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Exploring the antibacterial activity of endophytic bacteria from Andaliman (*Zanthoxylum acanthopodium*) against *Bacillus subtilis*

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Abstract. *Rizqoh D, Sipriyadi, Suryani UH, Putri CN, Agustin M, Taurustya H, Lestari N, Sariyanti M. 2024. Exploring the antibacterial activity of endophytic bacteria from Andaliman (*Zanthoxylum acanthopodium*) against* Bacillus subtilis*. Biodiversitas 25: 700-707.* Infectious diseases are still one of the global health problems. Increasing antibiotic resistance against several pathogenic microbes causes antibiotics to be less effective in treating infectious diseases. One pathogenic bacteria that causes infection is *Bacillus subtilis*, which causes bacteremia, septicemia, and endocarditis. Therefore, searching for new alternative antibiotics is urgently needed to overcome bacterial resistance. Andaliman (*Zanthoxylum acanthopodium* DC.) extract produces antimicrobial bioactive compounds. Thus, this study aims to explore endophytic bacteria from *Z. acanthopodium* and determine their antibacterial activity against *Bacillus subtilis*. This study used a qualitative experimental method, namely isolation of endophytic bacteria, characterization of endophytic bacteria colonies and cells, antagonistic test against *B. subtilis*, extraction of compounds from endophytic bacteria, and determine Minimum Inhibitory Concentration (MIC) of crude extract of endophytic bacteria against *B. subtilis*. This study showed that 16 endophytic isolates from *Z. acanthopodium* could inhibit the growth of *B. subtilis*, and potential isolates were EA7 and EA26, which have Minimum Inhibitory Concentration (MIC) at 40%, and EB6 at 80%.

Keywords: Antimicrobial, *Bacillus subtilis*, endophytic bacteria, *Zanthoxylum acanthopodium*

INTRODUCTION

In 2019, 3 million people died from infectious diseases caused by bacteria and viruses, from young children to older children and adolescents (GBD 2019 and Adolescent Communicable Disease Collaborators 2023). Based on Basic Health Research, the prevalences of several infectious diseases in Indonesia were as follows: Respiratory Tract Infection (RTI) was 25%, pneumonia (1.8%), and hepatitis (4.5%) had twice the prevalence rate in 2013 compared to 2007 that is 1.2%, meanwhile for the incidence and prevalence of diarrhea in all age in Indonesia is 3.5% and 7.0%, respectively (Ministry of Health RI 2013).

Bacillus subtilis is a bacteria species from the genus *Bacillus*. Other *Bacillus* species that cause infectious diseases are *Bacillus anthracis, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus brevis, Bacillus pumulus, Bacillus macerans, Bacillus subtilis, Bacillus sphaericus, and Bacillus thuringiensis*. Members of this genus are also organisms that cause food poisoning and enterotoxin-producing diarrhea (Yassin and Ahmad 2012). *Bacillus subtilis* cause disease bacteremia, septicemia and endocardidis (Bennett et al. 2020). This bacteria produces an extracellular toxin known as subtilisin, a protein compound capable of causing allergy reactions and hypersensitivity in repeated infections.

Bacteria that produce antibiotics possess several mechanisms against their antibiotics (Peterson and Kaur 2018). Excessive use of antibiotics caused many pathogenic bacteria to adapt to the environment and become resistant (Read and Woods 2014). Antibiotic resistance in pathogenic bacteria is a bacterial response to antibiotic exposure (Martinez 2014). Mak et al. (2014) reported that the genetic factors responsible for self-antibiotic resistance tend to be close to the genes involved in antibiotic production, and their expression is coordinated. Increasing antibiotic resistance causes the need for new potential antibacterial sources to overcome resistance problems. Searching for new antibacterial sources from plant origin is urgently needed.

Andaliman (*Zanthoxylum acanthopodium* DC.) is a spice used by the people of Batak Angkola and Batak Toba with the local names *Sinyar-nyar* (Angkola) and *Andaliman* (Toba) (Rizqoh 2021). The genus *Zanthoxylum* has several therapeutic properties. It has been traditionally used in diverse medicinal systems for treating numerous ailments, including stomachic, carminative, and anthelmintic (Phuyal et al. 2019). *Zanthoxylum acanthopodium* has antibacterial and antifungal activity (Yusoff et al. 2022). Various potential *Z. acanthopodium* is due to the presence of several chemical compounds, such as alkaloids, terpenoids, and

flavonoids. Flavonoid and terpenoid compounds are antibacterial against Gram-positive and Gram-negative bacteria (Yusoff et al. 2022).

