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Antibacterial Activity of Endophytic Bacterial Isolates from *Kaempferia galanga* Leaves

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Abstract

Indonesia has a high biodiversity, which can be relied upon as a potential source of medicinal materials. One of the medicinal plants in Indonesia is *Kaempferia galanga*, which demonstrates various pharmacological properties, including antibacterial, anti-inflammatory, antioxidant, anticancer, and antiangiogenic effects. However, directly extracting active compounds from plants requires a considerable amount of biomass. To address this challenge, utilizing endophytic bacteria associated with these plants presents a promising alternative. Consequently, the antibacterial activity of endophytic bacterial isolates from *K. galanga* leaves needs to be investigated. This study is an experimental laboratory investigation conducted in vitro. Two isolates demonstrated antibacterial activity: isolate code DR4 inhibited the growth of *Escherichia coli* (3 ± 0.5 mm), while isolate DR10 inhibited the growth of four bacteria: *Bacillus subtilis* (5 ± 0 mm), *Staphylococcus aureus* (1.5 ± 0.5 mm), *Escherichia coli* (1 ± 0 mm), and *Pseudomonas aeruginosa* (1 ± 0 mm). Disc diffusion tests using ethyl acetate extracts of isolate DR10 showed the highest antibacterial activity at a concentration of 10,000 ppm. The Minimum Inhibitory Concentration (MIC) was determined to be 156.2 ppm against *B. subtilis*, while the Minimum Bactericidal Concentration (MBC) was >625 ppm. Molecular identification showed that isolate DR 10 had 100% similarity to *Bacillus* sp. TS8. The active compound suspected to have antibacterial properties is pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, with the highest abundance.

1. Introduction

Renowned as an archipelagic nation, Indonesia holds exceptional biodiversity, supporting many plant life. It is home to 30,466 plant species across 2,968 genera and 317 families, representing 8.7% of the world's 351,180 vascular plant species. This remarkable diversity emphasizes Indonesia's crucial role in global biodiversity conservation and its value as a resource for scientific and medicinal advancements [1]. This can be relied on as a potential source to overcome antibiotic resistance. One of the plants that can be developed as a raw material for medicine is *Kaempferia galanga* (*K. galanga*) [2].

K. galanga is a plant used in traditional medicine to cure influenza, headache, diarrhea, and stomach inflammation [3]. While the rhizome is commonly utilized, the leaves are also used for sore throats, swollen

breasts, coughs, hair washing, and during pregnancy. Extracts from this plant have pharmacological effects such as anti-inflammatory, antioxidant, anticancer, antibacterial, and antiangiogenesis properties [4, 5]. Previous research has shown that the antibacterial activity of *K. galanga* essential oil inhibits the growth of bacteria that cause sore throats, namely *Streptococcus pyogenes* and *Staphylococcus aureus* [6]. The secondary metabolites found in this plant include terpenoids, phenolics, cyclic dipeptides, diarylheptanoids, flavonoids, polysaccharides, and essential oils [4].

Using endophytic bacteria from plants offers an alternative to using plants, as extracting active compounds from plants directly requires a large amount of biomass. Endophytic bacteria can be relied upon to produce secondary metabolites [7, 8]. Endophytic bacteria also provide many benefits without causing peripheral infections or adverse effects on their host [9]. These bacteria produce bioactive compounds with potential applications in discovering new antibiotics, anticancer agents, and treatments for diseases in humans, animals, and plants [10].

