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Antioxidant and Antiglycation Activity of Rhizosphere and Endophytic Actinobacteria of *Xylocarpus granatum*

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Abstract

Utilization of endophytic bacteria such as actinobacteria is one of the alternatives to obtain the bioactive compounds similar to the host plant. This study aimed to identify compounds produced by rhizosphere and endophytic actinobacteria isolated from *Xylocarpus granatum* for their antioxidant and antiglycation activity. Actinobacterial culture supernatant was extracted using ethyl acetate with a ratio of 1:1 (v/v). Antioxidant activity was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Whole TLC-bioautography and phytochemical tests were used to predict the group of active compounds. Antiglycation activity was identified based on the ability to inhibit the formation of advanced glycation end products (AGEs). Fifteen actinobacterial isolates had been isolated from rhizosphere (ten isolates), fruit (two isolates), and leaf (two isolates) of *X. granatum*. All isolates had been tested for their antioxidant and antiglycation activity. Isolate XR2 had the highest antioxidant activity with IC50 value of 1719.26 mg/L. Meanwhile, the highest antiglycation activity was obtained from isolate XR8 with IC50 value of 327.62 mg/L. This study informs that actinobacteria also live in the fruit, leaves and rhizosphere of *X. granatum*. The existence of rhizosphere and endophytic actinobacteria from *X. granatum* and their antioxidant and antiglycation activities contributes to the understanding of their diversity and potency as an antioxidant and antiglycation agent.

How to Cite

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INTRODUCTION

Xylocarpus granatum is an ancient mangrove plant spreads in the regions of Kalimantan and Sulawesi. Parts of this plant such as leaves, stem and fruit have a unique secondary metabolite, called limonoid which has anticancer, antihyperglycemic, antidyslipidemic, antidepressant, and neuroprotective activities (Srivastava et al., 2015; Gao et al., 2016, Zhou et al., 2016; Pejin & Glumac, 2018). Polyphenol compounds contained in ethanolic extract of *X. granatum* stem also have antioxidant and antidiabetic activities (Das et al., 2016; Liao et al., 2017). In the other side, secondary metabolite compounds are minor compounds contained in organisms, such as plants. Therefore, it requires a lot of samples used to produce this compound. This condition can cause environmental damage and ecosystem instability in nature. In addition, this can also cause the extinction of rare species. Apart from plants, bioactive compounds can also be obtained from bacteria. Bacteria are one of the microbes that can growth faster than yeast and mold. Thus, bioactive compounds can be obtained in a short time. Bacteria such as *Bacillus* sp. and *Lactobacillus* sp. are able to produce secondary metabolites which have antioxidants and α-glucosidase inhibitors activities (Coda et al., 2014). However, the metabolites produced are less diverse with limited activity.

One kind of bacteria that can be used to produce active secondary metabolite is actinobacteria. Actinobacteria are Gram-positive bacteria. These bacteria are widely distributed in nature, such as in soil, waters, and as endophytes in plants. Most soil actinobacteria are from the genus *Streptomyces* and 75% of this genus is capable of producing bioactive compounds. Production of bioactive compounds is influenced by the environment or ecosystem. Mangrove land is one of the unique ecosystems, located between land and waters. From this ecosystem, it is expected that actinobacteria can produce bioactive compounds. Endophytic bacteria are bacteria that can live inside plant tissues at specific periods without harming its host. Utilization of endophytic bacteria is an alternative way to produce various bioactive compounds from plants (Pimentel et al., 2011). Endophytic bacteria are expected to synthesize the same secondary metabolites as the host. Endophytic bacteria can be obtained in large quantities in a short time, so that, they can produce secondary metabolites in large quantities without damaging the environment, disrupting natural instability, and causing extinction. These bacteria have been known to produce secondary metabolites which have antibiotics, antitumor, anticancer, immunosuppressive, antioxidants and enzyme inhibitors activities (Pujiyanto et al., 2012; Fitriandini et al., 2017; Fitri et al., 2017).

