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

The Mangosteen (Garcinia mangostana L.), a tropical plant species endemic to Southeast Asia, particularly Indonesia, has been extensively studied for its medicinal properties. Traditionally, the fruit of the mangosteen has been utilized within local communities to address gastrointestinal issues such as diarrhea and dysentery, as well as ulcers [1]. Furthermore, the peel of the fruit is reputed for its efficacy in treating constipation, various respiratory disorders, skin infections, and inflammatory conditions. In addition, the roots of the mangosteen plant have been employed to correct irregular menstrual cycles [2]. A diverse array of chemical constituents has been identified within the mangosteen fruit, including xanthones, mangostin, garsion, flavonoids, and tannins. Among these, xanthone stands out for its potent antioxidant activity, specifically its ability to neutralize free radicals [3].

A highly efficient method of acquiring these compounds involves the utilization of endophytic microbes that are capable of producing the required compounds. This approach eliminates the need to extract the compounds from their host plants [4]. Endophytic bacteria are a type of bacteria that reside within the tissues of their host plants without causing any disease symptoms [5]. These bacteria typically penetrate plant tissues through roots, although plant parts that are directly exposed to air, such as flowers, stems, and cotyledons, can also serve as entry points for them. The symbiotic relationship between endophytic bacteria and plants is mutualistic in nature, with endophytic bacteria obtaining nutrients from the plant's metabolism and shielding the plant against pathogens [6], while the plant benefits from antioxidants due to genetic exchange and long-term evolutionary relationships [7].

Antioxidants are compounds that donate electrons and have the ability to counterbalance the harmful effects of free radicals. Free radicals are molecules with unpaired electrons in their outermost orbital, making them highly reactive and unstable. To achieve stability, free radicals interact with nearby atoms or molecules to acquire electron pairs. This ongoing process in the body can trigger chain reactions that harm cell structures, potentially leading to diseases if left unaddressed [8].

The presented study is designed to explore the antioxidative potential of secondary metabolites derived from endophytic bacteria residing in the roots of the mangosteen plant. This investigation employs the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, utilizing UV-Visible spectroscopy, to quantitatively assess the antioxidant capacity of these compounds.

2. MATERIAL AND METHODS

2.1 Isolation of Endophytic Bacteria from Mangosteen Roots

The roots of the Mangosteen were cleansed under a continuous stream of water for a duration of five minutes. The roots underwent a series of disinfection steps: initially, they were submerged in an ethanol solution with a concentration of 70% for a period of one minute. This was succeeded by immersion in a 5.25% Sodium Hypochlorite solution for five minutes, after which they were once again exposed to the 70% ethanol solution, this time for a shorter duration of thirty seconds. The roots were then subjected to a thorough rinse with distilled water for one minute. The process was repeated twice to ensure the removal of any residual sterilizing agents. Following this, the roots were placed atop sterile filter paper for two minutes to dry.

In preparation for further experimental procedures, the roots were segmented into lengths of two centimeters using sterilized scissors. These prepared segments were subsequently introduced into Nutrient Agar (NA) media to facilitate the growth of any endophytic bacteria present. The inoculated samples were incubated at a constant temperature of 25°C in the dark for a period of 48 hours. A critical evaluation was conducted after the initial 24 hours; the absence of microbial growth surrounding the samples was indicative of a successful surface sterilization.

Following the 48-hour incubation period, the procedure for culturing endophytic bacteria was initiated, with the aim of isolating a single bacterial colony for transfer onto fresh Nutrient Agar media. This step was crucial for the purification of the culture. Subsequent observations were made to identify distinct morphological characteristics of the endophytic bacteria, focusing on attributes such as the color, shape, and margins of the colonies.

2.2 Gram Staining of Endophytic Bacteria

A pure colony of the bacteria was carefully smeared onto a clean glass slide to create a thin film. This smear was subjected to fixation by gently passing the slide over the flame of a

Bunsen burner two to three times, ensuring the bacterial sample was affixed to the slide while preserving its structural integrity.

Subsequently, the fixed bacterial smear was stained with crystal violet solution for a duration of one minute to impart the primary color. Following this, the slide was thoroughly rinsed with distilled water to remove excess stain. An iodine solution, specifically Lugol's solution, was then applied for one minute as a mordant, enhancing the crystal violet stain uptake, after which the slide was again rinsed with distilled water.

The differentiation step involved treating the slide with a decolorizing solution, namely alcohol, for 10 to 20 seconds, to remove the primary stain from Gram-negative bacteria while retaining it in Gram-positive bacteria. This was immediately followed by counterstaining with safranin solution for 15 seconds, providing a contrasting color to those bacteria that were decolorized. After a final rinse with distilled water to remove excess counterstain, the slide was air-dried using filter paper.

