

RESEARCH ARTICLE

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Endophytic Bacteria in *Acalypha indica* L. Leaves and Their Antimicrobial Activity Against *Staphylococcus aureus* and *Candida albicans*

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Background: The anting-anting plant (*Acalypha indica* L.) is used in herbal medicine in the treatment of various diseases. The leaf extract of this plant is known for its antimicrobial activity, but the antimicrobial properties of the endophytic bacteria within its leaves have never been reported. This research aims to determine the antimicrobial activity of endophytic bacteria from the leaves of the anting-anting plant.

Materials and methods: The isolation of endophytic bacteria was performed using the spread plate method on nutrient agar (NA) media. Following isolation, the bacterial isolates were characterized through macroscopic and microscopic examination, as well as biochemical tests, which included indole production, hydrogen sulfide (H₂S) production, motility, Simmons citrate utilization, methyl red-Voges-Proskauer (MR-VP) test, catalase test, and triple sugar iron agar (TSIA) test. Identification of the bacterial isolates was conducted according to *Bergey's Manual of Systematic Bacteriology*. Additionally, the antimicrobial activity of the isolates was assessed using the diffusion method

Results: Fourteen isolates of anting-anting leaf endophytic bacteria were obtained (coded as BEDA 1 to BEDA 14). The BEDA 5 isolate exhibited the largest inhibitory zone diameter against *Staphylococcus aureus* (31.48 mm), while BEDA 9 showed a significant inhibitory zone diameter against *Candida albicans* (17.84 mm).

Conclusion: The two isolates (BEDA 5 and BEDA 9) exhibited significant antimicrobial activity, indicating their potential as promising candidates for alternative antimicrobial agents. These results suggest that endophytic bacteria from *Acalypha indica* may play an essential role in combating antibiotic resistance and in the development of new therapeutic strategies.

Keywords: endophytic bacteria, characterization, antimicrobial activity, *Acalypha indica*

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Introduction

Endophytic bacteria are bacteria that live in plant tissue and help plants adapt to biotic and abiotic environmental factors (e.g. pathogens, infections, drought, high salinity, and pollutants).¹ Secondary metabolites produced by endophytic bacteria can be similar to those of their host plants, exhibiting antibacterial and antifungal activity.² Endophytic bacteria can efficiently degrade organic contaminants such as petroleum hydrocarbons, chlorpyrifos, and 2,4,6-trinitrotoluene. Pseudomonaceae, Burkholderiaceae, Bacillaceae, and Enterobacteriaceae are the most common bacterial families found from the isolation of endophytic bacteria.³ The YCFE4 endophytic bacteria from the *Caulerpa* sp. can inhibit the growth of *Enterococcus faecalis* and *Escherichia coli* bacteria.⁴ The growth of *Staphylococcus aureus* bacteria can be inhibited by the M8 endophytic bacteria from the turmeric plant (*Curcuma longa* L.).⁵

Endophytic bacteria from the leaves of the anting-anting plant (*Acalypha indica* L.) plant have never been reported. However endophytic bacteria from its roots have been found to inhibit the growth of *Klebsiella pneumoniae* and were identified as *Luteimonas terrae*.⁶ *A. indica* is a widely available medicinal plant in Indonesia.⁷ Medicines derived from the anting-anting plant have been used for generations. The leaves of the *A. indica* plant are used as herbal medicine in the treatment of vomiting blood, indigestion, diarrhea, nosebleeds, dysentery, and blood in urine.⁸ A decoction of the *A. indica* plant's roots is used to treat spinal pain, headache, and wound medicine Bengkulu Province.⁹ A decoction of the *A. indica* plant's leaves is also used as medicine in the treatment of genitourinary disorders and cysts by the Tolaki Ethnic tribe community, Southeast Sulawesi Province.¹⁰