Free radicals are part of the reaction of lipid peroxidation in the body that can cause aging and promotion of cancer. Pollution exposure, ultraviolet light, and sun can also accelerate aging because it can increase free radicals and oxidative stress (Septiyorini, 2017). Therefore, antioxidant compounds are needed as substances that can inhibit radical formation. Aging in the body can also be caused by glycation reaction. Glycation is a reaction between an amine groups of a protein with a carbonyl group of a reducing sugar that will produce advanced glycation end products (AGEs). These AGEs compounds can cause aging in body tissues (Gkogkolou & Böhm, 2017). Although this reaction is a spontaneous reaction that occurs in the body, the presence of antiglycation compounds is expected to slow down the formation of AGEs. Relationship between antioxidants and glycation is found in the mechanism of formation of AGEs compounds. When the amine group reacts with the carbonyl group, it will produce a Schiff base which tends to form free radicals (Yeh et al., 2016). At this stage, antioxidants act as free radicals' reducer, so that the formation of AGEs will be inhibited. Based on these things, this study aimed to identify secondary metabolites of rhizosphere and endophytic actinobacteria isolated from *X. granatum* plant organs and ecosystem which have antioxidant and antiglycation activities. This research results will contribute to the understanding of diversity of rhizosphere and endophytic actinobacterial from *X granatum* and their potency as an antioxidant and antiglycation agent.

METHODS

Sample Collection and Preparation

Samples of soil, fruit and leaves of *X. granatum* were taken from the Mangrove Forest Area of Tarakan, North Kalimantan. Soil samples were dried using an oven with a temperature of 60 ℃ for 30 minutes. Dry fruit and leaf samples obtained from the ground were sorted and washed using water to clean the surface of the samples.

Isolation of Actinobacteria

Isolation of actinobacteria follows the procedure described by Coombs and Franco (2003). The dried soil samples were weighed as much as

1 gram and added with 10 mL distilled water. This mixture was called a 10^{-1} dilution. Then, a series of dilutions of 10^{-2}, 10^{-3} and 10^{-4} was made and distributed in humic acid-vitamin (HV) agar which contained cycloheximide 50 ppm and nalidixic acid 30 ppm. After that, they were incubated at room temperature for 14 days. Actinobacterial colonies from soil that have grown were purified in yeast malt extract (YM) media. Leaf and fruit surfaces were sterilized by soaking them in alcohol solution for 60 seconds, then soaking them in H_2O_2 for 5 minutes, then re-soaking them in alcohol solution for 60 seconds and rinsing with distilled water. Sterile sample surfaces were crushed using mortar aseptically. The vial sample was finely inserted, enough distilled water was added and they were homogenized using vortex. About 0.1 mL that mixture was distributed in HV agar which contained 50 ppm of cycloheximide and 30 ppm of malidixic acid. After that, they were incubated at room temperature for 14 days. Actinobacterial colonies from soil, fruit, and leaves that have grown were purified on yeast starch agar (YSA) media.

Five spheres (diameter of 0.5 cm) of isolated actinobacteria were put in 100 mL of liquid medium. Incubation was carried out at room temperature using a shaker at a speed of 75 rpm for 10 days. The culture was centrifuged at a speed of 4000 rpm for 30 minutes at 4 □ . The supernatant produced by the centrifugation was tested for its antioxidant and antiglycation activity. Potential supernatants were extracted with ethyl acetate.

Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) Method

This antioxidant activity test followed the procedure described by Batubara et al. (2009). DPPH solution was reacted with 100 µL of sample solution. The mixture was incubated for 30 minutes. After incubation, the absorbance of the mixture was measured at a wavelength of 517 nm. Ascorbic acid was used as a positive control, while ethanol was used as a solvent and negative control.

2,2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS) Method

In addition to using DPPH method, antioxidant activity was also tested using the ABTS method by following the procedure described by Fogarasi et al. (2015). ABTS solution was oxidized using potassium peroxydisulfate for 12-16 hours. ABTS radicals were reacted with 20 µL of samples and incubated for 15 minutes on a 96 well plate. Furthermore, the absorbance of the mixture was measured at a wavelength 734 nm. Measurement results in the form of trolox equivalent antioxidant capacity (TEAC) (w/w).

