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Endophytic Bacteria From Bidara (*Ziziphus mauritiana* L.) Leaves and Its Activity Against the Pathogens *Salmonella typhi* and *Escherichia coli*

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Abstract

Background: *One possible source of bioactive substances is endophytic bacteria. These bacteria reside in plant tissues without harming the host plant. By generating unique chemicals in plant tissues and boosting the body's defense mechanisms against host plants, endophytic bacteria can manage infections. The purpose of this study is to investigate and describe the ability of endophytic bacteria from bidara leaves to create antimicrobial substances via their metabolite activities.* **Methodology:** *Nutrient Agar (NA) media was used exclusively for the isolation and culture of endophytic bacteria. Endophytic bacterial colonies were morphologically characterized both macroscopically and microscopically. Salmonella typhi and Escherichia coli growth inhibition was evaluated for endophytic bacterial metabolites (supernatants) with disc diffusion method. Chloramphenicol 0.3% used as positive control, and steriled aquadest as negative control.* **Findings:** *END01, END02, and END03 are the three endophytic bacterial isolates that were recovered. The three isolates differed in their edges and forms; END01 was Gram negative, whereas END02 and END03 were Gram positive. Isolate END01 and END03 selected for strong inhibition in antagonist tests against S. typhi (16.44 mm and 12.57 mm, respectively) compared to chloramphenicol control (45.86 mm). Their secondary metabolites were harvested at stationary phase, and chemical screening confirmed positive flavonoids in cell-free supernatants. The END01 supernatant showed the highest antimicrobial activity, with inhibition zones of 26.30 mm against S. typhi and 11.34 mm against E. coli, categorized as very strong and strong inhibition, respectively.* **Contribution:** *Endophytic bacteria isolated from the bidara leaves have the potential to inhibit the growth of pathogenic Salmonella typhi and Escherichia coli. This study represents the first reported exploration of endophytic bacteria from bidara leaves as flavonoid-producing antimicrobial agents against these clinically significant pathogens.*

Keywords: *Antibacterial compounds; Endophytic bacteria; Escherichia coli; Salmonella typhi; Ziziphus mauritiana*

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INTRODUCTION

One of the reasons of potentially harmful complications in the body is infectious disorders brought on by pathogenic bacteria. The increase in antibiotic resistance makes pathogenic illnesses worse. *Salmonella typhi* and *Escherichia coli* are two hazardous bacteria that should be avoided because they cause numerous deaths. Although both of these bacteria are common in the human gut, they can become pathogenic and result in gastrointestinal infections when present in specific amounts and circumstances (Dewi et al., 2021). World Health Organization estimates in 2019, around 11 million deaths occur annually due to typhoid fever caused by *S. Typhi*. On the other hand, antibiotic-resistant *E. coli* is also reported to cause bacterial infections, causing more than 800,000 deaths (Naghavi et al., 2024). Therefore, exploration of new bioactive compounds is necessary to overcome these problems.

Endophytic bacteria are a potential source of bioactive compounds. These bacteria live within plant tissues without causing disease in the host plant. They have the ability to control pathogens by producing specialized metabolites within the plant tissue and stimulating the host's defenses (Govindappa, 2011). In addition, this bacteria is thought to be involved in the secondary metabolite synthesis pathway of the host plant so that it is able to produce the same metabolite compounds as those produced by the host plant. Endophytic bacteria biosynthesize secondary metabolites via diverse biosynthetic gene clusters (BGCs), producing compounds like alkaloids, flavonoids, and lipopeptides through symbiotic interactions that activate host pathways or enable independent microbial factories (Semenzato & Fani, 2024). These offer advantages over plant extracts, including higher yields, consistent production unaffected by environmental variability, and novel derivatives with enhanced bioactivity, providing sustainable alternatives for drug discovery (Narayanan & Glick, 2022). The use of medicinal plants as raw materials for traditional medicines is an important source of information for selecting host plants for endophytic bacteria. One natural ingredient widely used as a medicinal ingredient is the bidara plant (*Ziziphus mauritiana* L.).