A. indica plant extract is known to have anti-diabetes mellitus activity, antioxidant properties that inhibit free radicals, as well as anticancer activity, cytotoxicity, antimalarial and antibacterial effects.⁷ *A. indica* leaf extract can inhibit the growth of bacteria such as *Staphylococcus aureus*,⁷ *Bacillus cereus*, *Escherichia coli*, *Staphylococcus epidermidis*, *Salmonella typhi*,¹¹ *Pseudomonas aeruginosa*, *Staphylococcus pyogenes*,¹² *Sarcina lutea*, *Xanthomonas campestris* and *Proteus vulgaris*.¹³ The anting-anting plant also can inhibit the growth of fungi, namely *Candida albicans*, *Candida tropicalis*, *Trichophyton rubrum*, and *Trichophyton tonsurans*.¹² However, obtaining extracts

from these plants requires large specimens, so alternatives are sought using other sources, such as the use of endophytic bacteria. Given the numerous benefits of endophytic bacteria and the potential of *A. indica*, this research is necessary to carry out this research to determine the antimicrobial activity from endophytic bacteria associated with the leaves of *A. indica*. Therefore, this study was conducted to evaluate the antimicrobial activity of the endophytic bacteria isolated from the leaves of *A. indica*.

Materials and methods

Study Design

Plant samples were collected in the Kopelma Darussalam, Syiah Kuala, Banda Aceh, Aceh, Indonesia. This study was conducted at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala. Fourteen bacterial isolates of *A. indica* leaves endophytic bacteria (BEDA) used was obtained based on the isolation stages.

Isolation of Endophytic Bacteria

Five grams of *A. indica* leaves were collected by picking the third leaf from the shoot. The surface of the leaf was cleaned using running water, then sterilize the surface by soaking it in 70% ethanol (OneMed, PT Jayamas Medica Industri, Sidoarjo, Indonesia) for 1 minute, followed by soaking in 3% sodium hypochlorite (NaOCl) solution (Bayclin, PT SC Johnson, Jakarta, Indonesia) for 3 minutes. The leaf was then soaked again in 70% ethanol for 30 seconds and washed with distilled water 3 times. The water from the final rinse was used as a control and used in a 0.1 mL sample on nutrient agar (NA) media (Cat. No. 105450 Merck KGaA, Darmstadt, Germany) media using the spread plate method. The leaf samples were then crushed and inoculated into three 0.1 mL NA media, which had been supplemented with nystatin (50 µg/mL) (PT Ifars, Karanganyar, Indonesia), and incubated at 37 °C for 24 hours (Incubator Memmert, Germany). All bacterial colonies that grew on the media with different characteristics were selected for next stage.

Macroscopic and Microscopic Characterization of Endophytic Bacteria

Bacterial colonies growing on NA media were purified using the streak plate method. Colonies were picked with Ose and inoculated onto fresh NA medium. The color, shape, elevation, edges, and size of the colony were then observed.

Microscopic characteristics were determined using a Gram staining kit (HiMedia, PT Smart Lab Indonesia, Tangerang, Indonesia). Gram staining was performed with crystal violet dye for 1 minute, iodine solution for 1 minute, 95% alcohol for 20 seconds, and safranin dye for 45 seconds. Observations were made under a microscope. Gram-negative bacteria appeared pink, while Gram-positive bacteria appeared purple.

Biochemical Test

The bacterial isolates obtained were subjected to biochemical tests, including indole test, motility test, hydrogen sulfide (H_2S) test (SIM medium Merck KGaA, Darmstadt, Germany), catalase test, triple sugar iron agar (TSIA) test (TSIA medium Merck KGaA, Darmstadt, Germany), methyl red-Voges-Proskauer (MR-VP) test (MR-VP broth Merck KGaA, Darmstadt, Germany), and Simmons citrate (SC) test (SCA medium Merck KGaA, Darmstadt, Germany).

Genus Identification

The genus of the endophytic bacterial isolates was identified by referring to *Bergey's Manual of Systematic Bacteriology*, based on observations of the macroscopic characteristics of the colonies, microscopic characteristics of the cells, and the results of the biochemical tests.

