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Alternative Antibacterial Agents from Endophytic Bacteria of Curry Leaves (*Murraya koenigii* L. Spreng) for Inhibiting Diarrhoea-Causing Bacteria

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ABSTRACT

This study focused on the isolation and characterization of endophytic bacteria from curry leaves (Murraya koenigii L. Spreng), a medicinal plant known for its health-promoting properties. The research aimed to identify bacterial isolates based on their colony morphology, biochemical characteristics, Gram and endospore staining, and to assess their antibacterial activity against Bacillus cereus ATCC 11788 and Shigella sonnei. Leaf segments (1 cm) were surface-sterilized using 2% sodium hypochlorite three times and cultured on Nutrient Agar supplemented with 0.01% nystatin, followed by incubation at 37 °C for 24 hours. A total of nine bacterial isolates were obtained, all showing Gram-positive reactions and positive endospore formation, indicating their classification within the Bacillus genus. The antibacterial activity was tested using the Kirby-Bauer disk diffusion method, revealing that the endophytic isolates exhibited weak inhibitory effects against both tested bacterial strains. These findings suggest that while Bacillus endophytes from M. koenigii possess limited antibacterial potential under the tested conditions, they still hold promise for further investigation as potential sources of bioactive compounds with therapeutic applications

INTRODUCTION

Diarrhea remains one of the leading causes of mortality in children under five years old, accounting for an estimated 525,000 deaths annually worldwide (Ministry of Health, 2021). In Indonesia, diarrhea is classified as an endemic disease with the potential to cause outbreaks, as reflected by ten recorded outbreaks in eight provinces in 2018, resulting in 756 cases and 36 deaths. In the Aceh Province, the coverage of healthcare services for children with diarrhea remains suboptimal at 16%, below the targeted 22% (Aceh Health Office, 2021). These data indicate that diarrhea management in Aceh requires significant improvement to reduce the burden of this disease.

Diarrhea etiology is classified into infectious and non-infectious causes, with pathogenic bacteria such as Bacillus cereus and Shigella sonnei identified as major infectious agents. *Bacillus cereus*, a Gram-positive bacterium, causes food poisoning through the production of enterotoxins, while *Shigella sonnei*, a Gram-negative bacterium, is a predominant agent of diarrhea in industrialized countries (Schnupf and Sansonetti, 2019). Although chemical treatments are common, traditional medicinal plants like guava and dewandaru leaves have been used by communities due to their antibacterial properties.

Murraya koenigii, commonly known as curry leaf, is widely utilized in Indonesia and especially in Aceh, and possesses promising antibacterial potential. It contains various phytochemicals such as alkaloids and flavonoids known for their pharmacological activities (Tripathi et al., 2018). Previous studies revealed that endophytic bacteria residing in curry leaves have antibacterial activity against several pathogens, including *B. cereus* and *S. sonnei* (Pharba et al., 2018).

Based on this potential, the present study aims to isolate endophytic bacteria from Murraya koenigii leaves and evaluate their inhibitory effects against *B. cereus* and *S. sonnei*, which are common causative agents of diarrhea. This research may contribute to the development of natural plant-based alternatives for diarrhea treatment.

METHOD

The research employed a qualitative approach to observe and identify endophytic bacteria present in the leaves of *M. koenigii* L. Spreng. Additionally, a quantitative method was used to measure the inhibition zones formed by the endophytic bacteria against pathogenic bacteria.

The curry leaf samples (*M. koenigii* L. Spreng) used in this study were collected from Gampong Blang Krueng, Aceh Besar Regency. The samples consisted of young leaves that were intact, free from holes or defects, and exhibited a uniform green color. According to Putri et al. (2018), young leaves generally harbor a higher number of endophytic bacterial isolates compared to mature leaves, as they contain greater amounts of secondary metabolites.