For microscopic examination, the dried slide was observed under 100x magnification. The staining outcome, characterized by the coloration of the bacteria, allowed for their classification based on Gram status: bacteria appearing purple were identified as Gram-positive, whereas those appearing red were classified as Gram-negative

2.3 Production of Secondary Metabolites by Endophytic Bacterial Isolates

To extract secondary metabolites, a colony is introduced into 250 mL of NB media and incubated in a shaker at 27°C and 170 rpm for 48 hours. The resulting culture undergoes sonication for 30 minutes and centrifugation at 5000 rpm for 10 minutes. Further, the supernatant is fractionated with ethyl acetate and then concentrated using a water bath at 40°C until the secondary metabolites are isolated from the solvent.

2.4 Phytochemical Screening

the specimen is carefully transferred into a test tube. Next, 10 mL of chloroform: water (1:1) solution is added. The mixture is then thoroughly shaken within the test tube and permitted to settle momentarily until two distinguishable layers' form. The aqueous layer is then analyzed for flavonoids, phenolics, and saponins, while the chloroform layer is examined for terpenoids, steroids, and alkaloids.

2.1.1 *Phenolic Examination*

One to two drops of the aqueous layer are combined with an equivalent volume of ferric chloride (FeCl3) solution within a test tube. The emergence of a blue hue serves as an indicative marker for the presence of phenolic compounds.

2.1.2 *Flavonoid Examination*

One to two 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 emergence of a reddish-orange hue serves as an indicative marker of the presence of flavonoid compounds within the sample.

2.1.3 *Terpenoid and Steroid Examination*

One to two drops of 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 vial is filled with 4 ml of DPPH solution at 35 µg/ml. Next, a 2 ml mixture of ethanol and distilled water in a 1:1 ratio is added to the vial. The vial is then sealed and placed in a dark area for 30 minutes. After that, the absorption of the solution is measured using a UV-Vis spectrophotometer, with wavelengths ranging from 400 to 800 nm.

2.6 Determining Gallic Acid Antioxidant Activity

To obtain concentrations of 1, 1.5, 2, 2.5, and 3 µg/mL, 0.2, 0.3, 0.4, 0.5, and 0.6 ml are pipetted into 10 ml volumetric flasks from a 50 µg/mL concentration. After adding a distilled water and ethanol mixture (1:1) to the mark, 2 ml of each concentration is pipetted into separate vials and 4 ml of DPPH solution is added. These vials are then left to stand for 30 minutes in the dark. Using a UV-Visible spectrophotometer, the absorption of the solutions is measured at the maximum wavelength. The percentage inhibition is calculated using the following formula from the absorbance values of the gallic acid reference and the control:

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

2.7 Determination of Sample Antioxidant Activity (Mosquera, 2007)

A 500 mg quantity of a secondary metabolite sample derived from endophytic bacteria was accurately weighed and subsequently solubilized in methanol within a 100 mL volumetric flask to the calibrated mark, achieving a concentration of 5000 µg/mL. Aliquots of the methanolic solution, specifically (0.2; 0.5; 1; 1.5; 2) mL, were pipetted out. Following this, a methanol: water solution in a 1:1 ratio was added to a 10 ml volumetric flask until the designated mark was reached, thereby yielding sample solutions with concentrations of (100, 250, 500, 750, 1000) µg/mL. Subsequently, 2 mL of each concentration from the sample solutions was carefully transferred into individual vials, followed by the addition of 4 ml of a DPPH solution at a concentration of 35 µg/mL. The resulting mixtures were thoroughly homogenized and allowed to stand for a duration of 30 minutes in an environment devoid of light. The absorption rates were then quantitatively measured using a UV-Vis spectrophotometer across a wavelength range of 400 to 800 nm. The antioxidant activity inherent within the sample was quantitatively assessed based on the degree of inhibition observed in the DPPH radical absorption, employing the calculation of the percentage inhibition of DPPH absorption as a metric. This methodology underscored the significance of meticulous preparation and measurement in the empirical determination of the sample's antioxidant efficacy.

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

The initial step in the isolation of endophytic bacteria from a given sample involves the process of isolation itself. The specific sample utilized in this context comprises fresh roots. Prior to the commencement of isolation, it is imperative that the sample undergoes a thorough cleaning to remove any impurities and soil that may be adhering to it, accomplished through the use of clean, flowing water. Concurrently, the critical step of surface sterilization is undertaken to eliminate epiphytic microorganisms present on the sample. This is achieved by immersing the sample in a solution of 70% ethanol and 5.25% sodium hypochlorite (NaOCl), thereby ensuring the removal of external microbial flora without compromising the integrity of the endophytes sought for isolation. [9].