Endophytic bacteria live inside plant tissues and form colonies without harming the host (Podolich et al. 2015). These microbial communities substantially promote plant health (Kandel et al. 2017). Endophytes have adaptation mechanisms to their specific host plant. Endophytes' metabolic capabilities are thought to differ from those of microbes that reside in the soil (Brader et al. 2014). Several endophytic bacteria produce antimicrobial compounds (Castillo et al. 2003). However, there is no information regarding the antimicrobial activities of endophytic bacteria from *Z. acanthopodium*. Endophytic bacteria can produce unique natural compounds that are sometimes similar to the compounds produced by their host plants, one of which is the ability to synthesize antibacterial compounds as their host plants (Kusumawati et al. 2014). Based on the potential possessed by *Z. acanthopodium*, it is crucial to study the antimicrobial activity of *Z. acanthopodium* endophytic bacteria against *B. subtilis*. If there is an excellent antibacterial activity of endophytic bacteria from *Z. acanthopodium*, these endophytic bacteria could be studied further to overcome infectious diseases caused by pathogenic bacteria. This research aims to isolate endophytic bacteria from *Z. acanthopodium* and determine the antimicrobial activity against *B. subtilis*.

MATERIALS AND METHODS

This study isolates endophytic bacteria from *Z. acanthopodium* and determines their antimicrobial activity against *Bacillus subtilis*. This study was conducted at the Basic Science Laboratory of the Mathematics and Natural Science Faculty, Universitas Bengkulu, and the Microbiology Laboratory of the Medicine and Health Sciences Faculty.

Isolation of bacterial endophytes

Leaf, stem, and root samples from three *Z. acanthopodium* plants were obtained from Sidikalang, Dairi District, North Sumatra. Fresh and healthy plant samples were collected. Samples were cleaned with running water, cut into 2-5 cm sizes pieces, and separated according to plant parts. Samples were soaked in 70% alcohol for 1 minute, 5.25% sodium hypochlorite solution for 5 minutes, and washed with alcohol 70% as much three times for surface sterilization. After surface sterilization, the samples were cut into smaller pieces, implanted in King's B media, then incubated at room temperature. The growth of bacterial colonies around samples was observed daily.

Morphological characters of bacterial colony bacteria

Bacteria colonies from *Z. acanthopodium* were identified based on morphological characteristics (Leboffe and Pierce 2012). Colony shapes formed circular, irregular, or punctiform, while colony margins formed entire, undulate, lobate, filamentous, irregular, or rhizoid. Elevation of the colony forms is flat, raised, convex, pulvinate, or umbonate. The texture of the colony is moist, mucoid, or dry. Pigment formation can also be combined with appearance colors such as opaque, translucent, shiny, or dull. Gram staining was performed for Gram classification and cell morphology characterization.

Antagonist test of endophytes against *B. subtilis*

Antagonist test of endophyte isolates against *B. subtilis* using double-layer methods in Nutrient Agar (NA) media. *B. subtilis* was mixed using semi-solid media and then poured into solid media on a Petri dish. Endophyte bacteria were inoculated onto *B. subtilis* media with spot screening method and incubated for 3x24 hours at room temperature (Rizqoh et al. 2016). Endophytic bacteria that can inhibit the growth of *B. subtilis* formed a clear zone in the antagonist test. The diameter of the inhibitory zone is classified as follows (Table 1).

Test of antibacterial activity of the supernatant and pellets of endophytic bacteria

Three isolates of endophytic bacteria have good antibacterial activity. These isolates were analyzed further. These isolates were cultured in NB medium, incubated for 24 hours at room temperature, and then centrifuged at 10,000 rpm for 30 minutes (Rizqoh et al. 2016). The supernatant and pellets were separated and tested against *B. subtilis* using a paper disc method. The pellet was dissolved using sterile aquadest before being inoculated. 20 µL supernatant, or pellet, was transferred to paper discs, placed on NA inoculated with *B. subtilis*, and then incubated at room temperature for 24 hours. The diameter of the inhibition zone was measured after 24 hours.

Ethyl acetate extraction of potential endophyte isolate from *Z. acanthopodium*

The three selected endophytic bacteria were recultured in 500 mL NB medium incubated on a shaker incubator at 100 rpm at 30°C for 3 x 24 hours. Then, the culture was extracted with ethyl acetate with a ratio of 1:1, incubated at 30°C for 24 hours, stirring for 20 minutes, then centrifuged. The ethyl acetate extract was evaporated using a rotary evaporator. The concentrated extract is stored at -20°C for subsequent use.