The isolation of endophytic bacteria from curry leaves (*Murraya koenigii* L. Spreng) involved several steps. First, young, intact leaves were collected from Gampong Blang Krueng, Aceh Besar, and cleaned with running water before being cut into 1x1 cm pieces. The leaf

samples were sterilized with 70% alcohol, rinsed in 2% sodium hypochlorite solution for 1 minute, and then washed three times with distilled water. The sterilized leaf pieces were placed on Nutrient Agar (NA) medium, with the leaf veins in contact with the medium containing 0.01% nystatin, and incubated at 37 °C for 24 hours to allow for bacterial growth. Different bacterial colonies were then selected based on their morphology and purified using the streak quadrant method on NA medium, followed by another incubation at 37 °C for 24 hours. The endophytic bacteria were identified through macroscopic and microscopic observations, including colony characteristics and Gram staining to differentiate between Gram-positive and Gram-negative bacteria. Biochemical tests, such as indole, motility, catalase, methyl red, Voges-Proskauer, citrate, and Triple Sugar Iron Agar (TSIA) tests, were conducted for further identification of the isolates.

For the antibacterial activity test, the test bacteria *B. cereus* and *S. sonnei* were cultured and standardized to a McFarland 0.5 turbidity standard. The endophytic bacterial isolates were then tested for their antibacterial activity using the agar diffusion method with paper disks, where 20 µL of the endophytic suspension was applied to the disks placed on Mueller-Hinton Agar (MHA) inoculated with the test bacteria. Chloramphenicol (30 µg) served as a positive control, while sterile distilled water was used as a negative control. After incubation at 37 °C for 24 hours, the inhibition zones around the endophytic isolates were measured to determine their potential as antibacterial agents against the pathogens.

After a 24-hour incubation period, the inhibition zones around the paper disks were observed to assess bacterial sensitivity to the antibacterial agents tested. The clear zones indicated the effectiveness of the antibacterial substances, and their diameters were measured using a caliper, recording both horizontal and vertical measurements in millimeters (Siregar et al., 2020). The calculated diameter of the inhibition zone was then categorized based on the following criteria: weak (≤ 5 mm), moderate (6-10 mm), strong (11-20 mm), and very strong (≥ 20 mm).

RESULTS AND DISCUSSION

Based on the research findings, nine endophytic bacterial isolates were identified from curry leaves, each exhibiting distinct morphological characteristics. The isolates were designated as EK1 through EK9. The observed morphological characteristics included colony shape, elevation, margin, and color of the bacteria. These morphological characteristics are summarized in Table 1 as follows:

Table 1. Morphological Characteristics of Endophytic Bacteria from Curry Leaf (*Murraya koenigii* L. Spreng)

No.	Isolate code	Colony shape	Margin shape	Elevation	Color
1.	EK1	Coccus	spherical	flat	creamy white
2.	EK2	Rhizoid	lobed	flat	creamy white
3.	EK3	Rhizoid	filamentous	flat	creamy white
4.	EK4	Irregular	lobed	flat	Cream
5.	EK5	Irregular	filamentous	flat	Cream
6.	EK6	Irregular	serrated	raised flat	Cream
7.	EK7	Irregular	wavy	flat	White
8.	EK8	Irregular	wavy	flat	creamy white
9.	EK9	Coccus	filamentous	Flat	creamy white

Identification of endophytic bacteria was carried out through both macroscopic and microscopic characterization. Following microscopic characterization, Gram staining was performed on the endophytic bacteria, as shown in Figure 1 below:

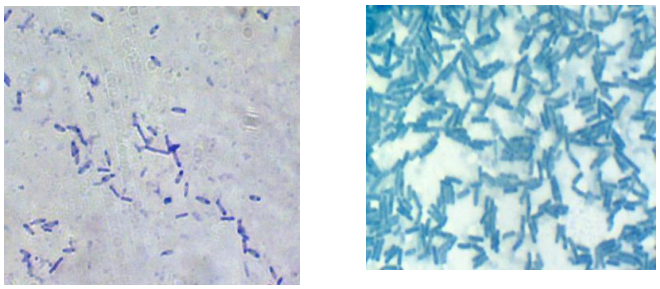

Figure 1. Shape of Gram-Positive Bacilli Cells and Endospore Staining

Based on the image above, the results of the Gram staining performed on 9 isolates of endophytic bacteria from curry leaves (*M. koenigii*) revealed Gram-positive bacteria, characterized by rod-shaped cells that appear purple. Meanwhile, the results of the spore staining can be seen in Figure 1.