Thin Layer Chromatography (TLC) Bioautography

Determination of active antioxidant components by TLC bioautography followed the step in Gu et al. (2009). Sample extract was spotted on TLC plate, dried and eluted with dichloromethane (DCM): butanol 3:7 (v/v). Detection of separation results was performed under 254 and 366 nm UV. Then, the plate was sprayed with DPPH in methanol. Active samples was observed in the form of yellow spots on purple plates. Phytochemical test was carried out using TLC with the same methods and detected with specific reagent for alkaloid, polyphenol, flavonoid, and steroid-terpenoid.

Antiglycation Activity

Antiglycation activity was measured based on the method described by Povichit et al. (2010). Bovine serum albumin (BSA) solution with concentration of 20 mg/mL, 235 mM glucose, fructose 235 mM, and 50 µL of sample or positive control are mixed in phosphate buffer 0.2 M pH 7.4. The mixture was incubated for 40 hours at 60 ℃. After incubation, the fluorescence excitation intensity was measured at a wavelength of 370 nm and the emission at a wavelength of 440 nm. Fluorometer used was from Fluorostar BMG LABTECH. Aminoguanidine was applied as a positive control. The correction solution was prepared in the same way, using water to replace the sample and positive control.

RESULTS AND DISCUSSION

Actinobacteria in this study were isolated from the rhizosphere, leaves and fruit of *X. granatum* obtained from the Mangrove Forest Area of Tarakan, North Kalimantan. Actinobacterial isolation of rhizosphere *X. granatum* produced 10 pure isolates, namely XR1, XR2, XR3, XR4, XR5, XR6, XR7, XR8, XR9, and XR10. These isolates grew well in YM media. Actinobacteri growth was characterized by the formation of pink aerial mycelia (Figure 1). This growth took 14-16 days. Five isolates from 10 pure isolates, namely XR2, XR4, XR5, XR8, and XR9 were selected based on the color and thickness of the colony to be proceed at the stage of crude extract production.

Meanwhile, isolation of leaves and fruit of *X. granatum* produced 5 isolates, three isolates

from leaves (XD1, XD2, and XD3) and two isolates from fruit, (XB1 and XB2). These isolates grew well on YSA media. Actinobacterial growth was characterized by the growth of white aerial mycelia for isolates XB1, XB2, XD1, and light yellow for isolates XD2 and XD3 (Figure 2). The five isolates were selected to continue to the stage of crude extract production.

Antioxidant and Antiglycation Activity

Screening of antioxidant and antiglycation activities was carried out on ten supernatant of endophytic and rhizosphere actinobacterial cultures. It aimed to determine the supernatant which has more than 50% antioxidant and antiglycation activity. Furthermore, the supernatant was extracted using ethyl acetate with a ratio of 1:1 (v/v). The ethyl acetate extract was again tested for antioxidant and antiglycation activity to determine the inhibition concentration of 50% population (IC_{50}) value. The results of screening of supernatant antioxidant activity showed that there were 5 isolates that had antioxidant activity of more than 50% namely XR2, XR4, XB1, XB2, and XD1 (Table 1). The highest antioxidant activity was owned by XB1 isolates with 93.21% percent inhibition. ANOVA test results showed that the type of isolate had an effect on percent inhibition ($F_{count} > F_{table}$). Meanwhile, the results of screening of supernatant anti-glycation activity

