NRRL B-5414 (T)	98.95	JOFT01000080
AGM2.1	*Streptomyces goshikiensis*	NBRC 12868 (T)	99.52	AB184204

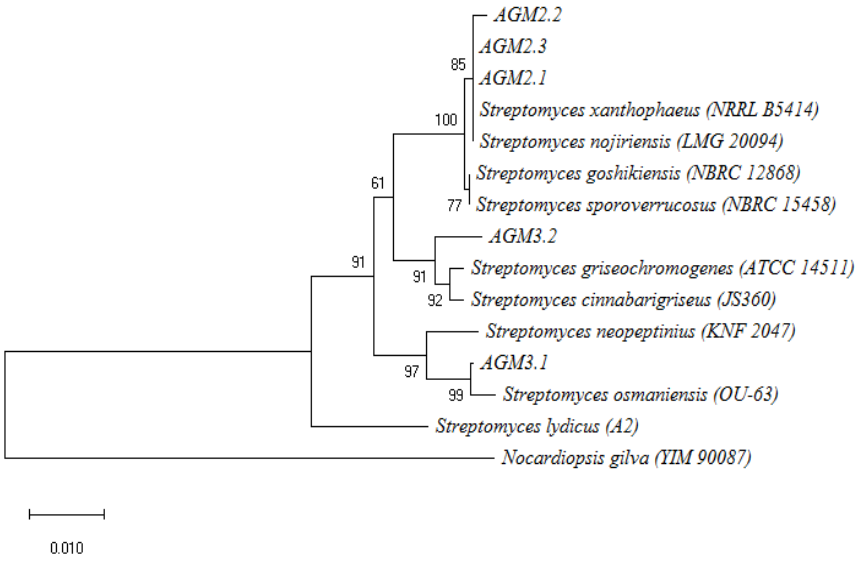

Figure 3. Phylogenetic tree of endophytic actinobacteria isolated from mangosteen peel according to 16S rRNA gene. Percentage of bootstrap at 1000 repetitions is indicated by value in each branch point. Bar 0.010 indicates substitution of nucleotide for each location

Based on phylogenetic tree construction (Figure 3) AGM3.2 isolates has similarity to *S. griseochromogenes*, with a bootstrap value of 91%. AGM3.1 has homology with *S. osmaniensis*, with a bootstrap value of 99%. Showed that the strains AGM2.3, AGM2.2, and AGM2.1 isolates were similar to *S. xanthophaeus* with bootstrap values of 85%. *Nocardiopsis gilva* (YIM 90087), non-*Streptomyces*, have been incorporated as outgroups in phylogenetic reconstruction.

Antioxidant activity of endophytic actinobacteria supernatant

The activity of antioxidant compounds in scavenging DPPH and ABTS radicals is expressed in % inhibition. The results showed that, the five endophytic actinobacterial supernatants of mangosteen peel showed an average % inhibition ranging from 11.36% to 36.96% (using DPPH) and an average % inhibition ranging from 95.86% to 98.50% (using ABTS). Based on the results of the analysis of variance showed that there was a significant difference between the types of isolates against the average value of % inhibition $P < 0.05$. Tukey test suggested that the five actinobacterial supernatants had an average % inhibition using DPPH and ABTS that were significantly different from the average of % ascorbic acid inhibition. Using both methods AGM2.1 showed to have the highest antioxidant activity of 36.96% and 98.50% for DPPH and ABTS respectively, significantly different from the other four isolates (Figure 4).

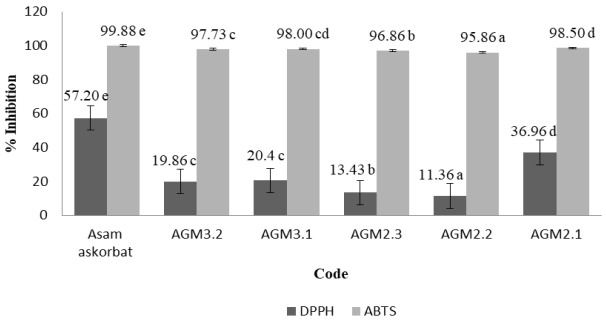

Figure 4. Percent inhibition of mangosteen peel endophytic actinobacterial supernatant. The numbers followed by the same letters in the same test method show no significant difference based on the Tukey test P<0.05