Test of antibacterial activity of endophytic bacterial extract from *Z. acanthopodium* against *Bacillus subtilis*

Bacillus subtilis was cultured in an NB medium and then incubated at 37°C for 24 hours. Before the test, the turbidity of the *B. subtilis* culture was measured (OD = 0.3). The 0,5 mL of *B. subtilis* culture derived from NB media was put into 50 mL of semi-solid NA medium at 35-40°C, poured into a petri dish of ±10 mL, and solidified. The Amoxicillin at 50 µg/mL concentration was used as a positive control. The test was carried out using the disc diffusion method. 50 µL of crude extract (100 mg/mL) and control positive Amoxicillin were dripped onto paper discs, then placed on the surface of the semi-solid NA agar medium containing *B. subtilis* cultures and incubated for 24 hours. Positive test results showed the formation of a clear zone (Rizqoh et al. 2016). The diameter of the inhibition zone around the test disc is measured.

Table 1. Category of inhibitory zone

Inhibition zone diameter	Category
≥20 mm	Very strong
10.1-20mm	Strong
5.1-10 mm	Moderate
≤ 5 mm	Weak

Minimum Inhibitory Concentration (MIC) test

The Minimum Inhibitory Concentration (MIC) test was conducted using the disc diffusion method (Cappucino 2013). The *B. subtilis* suspension culture was mixed into warm liquid NA media, homogenized with a magnetic stirrer, and poured into petri discs. Paper discs with a diameter of 6 mm were put into a petri dish containing the test bacteria using tweezers aseptically. The paper discs were dripped with 5 µL of endophytic bacterial extract at different concentrations and incubated for 48 hours at 37°C. After incubation, the clear zone around the disc paper was measured. The extract that inhibits the growth of *B. subtilis* is indicated by establishing a clear zone. The bioactive activity effectiveness of the crude extracts was determined using the diameter of the inhibitory zone.

Twelve concentrations of crude extract were used to determine the MIC value, i.e., 10 % (0.1 g/mL), 20 % (0.2 g/mL), 30% (0.3 g/mL), 40% (0.4 g/mL), 50% (0, 5 g/mL), 60% (0.6 g/mL), 70% (0.7 g/mL), 80% (0.8 g/mL), 90% (0.9 g/mL) and 100% (1 g/mL). Each concentration was repeated thrice. Extract *Z. acanthopodium* endophyte bacteria isolates were diluted using 7% DMSO. Amoxicillin was used as a positive control, and a negative control was 7% DMSO. The clear zone around the paper disc indicated that the *Z. acanthopodium* endophytic bacterial extract could inhibit the growth of *B. subtilis*. The inhibition zones were measured. The classification of the inhibition zone is described in Table 1.

Phytochemical test

Three isolates, i.e., EA26, EA7, and EB6, were analyzed for their phytochemical content, i.e., flavonoid, tannin, alkaloid, and saponin. EA26 and EA7 were the potential isolates from the root, and EB6 was isolated from the stem. They are the best isolate of endophytic bacteria from *Z. acanthopodium* endophyte bacteria because they can inhibit several pathogenic bacteria (Rizqoh, 2020 unpublished data). The phytochemical test solution was made by mixing 150 µL of ethyl acetate extract of endophytic bacteria from *Z. acanthopodium* with 15 µL of ethyl acetate solvent (Putri et al. 2014).

Flavonoid examination was conducted using Wilstater's reagent, namely 10 µL of crude extract solution added with 0.1 mg magnesium powder or 0.5 cm long magnesium tape and 10 µL concentrated HCL. The presence of the flavonoid group is indicated by changing the color of the solution to yellow or pink (Theodora et al. 2019).

Determination of alkaloids: 20 µL of the crude extract solution was evaporated over a cup, allowed to cool, and the filtrate was taken for the next step. 20 µL of concentrated HCl was added to the filtrate and dissolved. The solution was divided into three test tubes. Tube 1, as a blank, was added with dilute acid. Tube 2 was added with 3 drops of Dragendroff's reagent. Tube 3 was added with 3 drops of Mayer's reagent. An orange-colored precipitate indicated the presence of an alkaloid in tube 2. Meanwhile, alkaloids in tube 3 are characterized by a yellow or white precipitate (Putri et al. 2014).

Determination of saponin: 100 µL of crude extract solution was shaken vigorously vertically for 10 seconds and then left for 10 seconds. The appearance of stable foam indicates the presence of saponin. Adding 1 drop of concentrated HCl causes the foam to be more stable and not disappear (Dewi et al. 2021).

Determination of tannin: 20 µL of crude extract solution was divided into two tubes. Tube A was used as a blank, and tube B was added with 10 µL of 5% FeCl3 reagent to 10 µL of extract. The formation of a dark blue, blackish blue, or greenish-black color indicated the presence of tannins (Dewi et al. 2021).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria from *Z. acanthopodium*

The results of the isolation of endophytic bacteria produced 252 colonies. The number of colonies from root were 150 colonies (59.52%), 81 colonies from stem (32.14%), and 21 colonies from leaf (8.33%) (Table 2).