Several studies have been conducted on endophytic from *K. galanga*. In the study by Efendi *et al.* [11], endophytic fungi isolated from leaves and rhizomes of *K. galanga* were successfully identified as *Torulla* sp. (KG001), *Fusarium* sp. (KG003), and *Drechcera* sp. (KG005) have antibacterial activity against Gram-positive bacteria (*S. aureus* and *Vibrio cholera*) and Gram-negative bacteria (*Bacillus subtilis* and *Escherichia coli*). Azizah *et al.* [12] used the endophytic bacterium *Streptomyces vellosus* from *K. galanga* rhizomes, which exhibited antibacterial properties. However, research on endophytic bacteria from *K. galanga* leaves remains limited, even though *K. galanga* leaves also have the potential as a source of bioactive compounds [4, 13]. Therefore, this research aims to investigate the antibacterial activity of endophytic bacterial isolates from *K. galanga* leaves.

2. Experimental

2.1. Materials

The *K. galanga* leaf samples were obtained fresh from the Cibunar area, Sukabumi. The test bacteria used were *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 9027 (from the collection of the Microbiology Laboratory, Center for Research on Raw Materials for Medicines and Traditional Medicines, BRIN, Serpong). Other materials included tetracycline, dimethyl sulfoxide (DMSO), bacterial growth media such as nutrient broth (NB), nutrient agar (NA), Luria agar (LA), Mueller Hinton broth (MHB), Mueller Hinton agar (MHA), sterile distilled water, nystatin, 0.5% sodium hypochlorite (NaOCl), 95% alcohol, 70% alcohol, sodium chloride (NaCl) solution, ethyl acetate ($CH_3COOCH_2CH_3$), disc paper, elution buffer BE, lysis buffer B3, buffer B5, buffer BW, proteinase K, phosphate-buffered saline (PBS), lysis buffer B3, nuclease-free water (NFW), 1X TAE buffer, ethanol, forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3'), reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3'), GoTaq Green® Master Mix (Promega), n-hexane, and helium gas.

2.2. Instruments and Tools

Laminar air flow, Biosafety cabinet II, autoclave, incubator shaker, hot plate, magnetic stirrer, 96-well microplate, colony counter, L-shaped rod, Petri dishes, micropipettes, microtips, oven, evaporator, analytical balance, Erlenmeyer flasks, beakers, measuring cylinders, scissors, dropper pipettes, test tubes, test tube racks, Bunsen burner, matches, knives, tweezers, mortar and pestle, inoculating loop, trays, ruler, separatory funnel, tripod, column chromatography, thin-layer chromatography, vials, water bath, 1 mL Eppendorf tubes, 1.5 mL Eppendorf tubes, 2 mL collection tubes, DNA binding columns, centrifuge, vortex, PCR (Applied Biosystems™ 2720), electrophoresis, UV transilluminator, FirstBase Malaysia services, BioEdit software, BLAST-N program (NCBI) (http://ncbi.nlm.nih.gov/), MEGA 11.0 software, GC-MS (Agilent 19091S-433: 93.92873), HP-5ms column (5%-phenyl)-methylpolysiloxane 0°C-325°C (325°C): 30 m × 250 µm × 0.25 µm, MSD ChemStation Data Analysis software.

2.3. Experiment

2.3.1. Isolation of Endophytic Bacteria

The *K. galanga* leaves were washed with tap water, rinsed with sterile distilled water, then soaked in 70% ethanol solution for 1 minute, followed by 0.5% NaOCl solution for 3 minutes, and finally soaked in 96% ethanol solution for 30 seconds. After that, the leaves were rinsed with sterile distilled water and plated on NA and LA media to evaluate the success of sterilization. Serial dilutions were performed up to 10^{-2}, and 0.1 mL of each dilution was plated on NA and LA media containing 100 ppm of the antifungal nystatin, then incubated at ±37°C for 24-48 hours. The grew colonies were isolated on NA media to obtain pure cultures of endophytic bacterial isolates [14].

2.3.2. Morphological Identification

After reaching the growth stage, each endophytic bacterial colony was assessed based on various morphological parameters: colony color (such as yellow, white, pink, green, or clear), shape (round or irregular), colony size (small, medium, or large), surface texture (rough or smooth), and colony edge (even or uneven). In addition, the speed or growth rate of each colony was also recorded. All observation results were recorded and arranged in a table to facilitate analysis. Each colony was given a unique label and inoculated using a single-strike loop on NA media for stock supplies, which were then stored at 4°C to maintain the sustainability of the bacterial colonies [14].