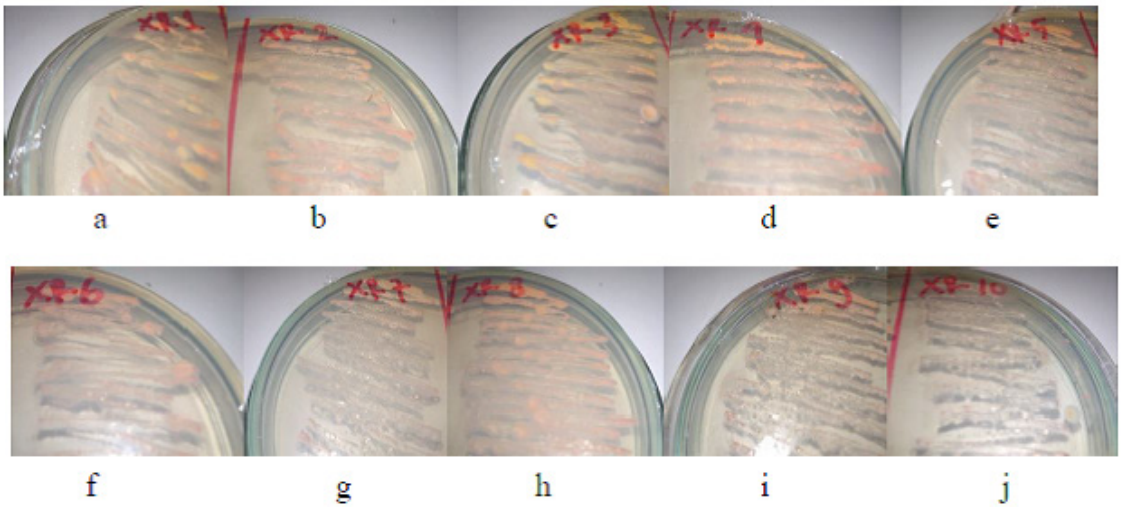

Figure 1. Pure Isolates of Rhizosphere Actinobacteria from *X. Granatum*, XR1 (a), XR2 (b), XR3 (c), XR4 (d), XR5 (e), XR6 (f), XR7 (g), XR8 (h), XR9 (i), and XR10 (j)

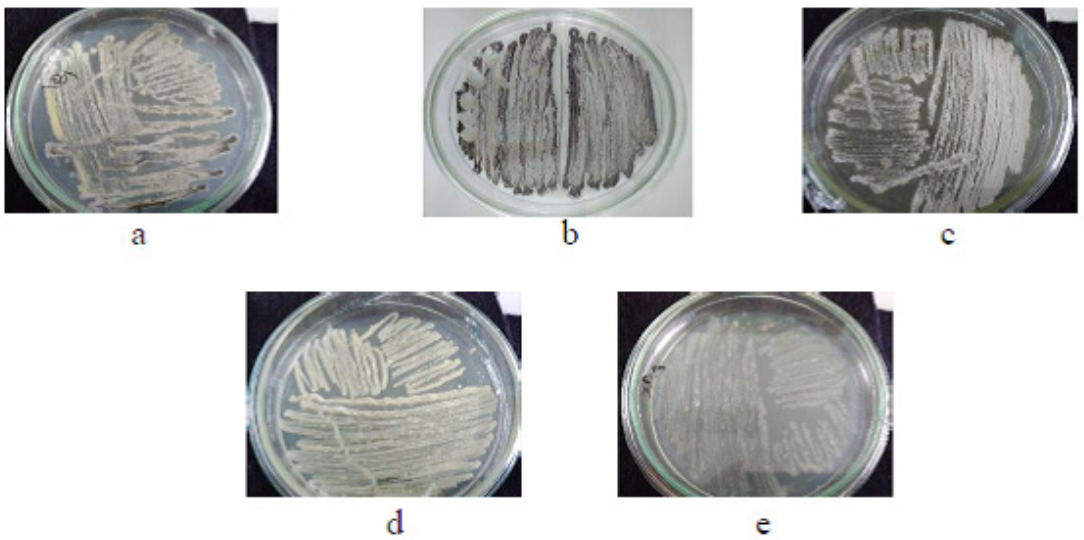

Figure 2. Pure Isolates of *X. granatum* Endophytic Actinobacteria, XB1(a), XB2(b), XD1(c), XD2(d), XD3(e)

showed that there were 7 isolates that had more than 50% anti-glycation activity (XR4, XR8, XR9, XB1, XB2, XD1, and XD3). Supernatant screening results from isolates XR4, XR8, XR9, and XB1 showed an inhibition of more than 100% (Table 1). This can be caused by several components contained in the supernatant having the same excitation wavelength and fluorescence emission, so that their presence was also detected when measuring. ANOVA test results showed that the type of isolate had an effect on percent inhibition ($F_{count} > F_{table}$).