Antimicrobial Activity Assay

The turbidity of the test microbial suspension was adjusted to match the standard 0.5 McFarland solution. The bacterial suspension (*E. coli* and *S. aureus*) (Microbiology Laboratory,

USK, Banda Aceh, Indonesia) were evenly applied using a sterile cotton swab and streaked onto Mueller-Hinton agar (MHA) media (Cat. No. 103872, Merck KGaA, Darmstadt, Germany), while the fungal suspension (*C. albicans*) (Microbiology Laboratory, USK, Banda Aceh, Indonesia) was streaked onto Sabouraud dextrose agar (SDA) media (Cat. No. 105438, Merck KGaA, Darmstadt, Germany). The endophytic bacterial isolate was transferred using a cork-borer and placed onto the surface of the media. Chloramphenicol discs (Thermo Scientific Oxoid, UK) were used as controls for bacterial test, and nystatin was used for fungal test. The inhibition zone diameter was calculated using the following formula¹⁴:

$$\text{Diameter of inhibition zone (mm)} = (D_v - D_c) + (D_h - D_c)/2$$

Where D_v is diameter of vertical, then D_c is diameter of the cork borer, and D_h is diameter of horizontal.

Results

Macroscopic and Microscopic Characteristics of Endophytic Bacteria from *A. indica* Leaves

A total of 14 isolates of BEDA were successfully isolated. The isolates exhibited diverse macroscopic characteristics, which were observed based on color, elevation, edges, shape and size of bacterial colonies. The isolates displayed colony colors including cream, white, yellowish-white, yellow and white with varying transparency. The colonies were round or irregular in shape, with smooth edges and elevation that were either flat and raised (Figure 1, Table 1). The microscopic characteristics showed rod-shaped and

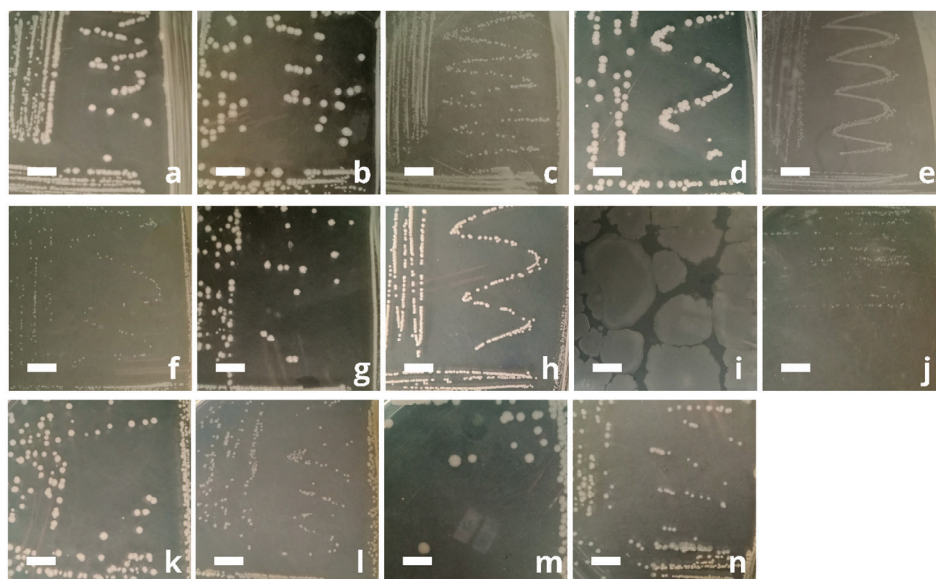


Figure 1. Macroscopic characteristics of BEDA isolates. (a) BEDA 1; (b) BEDA 2; (c) BEDA 3; (d) BEDA 4; (e) BEDA 5; (f) BEDA 6; (g) BEDA 7; (h) BEDA 8; (i) BEDA 9; (j) BEDA 10; (k) BEDA 11; (l) BEDA 12; (m) BEDA 13; (n) BEDA 14. White bar: 7.5 mm.

Table 1. Macroscopic and microscopic characteristics of bacterial isolates.