Upon inoculating plant tissue sections onto NA media for about 48 hours, endophytic bacteria from mangosteen roots displayed growth. Subsequently, morphological characteristics of the isolated endophytic bacteria were observed, including their smooth-edged, rod-shaped, and gram-negative colonies (Fig 1).

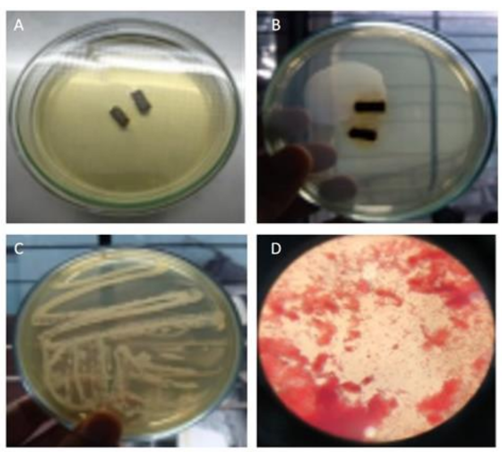

Fig. 1. The growth of endophytic bacteria isolated from mangosteen root tissues. (A) Mangosteen planted on NA media (B) Endophytic bacteria growing on mangosteen roots (C) Purified endophytic bacteria (D) Gram staining results at 40x magnification

Upon organoleptic assessment, the sample exhibits a cream hue and a unique scent. Further analysis through phytochemical screening confirms the presence of flavonoids, which are phenolic compounds known for their antimicrobial, antiviral, respiratory inhibiting, and antioxidant properties. Additionally, the sample contains saponins, potent surface-active agents that create foam when agitated in water and also possess antimicrobial properties. [10].

Based on our measurements, we discovered that the antioxidant activity of the secondary metabolite sample of endophytic bacteria had a maximum wavelength of 520 nm and an absorbance of 0.657. Using these absorbance measurements, we were able to calculate the percentage inhibition (or percentage of antioxidant compound immersion against DPPH). Our results showed that the percentage inhibition of the secondary metabolite sample of endophytic bacteria ranged from 2.508% to 5.734%, with a positive correlation between concentration and % inhibition value. These differences in antioxidant activity can be attributed to variations in the amount of flavonoid compounds, which provide hydrogen atoms [11,12].

The inhibitory percentages of gallic acid were 25.806%, 31.899%, 41.397%, 50.896%, and 58.243%. The difference in inhibitory ability between the bacterial isolate's secondary metabolites and gallic acid is due to the latter's hydroxyl groups, which are known to donate protons. The bacterial isolate's sample, on the other hand, is a solid secondary metabolite that has not yet been purified, resulting in suboptimal free radical neutralization [13]. Additionally, the flavonoid content in the bacterial isolate's secondary metabolites affects their inhibitory ability, as the presence of hydroxyl groups can influence the antioxidant ability of flavonoids. The flavonoid compounds in the bacterial isolate's secondary metabolites are suspected to have few free OH groups, resulting in an insufficient number of hydrogen atoms donated to neutralize the activity of DPPH radicals [14]. A study has also shown that the radical scavenging activity of flavonoids is related to the number and position of hydroxyl group bonds in the molecule [15].

Based on the percentage inhibition, the concentration of antioxidant compounds that can provide 50% inhibition can be determined, revealing the point at which the antioxidant can inhibit free radicals by 50%. The IC50 value of gallic acid has been calculated to be 2.498 g/mL. In accordance with Molyneux (2004) findings, IC50 values < 50 µg/mL indicate very strong antioxidant activity, while IC50 values of 50-100 µg/mL and 101-150 µg/mL indicate strong and moderate antioxidant activity, respectively. Conversely, IC50 values > 150 µg/mL indicate weak antioxidant activity. As such, the antioxidant activity of secondary metabolites from endophytic bacteria in mangosteen is considered weak and cannot serve as a viable alternative or solution for antioxidant production, as Table 1 illustrates.

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	

Based on the t-test data analysis results, it has been determined that there is a noteworthy variance between the antioxidant activity of gallic acid % inhibition and the secondary metabolites of endophytic bacteria in mangosteen, with a significance value of 0.013 ($P < .05$). Thus, it can be concluded that there exists a notable difference between the % inhibition of gallic acid and the secondary metabolites of endophytic bacteria in mangosteen.

4. CONCLUSION

The investigation into the endophytic bacteria of the mangosteen plant roots revealed a gram-negative strain with a cream-colored appearance, with smooth-edged and convex colonies. The antioxidant potency of secondary metabolites produced by endophytic bacteria was evaluated and yielded an IC50 value of 14,047.912 g/mL. This suggests that, in their current state, the metabolites are not a viable option for antioxidant production. Further research is required to explore the practical uses of endophytic bacteria from mangosteen roots and improve their antioxidant efficacy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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