Table 1. Antioxidant and Antiglycation Activity of Rhizosphere and Endophytic Actinobacteria

Isolate Code	Inhibition (%)	
	Antioxidant	Antiglycation
XR2	51.49 ± 0.55^{f}	49.49 ± 4.88^{b}
XR4	55.82 ± 0.93^{g}	131.40 ± 25.23^{gh}
XR5	15.34 ± 0.53^{a}	18.53 ± 1.83^{a}
XR8	28.57 ± 0.79^{cd}	116.00 ± 11.45^{efg}
XR9	27.43 ± 0.66^{c}	100.00 ± 9.88^{cde}
XB1	93.21 ± 1.10^{j}	124.47 ± 21.33^{fgh}
XB2	79.28 ± 1.07^{h}	99.29 ± 0.07^{cd}
XD1	90.74 ± 0.69^{i}	88.20 ± 3.34^{c}
XD2	20.02 ± 1.00^{b}	45.43 ± 5.26^{b}
XD3	41.80 ± 1.73^{e}	110.77 ± 1.03^{ef}

Description: values with different letters (a-j) in the same column show significant different statistically (p = 0.05)

The IC_{50} value of five selected ethyl acetate extract and ascorbic acid as a positive control was then determined. The results (Table 2) showed that the ethyl acetate extracts of rhizosphere actinobacteria (XR2, XR4) have higher antioxidant activity because they had smaller IC_{50} value than endophytic actinobacteria. Meanwhile, IC_{50} values of endophytic actinobacterial were more than 10.000 mg/L, except for XD1 which had an IC_{50} value of 4447.07 mg/L (Table 2). However, ethanol extract of *X. granatum* leaves has a higher antioxidant activity with IC_{50} 110.4 mg/L which is caused by the presence of tannin and flavonoid compounds (Das et al., 2016). Ethyl acetate extract of XR2 isolates had the highest activity with IC_{50} value of 1719.26 mg/L. However, this value is still far greater than ascorbic acid as a positive control. This condition can be caused by not all active compounds can be extracted by ethyl acetate solvents. ANOVA test results showed that the type of isolate influenced IC_{50} values ($F_{count} > F_{table}$). The antioxidant capacity of the five extracts that have been tested for antioxidants has different results. Unlike antioxidant activity, the antioxidant capacity of extracts of endophytic actinobacteria was better than the extract of rhizosphere actinobacteria. This was indicated by the greater value of TEAC owned by endophytic actinobacteria extract than the extract of rhizosphere actinobacteria (Table 2). The opposite results between activity and antioxidant capacity can be caused by differences in radical molecules that will affect the sample reactivity, reaction kinetics, and sample capabilities to react with ABTS radicals or inhibit the oxidation process (Karadag et al., 2009).

The seven ethyl acetate extracts from the supernatant were selected, then a series of concentrations was made to determine the IC_{50} value and aminoguanidine was used as a positive control. The results of IC_{50} determination on the seven selected extracts again showed that the ethyl acetate extract of rizosphere actinobacteria had better activity. This is indicated by the IC_{50} value of XR8 isolates that was smaller three to eight times than the IC_{50} value of endophytic actinobacteria (Table 2). However, the IC_{50} value of XR8 isolates was still greater than aminoguanidine as a positive control with IC_{50} value of 83.26 mg/L. Aminoguanidine has four amine groups which can replace amine groups from proteins when reacting with glucose, so that it can inhibit the glycation reaction. ANOVA test results indicate that the type of isolate affects the IC_{50} value (Fcount > Ftable).

In this study, compound profiles of five ethyl acetate extracts from the selected actinobacteria as antioxidants were determined seen from spot color on UV 254 nm and 366 nm. Figure 3A is spot detection in white light or visible light. This detection does not indicate a spot on the TLC plate. Detection of UV 254 nm produces a black spot, which indicates the presence of flavonoids, lignans, alkaloids and triterpene compounds (Fernand, 2003) (Figure 3B). Meanwhile, UV detection at 366 nm produces a blue spot, which indicates the presence of alkaloid and flavonoid compounds (Julkunen-Tiitto et al, 2015; Esmaiili & Shetab-Boushehri, 2017) (Figure 3C). After the TLC plates were sprayed with DPPH radicals, all the first spots on the selected actinobacterial ethyl acetate extract showed a yellow color change (Figure 3D). It shows that the spot contains compounds that have the potential as antioxidants. However, the color changes shown were very weak. This means that on the spot there were compounds that were not too active as