Characterization morphology colony bacteria

Based on the colony morphology from 85 isolates, there were 28 isolates from the root, 40 from the stem, and 17 from the leaf. Morphological diversity of the endophytic colony from *Z. acanthopodium* could be obtained in every part of the plant (Figure 1). There are 15 different colony morphology from the root, 14 from the stem, and 15 from the leaf.

Three shape variations were observed, i.e., circular, irregular, and punctiform. The most common shape of endophytic bacteria from the leaves, roots, and stems is irregular. Colony margins are more varied, such as entire, filamentous, irregular, rhizoids, and undulate. The most common margin shape of endophytic bacteria from leaves and roots is entire, while from roots, it is irregular. Variations in elevation form are convex, flat, and raised. Flat elevation was obtained from the most colonies from the leaves, roots, and stems. The colony texture is generally dry and moist. Most colonies on three parts of the plant had a moist surface. Endophytic bacterial colonies also have various pigments, such as dull, opaque, shiny, and transparent. The majority of pigment of endophytic colonies was opaque. Different colors are found in endophytic bacterial colonies, such as chocolate, white, yellow, and pink. In general, endophytic bacterial colonies are white.

Table 2 Total number of *Z. acanthopodium* isolation colonies

No.	Root		Stem		Leaf	
	I	II	I	II	I	II
1	50	20	11	11	6	3
2	30	17	10	34	5	5
3	27	6	7	8	0	2
Sub-total	150		81		21	
Total	252					

Bacterial cell shape and colony features are insufficient to identify a species of bacteria. Molecular identification or the identification of biological substances can be used to identify microorganisms. Therefore, more investigation is required to identify the bacteria at the species level.

Antagonist test of endophytic bacteria from *Z.acanthopodium* against *B. subtilis*

Due to the sizes of the isolates not being the same and not evenly distributed, the diameter of the inhibition zone is measured from the distance from the edge of the tested endophytic bacteria to the end of the clear zone. The average diameter of the inhibition zone was calculated from measurements of three different sides of the inhibition zone. The results show that 16 isolates have the potential to inhibit *B. subtilis* (Table 3, Figure 2), namely 13 isolates (81.25%) from the roots, 1 isolate (6.25%) from the stems, and 2 isolates (12.5%) from the leaf.

Grams stain of potential isolates

The result of the Gram Stain analysis of potential isolates showed that all isolates are Gram-positive bacteria with different cell shapes: 9 isolates (56.25%) of bacilli, 3 isolates (18.75%) of coccus, and 4 isolates (25%) of cocobacilli (Table 4).

Antibacterial activity of the supernatant and pellets of endophytic bacteria from *Z. acanthopodium*

The pellet and supernatant of three isolates with the most potential antibacterial activity from the antagonist test endophyte bacteria (Table 3) were re-tested against *B. subtilis.* The results were presented in Table 5 for supernatant and Table 6 for pellet.

Extraction of endophytic bacteria from *Z. aconthopodium*

The culture of three *Z. acanthopodium* endophytic bacteria isolates, i.e., EB6, EA7, and EA26, were subjected to extraction using ethyl acetate as a solvent. These three isolates were selected for extraction because they exhibited antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Rizqoh, 2020 unpublished data). Although EB6 was a weak category, it was the only isolate from the stem that had the potential to inhibit *Bacillus subtilis*. The extracts were evaporated in an oven at 50°C for 1 hour to remove ethyl acetate residue. The extracts obtained were 7 mL of isolate EA26, 8 mL of isolate EA7, and 10 mL of isolate EB6.

Minimum inhibitory concentration crude extract of endophytic bacteria from *Z. acanthopodium*

Figure 3 shows the inhibitory zone of crude extract of EA26, EA7, and EB6 against pathogenic *B. subtilis*. The growth inhibition of endophytic bacteria of EA26 and EA7 started at 40%, categorized as a weak antibacterial; however, their antibacterial activity at a concentration of 100% was classified as moderate. The extract of EB6 isolate started to form an inhibitory zone at 80%; therefore, it was classified as a weak antibacterial (Table 7).

Phytochemical test

The results of the phytochemical showed that the crude extract of EA26 contains flavonoids, alkaloids, saponin, and tannin. The crude extract EA7 contained flavonoid and saponin, and EB6 contained alkaloid and saponin (Table 8).