The benefits of bidara are supported by the abundance of metabolite compounds contained in the leaves. Bidara leaves are generally used traditionally to treat various ailments, such as skin infections, fever, diarrhea, cholesterol, and cancer (Rosalina et al., 2023). Furthermore, several in vitro studies have also shown antibacterial activity in bidara leaves. Ethanol extract of bidara leaves can inhibit the growth of *Escherichia coli, Staphylococcus aureus, Bacillus cereus*, and *Salmonella* sp. (Daris et al., 2023). Ethyl acetate extract of bidara leaves can inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*. Bidara leaf and fruit extracts have also been reported to inhibit the growth of Klebsiella pneumonia, *Bacillus subtilis, S. aureus*, and *Pseudomonas aeruginosa* (Javed et al., 2016).

However, the exploration of endophytic bacteria from bidara leaves as antibacterial agents has never been reported. The use of endophytic bacteria is a potential source of new bioactive compounds. Therefore, exploring endophytic bacteria in bidara leaves allows for the discovery of various types of endophytic bacteria with different secondary metabolites. The aim of this study was to explore endophytic bacteria from bidara leaves by observing their metabolite activity against

the growth of pathogenic bacteria *S. typhi* and *E. coli*. Therefore, this research contributes novel insights into endophytic bacteria from bidara leaves as sustainable sources of flavonoid-based antimicrobials, offering potential benefits for developing natural alternatives to combat *Salmonella typhi* and *Escherichia coli* infections.

METHOD

Isolation and Purification of Endophytic Bacteria

Samples of bidara leaves were taken in fresh and green condition, around Bandar Lampung, Lampung Province. The samples were washed under running water until free from any adhering dirt and weighed as much as 1 gram. Surface sterilization was carried out by soaking bidara leaves in 70% alcohol for 3 minutes, then rinsing with sterile distilled water, after that continued with soaking in NaOCl for 1 minute, and finally rinsed again with sterile distilled water 3 times. The samples were then ground using a sterile mortar and mortar until smooth and then added as much as 10 mL of sterile distilled water. From the results of grinding and the addition of sterile distilled water, as much as 1 mL was taken and then carried out a dilution of 10^{-1} to 10^{-7}. The results of the 10^{-7} dilution were spread as much as 0.5 ml on NA media using the spread plate method and then incubated for 24 hours at 28°C. The results of the isolation were then purified using the streak method on NA media, then incubated again for 24 hours at 28°C (Pradana et al., 2016).

Endophytic Bacteria Characterization

Identification of endophytic bacteria was performed macroscopically and microscopically. Endophytic bacteria were identified using the streak method. Macroscopic observations were based on bacterial morphology, including colony shape, margins, elevation, and color. Microscopic observations, on the other hand, were based on cell shape and Gram staining. Gram staining in this observation aimed to determine whether the bacteria were Gram-negative or Gram-positive (Pranoto et al., 2014).

Antagonism Test

Testing the antibacterial activity of endophytic bacteria against pathogenic bacteria was carried out using the well method in three replication. One loop of endophytic bacteria was taken and then placed in a 0.85 % NaCl solution. The endophytic bacterial suspension was then shaken and its turbidity was compared with the McFarland 0.5 turbidity standard. The same thing was done for the test bacteria. After the endophytic and pathogenic bacterial suspensions had been prepared, the pour plate method was carried out by taking 100 µL of the test bacterial suspension and placing it in a petri dish. After the NA medium containing the test bacteria had hardened, 3 wells were made using a sterile blue tip with a diameter of 5 mm. 50 µL of the endophytic bacterial suspension, 0.3 % chloramphenicol (+), and sterile distilled water (-) were inserted into the well holes, then incubated at 37 °C for 24 hours. The clear zone around the well formed in the test bacterial culture was observed, and the diameter of the clear zone was then measured. The categorization of the inhibition

zone was carried out based on Nuryanti et al., (2021). The diameter of the inhibition zone, as referenced by Nuryanti et al. (2021), is categorized as follows: 0 mm indicates no activity, 5-10 mm indicates weak activity, 11-15 mm indicates moderate activity, 16-20 mm indicates strong activity, and greater than 20 mm indicates very strong activity.