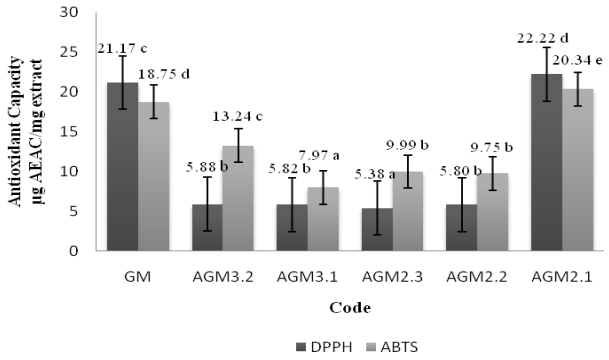

Figure 5. Antioxidant capacity of ethyl acetate extract of mangosteen peel (GM) and actinobacteria (AGM3.2-AGM2.1). The numbers followed by same letters within same assay show no significant difference based on Tukey test at P<0.05

Antioxidant capacity of ethyl acetate extract of actinobacteria and mangosteen peel

Based on the assay results, it is noted that the sample studied contains a significant amount of antioxidative compounds able to scavenge DPPH and ABTS radicals. The antioxidant capacity value of actinobacterial ethyl acetate extracts ranged from 5.38-22.22 µg AEAC/mg extract (DPPH assay) and from 7.97-20.34 µg AEAC/mg extract (ABTS assay). In terms of mangosteen peel extract, the antioxidant capacity was found at range of 21.17 µg AEAC/mg extract and 18.75 µg AEAC/mg extract using similar assay (Figure 5). ANOVA revealed that antioxidant capacity of samples measured by DPPH and ABTS method showed significant difference (P<0.05). Tukey test suggested that all five ethyl acetate extracts of actinobacteria significantly differed compared to those from mangosteen peel in both assays. Ethyl acetate extract of AGM2.1 was evidenced to have the highest antioxidant capacity, i.e. 22.22 µg AEAC/mg extract (DPPH assay) and 20.34 µg AEAC/mg extract (ABTS assay) compared to other isolates of actinobacteria, and being slightly higher than ethyl acetate extract of mangosteen peel. AGM2.1 isolates are thought to have the ability to produce bioactive compounds similar to those produced by host plants.

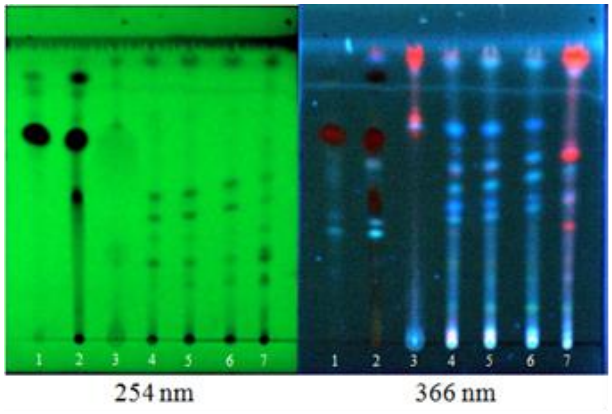

Figure 6. Chromatogram of mangosteen peel ethyl acetate extract and actinobacterial ethyl acetate extract using the mobile phase of chloroform: methanol (9 : 1 v/v). Samples 1-7 (alpha mangosteen standard, mangosteen peel extract, ethyl acetate extract AGM3.2, AGM3.1, AGM2.3, AGM2.2, AGM2.1)

Thin-layer chromatography profile

Detection using UV 254 nm only showed a few dark spots, whereas detection using UV 366 nm spot luminescent color of the compound was more observed in each sample. The results of observations using UV 366 nm showed that the ethyl acetate extract of mangosteen peel and ethyl acetate extract of actinobacteria compared to alpha mangosteen standard had different spot color compounds. The alpha mangosteen standard has one dark red compound spot. Mangosteen peel extract was observed as many as seven spots of blue and dark red compounds. Meanwhile, ethyl acetate extract AGM3.2 has three spots of blue and red compounds. Ethyl acetate extract of AGM3.1, AGM2.3, AGM2.2 isolates had nine spots of blue and slightly greenish compounds while the ethyl acetate extract AGM2.1 has eight spots of compounds in blue, red and green (Figure 6).

