ISSN: -

Journal Biology & Health Innovations

ANTIOXIDANT ACTIVITY OF ENDOPHYTIC BACTERIAL METABOLITE EXTRACTS FROM MORINGA OLEIFERA STEM BARK

Article Type : Article Text

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ABSTRACT

Endophytic bacteria from the stem bark of *Moringa oleifera* are microorganisms that live within plant tissues and are capable of producing bioactive compounds similar to those of their host. The antioxidant potential of endophytic bacteria associated with Moringa plants may provide beneficial health effects. This study aimed to determine the antioxidant activity of endophytic bacterial isolates from *Moringa oleifera* stem bark and to identify the isolates morphologically. A true experimental design was used in this study. Surface sterilization was performed to ensure that the bacteria tested were endophytic in origin. Microscopic identification was carried out using Gram staining, followed by catalase test screening. The selected isolates were fermented in Nutrient Broth and extracted using Ultrasound-Assisted Extraction (UAE). The resulting extracts were evaluated for antioxidant activity using the DPPH method to determine IC_{50} values and classify antioxidant strength. Four endophytic bacterial isolates were successfully obtained from *Moringa oleifera* stem bark, showing relatively similar colony morphology on Nutrient Agar medium. Microscopic identification revealed that all isolates were Gram-positive single bacilli, and catalase screening results were positive. The metabolite extracts tested using the DPPH method yielded a mean IC_{50} value of 430.924 ± 24.133 µg/mL, indicating very weak antioxidant activity. Based on morphological and biochemical identification, the isolates were suspected to belong to the genus Bacillus. In conclusion, the metabolite compounds produced by endophytic bacterial isolates from *Moringa oleifera* stem bark exhibited weak antioxidant activity.

Keywords Antioxidant, Endophytic Bacteria, DPPH, Fermentation, *Moringa oleifera*

Submitter Date: 17 January 2026
Accepted Date : 31 May 2026
Published Date : 01 June 2026

INTRODUCTION

Oxidative stress is a condition caused by an imbalance between free radicals and the antioxidant system in the body, which can interfere with normal physiological functions such as the immune responses, hormone synthesis, and cellular signaling (1). Excessive accumulation of free radicals can damage lipids, proteins, and DNA, thereby contributing to the development of degenerative and chronic diseases, including hypertension, coronary heart disease, stroke, diabetes mellitus, and chronic kidney disease (2). According to data from the Ministry of Health of the Republic of Indonesia, cardiovascular diseases account for approximately 651,481 deaths annually in Indonesia. Furthermore, the 2018 Riskesdas report showed that the prevalence of hypertension reached 34.1%, coronary heart disease 1.5%, and chronic kidney disease 0.38%. The high prevalence of these diseases highlights the importance of preventive strategies through the utilization of antioxidant compounds capable of scavenging free radicals and reducing oxidative stress (3).

Antioxidants are compounds capable of donating electrons to stabilize free radicals and minimize oxidative damage in the body. Natural antioxidants can be obtained from various medicinal plants, including *Moringa oleifera*, which is known to contain bioactive compounds such as flavonoids, alkaloids, tannins, saponins, polyphenols, and steroids with antioxidant potential Although *Moringa oleifera* has been widely studied for its pharmacological properties, the direct utilization of plant materials as a source of bioactive compounds requires large amounts of biomass and may affect the sustainability of plant resources.

One alternative source of bioactive compounds is endophytic microorganisms, particularly endophytic bacteria. Endophytic bacteria are microorganisms that inhabit plant tissues without causing damage to their host plants. These microorganisms are known to produce secondary metabolites similar to those of their host plants due to coevolution and genetic transfer between plants and endophytic microbes (5). In addition, endophytic bacteria offer several advantages, including rapid growth, ease of cultivation, and greater efficiency compared to direct plant exploitation (6).