Growth curve of endophytic bacteria

The growth curve was obtained based on observations of OD (Optical Density) values, conducted in triplicate for statistical reliability. Observations of OD values and biomass analysis were performed every 2 hours over 24 hours (from 0 to 24 hour), using sterile NB media as blank control. One loop of purified endophytic bacterial culture from NA media was inoculated into an Erlenmeyer flask containing 50 mL sterile NB media, shaken for 24 hours at 37 °C; then 300 µL was subcultured into 150 mL fresh NB media, shaken again, with 3 mL sampled every 2 hours for OD measurement at 620 nm using a spectrophotometer.

Production of endophytic bacterial metabolites

Secondary metabolite compounds in endophytic bacteria are produced during the stationary phase. One loop of endophytic bacterial culture was inoculated into 50 mL NB production medium, shaken and incubated for the optimum time of 18 hours for END01 and 16 hours for END03 (determined from growth curve measurements showing stationary phase entry). Sterile NB medium without inoculum served as negative control to confirm metabolite activity originated from bacterial cells. Metabolites were harvested by centrifugation at 5,000 rpm for 20 minutes to obtain cell-free supernatant (Sugireng & Lio, 2020).

Preliminary screening of endophytic bacterial metabolites

Metabolite compound identification was carried out in two chemical compound tests, namely the flavonoid compound test and the alkaloid compound test. In the flavonoid test, 1 mL of the supernatant sample was combined with 0.1 mg of magnesium powder and 0.4 mL of amyl alcohol, which consists of a mixture of 37% hydrochloric acid and 95% ethanol in equal volumes. Subsequently, 4 mL of 95% ethanol was added, and the mixture was shaken. A positive reaction was indicated by the formation of an orange or pale yellow color. Meanwhile, in the alkaloid test, it was carried out using Mayer and Dragendorff reagents. Mayer reagent test: 1 mL of the sample was added with 1 mL of 2N HCl and 1 mL of Mayer reagent. Dragendorff reagent test: 1 mL of the sample was added with 1 ml of Dragendorff reagent. A positive reaction was indicated by the formation of a white precipitate and an orange precipitate respectively in each test (Wardhani et al., 2018).

Antibacterial Activity

Antibacterial activity was assessed using disc diffusion method with 6 mm sterile paper discs. Pure suspensions of *S. typhi* and *E. coli* were standardized to McFarland 0.5 turbidity, streaked onto NA media using sterile cotton swabs, and 100 µL endophytic bacteria supernatant was impregnated onto discs placed on inoculated

plates (chloramphenicol 0.3% as positive control, sterile aquadest as negative control), performed in triplicate. Plates were incubated at 37 °C for 24 hours. The diameters of the inhibition zones were measured as the average of the horizontal and vertical dimensions and interpreted the criteria refers to Nuryanti et al. (2021): 0 mm indicates no activity, 5-10 mm indicates weak activity, 11-15 mm indicates moderate activity, 16-20 mm indicates strong activity, and > 20 mm indicates very strong activity. The zone data were subjected to analysis using a one-way ANOVA with a significance level of α=0.05, followed by a Duncan post-hoc test, conducted using R version 4.3.1, to determine significant differences among the treatments, as referenced in Astriani & Dwijayanti (2022).

RESULT AND DISCUSSION

Macroscopic and Microscopic Characterization of Endophytic Bacteria

Three isolates of endophytic bacteria successfully isolated from bidara leaves showed diversity, both macroscopically and microscopically. Macroscopically, the three isolates showed that in terms of shape and edges, all isolates were different. Isolates END02 and END03 had the same morphological character in terms of elevation, namely flat. Then the next similarity in morphological characters was that isolates END01 and END03 had a yellowish color. According to research by Pranoto et al., (2014), reported that the morphology of endophytic bacterial colonies tends to have an irregular or round shape, flat or rough edges, filamentous, and have a flat to convex elevation or height, white, milky white to yellowish white. The morphological characteristics of endophytic bacterial colonies macroscopically are presented in Table 1 and Figure 1.