Isolate	Macroscopic Characteristics					Microscopic Characteristics	
	Color	Shape	Edge	Elevation	Size	Gram	Shape
BEDA 1	Cream	Round	Smooth	Rise	Moderate	Positive	Coccus
BEDA 2	Cream	Round	Smooth	Rise	Moderate	Negative	Coccus
BEDA 3	Cream	Round	Smooth	Rise	Small	Negative	Rod
BEDA 4	White	Round	Smooth	Flat	Moderate	Positive	Coccus
BEDA 5	White and transparent	Round	Smooth	Rise	Punctiform	Positive	Rod
BEDA 6	Yellowish-white	Round	Smooth	Rise	Punctiform	Positive	Rod
BEDA 7	Cream	Irregular	Smooth	Rise	Moderate	Positive	Coccus
BEDA 8	White	Round	Smooth	Rise	Small	Positive	Coccus
BEDA 9	White and transparent	Irregular	Wavy	Flat	Big	Negative	Rod
BEDA 10	Yellow	Round	Smooth	Rise	Punctiform	Positive	Rod
BEDA 11	Cream	Round	Smooth	Rise	Moderate	Positive	Rod
BEDA 12	Yellow	Round	Smooth	Rise	Punctiform	Negative	Coccus
BEDA 13	White	Round	Smooth	Rise	Moderate	Positive	Rod
BEDA 14	Yellowish-white	Round	Smooth	Rise	Small	Negative	Rod

coccus-shaped cells, with both Gram-positive and Gram-negative reactions (Figure 2, Table 1).

Biochemical Characteristics and Genus Identification

BEDA isolates exhibited various physiological characteristics based on biochemical properties. Biochemical tests showed positive results, including a red ring formation in the indole test, cloudy media or growth moving away from the inoculation point in the motility test, a black precipitate in the H₂S test, air bubble formation in the catalase test, a red-to-yellow color change in the TSIA test, a reddish color change in the MR-VP test, and a green-to-blue color change in the SC test. The results of biochemical testing of BEDA isolates and genus identification (Table 2) were identified.

Antimicrobial Activity

Most of the isolates tested showed small zone of inhibition in their ability to inhibit the growth (Table 3). However,

some isolates displayed larger inhibition zones, suggesting significant antimicrobial activity, with inhibition zone sizes exceeding those of the controls (Figure 3).

Discussion

The number of BEDA isolates obtained was 14 different isolates, with several researchers successfully isolating eight isolates of endophytic bacteria from *Momordica charantia* L. roots,¹⁶ 20 isolates from leaves of potato plants (*Solanum tuberosum* L.),¹⁷ 30 isolates of endophytic bacteria from *Fraxinus hupehensis*,¹⁸ five isolates of endophytic bacteria from cassava leaves (*Manihot esculenta* Crantz),¹⁹ 18 isolates of endophytic bacteria from *Kochia prostrata* (L.) Schrad leaves,²⁰ and eight isolates of endophytic bacteria from the *Cordia dichotoma* L. leaves.²¹ This variability in the number of endophytic bacteria obtained will vary depending on the type of plant.²²

