ANTIOXIDANT ACTIVITY OF ETHYL ACETATE EXTRACT FROM ENDOHYTIC BACTERIA OF MANGOSTEEN (*Garcinia mangostana* L.) ROOTS

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ABSTRACT

Aims: This study aims to obtain isolates of endophytic bacteria found in mangosteen roots, determine information regarding the chemical contents of secondary metabolites from endophytic bacteria, and obtain antioxidant activity data using the DPPH (1,1-Diphenyl-2-Picrylhydrazyl) method.

Place and Duration of Study: The research was conducted from June to September 2019 at the Microbiology Laboratory of Universitas Perintis and the Higher Education Coordinating Board Region X, West Sumatra, Indonesia.

Methodology: Isolation of endophytic bacteria, secondary metabolite production by endophytic bacteria, phytochemical screening, and antioxidant activity testing using the DPPH method.

Results: The results of measuring the antioxidant activity of secondary metabolite extracts from endophytic bacteria of mangosteen rootsusing the DPPH method yielded an IC_{50} of 14,047.912 g/ml and gallic acid solution of 2,498 g/ml. Furthermore, the T-test analysis between the % inhibition of secondary metabolites from endophytic bacteria of mangosteen roots and gallic acid yielded a significant value of 0.013.

Conclusion: The antioxidant activity of secondary metabolites from endophytic bacteria of mangosteen roots is considered weak and cannot be an alternative or solution for antioxidant production.

Keywords: Mangosteen Root, Garcinia mangostana L., Antioxidants, Endophytic Bacteria.

1. INTRODUCTION

Mangosteen (Garcinia mangostana L.) is a tropical plant species native to Southeast Asia, particularly Indonesia. In the community, mangosteen fruit is used to treat diarrhea, dysentery, and ulcers [1]. The fruit peel is utilized for treating constipation, respiratory disorders, skin infections, and inflammation. The roots are used to regulate irregular menstruation [2]. Chemical constituents found in mangosteen fruit include xanthones, mangostin, garsion, flavonoids, and tannins. One crucial component in mangosteen is xanthone, which functions to neutralize free radicals [3].

An efficient way to obtain these compounds is by utilizing endophytic microbes capable of producing the required compounds, thus eliminating the need to extract them from their host plants [4]. Endophytic bacteria are bacteria that live within the tissues of their host plants without causing any disease symptoms [5]. Endophytic bacteria typically enter plant tissues through roots, although plant parts directly exposed to air such as flowers, stems, and cotyledons can also serve as entry points for endophytic bacteria. The mutualistic symbiosis between endophytic bacteria and plants involves endophytic bacteria obtaining nutrients from the plant's metabolism and protecting the plant against pathogens [6], while the plant

benefits from antioxidants due to genetic exchange and long-term evolutionary relationships [7].

Antioxidants are electron-donating compounds that can mitigate the negative effects of free radicals. Free radicals are molecules containing one or more unpaired electrons in their outermost orbital, making them highly reactive and unstable. In an effort to achieve stability, free radicals react with atoms or molecules in their vicinity to obtain electron pairs. This reaction occurs continuously in the body, leading to chain reactions capable of damaging cell structures, and if left unchecked, can result in various diseases [8].

Based on the description above, this research will investigate the antioxidant activity of secondary metabolites from endophytic bacteria of mangosteen roots using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay with UV-Vis spectroscopy.

2. MATERIAL AND METHODS

2.1 Isolation of Endophytic Bacteria from Mangosteen Roots

Mangosteen roots were washed under running water for 5 minutes. Subsequently, they were sterilized in a Laminar Air Flow hood to prevent contamination from other bacteria. The roots were then immersed in 70% alcohol for 1 minute, transferred to 5.25% Sodium Hypochlorite solution for 5 minutes, and then placed in 70% alcohol for 30 seconds. Afterward, the roots were rinsed with distilled water for 1 minute, repeated twice, and placed on sterile filter paper for 2 minutes. The roots were then cut into 2 cm lengths using sterile scissors and planted in Nutrient Agar media. The samples that have been inoculated on NA media are then incubated at 25°C in the dark for 48 hours. If after 24 hours there is no microbial growth around the sample, surface sterilization is considered successful. After 48 hours of incubation, endophytic bacteria are cultured, and a single bacterial colony is transferred to Nutrient Agar (NA) media. Following purification, observations of endophytic bacterial morphology characteristics are conducted based on criteria such as color, colony shape, and colony margin.