Table 1. Morphological characteristics of endophytic bacterial colonies of bidara leaves

Morphology		**Endophyte Isolate**		
		END01	**END02**	**END03**
Macroscopic	Shape	Rhizoid	Circular	Irregular
	Edge	Undulate	Entire	Lobate
	Elevation	Umbonate	Flat	Flat
	Color	Yelowish	White	Yelowish
Microscopic	Gram	Negative	Positive	Positive
	Shape of cell	Coccus	Coccus	Coccobaccil

According to Bhore et al., (2010) endophytic bacteria originating from a single host plant generally consist of several genera and species, the diversity of endophytic bacteria is influenced by environmental conditions such as salinity, temperature, pH, nutrients and also the growth of the host plant. Zuraidah et al., (2020) Each bacterium has a different ability to adapt to survive in its environment, changes in the environment can affect the morphological and physiological properties of a bacterium. In addition, each type of plant has specific and distinctive endophytic bacteria that inhabit the plant, such as one example of endophytic bacteria isolated from bidara leaves. Observations of the characteristics obtained indicate that it is likely that isolates END01, END02, and END03 come from different groups.

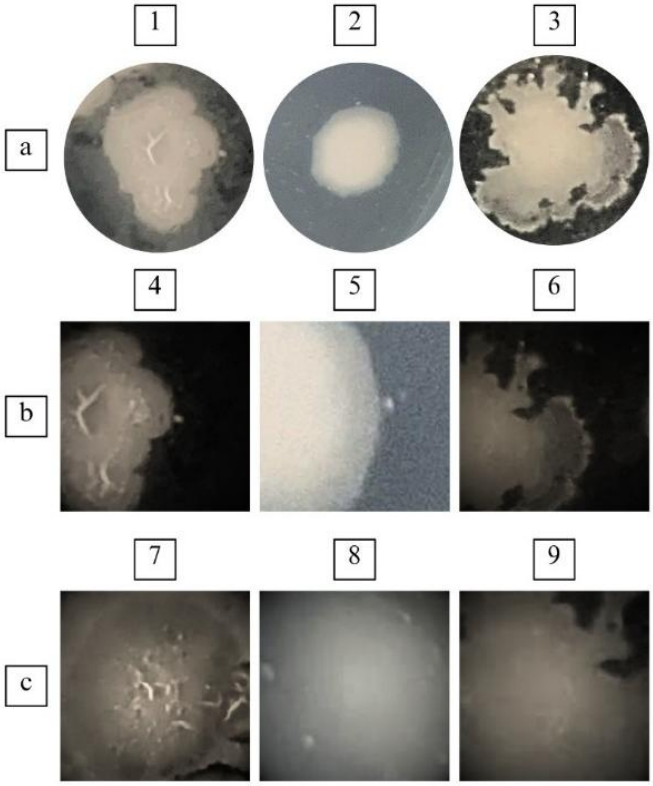

Figure 1. Morphological characteristics of endophytic bacterial colonies of bidara leaves. (A) Shape; *A1. Rhizoid; A2. Circular; A3. Irregular*; (B) Edge; *B4. Undulate; B5. Entire; B6. Lobate;* (C) Elevation; *C7. Umbonate; C8. Flat; C9. Flat*. Colony photos were taken from each colony grown on a 90 mm diameter petri dish.

Antagonistic Activity of Endophytic Bacterial Isolates against Pathogenic Bacteria *Escherichia coli* and *Salmonella typhi*

The characterization of endophytic bacteria as antibacterial-producing agents in NA media showed that three isolates of endophytic bacteria were each capable of inhibiting the growth of *S. typhi*, but did not show inhibitory activity against *E. coli*. The area of the inhibition zone formed can be seen in Table 2. The results of the antagonist test showed that isolate END01 had the greatest inhibitory ability against the growth of S. typhi with a clear zone diameter of 16.44 mm, which is included in the classification of a strong inhibitory response. As explained by Nuryanti et al., (2021) stated that antibacterial activity is divided into 4 groups, namely weak, moderate, strong, and very strong. Antibacterial activity is included in the weak group if it has a diameter <5 mm, moderate between 5-10 mm, strong between 10-20 mm, and very strong diameter >20 mm. On the other hand, the three endophytic bacterial isolates are thought to be relatively less active against *E. coli*. This could be caused by different sensitivity factors between *E. coli* and *S. typhi*.