Several studies have reported the presence of endophytic microorganisms in *Moringa oleifera*. Abna et al. (2024) identified endophytic fungi from the stem of *Moringa oleifera* that exhibited antibacterial activity. Other studies also successfully isolated endophytic bacteria such as *Bacillus cereus* and *Citrobacter freundii* from *Moringa oleifera* stems with potential biological activity against pathogenic bacteria (7). However, studies specifically investigating the antioxidant activity of metabolites produced by endophytic bacteria isolated from the stem bark of *Moringa oleifera* remain limited. In particular, information regarding the morphological characteristics and antioxidant potency of endophytic bacterial metabolites derived from stem bark tissues is still scarce.

Therefore, this study aimed to isolate and identify endophytic bacteria from the stem bark of *Moringa oleifera* and evaluate the antioxidant activity of their metabolite extracts using the DPPH method. The bacterial metabolites were obtained through fermentation in Nutrient Broth medium and extracted using the Ultrasound-Assisted Extraction (UAE) method. The findings of this study are expected to provide scientific information regarding the potential of endophytic bacteria from *Moringa oleifera* stem bark as an alternative natural source of antioxidant compounds.

MATERIALS AND METHODS

Study Design and Sample Collection

This study employed a true experimental in vitro design to evaluate the antioxidant activity of metabolites produced by endophytic bacteria isolated from the stem bark of *Moringa oleifera*. Stem bark samples were collected from healthy adult *Moringa oleifera* plants in the Jabodetabek area, Indonesia. The study was conducted from February 2025 to January 2026 at the Microbiology and Parasitology Laboratory and the Biochemistry Laboratory of Universitas Pembangunan Nasional Veteran Jakarta, as well as the Food Technology Laboratory of Universitas Djuanda Bogor.

Tools and Materials

The instruments used in this study included sterile forceps, Petri dishes, test tubes, micropipettes, inoculating loops, Bunsen burner, Laminar Air Flow cabinet, autoclave, centrifuge, microscope, ultrasonic water bath, Erlenmeyer flasks, and a UV-Vis spectrophotometer. The materials used were stem bark of *Moringa oleifera*, Nutrient Agar (NA), Nutrient Broth (NB), methanol, DPPH solution (0.4 mM), sodium hypochlorite (NaOCl), distilled water, hydrogen peroxide (H_2O_2), vitamin C as positive control, and endophytic bacterial isolates.

Isolation of Endophytic Bacteria

Stem bark samples were washed under running water for 10 minutes and cut into approximately 1 cm pieces. Surface sterilization was carried out inside a Laminar Air Flow cabinet by immersing the samples in 70% ethanol for 1 minute, followed by immersion in 5% NaOCl solution for 5 minutes, and rinsing three times with sterile distilled water. To validate the effectiveness of the sterilization process, the final rinse water was inoculated onto Nutrient Agar (NA) medium and incubated under the same conditions. The absence of microbial growth confirmed the success of

surface sterilization and indicated that the isolates originated from internal plant tissues. Sterilized stem bark samples were inoculated onto Nutrient Agar medium prepared by dissolving 4.2 g of NA powder in 150 mL distilled water. The medium was sterilized using an autoclave at 121°C and 15 psi for 15 minutes before being poured into sterile Petri dishes. The inoculated samples were incubated at 37°C for 2–3 days to obtain endophytic bacterial isolates.

Screening and Identification of Bacterial Isolates

The isolates were screened using the catalase test by adding hydrogen peroxide (H_2O_2) onto bacterial colonies. Isolates showing the highest bubble formation and fastest reaction time were selected for further analysis. Morphological identification was performed using Gram staining. Bacterial smears were fixed on glass slides and stained sequentially with crystal violet for 5 minutes, Lugol's iodine for 30 seconds, alcohol decolorizer, and safranin for 1–2 minutes. The stained preparations were then observed under a microscope to determine Gram characteristics and bacterial cell morphology.

Fermentation and Extraction of Bacterial Metabolites

Selected bacterial isolates were fermented by inoculating approximately 10% bacterial culture into 50 mL of sterile Nutrient Broth contained in 100 mL Erlenmeyer flasks. The fermentation process was carried out at 37°C for 24 hours under agitation using a shaker incubator at 120 rpm. The extraction process was performed directly on the fermentation broth using the Ultrasound-Assisted Extraction (UAE) method without prior separation of bacterial cells. An ultrasonic water bath was operated at 30–40°C with a frequency of 40 kHz for 20 minutes. Following sonication, the fermentation broth was centrifuged to separate bacterial cell pellets from the supernatant. The supernatant was collected as the metabolite extract for antioxidant testing.