Based on the image above, the spore staining results for the 9 isolates of endophytic bacteria from curry leaves (*M. koenigii*) showed spore-positive bacteria, characterized by rod-shaped cells stained green. The biochemical tests performed included TSIA, Methyl Red, Voges-Proskauer, Indole, Motility, Catalase, and Citrate tests. The results of the Gram staining and biochemical tests for the endophytic bacteria are presented in Table 2.

Table 2. Biochemical Tests of Endophytic Bacteria from Curry Leaves (*Murraya koenigii*)

Isolate code	Bentuk sel	Gram	Glucose	Lactose	Sucrosa	Motility	Indole	Citrate	Catalase	MR	VP	Endospore	Results
EK 1	Bacilli	+	+	-	-	-	-	-	-	+	-	+	*Bacillus* sp. 1
EK 2	Bacilli	+	+	-	-	-	-	+	-	+	-	+	*Bacillus* sp. 2
EK 3	Bacilli	+	+	-	-	-	-	-	-	+	-	+	*Bacillus* sp. 1
EK 4	Bacilli	+	+	+	+	-	-	+	-	+	-	+	*Bacillus* sp. 3
EK 5	Bacilli	+	+	-	-	+	-	-	-	+	-	+	*Bacillus* sp. 4
EK 6	Bacilli	+	+	+	+	-	-	+	-	+	-	+	*Bacillus* sp. 3
EK 7	Bacilli	+	+	+	+	+	-	+	-	+	-	+	*Bacillus* sp. 5
EK 8	Bacilli	+	+	+	+	+	-	+	-	+	-	+	*Bacillus* sp. 5
EK 9	Bacilli	+	+	-	-	+	-	+	-	+	-	+	*Bacillus* sp. 6

Note : EK= Endofit Kari; (+) = positive; (-) = negative

Table 3. Characterization of *Bacillus* sp. from various literature sources

Genus	Characteristics
Bacillus sp.	Bacilli Endospore (+) Catalase (-) Motility (+/-) Indole (-) *Methyl red* (+) *Voges proskaeur* (-) Glucosa(+) Sucrosa (+/-) Simmon citrate (+/-) (Bergey's, 1957) (Damayanti *et al*., 2019) (Nurmalasari *et al*., 2020) (Silalahi *et al*., 2020)

The results of the inhibition measurement are indicated by the presence of a clear zone.