Table 2. Antagonistic activity of endophytic bacteria against *Salmonella typhi* and *Escherichia coli* bacteria

Pathogen	Treatment	Inhibition zone (mm)	Category (Nuryanti et al., 2021)
Salmonella typhi	END01	16.44 ± 0.31[b]	Strong
	END02	4.43 ± 0.83[d]	Weak
	END03	12.57 ± 0.39[c]	Strong
	K+	45.86 ± 0.70[a]	Very strong
	K-	0 ± 0[e]	No inhibition
Escherichia coli	END01	0 ± 0[b]	No inhibition
	END02	0 ± 0[b]	No inhibition
	END03	0 ± 0[b]	No inhibition
	K+	48.36 ± 0.40[a]	Very strong
	K-	0 ± 0[b]	No inhibition

Description: *Different letter notations indicate significant differences in the DMRT test*

The formation of a clear zone around an endophytic bacterial colony indicates the possible presence of active bactericidal/antibiotic compounds capable of killing or at least inhibiting the growth of pathogenic bacteria. Endophytic bacteria from betel plants isolated from the roots, stems, and leaves have antibacterial activity characterized by the formation of a clear zone around the endophytic bacterial colony. Inhibition of pathogenic bacterial growth by endophytic bacteria through secondary metabolites can occur through several mechanisms, including damage to cell membranes, inhibition of cell wall synthesis, nucleic acid, and cellular respiration pathways, and inactivation of various bacterial metabolic pathways (Yuan et al., 2021).

The three endophytic bacterial isolates exhibited antibacterial activity, consistent with previous research using bidara leaf extract. Based on other research related to bidara leaf extract, it is known that bidara leaf extract inhibited the growth of *Streptooccus mutans, Salmonella typhi,* and *Bacillus cereus* (Shufyani & Dominica, 2022; (Nabella et al., 2025). Endophytic bacteria are known to produce the same metabolite compounds as those produced by host plants. This resulted in the endophytic bacteria isolated from bidara leaves being able to inhibit the growth of pathogenic bacteria. It is suspected that this antibacterial activity stems from the metabolite compounds produced.

Production of Metabolite Compounds from Endophytic Bacterial Isolates

Two of the three potential endophytic bacteria from bidara leaves exhibit antibacterial activity, the two isolates being END01 and END03. These potential isolates were further tested as candidates for producing antibacterial compounds by harvesting secondary metabolites. Secondary metabolite harvesting from bacteria can be performed during the stationary phase leading to death through a growth curve. The growth curve of isolate END01 is presented in Figure 2.

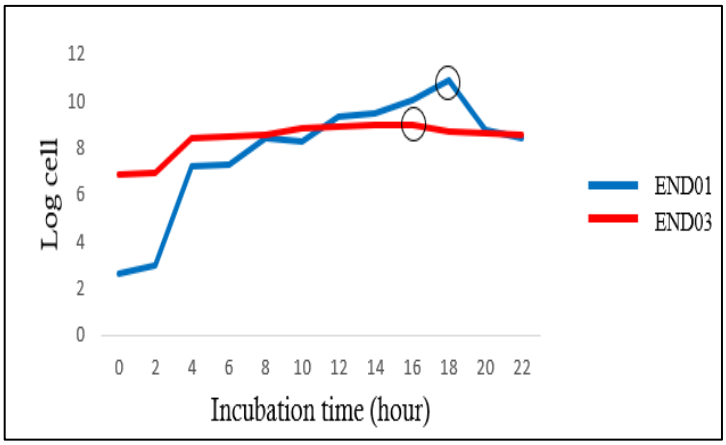

Figure 2. Growth curve of END01 and END03

Isolate END01 experienced a stationary phase at 18 hours (OD=0.484), while isolate END03 experienced a stationary phase at 16 hours (OD=0.471). This difference may be the cause of the difference in metabolic time of endophytic bacterial isolates in reaching the stationary phase. The difference in the length of time in each phase of bacterial growth can be caused by several factors, one of which is the difference in bacterial species. In general, each bacterial species has different metabolic capabilities (Wijanarka et al., 2016).