Table 2. IC_{50} and TEAC Value of Antioxidant and Antiglycation Activities of Ethyl Acetate Extract of Rizosphere and Endophytic Actinobacteria

Isolate Code	IC_{50} (mg/L)		TEAC (µg trolox equivalent/g extract)
	Antioxidant	Antiglycation	
XR2	1719.26[b]	-	47 ± 7.52 × 10^{-4}[a]
XR4	1813.49[b]	> 500[e]	24 ± 7.97 × 10^{-4}[a]
XR8	-	327.62[b]	-
XR9	-	> 500[e]	-
XB1	10705.94[d]	2249.81[d]	714 ± 0.060[c]
XB2	13739.09[d]	1352.90[c]	1026 ± 0.013[d]
XD1	4447.07[c]	2807.36[d]	597 ± 0.004[b]
XD3	-	1152.86[c]	-
Ascorbic acid	3.29[a]	-	-
Aminoguanidine	-	83.26[a]	-

Description: values with different letters (a-j) in the same column show significant different statistically (p = 0.05)

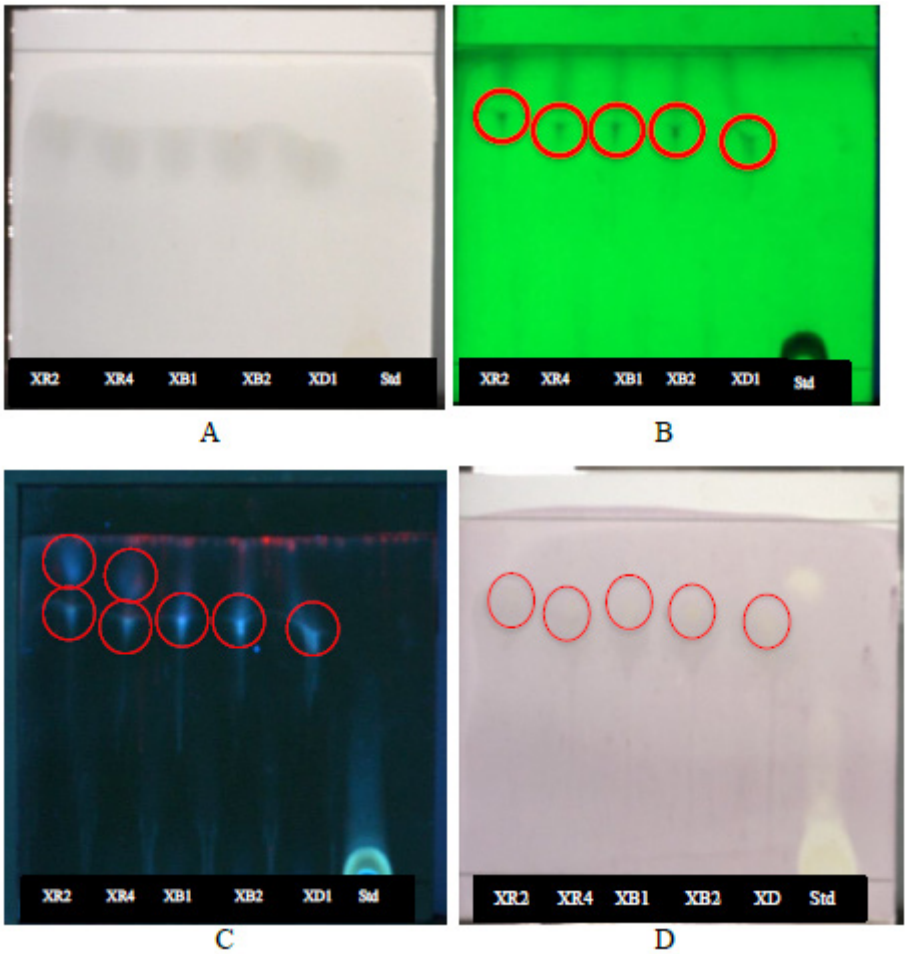

Figure 3. Bioautography Chromatogram of Selected Actinobacterial Ethyl Acetate Extract on Visible Light (A), UV 254 nm (B), UV 366 nm (C), Visible Light After Sprayed with DPPH (D) Solution with Mobile Phase DCM: butanol 3:7 (v/v)