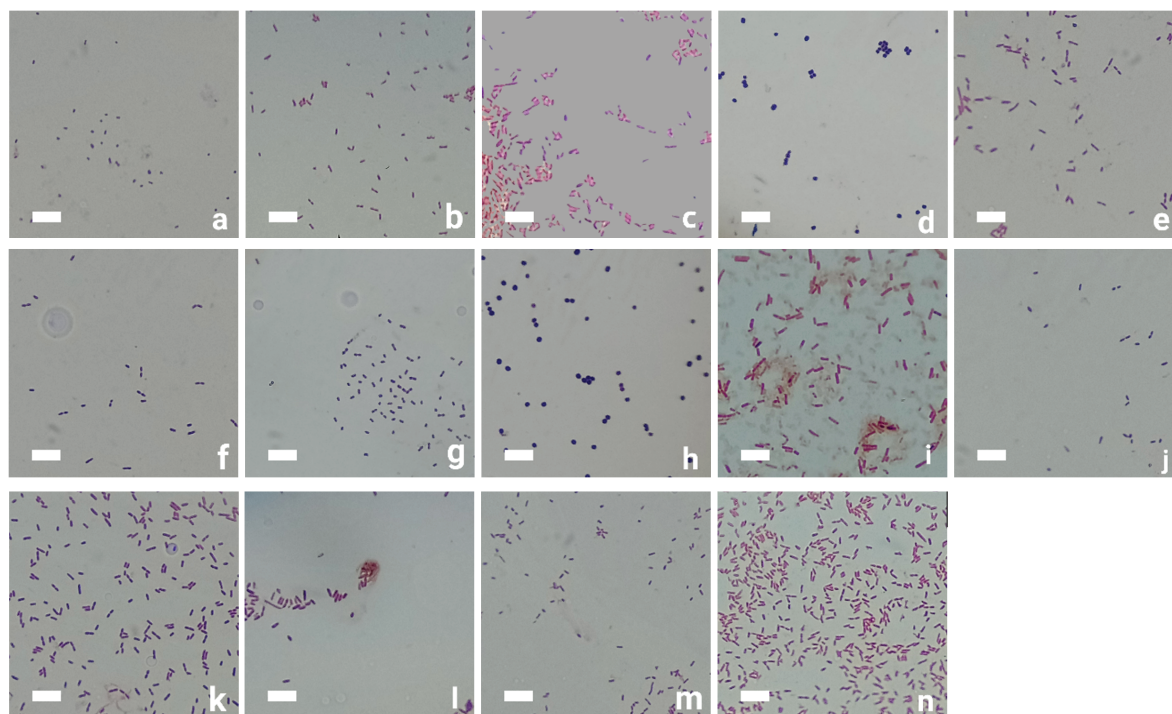


Figure 2. Microscopic characteristics of BEDA isolates. (a) BEDA 1; (b) BEDA 2; (c) BEDA 3; (d) BEDA 4; (e) BEDA 5; (f) BEDA 6; (g) BEDA 7; (h) BEDA 8; (i) BEDA 9; (j) BEDA 10; (k) BEDA 11; (l) BEDA 12; (m) BEDA 13; (n) BEDA 14. White bar: 5 μ m.

The characteristics of the BEDA isolate are microscopic. There are five Gram-negative bacterial isolates and nine Gram-positive bacteria isolates (Figure 2). Of these, eight isolates exhibit a rod shape, while six isolates are cocci. Endophyte bacteria with different forms and types of Gram have also been discovered, namely endophyte bacteria with the form of Gram-negative rods, Gram-positive rods, Gram-positive cocci 5 and Gram-negative cocci. Diversity in endophyte types of bacteria can be influenced by the climate. Climate conditions result in a change in the community of bacteria, interactions, and adaptation of endophyte bacteria in plants.²³

Identification of the genus of bacterial isolates is based on microscopic, macroscopic, and biochemical characteristics (Table 2), reference to *Bergey's Manual of Systematic Bacteriology*. BEDA 1 and BEDA 8 isolates were thought to belong to the genus *Staphylococcus*. Endophytic bacteria were also found in from *Ginkgo biloba* with a suspected affiliation to *Staphylococcus*.²⁴ Additionally, endophytic bacteria belonging to *Staphylococcus* were identified in grapevine plants (*Vitis vinifera*).²⁵

The BEDA 2 isolate is thought to belong to the genus *Neisseria*. Endophytic bacterial isolates obtained from buckwheat (*Fagopyrum tataricum*) are also thought to be

Neisseria.²⁶ The BEDA 3 and BEDA 9 isolates are thought to belong to the genus *Escherichia*.²⁷ Bacteria of *Escherichia* are often found in the leaves of the *Achnatherum inebrians* plant as endophytic bacteria.²⁸ BEDA 4 isolate are thought to belong to the genus *Enterococcus*. Endophytic bacterial isolates obtained from banana seeds (*Musa balbisiana*) are also thought to be *Enterococcus*.²⁹ The isolates BEDA 5, BEDA 10, BEDA 11, BEDA 13, and BEDA 14 are thought to belong to the genus *Bacillus*. Endophytic bacteria closely related to *Bacillus* have also been found.³⁰

The BEDA 6 isolate is thought to belong to the genus *Listeria*. Bacteria of the genus *Listeria* are often found in the *Aquilaria sinensis* plant as endophytic bacteria.³¹ The BEDA 7 isolate is thought to belong to the genus *Streptococcus*. The genus *Streptococcus* were also found in medicinal plant *Dialium guineense*.³² The BEDA 12 isolate is thought to belong to the genus *Klebsiella*. *Klebsiella* was also found as an endophytic bacteria of *Alternanthera philoxeroides*.³³

Endophytic bacteria can produce the same antibacterial compounds as their host plants.³⁴ A low capacity for antimicrobial activity or only producing a small number of antibacterial compounds will likely produce other active substances that are still unknown. The bacterial endophytic isolates also found were unable to inhibit the growth

Table 2. Biochemical characteristics and genus identification of BEDA isolates.