Table 3. The diameter of the inhibition zone of endophytic bacteria from *Z. acanthopodium* against *B. subtilis*

Isolate code	Inhibition zone diameter (mm)	Category of antibacterial activity
Root		
EA1	2.7 ± 0.3	Weak
EA4	5.1 ± 1.7	Moderate
EA5	8.0 ± 0.5	Moderate
EA7	7.0 ± 0.3	Moderate
EA9	4.7 ± 0.2	Weak
EA14	4.1 ± 0.6	Weak
EA15	4.7 ± 0.4	Weak
EA16	0.5 ± 0.0	Weak
EA24	2.2 ± 0.1	Weak
EA25	0.7 ± 0.3	Weak
EA26	5.1 ± 0.6	Moderate
EA27	0.6 ± 0.2	Weak
EA28	1.1 ± 0.5	Weak
Stem		
EB6	1.0 ± 0.6	Weak
Leaf		
ED10	0.5 ± 0.2	Weak
ED11	0.5 ± 0.1	Weak

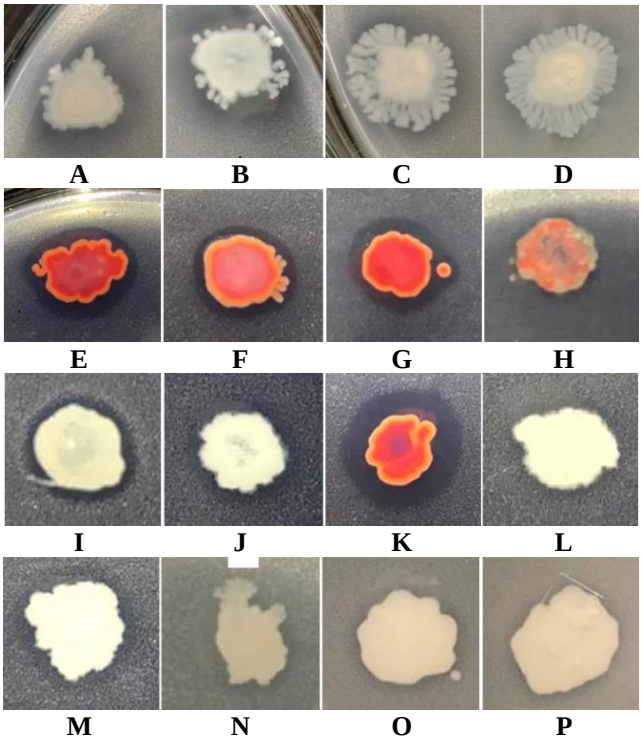

Figure 2. Inhibition zone of endophytic bacteria from *Z. acanthopodium* against. Note: *B.subtilis*; A. EA1; B. EA4; C. EA5; D. EA7; E. EA9; F. EA14; G. EA15; H. EA16; I. EA24; J. EA25; K. EA26; L. EA27; M. EA28; N. EB6; O. ED10; P. ED11