2.3.3. Screening of Antibacterial Activity Using the Antagonistic Method

The test bacteria (*B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 9027) were prepared by transferring three loops of each bacterium from the slanted NA stock into 50 mL of NB. The culture was incubated in a shaker incubator at 37°C and 120 rpm for 24 hours. Subsequently, 1% v/v of the bacterial culture was added to molten NA at

approximately 40°C. The mixture was poured into petri dishes and allowed to solidify. Once solidified, the endophytic bacterial isolate was streaked in a circular pattern with a diameter of 6 mm. The plates were incubated at room temperature for 24 hours, and the inhibition zones were observed [14].

2.3.4. Fermentation and Extraction

The endophytic bacteria were inoculated onto NA media using the four-quadrant streak method and incubated at room temperature for 24 hours. The fermentation starter was prepared by inoculating three loops of endophytic bacteria into liquid NB media and incubating it on a shaker at 120 rpm and 37°C. The fermentation process was initiated by inoculating 20 µL of the starter (1:100) into 2 liters of liquid NB media, which was then incubated on a shaker at 120 rpm and 37°C for 3 days. The supernatant was separated using a separating funnel and transferred to a dark glass bottle, while the extraction residue was mixed with ethyl acetate (1:1) and placed in an incubator shaker at 120 rpm and 37°C for 1 hour. This extraction process was repeated three times. All supernatant solutions collected in dark glass bottles were combined and evaporated until only a wet extract remained. The extract was then placed in an oven at 50°C until dry, and its weight was measured [15].

2.3.5. Disc Diffusion Test

The ATCC bacteria cultured for 24 hours were introduced into molten NA media at approximately 40°C (1% v/v), which was then poured into Petri dishes and allowed to solidify. Paper disks were prepared by applying 20 µL of the positive control (tetracycline at 200 ppm), the negative control (DMSO), and the DR10 extract at concentrations of 1,000 ppm, 5,000 ppm, and 10,000 ppm. The disks were placed onto the solidified NA media. The plates were incubated at room temperature for 24 hours, and the inhibition zones were observed [14].

2.3.6. Minimum Inhibitory Concentration (MIC) Test

The test bacteria were cultured for 24 hours, and the extracts were prepared at an initial concentration of 5,000 ppm. Wells were inoculated with the positive control (200 ppm tetracycline), negative control (100% DMSO), and the extract. In well A, 100 µL of Mueller Hinton Broth (MHB) was added, followed by serial dilutions, discarding the final 100 µL. A 1% bacterial suspension was prepared by mixing cultured bacteria with 20 mL of NaCl in a Falcon tube, thoroughly mixed, and transferred to a reservoir. Subsequently, 100 µL of the test bacteria were inoculated into wells A–H. The plate was incubated at 37°C and 120 rpm for 24 hours. After incubation, the clarity of the wells was observed to determine the extract's effective concentration [14].

2.3.7. Minimum Bactericidal Concentration (MBC) Test

A volume of 10 µL from the clear wells of the MIC test was transferred onto MHA in Petri dishes. The plates were incubated at 37°C for 24 hours, and bacterial growth was observed to evaluate the effect of the extract [14].

2.3.8. Molecular Identification

The selected endophytic bacterial isolate was processed using the GeneProof kit. The DNA sample was amplified for the 16S rRNA sequence through the PCR method, employing the forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and the reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') [10] on a PCR machine, targeting a DNA fragment of 1300 bp. A 50 µL PCR mixture was prepared by combining 5 µL of the forward primer (10 pmol), 5 µL of the reverse primer (10 pmol), 25 µL of 2X GoTaq Green® Master Mix (Promega), 2 µL of DNA template (100 ng/µL), and 13 µL of nuclease-free water. The PCR process involved pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute and 45 seconds, and post-PCR extension at 72°C for 10 minutes, over a total of 35 cycles.