2.2 Gram Staining of Endophytic Bacteria

A single colony of endophytic bacteria is smeared onto the surface of a glass slide. Bacterial fixation is carried out by passing the glass slide over a Bunsen burner flame 2-3 times. Then, crystal violet solution is added for 1 minute, followed by rinsing with water. Next, iodine solution (lugol solution) is applied for 1 minute, followed by rinsing with water. The slide is then treated with decolorizing solution (alcohol) for 10-20 seconds. After that, safranin solution is added for 15 seconds, followed by rinsing with water. The slide is then air-dried using filter paper. Examination is performed under a microscope at 100x magnification, and the observed color of the bacteria indicates their Gram staining status: red color indicates Gram-negative bacteria, while purple color indicates Gram-positive bacteria.

2.3 Production of Secondary Metabolites by Endophytic Bacterial Isolates

A single colony from the purified culture is inoculated into 250 mL of NB media and incubated in a shaker incubator at 27°C and 170 rpm for 48 hours. The culture is then sonicated for 30 minutes. After that, the culture is centrifuged at 5000 rpm for 10 minutes. The supernatant from the centrifuged culture is fractionated using ethyl acetate and then

concentrated using a water bath at 40°C to separate the secondary metabolites from the solvent until it becomes concentrated.

2.4 Phytochemical Screening

The sample is placed into a test tube, and then 10 ml of chloroform: water (1:1) is added. The mixture is shaken in the test tube and allowed to settle briefly until two layers were formed. The aqueous layer is examined for flavonoids, phenolics, and saponins. The chloroform layer is examined for terpenoids, steroids, and alkaloids.

2.1.1 *Phenolic Examination*

One to two drops of the aqueous layer are taken and mixed with one to two drops of $FeCl_3$ solution in a test tube. The formation of a blue color indicates the presence of phenolic compounds.

2.1.2 *Flavonoid Examination*

Take 1-2 drops of the aqueous layer and place them on a spot plate. Add a piece of Mg metal and 1-2 drops of concentrated HCl. The appearance of a reddish-orange color indicates the presence of flavonoid compounds

2.1.3 *Terpenoid and Steroid Examination*

1-2 drops of the chloroform layer are placed in a spot plate and allowed to dry. Then, anhydrous acetic acid with 2N H_2SO_4 (Lieberman-Burchard reagent) is added. A red color indicates the presence of terpenoid compounds, while a purple-blue color indicates the presence of steroid compounds.

2.1.4 *Saponin Examination*

The aqueous layer is taken and vigorously shaken in a test tube. The formation of permanent foam (approximately 15 minutes) indicates the presence of saponins.

2.1.5 *Alkaloid Examination*

A small amount of the chloroform layer is taken and mixed with 10 ml of 0.05 N ammonium chloroformate. The mixture is gently stirred, and then a few drops of 2N H_2SO_4 are added. The mixture is shaken gently and allowed to separate. To the acidic layer, a few drops of Mayer's reagent are added. A positive alkaloid reaction is indicated by the presence of white precipitate or white clouds.

2.5 Determining the Maximum Wavelength of DPPH

A freshly prepared solution of DPPH at 35 µg/ml is pipetted into a dark vial, totaling 4 ml. Then, a mixture of distilled water and ethanol (1:1) at 2 ml is added to the vial. The vial is left for 30 minutes in a dark place with the lid closed. The absorption of the solution is measured using a UV-Vis spectrophotometer at wavelengths ranging from 400 to 800 nm.