Generally, secondary metabolites in bacteria are produced during the stationary phase. During this phase, all cells stop dividing, or when living and dead cells reach equilibrium, the number of cell growth equals the number of cell deaths (Annisa et al., 2024). Bacteria survive by producing secondary metabolites, and some die due to toxicity from changing environmental conditions caused by the metabolites they produce. Harvesting was carried out based on the optimal time for isolates END01 and END03 to produce secondary metabolites, which was 18 and 16 hours of incubation. Antibacterial activity is due to the presence of antibacterial compounds included in secondary metabolites. Furthermore, during the stationary phase, secondary metabolites accumulate, some of which are used to maintain life. Therefore, harvesting is optimal if carried out at the end of the stationary phase.

Bacterial growth can be characterized by an increase in cell number and mass, while growth rate depends on environmental factors, namely physical and chemical factors. Secondary metabolites are produced by bacteria as a self-defense mechanism produced extracellularly, so to harvest secondary metabolites it is necessary to separate the bacterial cells in the supernatant to extract the secondary metabolites (Erlindawati et al., 2015).

Qualitative Identification of Flavonoids and Alkaloids

Based on the results of chemical compound screening, isolate END01 and isolate END03 were indicated as positive result for containing secondary metabolites in the form of flavonoids but had negative results in the alkaloid compound test. This is supported by research conducted by Aisyah et al., (2022); Jain et al., (2019) who reported that ethyl acetate extract of bidara leaves contains a number of phenolic metabolites in the form of flavonoids and tannins. As is known, the metabolites

produced by endophytic bacteria will be similar to the metabolites produced by the host plant. These bacteria are thought to be involved in the host's secondary metabolite synthesis pathway, so they can produce secondary metabolites similar to those produced by their host plant (Li et al., 2023).

Table 3. Qualitative chemical compound content of endophytic bacterial isolate

Isolate	Alkaloids			Flavonoids	
	Reagents		Results	Reagents	Results
	Reagen Mayer	Reagen Dragendorff		HCl + Mg	
END01	No white precipitate is formed	No orange to brown sediment is formed	-	Greenish yellow to pale yellow	+
END03	No white precipitate is formed	No orange to brown sediment is formed	-	Greenish yellow to pale yellow	+

Negative results in the alkaloid test can be caused by, among other things, the low alkaloid concentration found, making it undetectable during alkaloid screening. On the other hand, the use of plant parts as a source of isolates can also be a factor in undetectable alkaloid compounds. This may be due to the low alkaloid concentration in bidara leaves, resulting in undetectable alkaloid-containing endophytic bacteria isolated from the leaves. Secondary metabolites produced by endophytes such as flavonoids have been reported to contribute to antimicrobial activity against various pathogenic bacteria and fungi (Hnamte et al., 2024). Endophytic isolates from *Curcuma longa* rhizome were reported to have antibacterial activity and the presence of flavonoid/phenolic groups was detected in the extract through phytochemical screening (Sulistiyani et al., 2016). Furthermore, the antibacterial activity of bidara leaf endophytic bacteria may be due to several secondary metabolites other than alkaloids and flavonoids, which also have antibacterial activity. Supernatants that tested positive for flavonoids were then tested for antibacterial activity to confirm that the flavonoid chemical compounds have antibacterial activity.