Electrophoresis of the PCR product was conducted on a 1.5% agarose gel at 50 V using 1X TAE buffer for 50 minutes. DNA visualization was performed using fluorosafe dye and observed under a UV transilluminator. The remaining PCR product was sequenced by FirstBase Malaysia services. The nucleotide sequence obtained from the forward primer 63F and reverse primer 1387R was aligned and reconstructed using SeqMan II software.

The resulting sequences were input into the BLAST- N (NCBI) program (http://ncbi.nlm.nih.gov/) to obtain sequence data. Phylogenetic trees were constructed using isolate sequence data and comparison sequences retrieved from gene banks at http://ncbi.nlm.nih.gov/. Sequence data collection was performed through multiple alignments using BioEdit software. Subsequently, nucleotide sequence analysis was carried out using MEGA 11.0 software, applying the Neighbor-Joining Tree method with the Bootstrap Method and 1000 bootstrap replications [14].

2.3.9. Scanning Electron Microscope (SEM) Observation

The most promising isolates were visualized through SEM analysis. The bacterial cells were harvested and placed onto the surface of a single-polished silicon wafer (Sigma), then incubated at room temperature (29°C) for 18 hours. The SEM specimens were subsequently examined at a magnification of 5000×, a working distance of 5 µm, and an accelerating voltage of 10 kV using a JEOL JSM-IT200 SEM (JEOL, South Korea) [16].

2.3.10. Compound Analysis with GC-MS (Gas Chromatography-Mass Spectrometry)

The crude extract of the potential isolates was analyzed using a GC-MS machine (Shimadzu QP2010) to identify antibacterial compounds in the extract. The column used was a Capillary Phase Rtx-5MS, with a length of 60 m and a diameter of 0.25 mm. The instrument conditions included a column temperature of 50°C, helium gas as the carrier, an SPL temperature of 280°C, an MS interface temperature of 280°C, a pyrolysis temperature of 280°C, and an ion source temperature of 200°C [17].

3. Results and Discussion

3.1. Antibacterial Activity

3.1.1. Isolation Endophytic Bacteria from *K. galanga* leaves

A total of 27 types of endophytic bacterial isolates were successfully obtained from the leaves, with 19 isolates from NA media and 8 from LA media (Figure 1 and Table 1). The isolates were derived from different media, specifically NA and LA, which are commonly used for bacterial culture but have distinct characteristics. NA media contains meat extract, peptone, sodium chloride, and agar, while LA media contains peptone, yeast extract, sodium chloride, and agar. LA media generally has lower selectivity than NA media, which tends to support the growth of a broader range of endophytic bacteria. As shown in the study by Duhan *et al.* [18], LA media demonstrated a higher presence of endophytic bacteria in plant tissue compared to NA media. The difference in selectivity between LA and NA media is due to their nutrient composition. NA is a relatively simple medium with a balanced mix of peptone, yeast extract, and sodium chloride, which supports the growth of a wide range of bacteria, including both fastidious and non-fastidious strains.

Meanwhile, LA media has a higher concentration of certain nutrients, such as tryptone and yeast extract, making it richer in nitrogen sources. This can favor the growth of specific bacterial groups and result in lower overall selectivity [19, 20]. The selectivity between NA and LA media can influence the genetic expression and physiological activity of cultured endophytic bacteria, leading to variations in their growth, metabolism, and other characteristics [21]. In a study by Mamangkey *et al.* [22], Nineteen types of endophytic bacterial isolates were successfully obtained from Zingiberaceae rhizomes using NA media. However, no research has been found that isolates endophytic bacteria from *K. galanga* using LA media. According to research by Preveena and Bhore [23], 50 types of endophytic bacterial isolates were successfully obtained using LA media from the leaves and stems of Tridax procumbens Linn. Meanwhile, Yunita *et al.* [24] concluded that the addition of 1% peptone and *M. fragrans* filtrate to NA media yielded better results than previous studies, demonstrating stronger antibacterial activity.