2.6 Determining Gallic Acid Antioxidant Activity

From a concentration of 50 µg/ml, 0.2, 0.3, 0.4, 0.5, and 0.6 ml are pipetted into 10 ml volumetric flasks. Then, a mixture of distilled water and ethanol (1:1) is added to the mark to obtain concentrations of 1, 1.5, 2, 2.5, and 3 µg/ml. Next, 2 ml of each concentration (1, 1.5,

2, 2.5, and 3 µg/ml) is pipetted into separate vials, and 4 ml of DPPH solution is added to each vial. The vials are left to stand for 30 minutes in the dark. The absorption of the solutions is measured using a UV-Visible spectrophotometer at the maximum wavelength. From the absorbance values of the gallic acid reference and the control, the percentage inhibition is calculated using the following formula:

$$\text{Inhibition \%} = \frac{\text{Absorbance of Control} - \text{Absorbance (gallic acid/samples + DPPH)}}{\text{Absorbance Control}} \times 100\ \%$$

2.7 Determination of Sample Antioxidant Activity (Mosquera, 2007)

500 mg of secondary metabolite sample from endophytic bacteria is weighed and dissolved in methanol in a 100 ml volumetric flask up to the mark, resulting in a concentration of 5000 µg/ml. From the sample solution, (0.2; 0.5; 1; 1.5; 2) ml is pipetted out. Then, methanol:water (1:1) is added to a 10 ml volumetric flask up to the mark. Thus, samples with concentrations of (100, 250, 500, 750, 1000) µg/ml are obtained. 2 ml of each concentration of the sample solution is pipetted into separate vials, and then 4 ml of DPPH solution at 35 µg/ml is added. The mixture is homogenized and left to stand for 30 minutes in the dark. Absorption is measured using a UV-Vis spectrophotometer at wavelengths ranging from 400 to 800 nm. The antioxidant activity of the sample is determined by the extent of inhibition of DPPH radical absorption through the calculation of the percentage inhibition of DPPH absorption.

2.8 Data Analysis

Data obtained in this study will be statistically analyzed using the t-test. A significant difference will be indicated by a statistical result with a p-value of less than 0.05. Data analysis using the t-test will be conducted using the statistical software SPSS 23.0 for Windows Evaluation. The purpose is to compare the samples with the blank and determine whether the difference between them is statistically significant.

3. RESULTS AND DISCUSSION

Isolation is the first process in obtaining endophytic bacteria from the sample. The root sample used is fresh roots. Before isolation, the sample is cleaned from impurities and soil adhering to it using clean flowing water. Meanwhile, surface sterilization of the sample from epiphytic microorganisms is carried out by immersing the sample in 70% alcohol and 5.25% NaOCl [9].

Endophytic bacteria from mangosteen roots began to show growth after plant tissue sections were inoculated on NA media for approximately 48 hours (2 days). From this isolation, observations of the morphological characteristics of endophytic bacteria were conducted, such as: the colonies have smooth edges, are rod-shaped, and are gram-negative.

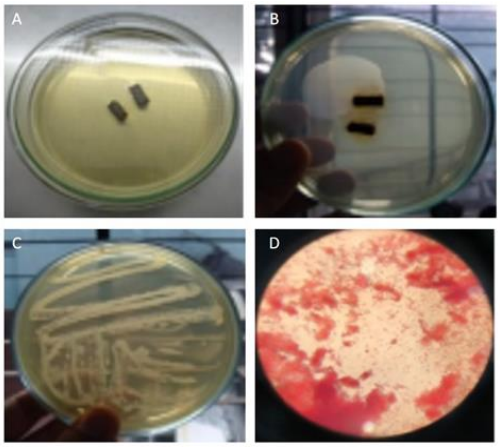

Fig. 1. Isolation Process of endophytic bacteria. (A) Mangosteen planted on NA media (B) Endophytic bacteria growing on mangosteen roots (C) Purified endophytic bacteria (D) Gram staining results at 40x magnification

From the bacterial isolation process, the production of secondary metabolites was conducted with the aim of determining the antioxidant activity of the bacteria. The production of secondary metabolites from bacterial isolates was carried out for 48 hours at a temperature of 27°C with a speed of 170 rpm using Nutrient Broth (NB) media in a shaker incubator. The obtained culture was sonicated for 30 minutes and then centrifuged at a speed of 5000 rpm for 10 minutes to separate bacterial cells and supernatant [10]. The supernatant obtained from centrifugation represents the crude sample of secondary metabolites. This sample was fractionated using ethyl acetate and then concentrated using a water bath at 40°C, resulting in a concentrated sample of endophytic bacterial secondary metabolites.