Antibacterial Activity of Bacterial Cell-Free Supernatant

Based on the results of the inhibitory data analysis, each treatment has an effect on antibacterial activity against the test bacteria. Isolate END01 had the greatest inhibitory activity against *S. typhi* bacteria, with inhibition zone 26.30 mm, followed by isolate END03 at 23.4 mm, where both of these numbers are almost close to the diameter of the inhibition zone in positive control, chloramphenicol, at 33.16 mm. Testing antibacterial activity using cell-free supernatants taken during the stationary phase resulted in high concentrations of secondary metabolite compounds (Rumidatul et al., 2021). This causes antibacterial activity to be twice as large, compared to using bacterial cells.

Table 3. Antibacterial activity of supernatants of endophytic bacterial isolates

Pathogen	Treatment	Inhibition zone (mm)	Category (Nuryanti *et al.*, 2021)
Salmonella typhi	END01	26.30 ± 0.63[b]	Very strong
	END03	23.40 ± 0.80[c]	Very strong
	K+	33.16 ± 0.85[a]	Very strong
	K-	0.00 ± 0[d]	No inhibition
Escherichia coli	END01	11.34 ± 0.97[b]	Strong
	END03	8.09 ± 0.45[c]	Moderate
	K+	31.43 ± 1.07[a]	Very strong
	K-	0.00 ± 0[d]	No inhibition

*Description: *Different letter notations indicate significant differences in the DMRT test*

The diameter of the inhibition zone in the test sample formed is thought to be the influence of the flavonoid content which was confirmed positive in the chemical compound screening test. Flavonoids have a working mechanism as an antibacterial which is divided into three types of inhibition, namely inhibiting nucleic acid synthesis, inhibiting cell membrane function, and inhibiting energy metabolism (Rahmadeni et al., 2019). The antibacterial mechanism of flavonoids according to Shufyani & Dominica (2022) states that flavonoid compounds can denature protein bonds in the cell membrane, so that the cell membrane will experience shrinkage if there are phenol compounds that successfully enter the cell nucleus, which results in bacteria being unable to grow.

The results of the antibacterial activity test of cell-free supernatants have a correlation with the antagonistic activity test against pathogenic bacteria. In the test using bacterial cells, it was seen that endophytic bacterial isolates had inhibitory power with the interpretation of the activity category "strong" in inhibiting *S. typhi* bacteria. This is in line with the results of the test using supernatants which stated that the antibacterial activity was doubled with the interpretation of the activity category "very strong" in both supernatants. Then the inhibition of *E. coli* bacteria using supernatants has strong activity in END01 and moderate activity in END03, which previously in the test using bacterial cells did not have inhibitory power. This could occur due to the possibility of differences in the concentration of metabolite compounds used during the antagonist test and the antibacterial activity test.

In addition, based on the diameter of the inhibition zone formed, it indicates that *S. typhi* is more sensitive to the test sample than *E. coli*. *S. Typhi* often appears to be more sensitive than *E. coli* because *E. coli* generally has more and more diverse intrinsic and plasmid-mediated resistance mechanisms (e.g., β-lactamases, efflux pumps, and porin alterations) (Nurjanah et al., 2020; Castro et al., 2025). In the ciprofloxacin diffusion test against *S. Typhi*, the average diameter of the inhibition zone can reach around 30-35 mm and is categorized as sensitive according to CLSI criteria (≥ 21 mm for sensitive) (Wulandari et al., 2025). In *E. coli* from various clinical samples or animal food, quite a number of isolates showed smaller inhibition zones or even entered the intermediate/resistant category to antibiotics such as fluoroquinolones and cephalosporins (Manishimwe et al., 2021; El Ftouhy et al., 2023).

CONCLUSION

This study represents the first documented exploration of endophytic bacteria from *Ziziphus mauritiana* (bidara) leaves, revealing END01 and END03 as potent flavonoid producers with exceptional antimicrobial activity. END01 supernatant achieving 26.30 mm inhibition zones against *Salmonella typhi*. These findings highlight bidara endophytes as promising natural alternatives against some pathogens, though metabolite identification remains qualitative. This research establishes a foundation for developing sustainable, plant derived antimicrobials with potential applications in food safety and clinical therapeutics.

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