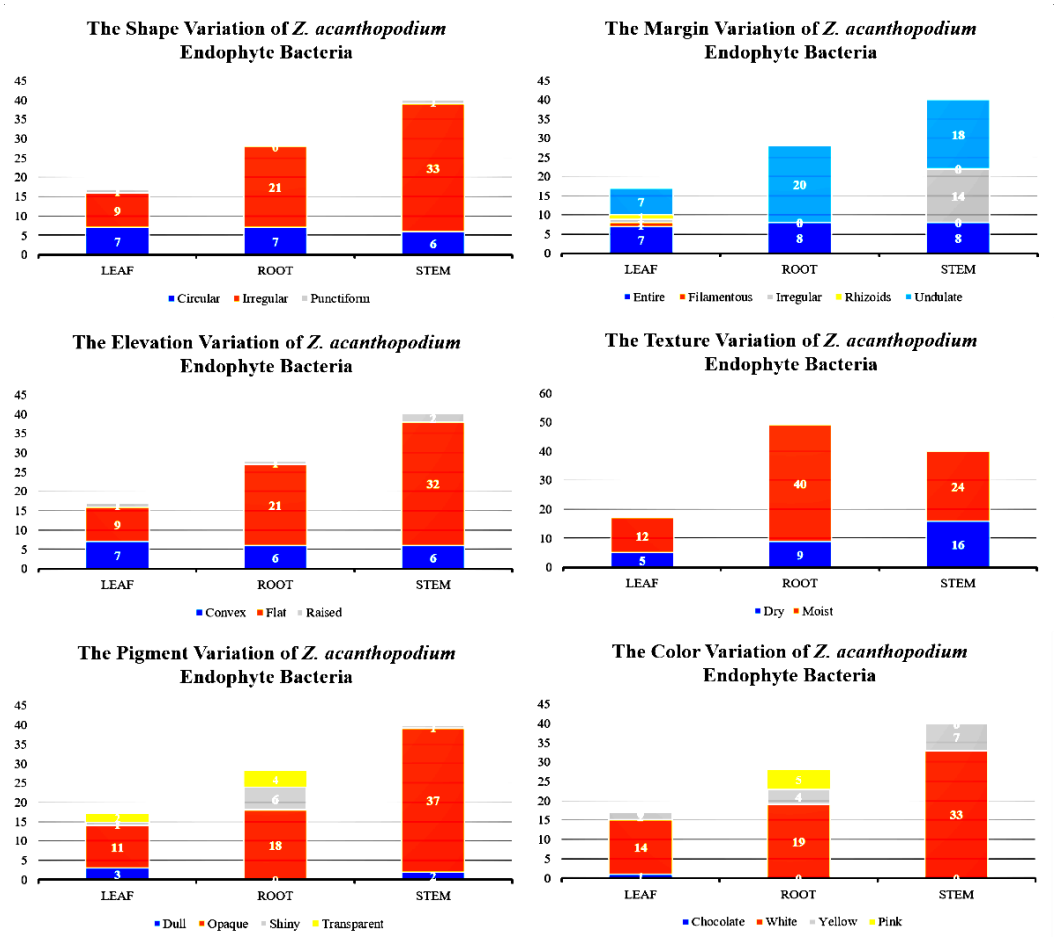

Figure 1. Diversity of shape, margin, elevation, texture, pigment, and color of endophytic bacteria from *Z. acanthopodium*

Table 4. Cell shapes of potential endophytic bacteria

Form	Isolate code
Bacilli	EA1; EA7; EA9; EA14; EA24; EA25; EA27; EA28; EB6
Coccus	EA16; ED10; ED11
Coccobacilli	EA4; EA5; EA15; EA26

Table 5. Diameter of inhibition zone of supernatant from endophytic bacteria from *Z. acanthopodium* against *B. subtilis*

Isolate code	Diameter of clear zone (mm)	Category of antibacterial activity
EA1	2.4 ± 0.2	Weak
EA24	5.6 ± 2.6	Moderate
EA26	14.2 ± 7.5	Strong

Table 6. Diameter of inhibition zone of pellet from endophytic bacteria from *Z. acanthopodium* against *B. subtilis*

Isolate code	Diameter of clear zone (mm)	Category of antibacterial activity
EA1	4 ± 0.6	Weak
EA24	6.9 ± 1.3	Moderate
EA26	16.3 ± 1.1	Strong

Table 7. The average inhibitory zone of endophytic bacteria crude extract from *Z. acanthopodium* against *B. Subtilis*

Treatment	Average zone of inhibition (mm) ± SD		
	EA 26	EA 7	EB 6
K+ (Amoxicillin)	7.91 ±2.19	8.16±1.19	3.16 ± 0.37
K- (DMSO 7%)	0.00 ±0.00	0 .00 ± 0.00	0 .00 ± 0.00
10 %	0.00 ±0.00	0 .00 ± 0.00	0 .00 ± 0.00
20 %	0.00 ±0.00	0 .00 ± 0.00	0 .00 ± 0.00
30 %	0.00 ±0.00	0 .00 ± 0.00	0 .00 ± 0.00
40 %	0.33 ±0.47	1.5 0±0.54	0 .00 ± 0.00
50 %	1.66 ±0.94	1 .00±0.89	0 .00 ± 0.00
60 %	1.83 ±0.68	1.33 ±1.21	0 .00 ± 0.00
70 %	3.16 ±1.21	0.66 ±0.51	0 .00 ± 0.00
80 %	3.5 0 ±0.50	3 .00±1.54	1.33 ±0.74
90 %	3.5 0 ±0.76	3.83 ±1.72	1.0 5 ± 0.95
100 %	7.91 ±2.19	8.15±1.19	3.16 ±0.37

Table 8. Phytochemical content of crude extract from endophytic bacteria from *Z. acanthopodium*

Phytochemical compound	EA 26	EA 7	EB 6
Flavonoid	+	+	-
Alkaloid	+	-	+
Saponin	+	+	+
Tannin	+	-	-