The sample appears cream-colored and has a distinctive odor based on organoleptic assessment. Meanwhile, phytochemical screening indicates that the sample contains flavonoids and. Flavonoids are compounds containing phenolic groups. Compounds belonging to the flavonoid group have the ability to act as antimicrobial, antiviral, strong respiratory inhibitors, and antioxidants. Saponins are powerful surface-active compounds that produce foam when shaken in water, and they can act as antimicrobials [11].

From the measurement results, the maximum wavelength of the antioxidant activity of the secondary metabolite sample of endophytic bacteria was found to be 520 nm with an absorbance of 0.657. The obtained absorbance measurements were then used to calculate the percentage inhibition or the percentage of antioxidant compound immersion against DPPH. The results of the percentage inhibition of the secondary metabolite sample of endophytic bacteria are 2.508%, 3.225%, 3.942%, 4.659%, and 5.734%. This indicates that the higher the concentration, the higher the % inhibition value [12]. The difference in antioxidant activity in the sample of secondary metabolites from endophytic bacteria is due to the difference in the amount of flavonoid compounds, which contribute hydrogen atoms [13].

Gallic acid exhibited inhibition percentages of 25.806%, 31.899%, 41.397%, 50.896%, and 58.243%. The difference in inhibitory ability between the secondary metabolites of bacterial isolates and gallic acid is because gallic acid is a compound known to have hydroxyl groups to donate protons, whereas the sample is a solid secondary metabolite that is not yet pure, resulting in suboptimal ability to neutralize free radicals [14]. The content of flavonoids in the secondary metabolites also affects the inhibitory ability, as the antioxidant ability of

flavonoids can be influenced by the presence of hydroxyl groups. The more free hydroxyl groups on flavonoids, the greater their antioxidant activity. It is suspected that flavonoid compounds in the secondary metabolites of bacterial isolates have few free OH groups, so the number of hydrogen atoms donated is insufficient to neutralize the activity of DPPH radicals [15]. A study has shown that the radical scavenging activity tested on flavonoids is related to the number and position of hydroxyl group bonds in the molecule [16].

From the percentage inhibition, the IC_{50} value can be determined, which allows for the determination of the concentration of antioxidant compounds that provide 50% inhibition, meaning that at this concentration, the antioxidant can inhibit free radicals by 50%. The IC_{50} value of gallic acid can be calculated as 2.498 g/mL. The antioxidant activity of secondary metabolites from endophytic bacteria in mangosteenis 14.047,912 g/mL Molyneux (2004) stated that IC_{50} values < 50 µg/mL indicate very strong antioxidant activity, IC_{50} values of 50-100 µg/mL indicate strong antioxidant activity, IC_{50} values of 101-150 µg/mL indicate moderate antioxidant activity, and IC_{50} values > 150 µg/mL indicate weak antioxidant activity. Therefore, the antioxidant activity of secondary metabolites from endophytic bacteria in mangosteen is considered weak and cannot be an alternative or solution for antioxidant production, as shown in the Table 1.

Table 1. Antioxidant activity of endophytic bacteria secondary metabolites

Sample Concentration (µg/mL)	Absorbance of DPPH	Absorbance of Sample + DPPH	Absorbance of Sample without DPPH	% Inhibition	IC50 (µg/mL)
100		0,544	0,000	2.508	
250		0,540	0,001	3.225	
500	0,657	0,536	0,001	3.942	**14,047.912**
750		0,532	0,000	4.659	
1000		0,526	0,002	5.734	

In the data analysis using the t-test, it is found that the antioxidant activity between the % inhibition of gallic acid and the secondary metabolites of endophytic bacteria in mangosteen is significant with a significance value of 0.013 (P = .05). Therefore, it can be concluded that there is a significant difference between the % inhibition of gallic acid and the secondary metabolites of endophytic bacteria in mangosteen.