Antioxidant Activity Assay

Antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Serial concentrations of metabolite extracts (5, 10, 20, and 40 µg/mL) were prepared using methanol. Vitamin C was used as a positive control. For the assay, 1 mL of extract solution was mixed with 3 mL of DPPH solution and incubated in the dark for 30 minutes at room temperature prior to absorbance measurement at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH radical inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{Ab - As}{\text{Ab}} \times 100$$

where A_b is the absorbance of the blank solution and A_s is the absorbance of the sample solution.

Data Analysis

The IC_{50} value, defined as the concentration required to inhibit 50% of DPPH radicals, was determined using linear regression analysis between concentration and percentage inhibition values. Data were analyzed descriptively and presented as mean and standard deviation from three replications. Antioxidant activity was categorized based on the obtained IC_{50} values.

DISCUSSION

Isolation of Endophytic Bacteria from *Moringa oleifera* Stem Bark

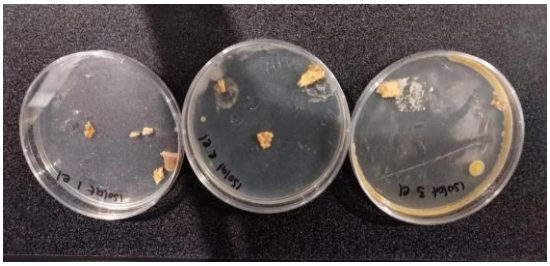

Figure 1. Isolation of endophytic bacteria from *Moringa oleifera* stem bark

The cultured stem bark samples of *Moringa oleifera* showed the growth of endophytic bacteria on Nutrient Agar (NA) medium after incubation. The colonies appeared whitish-yellow with medium-sized growth surrounding the stem bark segments, indicating successful isolation of endophytic bacteria from internal plant tissues. Nutrient Agar was selected because it provides a complex medium containing peptone, yeast extract, NaCl, and agar that supports the growth of endophytic bacteria (8). Prior to isolation, surface sterilization using 70% ethanol and 5% NaOCl was performed to eliminate epiphytic microorganisms and ensure that the isolates originated from internal plant tissues (9). The sterilization process is an important step in endophytic bacterial isolation because contamination from external microorganisms may affect the purity and validity of the isolates obtained. The appearance of bacterial colonies around the sterilized plant tissues confirmed that the isolated bacteria were able to survive and grow from the internal tissues of the stem bark.

The successful growth of endophytic bacteria from *Moringa oleifera* stem bark indicates that the plant tissues provide a suitable ecological niche for microbial colonization. Endophytic bacteria are known to establish mutualistic interactions with their host plants and may contribute to plant protection, growth promotion, and secondary metabolite production. These microorganisms are capable of synthesizing bioactive compounds similar to those produced by the host plant due to coevolutionary

adaptation and possible genetic interactions between the host and the microorganisms (5). Therefore, the isolation of endophytic bacteria from medicinal plants such as *Moringa oleifera* may provide alternative sources of natural bioactive compounds without requiring excessive plant biomass exploitation.

Morphological Identification of Endophytic Bacterial Isolates

Table 1. Morphological characteristics of endophytic bacterial isolates

Isolates	1	2	3	4
Pictures				
Color	Whitish-yellow	Whitish-yellow	Whitish-yellow	Whitish-yellow
Size	Medium	Medium	Medium	Medium
Shape	Circular	Circular	Circular	Unclear
Surface	Smooth	Smooth	Smooth	Smooth
Elevation	Convex	Convex	Convex	Convex
Margin	Regular	Regular	Regular	Regular

Morphological observation showed that all isolates had relatively similar colony characteristics, including whitish-yellow color, medium size, smooth surface, convex elevation, and regular margins. Most isolates showed circular colony shapes, while isolate 4 could not be clearly identified due to incomplete purification. The similarity in colony morphology suggested that the isolates may belong to closely related bacterial groups. Colony morphology is commonly used as an initial approach in bacterial identification because characteristics such as color, surface texture, elevation, and colony margins may reflect similarities in bacterial taxonomy and physiology.