The resulting isolates vary due to several factors, including the host plant environment, soil microbiota composition, interactions between endophytic bacteria and plants, and the unique genetic properties of the bacteria themselves. The number of endophytic bacteria depends on various factors, such as plant type, soil structure, plant age, geographic location, and sampling time. Although the primary entry route for endophytic bacteria is typically through the roots, plant parts directly exposed to air—such as leaves (through stomata), flowers, stems, branches, and cotyledons—can also serve as entry points. Additionally, endophytic bacteria can enter plants through wounds caused by both biotic and abiotic factors [9].

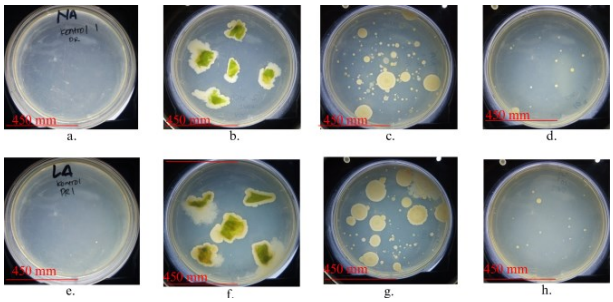

Figure 1. Endophytic bacterial isolates from *K. galanga* leaves on NA and LA media: a) control on NA media; b) planted leaves on NA media; c) concentration of 10^{-1} on NA media; d) the concentration of 10^{-2} on NA media; e) control on LA media; f) planted leaves on LA media; g) concentration of 10^{-1} on LA media; h) the concentration of 10^{-2} on LA media

3.1.2. Screening of Antibacterial Activity Using the Antagonistic Method

Endophytic bacterial isolates were screened using the antagonistic antibacterial test method, and the presence of antibacterial activity was observed through the formation of inhibition zones in Petri dishes. These zones were formed due to the ability of the endophytic bacteria to synthesize antibacterial compounds [25]. In this study, two isolates exhibited antibacterial activity against the test bacteria (*B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*) with different antibacterial spectra (Figure 2 and Table 2). The isolates inhibited at least one target strain, as indicated by a clear zone around the endophytic bacterial colony. The formation of the inhibition zone was also influenced by the type of test bacteria.

The difference in cell wall structure between Gram-positive and Gram-negative bacteria affects their susceptibility to antibacterial agents. Gram-positive bacteria have a relatively simpler peptidoglycan layer, making them more susceptible to antibacterial agents, while Gram-negative bacteria possess a more complex cell wall structure, including a lipopolysaccharide layer that acts as a barrier to antibacterial compounds. Consequently, Gram-negative bacteria are generally more resistant to antibacterial attacks than Gram-positive bacteria [26]. This was evident in isolate DR10, which formed a larger inhibition zone on Gram-positive test bacteria (*B. subtilis* and *S. aureus*) than on Gram-negative test bacteria (*E. coli* and *P. aeruginosa*).

However, despite the more complex cell wall structure of Gram-negative bacteria, some antibacterial agents can produce wider inhibition zones against Gram-negative bacteria than Gram-positive bacteria. Factors such as membrane permeability, the molecular structure of antibacterial agents, their mechanisms of action, and bacterial sensitivity can influence the effectiveness of antibacterial agents against both types of bacteria. Furthermore, the mechanism of action of antibacterial agents can determine their effectiveness, with some agents being more effective against Gram-negative bacteria. Therefore, despite their complex cell wall structure, some antibacterial agents can produce wider inhibition zones against Gram-negative bacteria than against Gram-positive bacteria [27].