Note: - : nor present, +: present

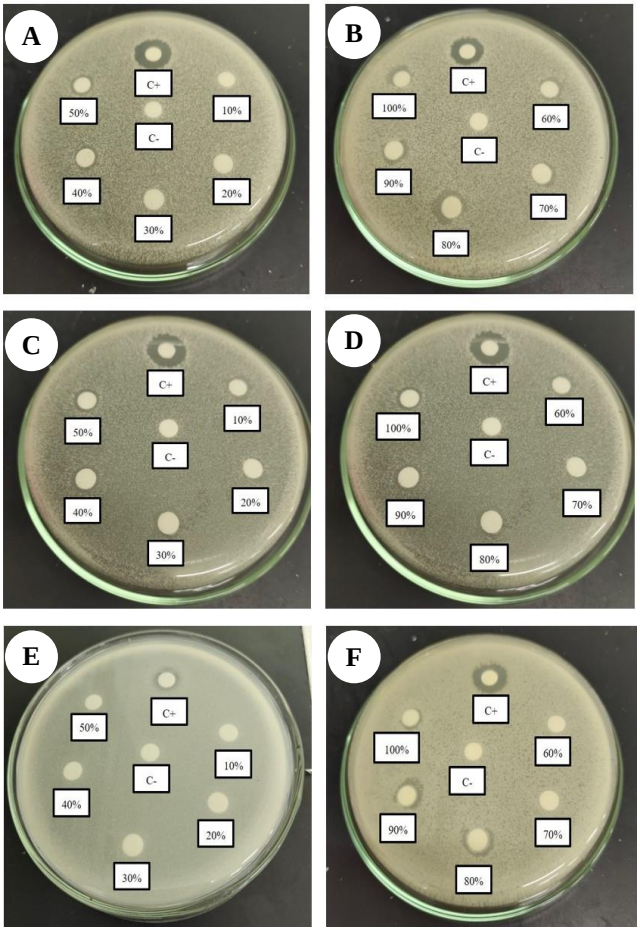

Figure 3. The inhibitory zone of extract of endophytic bacteria from *Z. acanthopodium* against *B. subtilis*. (a-b : crude extract of isolate; c-d : crude extract of isolate EA26; e-f : crude extract isolate EB6

Discussion

Isolation and characterization of Z. acanthopodium *endophyte bacteria*

Endophyte bacteria live in plant tissues without causing any disease to the host plant. Several species of endophyte bacteria produce active compounds with antibacterial and antifungal activities (Beck et al. 2003; Castillo et al. 2003). The isolation of endophytic bacteria from *Z. acanthopodium* showed that roots produce the highest colonies (59.52%) compared to stems and leaves. It might be due to the bacteria entering a plant through the root, then colonizing at the point of entry, or spreading throughout the plant through the xylem (Kandel et al. 2017). Environment, type, age, and condition of plant tissue are several factors that affect the number of endophyte bacteria in plant organs (Gouda et al. 2016). Endophytic bacteria from *Z. acanthopodium* have diverse colony morphology. Plant growth conditions give rise to the diversity of endophyte colonies. Furthermore, endophytic bacteria are more varied (Rizqoh et al. 2016).

Antagonist test of *Z. acanthopodium* endophyte bacteria against B. subtilis

An antagonist test of endophytic bacteria from *Z. acanthopodium* against *B. subtilis* was carried out using Nutrient Agar (NA). Nutrient agar (NA) is often used for growing and culturing bacteria because it contains carbohydrates, protein, and peptone, essential substrates for bacterial metabolism (Cappucino 2013). The double-layer methods carried out the antibacterial test because its inhibition zones were more clearly visible (Rizqoh et al. 2016).

Sixteen endophytic bacteria from *Z. acanthopodium* inhibited the growth of *B. subtilis*. The findings of this study support other research showing that phyllosphere bacteria of *Z. acanthopodium* extract possess antimicrobial properties (Rizqoh et al. 2023, unpublished data). These potencies are due to several chemical compounds contained in the phyllosphere bacteria of *Z. acanthopodium*, such as saponin compound (Bis(2-ethylhexyl) phthalate), ester compound (Methyl butanoic and Propylphosphonic acid, and di-(2-methyl propyl) ester), fatty acid (Dodecanoic acid, Tetradecanoic acid, n-Hexadecanoic acid, dan Hexadecanoic acid, 1-[[[(2-aminoethoxy) hydroxyphosphinyl] oxy]methyl]-1,2ethanediyl ester), organic compound (2,3-Butanediol and Phthalic anhydride), and diester compounds (2-Oxo-2-(pentyloxy)ethyl 2-(1,3-dioxoisoindolin-2-yl)acetate) (Rizqoh et al. 2023, unpublished data). The endophytic bacteria from *Z. acanthopodium* contained flavonoids, alkaloids, saponin, and tannin. The primary secondary metabolites and active ingredients in many traditional medications are saponins. The chemical structures of different saponins determine their biological properties, including antiviral, antifungal, antibacterial, and anti-inflammatory properties (Dong et al. 2020).