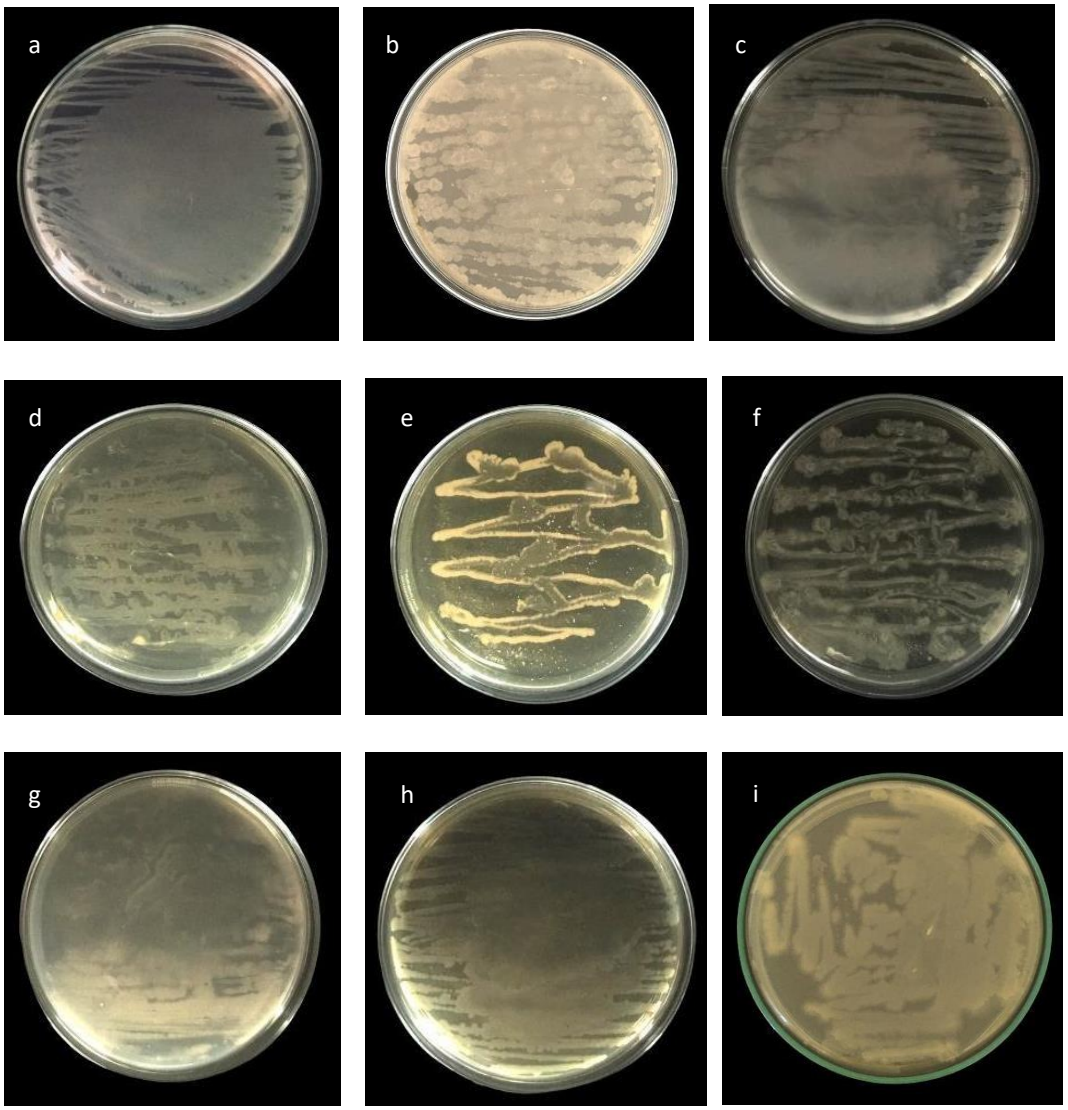

Figure 2. Endophytic bacteria isolate

Table 4. Inhibition zone activity of endophytic bacteria from Curry Leaves (*Murraya koenigii* L. Spreng) against *Bacillus cereus*.

Isolate code	Inhibition of diameter		Inhibition criteria	
	B. cereus	*S. sonnei*	*B. cereus*	*S. soneii*
EK1	2,02	2,00	weak	weak
EK2	1,30	4,28	weak	weak
EK3	1,81	1,61	weak	weak
EK4	1,53	1,65	weak	weak
EK5	2,12	0,18	weak	weak
EK6	1,47	2,85	weak	weak
EK7	0,14	2,22	weak	weak
EK8	1,46	4,92	weak	weak
EK9	4,76	4,40	weak	weak
control	16,55	22,02	strong	very strong

Based on the research results, the activity test of endophytic bacteria from curry leaves (*Murraya koenigii* L. Spreng) demonstrated antibacterial activity capable of inhibiting the growth of *B. cereus* and *S. sonnei*, as shown in Figure 3.

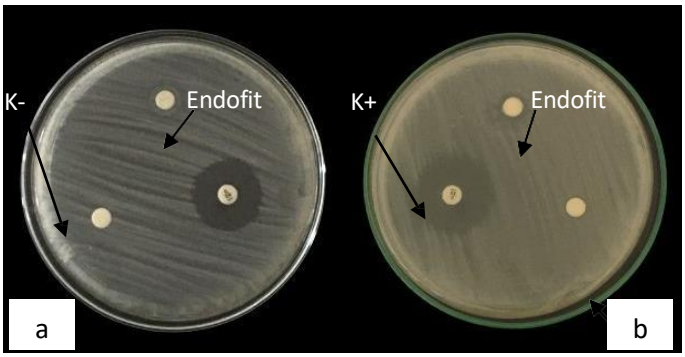

Figure 3. Results of Endophytic Curry Activity Test on Test Bacteria: a). *B. cereus* on isolate EK9 and b). *S. sonnei* on isolate EK8.