The whitish-yellow coloration observed in all isolates may indicate pigment production associated with bacterial metabolic activity. Smooth colony surfaces and convex elevations are commonly observed among members of the genus Bacillus, which are known to form regular and relatively large colonies on Nutrient Agar medium. However, morphological observation alone is insufficient for species-level identification because colony appearance may vary depending on environmental conditions and culture media composition. Therefore, Gram staining was performed to further characterize the isolates microscopically.

Table 2. Microscopic characteristics of Gram staining results

Isolates	1	2	3	4
Pictures				
Shape	Bacillus	Bacillus	Bacillus	Bacillus
Arrangement	Single	Single	Single	Single
Color	Purple	Purple	Purple	Purple
Gram Characteristic	Gram-positive	Gram-positive	Gram-positive	Gram-positive

Gram staining results showed that all isolates were Gram-positive bacteria with rod-shaped (bacillus) cells arranged singly. The purple coloration indicated that the isolates retained the crystal violet-iodine complex due to the thick peptidoglycan layer characteristic of Gram-positive bacteria (10). During the Gram staining procedure, alcohol decolorization causes dissolution of membrane lipids in Gram-negative bacteria, allowing the primary stain to be released. In contrast, Gram-positive bacteria possess thick peptidoglycan layers that retain the crystal violet stain, resulting in purple-colored cells after staining.

Based on the morphological and microscopic characteristics, the isolates were presumed to belong to the genus Bacillus. Members of this genus are commonly isolated as endophytic bacteria from medicinal plants and are widely reported to produce various bioactive secondary metabolites, including antimicrobial, antioxidant, and enzyme-producing compounds. Several studies have also identified Bacillus species from *Moringa oleifera* tissues, supporting the possibility that the isolates obtained in this study belong to the same genus (7). The presence of Gram-positive Bacillus-like bacteria may indicate their adaptability to survive within plant tissues and produce metabolites that potentially contribute to plant defense mechanisms.

Catalase Test Screening

Figure 2. Catalase test results, where isolate 1, isolate 2, isolate 3, and isolate 4 are represented by numbers 1, 2, 3, and 4, respectively.

Table 3. Comparison of catalase test results among isolates

Sample	Bubble Formation	Time of Bubble Formation
Isolate 1	+++	0.4 seconds
Isolate 2	+	1.2 seconds
Isolate 3	++	1 second
Isolate 4	+	2 seconds

All isolates showed positive catalase activity indicated by bubble formation after the addition of hydrogen peroxide. Catalase is an enzymatic antioxidant that decomposes hydrogen peroxide into water and oxygen, thereby reducing reactive oxygen species (ROS) (11). The formation of oxygen bubbles after hydrogen peroxide addition indicated that the isolates possessed catalase enzymes capable of detoxifying peroxide compounds. Catalase activity is important because hydrogen peroxide is one of the reactive oxygen species that can damage cellular structures if accumulated excessively.

Isolate 1 demonstrated the highest catalase activity with the greatest bubble formation (+++) and the fastest reaction time of 0.4 seconds. Therefore, isolate 1 was selected for further fermentation and antioxidant activity testing. The rapid formation of bubbles suggested higher catalase enzyme activity compared to the other isolates. Catalase-positive bacteria are often associated with increased tolerance to oxidative stress because they are capable of neutralizing toxic peroxide compounds. Similar studies have reported that catalase-positive endophytic bacteria potentially possess antioxidant-related activity due to their ability to neutralize oxidative compounds (8).

The catalase screening method used in this study served as an initial selection step to identify isolates with potentially higher antioxidant activity. Although catalase activity alone does not directly determine overall antioxidant capacity, the presence of active antioxidant enzymes may indicate the ability of bacteria to produce antioxidant metabolites. Based on morphological and biochemical characteristics, the isolates were presumed to belong to the genus Bacillus (12), which is known for producing various extracellular enzymes and bioactive compounds.