Antioxidant bioautography

Bioautographic analysis showed that the alpha mangosteen standard had one active fraction with an Rf value of 0.64 Mangosteen peel extract has five active fractions with an Rf value (0,09, 0.15, 0.55, 0.64, 0.84). In Figure 7, it can be seen that one compound spot on the mangosteen peel ethyl acetate extract shows the same spots as the alpha mangosteen standard, each having an Rf value of 0.64. Actinobacterial ethyl acetate extract AGM3.2 has one spot of active compound with an Rf value of 0.09. In addition, AGM3.1 and AGM2.3 extracts had eight spots of active compound with Rf value followed: (0.09, 0.15, 0.21, 0.41, 0.42, 0.45, 0.55, and 0.64). AGM2.2 extract had eight compound spots with Rf values respectively (0.09, 0.15, 0.21, 0.42, 0.44, 0.45, 0.56, 0.64) and AGM2.1 extract contained three spots of active compound with Rf values of (0.09, 0.27, and 0.64).

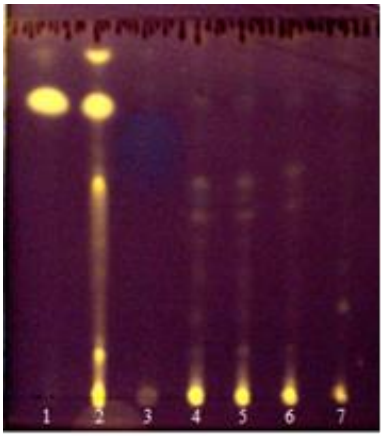

Figure 7. Bioautography antioxidant extract of mangosteen peel ethyl acetate and actinobacterial ethyl acetate extract. Samples 1-7 (alpha mangosteen standard, mangosteen peel extract, ethyl acetate extract AGM3.2, AGM3.1, AGM2.3, AGM2.2, AGM2.1)

Discussion

In this research, a total of five isolates of endophytic actinobacteria were successfully isolated from mangosteen peel. The five isolates had difference in color of aerial mycelium, substrate mycelium, and shape of colonies. Each of the mangosteen peel actinobacteria isolates did not produce color pigments on four different media. Difference in aerial mycelium and substrate mycelium colors is affected by characteristics of species, source of carbon and nitrogen in growth medium (Li et al. 2016). In addition, to nutrition availability, other factors such as temperature and pH also provide effects on characteristics of actinobacteria (Palanichamy et al. 2011). The optimum condition for *Streptomyces* is 25°C and pH of 6-9 (Barka et al. 2016). Macroscopic and microscopic morphological characteristics of actinobacteria isolate mangosteen peel endophytes can be considered as an initial identification to describe the differences between isolates.