Isolate	Biochemical Test								Genus
	H ₂ S	Indole	Motility	SC	MR	VP	Catalase	TSIA	
BEDA 1	-	+	-	+	-	-	+	R/R	<i>Staphylococcus</i>
BEDA 2	-	+	-	-	+	-	+	R/Y	<i>Neisseria</i>
BEDA 3	-	-	+	+	-	-	-	Y/Y	<i>Escherichia</i>
BEDA 4	-	+	-	-	-	-	+	Y/R	<i>Enterococcus</i>
BEDA 5	-	-	+	+	+	-	+	Y/Y	<i>Bacillus</i>
BEDA 6	-	-	+	+	-	-	-	R/R	<i>Listeria</i>
BEDA 7	-	-	-	-	-	-	-	R/Y	<i>Streptococcus</i>
BEDA 8	-	+	-	-	-	-	+	R/R	<i>Staphylococcus</i>
BEDA 9	-	+	-	-	-	-	+	R/Y	<i>Escherichia</i>
BEDA 10	-	-	-	-	-	-	-	R/R	<i>Bacillus</i>
BEDA 11	-	+	-	+	+	-	+	Y/Y	<i>Bacillus</i>
BEDA 12	-	+	-	+	-	-	+	R/R	<i>Klebsiella</i>
BEDA 13	-	+	+	+	+	-	+	R/Y	<i>Bacillus</i>
BEDA 14	-	+	-	+	-	-	-	R/R	<i>Bacillus</i>

SC: Simmons citrate; MR: methyl red; VP: Voges-Proskauer; R: red; Y: yellow.

of *S. aureus* and *E. coli*.³⁵ The results from another study also showed that the endophytic bacteria obtained were unable to inhibit the growth of *C. albicans*.³⁶

The abilities of BEDA 1, BEDA 2, BEDA 5, BEDA 6, BEDA 7, BEDA 9, BEDA 10, BEDA 12, and BEDA 13 isolates fall into the narrow-spectrum category. The isolates

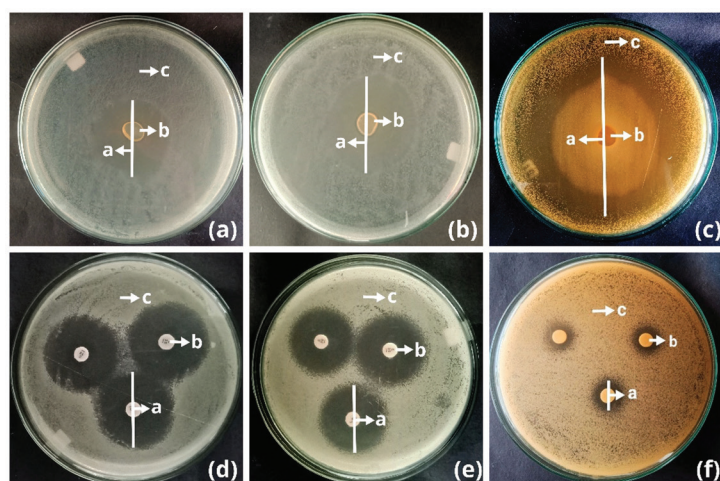


Figure 3. Antimicrobial activity test results. (a) a. Inhibition zone diameter (23.01 mm), b. BEDA 9 isolate diameter (8.5 mm), c. *E. coli*; (b) a. Inhibition zone diameter (41.76 mm), b. BEDA 5 isolate diameter (8.5 mm), c. *S. aureus*; (c) a. Inhibition zone diameter (66.85 mm), b. BEDA 9 isolate diameter (47 mm), c. *C. albicans*; (d) a. Diameter of the control inhibition zone (31.47 mm), b. Chloramphenicol disc diameter (6 mm), c. *E. coli*; (e) a. Diameter of the control inhibition zone (31.11 mm), b. Chloramphenicol disc diameter (6 mm), c. *S. aureus*; (f) a. Diameter of the control inhibition zone (14.87 mm), b. Nystatin disc diameter (6 mm), c. *C. albicans*.