4. CONCLUSION

The morphological characteristics from a single isolate of bacteria from the roots of mangosteen plantswere observed to be cream-colored, convex in shape with smooth colony edges, and classified as gram-negative bacteria. The antioxidant activity of secondary metabolites from endophytic bacteriawas demonstrated with an IC_{50} value of 14,047.912 g/mL.The secondary metabolite antioxidants from endophytic bacteria in mangosteen are considered weak and cannot serve as an alternative or solution for antioxidant production.

These findings suggest that while endophytic bacteria from mangosteen roots show potential in producing secondary metabolites with antioxidant activity, further research is needed to explore their practical applications and to enhance their antioxidant properties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Priya V V, Mallika J, Mohan SK, Saraswathi P, Gopan CSV. Antimicrobial Activity of Pericarp Extract of Garcinia Mangostana Linn. Int J Pharma Sci Res. 2010;1:278–81.

2. Bi C, Xu H, Yu J, Ding Z, Liu Z. Botanical characteristics, chemical components, biological activity, and potential applications of mangosteen. PeerJ. 2023;11.

3. Abate M, Pagano C, Masullo M, Citro M, Pisanti S, Piacente S, et al. Mangostanin, a Xanthone Derived from Garcinia mangostana Fruit, Exerts Protective and Reparative Effects on Oxidative Damage in Human Keratinocytes. Pharmaceuticals. 2022;15.

4. Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. The Use of Endophytes to Obtain Bioactive Compounds and Their Application in Biotransformation Process. Biotechnol Res Int. 2011;2011:1–11.

5. Gouda S, Das G, Sen SK, Shin HS, Patra JK. Endophytes: A treasure house of bioactive compounds of medicinal importance. Front Microbiol. 2016;7:1–8.

6. Wu W, Chen W, Liu S, Wu J, Zhu Y, Qin L, et al. Beneficial Relationships Between Endophytic Bacteria and Medicinal Plants. Front Plant Sci. 2021;12:1–13.

7. Kasote DM, Katyare SS, Hegde M V., Bae H. Significance of antioxidant potential of plants and its relevance to therapeutic applications. Int J Biol Sci. 2015;11:982–91.

8. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4:118–26.

9. Radji M, Sumiati A, Rachmayani R, Elya B. Isolation of fungal endophytes from Garcinia mangostana and their antibacterial activity. African J Biotechnol. 2011;10:103–7.

10. Fuchs A, P. Tripet B, Cloud B. Ammons M, Copie V. Optimization of Metabolite Extraction Protocols for the Identification and Profiling of Small Molecule Metabolites from Planktonic and Biofilm Pseudomonas aeruginosa Cultures. Curr Metabolomics. 2015;4:141–7.

11. Rai S, Kafle A, Devkota HP, Bhattarai A. Characterization of saponins from the leaves and stem bark of Jatropha curcas L. for surface-active properties. Heliyon. Elsevier Ltd; 2023;9:e15807.

12. Mohamad H, Andriani Y, Bakar K, Siang CC, Fitrya D. Research Article Effect of drying method on anti-microbial , anti-oxidant activities and isolation of bioactive compounds from Peperomia pellucida (L) Hbk. 2015;7:578–84.

13. Sarjono PR, Putri LD, Budiarti CE, Mulyani NS, Ngadiwiyana, Ismiyarto, et al. Antioxidant and antibacterial activities of secondary metabolite endophytic bacteria from papaya leaf (Carica papaya L.). IOP Conf Ser Mater Sci Eng. 2019;509.

14. Pince S, Muharram, Rika F. Antibacterial activity of secondary metabolite compounds in ethyl acetate extract of rumput mutiara (Hedyotis corymbosa (l.) lamk). Mater Sci Forum. 2019;967 MSF:38–44.

15. Kose LP, Gulcin İ. Evaluation of the antioxidant and antiradical properties of some phyto and mammalian lignans. Molecules. 2021;26.

16. Banjarnahor SDS, Artanti N. Antioxidant properties of flavonoids. Med J Indones. 2014;23:239–44.

17. Molyneux P. The Use of the Stable Free Radical Diphenylpicryl-hydrazyl (DPPH) for Estimating Antioxidant Activity. Songklanakarin J Sci Technol. 2004;26:211–9.