In this research, supernatant and pellet of endophytic bacteria from *Z. acanthopodium* have the potential to inhibit the growth of *B. subtilis*. It is due to the Gram-positive bacteria's cell wall structure, which is dominated by peptidoglycan and susceptible to antibacterial compounds in supernatant and pellets of endophytic bacteria. Endophytic bacteria from *Z. acanthopodium* are possibly capable of producing antibacterial compounds (Singh et al. 2012). Bioactive compounds from the supernatant were excreted by bacterial cells into the medium (extracellular) completely (or partly). The antibacterial activity of the pellet showed that bioactive compounds were produced and secreted intracellularly (Sarker et al. 2006).

Extraction of endophytic bacteria

The endophytic bacteria were grown in a Nutrient Broth (NB) using a shaker incubator for three days (3 x 24 hours) to obtain optimal biomass cells. The incubation time influences the production of secondary metabolites. In the stationary phase, the number of bacteria cells is constant, i.e., the number of dead bacteria is the same as that of live bacteria. In limited nutrition, competition occurs, and bacteria produce secondary metabolites for their defense mechanism (Yadav 2021).

The solvent used for extraction is ethyl acetate because it evaporates quickly, is non-hygroscopic, has low toxicity, and is semi-polar to extract semi-polar compounds (Shasti 2017). Alkaloids, glycosides, phenolic compounds, terpenoids, and aglycones can be extracted using semi-polar solvents. Metabolites produced by endophytes can secreted into liquid culture media to obtain extracellular metabolites (Yadav 2021).

MIC of crude extract of endophytic bacteria from Z.acanthopodium

In the determination of MIC value, amoxicillin was used as the control-positive. Amoxicillin is a broad-spectrum β-lactam antibiotic frequently used to treat various infectious diseases caused by Gram-positive and Gram-negative bacteria as bactericidal (Bennett et al. 2020). Amoxicillin prevents the cross-linked process of peptidoglycan at the end stage of cell wall synthesis by inhibiting penicillin-binding proteins. The MIC test results showed that three crude extracts of endophytic bacteria from *Z. acanthopodium* inhibit the growth of *B. subtilis*. These crude extracts showed different inhibition abilities against *B.subtilis*. The antibacterial activity of EA7 and EA26 was classified as moderate antibacterial, and isolate EB6 had weak antibacterial activity.

Endophyte bacteria produce secondary bioactive compounds that are sometimes similar to bioactive compounds produced by their host plants, such as antibacterial compounds (Yadav 2021). Bacteria produce secondary metabolites at the end of the log phase and during the stationary phase (Rana et al. 2019). This study showed that crude extracts of endophytic bacteria from *Z. acanthopodium* only contain saponin. Saponin reduces the surface tension of bacterial membrane cells and damages membrane permeability. Lysis can result in cell death, so that saponins can be antibacterial (Yusoff et al. 2022).

The results differ between the antagonist test and the MIC of endophytic bacteria from *Z. acanthopodium*. The amount of ethyl acetate solvent used during extraction cannot extract all secondary metabolite compounds produced by the endophyte isolate, so only several compounds are extracted. The choice of solvent polarity determines the type of extracted compounds. Polar compounds tend to be extracted by polar solvents; conversely, non-polar compounds are extracted by non-polar solvents (Buhian et al. 2016). In addition, impurity substances that contain various compounds in the extract may inhibit the activity of pure antimicrobial compounds (Rizqoh et al. 2016).

Variations in the inhibitory zone diameter are caused by the different diffusion rates of the compounds and the mechanism of compounds/ extract against pathogenic microbes. The differences in bacterial growth that occur at each fermentation time are caused by the different abilities of bacteria to reproduce, depending on the growth media and available nutrients. The specific growth rate for each bacterium is different because the enzyme content in each bacterium is different, which influences the metabolic process of the bacterium in producing secondary metabolites. Several genera of endophytic bacteria are known to produce secondary metabolite compounds such as antibiotics, anticancer compounds, antifungals, antivirals, and insecticidal agents (Singh et al. 2017).

In conclusion, there are 252 endophyte bacteria isolates obtained from *Z. acanthopodium*. Sixteen endophytic bacteria possessed antibacterial activity against *B. subtitlis*. Three selected isolates, EA1, EA24, and EA26, have the most potent antibacterial activities compared to other isolates. The supernatant and pellets of the three isolates also have inhibition activity against *B. subtilis*. Crude extract of isolate EA7 and EA had moderate antibacterial, while EB6 had weak antibacterial activity. The results of phytochemical analysis showed that endophytic bacteria contain flavonoid, alkaloid, saponin, and tannin compounds.

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