Table 3. The Groups of Compounds Contained in The Actiobacterial Selected as Antioxidants

Isolate Code	Compound Group			
	Alkaloid	Flavonoid	Polyphenol	Terpenoid/Steroid
XR2	+	-	-	-
XR4	+	-	-	-
XB1	+	-	-	-
XB2	+	-	-	-
XD1	+	-	-	-

antioxidants, so that, to cause strong color changes higher concentrations were needed.

The group of compounds in extracts of natural ingredients can be predicted using a series of phytochemical tests. In this study, phytochemical tests were carried out by spraying certain reagents to detect certain classes of compounds. After being detected with UV 254 nm, there was a black spot which showed the presence of alkaloids and flavonoids. Detection of UV 366 nm shows a blue spot, which means that the spot contains alkaloids and flavonoids. These results were then strengthened by Dragendorff reagent spraying for the detection of alkaloids and ammonia vapor for the detection of flavonoids. Spraying with Dragendorff reagent caused an orange spot on the visible light, which means that the spot contains alkaloid compounds. Spraying with ammonia vapour did not cause color to the visible light, which means that the spot did not contain flavonoids. Overall, the selected actinobacteria contains only antioxidants from alkaloid compounds and did not contain flavonoids, polyphenols, terpenoids, or steroids (Table 3).

Natural products have long been recognized as an important source of therapeutically effective medicines. Of the 520 new drugs approved between 1983 and 1994, 39% were natural products or derived from natural products and 60–80% of antibacterial and anticancer drugs were also derived from natural products. Plants offer a vast source of compounds that shows different effects in human. The pharmacological studies in alkaloids have been largely concerned with the effect of alkaloids on physiological processes other than inflammation. Isoquinoline, indole and diterpene alkaloids were the most studied about their activities on inflammation. The alkaloids tend to be rather toxic, although the toxicity appears to be well below the therapeutic levels. The alkaloids appear to offer the considerable promise for further investigation as anti-inflammatory compounds, and some appears to be remarkably active (Filho et al., 2006). Alkaloid compounds isolated from *Jishengella endophytica* 16111, an endophytic organism from *X. granatum*, have antiviral activity (Wang et al., 2014). Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants. The antioxidant activity of flavonoids depends on the arrangement of functional groups in their structure. Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hepatoprotective activities. Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity (Kumar & Pandey, 2013).

Polyphenols are secondary compounds widely distributed in the plant kingdom. They are divided into several classes i.e. phenolic acids, flavonoid, stilbenes, and lignans. The beneficial effects of polyphenols have been ascribed to their strong antioxidant activity that is their ability to scavenge oxygen radicals and other reactive species. This feature make phenols a potentially interesting material for the development of functional foods or possible therapy for the prevention of some diseases (Gharras, 2009). Terpenoids are widely used as industrially relevant chemicals, including many pharmaceuticals, flavours, fragrances, pesticides and disinfectants, as well as large-volume feedstocks for chemical industries. Recently, there has been a renaissance of awareness of plant terpenoids as a valuable biological resource for societies that will have to become less reliant on petrochemicals (Bohlmann & Keeling, 2008). This study provides the information about diversity, antioxidant and antiglycation activities of rhizosphere and endophytic actinobacteria from leaves and fruit of *X. granatum*.

CONCLUSION

XR2 isolate has the highest antioxidant activity with IC_{50} value of 1719 mg/L, but has a small TEAC value, which is 47 µg of trolox equivalent/g extract. Based on the results of TLC bioautography and phytochemical tests, compounds that provide antioxidant activity are alkaloid group compounds. The XR8 isolate has the highest antiglycation activity with an IC_{50} value of 327 mg/L.

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