Table 1. Morphology identification of endophytic bacterial isolates from *K. galanga* leaves (DR) macroscopically

No.	Code	Colony color	Colony shape	Colony size	Colony surface	Colony edge	Growth rate (days)
1	DR1	Yellow	Round	Small	Rough	Uneven	1
2	DR2	White	Irregular	Large	Rough	Uneven	1
3	DR3	White to Green	Round	Small	Smooth	Even	2
4	DR4	Yellow	Round	Small	Smooth	Even	1
5	DR5	Yellow	Irregular	Medium	Smooth	Even	1
6	DR6	Yellow	Round	Small	Smooth	Even	1
7	DR7	White	Irregular	Large	Rough	Uneven	1
8	DR8	White	Irregular	Large	Rough	Even	1
9	DR9	Yellow	Irregular	Large	Rough	Uneven	1
10	DR10	White	Irregular	Large	Rough	Uneven	1
11	DR11	White to Yellow	Irregular	Large	Rough	Uneven	1
12	DR12	White	Round	Medium	Rough	Even	1
13	DR13	Pink	Round	Small	Rough	Uneven	1
14	DR14	White	Round	Medium	Rough	Even	1
15	DR15	Yellow	Round	Medium	Smooth	Uneven	1
16	DR16	White to Yellow	Round	Small	Rough	Uneven	1
17	DR17	White	Round	Small	Rough	Even	1
18	DR18	Clear	Round	Small	Smooth	Even	2
19	DR19	Yellow	Round	Small	Smooth	Even	2
20	DR20	Yellow	Round	Small	Smooth	Even	1
21	DR21	White	Round	Medium	Rough	Even	1
22	DR22	White to Yellow	Round	Small	Smooth	Even	1
23	DR23	White	Round	Small	Smooth	Even	2
24	DR24	Yellow	Round	Small	Smooth	Even	2
25	DR25	Pink	Round	Small	Smooth	Even	2
26	DR26	White	Round	Large	Smooth	Even	1
27	DR27	Orange	Round	Small	Smooth	Even	1

Note: Small colony size: 1-2 mm, medium: 3-4 mm, large: ≥ 5 mm.

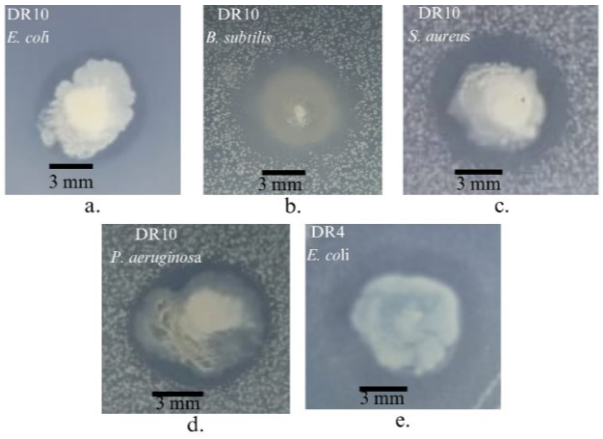

Figure 2. Antibacterial activity of bacterial isolates from *K. galanga* leaves (antagonist method)

Table 2. Results of antagonist method from endophytic bacterial isolates from *K. galanga* leaves (DR) against test bacteria (*B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*)

Code	Inhibition zone (mm)			
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
DR4	-	-	+ 3.00 ± 0.50	-
DR10	++ 5.00 ± 0.00	+ 1.50 ± 0.50	+ 1.00 ± 0.00	+ 1.00 ± 0.00

Note: (-) no activity, (+) weak activity (inhibition zone <5.00 mm), (++) moderate activity (inhibition zone 5.00-10.00 mm), (+++) strong activity (inhibition zone >10.00 mm).