Further identification using molecular approach was conducted by using 16S rRNA gene. Based on BLAST analysis, the five isolates showed closed relationship to *Streptomyces*. This is also in accordance with results of morphological analysis (macroscopic and microscopic characters) of of actinobacteria colonies present in mangosteen peel. *Streptomyces* is the biggest genus in actinobacteria phylum, which is reported capable of producing many secondary metabolites (Berdy 2005). The results of phylogenetic tree construction showed that AGM2.1, AGM2.2, and AGM2.3 isolates were in the same group and were closely related to *S. xanthophaeus*. Previous studies reported that various metabolite compounds such as post proline endopeptidase, benarthin, β-galactosidase that inhibit isoflavonoids and geomycins were obtained from *S. xanthophaeus* (Hazato et al. 1979). *S. xanthophaeus* is known to have antibacterial and antifungal activity (Sigh et al. 2016). *S. xanthophaeus* isolated from soil samples in Mount Taibai, Shaanxi Province, China was reported to have potential α-glucosidase inhibitors and phytotoxic effects (Wei et al. 2016). Based on this current study, the potency of *S. xanthophaeus* as antioxidant agent can be considered as first report. Besides being similar to *S. xanthophaeus*, the three isolates were also similar to *Streptomyces nojiriensis*. According to Miyashiro et al. (1983), *S. nojiriensis* C-13 produces antibiotic compounds of the streptothricin group which has antibacterial activity. Phylogenetic tree construction results differ from the results of BLAST which showed that the isolate AGM2.1 was more similar to *S. goshikiensis*. That is because, the short sequence of endophytic actinobacterial samples so that it affects the phylogenetic tree construction. The similarity of actinobacterial isolate was only assumed using the morphological characteristic approach and based on molecular analysis of the 16S rRNA gene. This is not enough to prove that the actinobacterial isolate is the same as the homologous species. Therefore, to prove that the five endophytic actinobacterial isolates are the same as the homologous species, it is necessary to approach the polyphasic method. The principle of the polyphasic method is to combine the characteristics of all genotypes, phenotypes, and phylogenetic information (Vandame et al. 1996). Moreover, AGM3.1 isolate has similarity with *S. osmaniensis* OU-63 at 98.35%. *S. osmaniensis* OU-63 was firstly isolated from garden soil samples on the campus of Osmania University, Hyderabad, India (Reddy et al. 2010). Samy et al. (2019) succeeded in isolating *S. osmaniensis* CA-244599 from soil samples in a savanna environment in Bangouamafsakoa, in the Comoros Islands. *S. osmaniensis* CA-244599 is known to be able to produce osmanicin compounds, streptazoline, streptazone C, streptazone B1, streptenol, nocardamine, and desmethylenyl-nocardamine. It is known that osmanicin has the strongest activity with IC_{50} value of 3.7 µM. Osmanicin compounds are known to be effective in antiaging treatment of the skin (Samy et al. 2019). AGM3.2 isolate had similarity with *S. griseochromogenes* of 99.06%. Previous studies have successfully isolated tautomycetin compound (TTN) which is a protein phosphatase inhibitor (Li et al. 2009). Blasticidin S (BS) compounds from *S. griseochromogenes* have the potential as strong fungicides against rice pathogens caused by *Pyricularia oryzae* (Gould and Guo 1994) and as cytotoxic antibiotics (Zabriskie et al. 2003). *Nocardiopsis gilva* (YIM 90087) namely the non-*Streptomyces* groups have been included as outgroups and used as comparative isolates that are known to have antioxidant activity. *N. gilva* (YIM 90087) is a non-*Streptomyces* actinobacterial group reported by Tian et al (2013) having antioxidant activity against DPPH and ABTS.

The results of this study clearly showed that the endophytic actinobacterial supernatant on mangosteen peel showed inhibitory activity ranging from 11.36%-36.96% (DPPH assay) and 95.86%-98.50% (ABTS assay). Bioactive compounds produced from the supernatant of each actinobacterial isolate have different abilities to reduce the two radicals. The difference in inhibition values in each actinobacterial supernatant is suspected, because the mechanism of bioactive compounds that have the ability as antioxidants in reducing DPPH and ABTS radicals is not the same. This results in a higher average %

inhibition value for certain types of radicals. (Figure 3). This study uses the DPPH and ABTS methods. Both of these methods have advantages and disadvantages. The DPPH method has several advantages which are technically simple, practical, and fast. However, it has several disadvantages, such as being sensitive to acidic pH, samples containing anthocyanins cause too low antioxidant activity, and DPPH radicals react slowly with samples (Arnao 2000). The ABTS method has advantages such as, it can be used at different pH levels so it is useful for measuring the antioxidant activity of samples extracted with acid solvents, providing specific absorbance, and faster reaction times. However, it has disadvantages that testing using ABTS does not found in biological systems and is not similar to radicals found in those systems so that ABTS can only be used as a comparison method (Karadag 2009).

The five crude extracts of actinobacteria were obtained by extraction using maceration method. Ethyl acetate as a semipolar solvent was chosen with the expectation that the components of the bioactive compounds obtained varied, both polar and non-polar groups of compounds. Based on the results of antioxidant capacity testing using DPPH and ABTS methods, AGM2.1 ethyl acetate extract was found to have the highest antioxidant capacity compared to the four other isolates and was slightly higher than the mangosteen peel ethyl acetate extract. This is related to the role of endophytic actinobacteria that live in plant tissues. Endophytic microbes are known to be able to produce identical bioactive compounds, even have higher activity compared to their host plants. The value of antioxidant capacity in each sample in reducing DPPH and ABTS radicals is not the same. This can be expected because of the ability of bioactive compounds that have antioxidant activity from the ethyl acetate extract of mangosteen peel and actinobacterial ethyl acetate extract in reducing DPPH and ABTS radicals have different proton and electron transfer mechanisms. DPPH radicals are known capable of retrieving hydrogen atoms through mechanism of electron transfer (Molyneux 2004). Whereas, principle of antioxidant activity using ABTS radicals is of action for radical scavenging is based on the ability of antioxidant compounds to donate proton, then reacting with cation radical in ABTS radicals through mechanism of proton transfer (Re et al. 1999).