DPPH Antioxidant Activity Assay

The DPPH assay is one of the most commonly used methods for evaluating antioxidant activity. DPPH acts as a stable free radical that reacts with antioxidant compounds present in the sample. The antioxidant activity was indicated by the reduction of purple-colored DPPH radicals into a yellow-colored stable form (DPPH-H) (13). Antioxidant capacity was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm (14).

The extract samples were prepared in five concentration levels prior to absorbance measurement. Percentage inhibition values were calculated from the absorbance results, and the assay was performed in triplicate using the selected bacterial isolate extract. Linear regression equations were then generated from each replication.

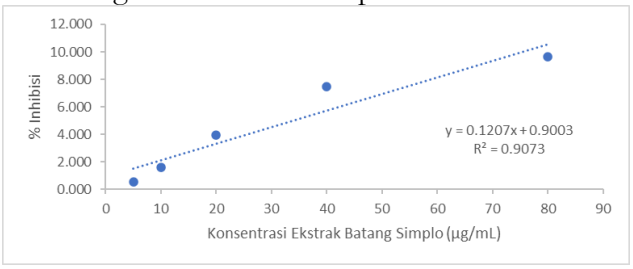

Figure 3. Linear regression equation of first replication stem bark extract

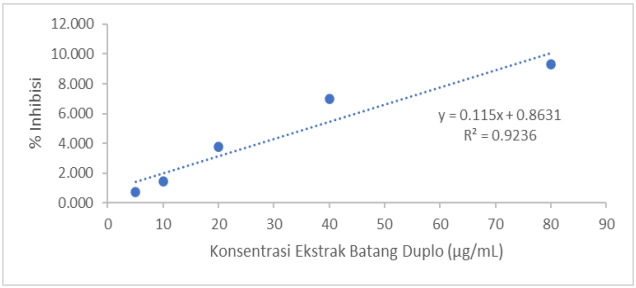

Figure 4. Linear regression equation of second replication stem bark extract

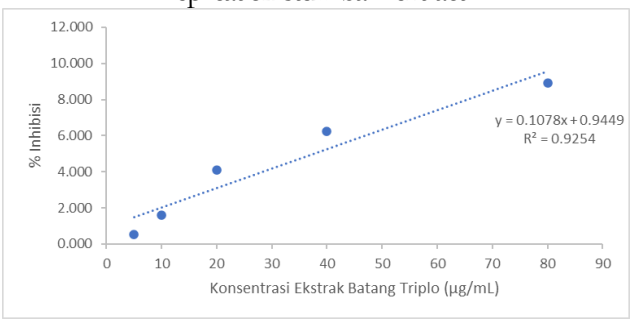

Figure 5. Linear regression equation of third replication stem bark extract.

The obtained R^2 values from the three replications were 0.9073, 0.9236, and 0.9254, respectively. R^2 values greater than 0.9 indicated that the regression equations were valid and suitable for determining IC_{50} values. Increasing extract concentration resulted in higher percentage inhibition values, indicating an increase in antioxidant activity due to the presence of secondary metabolites (15). The relatively high R^2 values also suggested that the concentration-response relationship was sufficiently linear and reliable for antioxidant analysis.

Table 4. Antioxidant activity classification and IC_{50} values

Extract	% Inhibition ± SD	IC_{50} Value (µg/mL)	Antioxidant Activity
Extract 1	4.643 ± 3.457	406.791	Very weak
Extract 2	4.428 ± 3.264	427.277	Very weak
Extract 3	4.286 ± 3.056	455.057	Very weak
Ascorbic acid	-	9.459	Strong
Methanol	0.00	-	No antioxidant activity
Mean ± SD		430.924 ± 24.133	
Coefficient of variation		12.626	

The results showed that the metabolite extracts of endophytic bacteria from *Moringa oleifera* stem bark exhibited very weak antioxidant activity, with a mean IC_{50} value of 430.924 ± 24.133 µg/mL. According to previous studies, antioxidant activity is categorized as very weak when the IC_{50} value exceeds 200 µg/mL (15). In comparison, ascorbic acid showed a strong antioxidant activity with an IC_{50} value of 9.459 µg/mL, while methanol showed no antioxidant activity. Although the antioxidant activity of the bacterial extract was low, the coefficient of variation value below 15 indicated that the measurements were relatively precise and reproducible across replications.