The inhibitory strength of the 9 endophytic bacterial isolates against B. cereus and S. sonnei is considered weak, as the diameter of the inhibition zone is less than 5 mm. The positive control used chloramphenicol at 30 µg, which produced a strong average inhibition zone. Meanwhile, the negative control using distilled water did not produce any inhibition zone.

In this study, curry leaves (Murraya koenigii L. Spreng) were used as the source for isolating endophytic bacteria, specifically young leaves due to their higher secondary metabolite content compared to older leaves (Putri et al., 2018). The endophytic bacteria were cultivated on Nutrient Agar (NA), which provides a complex medium similar to the plant's internal conditions, and the addition of nystatin effectively inhibited fungal contamination (Irene et al., 2020). After 24 hours of incubation, nine endophytic bacterial isolates were obtained, all belonging to the genus *Bacillus*, characterized by various colony morphologies. Biochemical tests, including TSIA, citrate, indole, motility, catalase, methyl red, and Voges-Proskauer, were conducted to further identify the isolates. Results indicated that isolates EK1, EK2, EK3, EK5, and EK9 could ferment glucose but not sucrose or lactose, while EK4, EK6, EK7, and EK8 fermented all carbohydrates. The citrate test showed positive results for several isolates, while all isolates tested negative for indole and catalase. The methyl red test was positive for all isolates, indicating mixed acid fermentation, whereas the Voges-Proskauer test was negative. The endophytic bacteria, primarily *Bacillus* sp., are known for their role in plant colonization, biocontrol of pathogens, and promoting plant growth through various mechanisms, including nitrogen fixation and hormone production (Afzal et al., 2019; Qi et al., 2021; Miljakovic et al., 2020). Environmental factors such as temperature, humidity, and soil conditions significantly influence the diversity and effectiveness of these endophytic bacteria in plants (Namdar et al., 2019; Ek-Ramos et al., 2019).

The antibacterial activity of endophytic bacteria from curry leaves (*M. koenigii*) against the test bacteria *B. cereus* ATCC 11788 and *S. sonnei* resulted in clear inhibition zones around the paper disks, indicating that several endophytic isolates inhibited the growth of these

pathogens. The inhibition zones for *B. cereus* ranged from 0.14 to 4.76 mm, while for *S. sonnei*, they ranged from 0.18 to 4.92 mm, categorizing the activity as weak for isolates EK1 to EK9. Previous studies, such as those by Islam et al. (2019), found that endophytic isolates from *Ginkgo biloba* showed no antibacterial activity against *B. cereus*, suggesting an inability to penetrate bacterial cell membranes and induce cell lysis. Similarly, Sousa et al. (2017) reported weak antibacterial activity (2.50 mm and 3.20 mm) from endophytic isolates of *Miconia albicans* against *S. sonnei*. The limited inhibition observed may be attributed to factors such as culture medium composition, incubation processes, agar diffusion rates, and organism sensitivity (Husain et al., 2022). The degree of inhibition depends on the endophytic bacteria's ability to produce antibacterial compounds. The small inhibition zones suggest that the endophytic bacteria may not produce sufficient secondary metabolites, or that the flavonoid content in *M. koenigii* leaves, which can inhibit pathogen growth by disrupting cell wall synthesis, is low. This disruption can lead to lysis of the bacterial cells, affecting their functionality (Purwatiningsih et al., 2021). Thus, the clear zones produced by the endophytic isolates indicate their antibacterial activity, as they can damage critical components of both *B. cereus* and *S. sonnei* cells.

CONCLUSION

Nine endophytic bacterial isolates were found in curry leaves (*M. koenigii* L. Spreng) that were identified to be related to the genus Bacillus. The endophytic bacteria in curry leaves (*M. koenigii* L. Spreng) exhibited antibacterial activity against the test bacteria *B. cereus* and *S. sonnei*, although with a weak inhibition response.

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