Previous studies reported that antioxidant capacity of ethyl acetate extract of mangosteen (*Garcinia mangostan*a L.) peel was reported at 33.32 µg/mg equivalent to Trolox, measured using DPPH assay (Suttirak dan Manurakchinakorn 2014). In addition, the radical activity of methanolic extract of *Garcinia parvifolia* peel towards ABTS radicals reached 22.6 mg AEAC/g sample (Hasan et al. 2013). Based on the results of this study the antioxidant capacity of mangosteen peel ethyl acetate extract was obtained with a DPPH of 21.17 and with ABTS of 18.75. When compared with the antioxidant capacity of previous studies, mangosteen peel skin ethyl acetate extract has a slightly lower antioxidant capacity. However, as compared to the antioxidant capacity of mangosteen peels obtained from this study the antioxidant capacity of AGM2.1 was higher. This is presumably because, the components of the bioactive compound from mangosteen peel extract are different from the components of the bioactive compound produced from the actinobacteria extract, so the antioxidant capacity is different in reducing free radicals. This predicted phenomenon is supported by the result from thin layer chromatography and bioautography.

Spot detection of compounds in the sample using UV 254 nm only appears dark spots, because the absorption of compounds to light will be reduced and there will be a blackout process (diminish). The stationary phase in the form of silica gel contains a fluorescence indicator that can absorb light at a wavelength of 254 nm, so that only the mobile phase is fluorescent (Sherma and Fried 2003). Based on the results of the detection of compounds with UV 366 nm in Figure 5 shows that the five ethyl acetate extracts of actinobacteria have different compound components compared to the alpha mangosteen standard. As stated by Markham (1982), the fluorescence spot in TLC plate appeared as blue color when observed at wavelength of 366 nm, indicating that they represented flavon, flavanon, flavonol, and isoflavon compounds, while red color indicated presence of anthocyanidin and greenness is auron and flavon compounds.

Bioautography with TLC was carried out to qualitatively analyze the active compound band in reducing DPPH radicals from ethyl acetate extract from mangosteen peel and ethyl acetate extract from actinobacteria compared to alpha mangosteen standard. Alpha mangosteen compound is a xanthone derivative compound that is extracted from mangosteen peel. In addition to xanthone compounds, other compounds that have antioxidant activity are flavonoids, and phenolics that have been successfully extracted from mangosteen plants (Ibrahim et al. 2018). Based on the results of this study indicate that the ethyl acetate extract of mangosteen peel contained alpha mangosteen compounds and several other compounds (Figure 7). Meanwhile, the actinobacterial ethyl acetate extracts AGM3.1, AGM2.3, AGM2.2, and AGM2.1 have compound spots with Rf values equal to the alpha mangosteen standard. However, the spot color of the alpha mangosteen compound is dark red while the ethyl acetate extracts AGM3.1, AGM2.3, AGM2.2, and AGM2.1 have blue colored spots. Blue spots indicate groups of compounds flavone, flavanone, flavonol, and isoflavones. These compounds include a subgroup of flavonoid compounds. Flavonoids are known to have antioxidant activity, are able to ward off free radicals, overcome oxidative stress and play a role in the process of preventing and healing various diseases (Kumar and Pandey 2013). Ethyl acetate extract AGM3.2 does not have spot compounds with the same Rf value as the alpha mangosteen standard. If observed in Figure 6, the spot of the active compound in mangosteen peel extract with an Rf value of 0.09 was also found in ethyl acetate extract isolates AGM3.1, AGM2.3, AGM2.2, and AGM2.1 with blue spots. Scientific reports about endophytic actinobacteria from mangosteen peels having antioxidant activity as described in this study can be considered as firstly published data.

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