The weak antioxidant activity observed in this study may be associated with several factors, including the extraction process, bacterial fermentation conditions, and environmental factors affecting the host plant. The very weak antioxidant activity observed in this study may also be associated with nutritional limitations of the fermentation medium. Nutrient Broth (NB) is a general-purpose medium primarily designed to support bacterial growth rather than stimulate the production of specialized secondary metabolites. The medium may lack specific nutritional precursors, carbon sources, amino acids, or environmental stress conditions required to activate biosynthetic pathways involved in phenolic, flavonoid-like, or polyketide compound production. Consequently, the endophytic bacterial isolates may have produced only limited amounts of antioxidant metabolites under the fermentation conditions used in this study.

Previous studies also reported low antioxidant activity from endophytic bacteria isolated from *Moringa oleifera*, suggesting that crude bacterial metabolites may contain only small amounts of antioxidant compounds mixed with non-antioxidant substances (16). Another factor contributing to the weak antioxidant activity may be the use of crude metabolite extracts without further purification. The obtained supernatant likely contained various non-antioxidant extracellular components, including residual Nutrient Broth constituents, structural proteins, extracellular polymeric substances, and other inactive metabolites. These compounds may dilute the concentration of active antioxidant molecules, resulting in relatively high IC_{50} values. Further purification and fractionation processes may increase the concentration of active antioxidant compounds and improve antioxidant activity. Additionally, antioxidant compounds produced by endophytic bacteria may require further purification to achieve stronger antioxidant effects.

The extraction method and fermentation process may also influence metabolite production. In this study, bacterial fermentation was performed for 24 hours, which likely corresponded to the stationary growth phase where bacterial growth becomes balanced between living and dying cells (17). Although secondary metabolites are often produced during the stationary phase, the production level may vary depending on bacterial species, nutrient availability, pH, temperature, and oxygen conditions. The use of a simple Ultrasound-Assisted Extraction (UAE) method may also have affected extraction efficiency and metabolite concentration.

Environmental factors associated with the host plant may additionally contribute to the low antioxidant activity observed. The *Moringa oleifera* samples used in this study were collected from roadside areas, where exposure to pollution and environmental stress may influence plant metabolism and the composition of endophytic microorganisms. Previous studies reported that environmental conditions such as soil nutrients, humidity, light intensity, altitude, and air pollution significantly affect the production of phenolic and flavonoid compounds in plants (18,19). Since endophytic bacteria are closely associated with their host plants, variations in plant environmental conditions may indirectly affect the metabolic capabilities of the bacterial isolates.

Another possible explanation is that the bacterial isolates may possess biosynthetic genes encoding antioxidant metabolites that were not fully expressed under the culture conditions used in this study. Certain endophytic bacteria

require specific precursors or environmental stimuli to activate secondary metabolite biosynthesis pathways (10), optimization of culture media, fermentation duration, extraction techniques, and metabolite purification may improve antioxidant activity in future studies.

CONCLUSION

Endophytic bacteria isolated from the stem bark of Moringa oleifera showed morphological and biochemical characteristics of Gram-positive bacilli and were presumed to belong to the genus Bacillus. The metabolite extract from the selected isolate exhibited very weak antioxidant activity in the DPPH assay, with a mean IC_{50} value of 430.924 µg/mL. These results indicate low antioxidant potential under the experimental conditions, which may be related to the use of a general growth medium and crude extract that still contains non-active components. Further studies are needed to optimize fermentation conditions, improve extraction and purification methods, and perform molecular identification for more accurate characterization of the isolates.

ACKNOWLEDGMENT

The authors would like to thank Universitas Pembangunan Nasional Veteran Jakarta and Universitas Djuanda Bogor for the laboratory facilities and support provided throughout this research. Appreciation is also extended to all parties who contributed to this study.

DECLARATIONS

1. Declarations (Author Contribution, Funding Statement, Conflict of Interest)
2. Declarations (Author Contribution, Funding Statement, Conflict of Interest)
3. Declarations (Author Contribution, Funding Statement, Conflict of Interest)
4. Declarations (Author Contribution, Funding Statement, Conflict of Interest)

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