Table 3. Inhibition zone sizes from antimicrobial activity test results.

Isolate	Inhibition Zone (mm)		
	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
Control	24.92±0.55 ^{****} (Chloramphenicol)	24.82±0.29 ^{****} (Chloramphenicol)	8.10±0.77 ^{**} (Nystatin)
BEDA 1	0	0.51±0.06 [*]	0
BEDA 2	0	3.83±0.58 [*]	0
BEDA 3	7.86±2.93 ^{**}	26.95±0.23 ^{****}	0.85±0.26 [*]
BEDA 4	0.47±0.26 [*]	0.27±0.15 [*]	0.58±0.33 [*]
BEDA 5	0	31.48±1.78 ^{****}	0
BEDA 6	1.55±0.18 [*]	0	0.45±0.39 [*]
BEDA 7	0.45±0.14 [*]	0.73±0.48 [*]	0
BEDA 8	1.13±0.35 [*]	1.95±0.44 [*]	0.63±0.47 [*]
BEDA 9	12.16±2.35 ^{***}	0	17.84±2.01 ^{***}
BEDA 10	0.53±0.23 [*]	0	0
BEDA 11	4.24±0.66 [*]	1.93±0.33 [*]	0.50±0.22 [*]
BEDA 12	0	0.07±0.09 [*]	0
BEDA 13	0	1.99±0.14 [*]	0.71±0.29 [*]
BEDA 14	0.50±0.07 [*]	0.85±0.21 [*]	0.57±0.52 [*]

*Inhibition categories.¹⁵ *: Weak; **: Moderate; ***: Strong; ****: Very strong.

BEDA 3, BEDA 4, BEDA 8, BEDA 11, and BEDA 14 based on their ability are categorized as broad-spectrum antibiotics. Based on the spectrum of action, antibiotics can be divided by those that have a narrow-spectrum or a broad-spectrum. Broad-spectrum antibiotics are able to actively inhibit or kill many Gram-positive, and Gram-negative bacteria and fungi. Narrow-spectrum antibiotics are only active against some types of bacteria and fungi.³⁷ The different effects on Gram-negative and -positive bacteria are due to variations in the composition and structure of their cell walls and membranes. All bacteria and fungi have cell membranes.³⁸ Substances from secondary metabolites have the ability to disrupt binaries fission, to interact with extracellular proteins and damage bacterial cell wall integrity.³⁹

The inhibition zone produced by isolates BEDA 3, BEDA 5, and BEDA 9 was greater than the size of the inhibition zone by the controls. BEDA 5 isolate has better activity than BEDA 3 isolate against *S. aureus*. Additional research found endophytic bacteria with a strong ability to inhibit the growth of *S. aureus*.³⁵ The endophytic bacteria also can inhibit the growth of *C. albicans*; the largest inhibitory zone size is 24 mm.⁴⁰ BEDA 5 and BEDA 9 isolates have good potential for further antibiotic development.

This research only reaches the potential stage for the bacterial isolates. The diffusion method used does not provide a complete representation of the effectiveness of therapeutic compounds, especially in determining the level of inhibition. Potentially beneficial bacterial isolates need

extensive testing for therapeutic applications. Therefore, for further studies should include more in-depth tests, such as measuring the minimum inhibitory concentration (MIC), to gain deeper insights into the activity being studied.

Conclusion

Fourteen isolates of endophytic bacteria were obtained from *A. indica* leaves, labeled BEDA 1 to BEDA 14, and each exhibited remarkable antimicrobial activity. Two important outcomes were observed: The BEDA 5 isolate demonstrated a strong inhibition zone against *S. aureus* (31.48 mm), while the BEDA 9 isolate showed a strong inhibition zone against *C. albicans* (17.84 mm). These endophyte bacterial isolates have the potential to serve as antimicrobial agents.

Authors' Contributions

AS, LF, and S were involved in conceptualizing and planning the research. AS performed data acquisition, calculated the experimental data, and conducted the analysis. AS drafted the manuscript and designed the figures. AS also aided in interpreting the results. AS, LF, and S participated in providing critical revisions to the manuscript.

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