Table 3. Antibacterial activity of ethyl acetate extract from endophytic bacteria from *K. galanga* leaves

No.	Code	Treatment (ppm)	Inhibition zone (mm)			
			B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*
1	(+)	Tetracycline	4.17±0.24	4.33±0.47	2.00±0.00	4.50±0.41
2	(-)	DMSO	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	DR10	10.000	1.83±0.24	2.50±0.00	2.83±0.24	3.00±0.41
4	DR10	5.000	1.33±0.24	2.33±0.24	2.00±0.00	2.33±0.24
5	DR10	1.000	0.00±0.00	0.17±0.24	1.83±0.24	1.83±0.62

Note: DR10: endophytic bacterial isolate from *K. galanga* leaves, (+): Positive control- tetracycline 200 ppm and (-): Negative control- 100% DMSO.

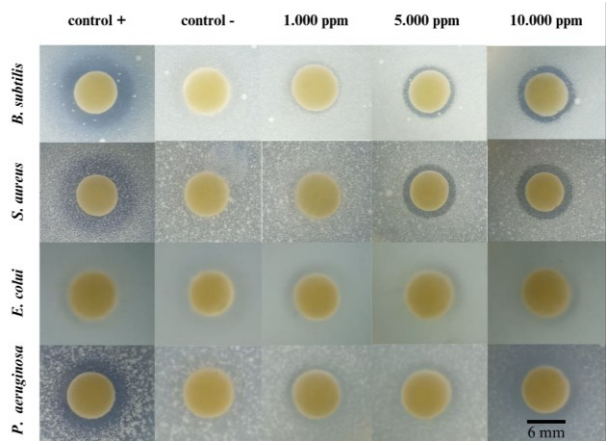

Figure 3. Antibacterial activity of ethyl acetate extract of endophytic bacterial isolate DR10. (control +): 200 ppm tetracycline and (control -): 100% DMSO

3.1.3. Disc Diffusion Test

Among the isolated bacteria, the isolate with code DR10 exhibited broad antibacterial activity against all four test bacteria (Figure 3 and Table 3), making it the isolate selected for further study. As shown in Table 3, the ethyl acetate extract of isolate DR10 demonstrated that higher concentrations resulted in better antibacterial activity against the test bacteria, with the highest activity observed at 10,000 ppm and the lowest at 1,000 ppm. The ethyl acetate extract of isolate DR10 had the largest inhibition zone against *P. aeruginosa*, a Gram-negative bacterium, whereas, in the antagonistic test, DR10 showed higher activity against Gram-positive bacteria than Gram-negative bacteria.

In the disc diffusion test, a disc impregnated with the ethyl acetate extract from the endophytic bacterial isolate is placed on the agar surface. Antibacterial agents in the extract may differ from those in unextracted isolates in terms of strength, activity spectrum, or ability to penetrate bacterial cell walls. Although Gram-negative bacteria have a more complex cell wall, once the antibacterial agent (ethyl acetate extract of endophytic bacterial isolates) penetrates their outer membrane, it may interact with more targets, causing a stronger effect. In contrast, Gram-positive bacteria may be more resistant or have fewer targets, leading to a smaller inhibition zone. The difference in inhibition zone formation may be due to the sensitivity and structural characteristics of the bacteria and the nature of the antibacterial agent [28, 29].

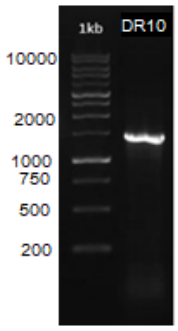

Figure 4. Visualization of DNA bands from the 16S rRNA gene (1300 bp) from endophytic bacterial isolate DR10

3.1.4. Minimum Inhibitory Concentration (MIC) Test and Minimum Bactericidal Concentration (MBC) Test

Furthermore, the MIC (minimum inhibitory concentration, which is the lowest concentration of an antibacterial substance that inhibits bacterial growth) and MBC (minimum bactericidal concentration, which is the lowest concentration of an antibacterial compound that kills 99.9% of bacteria) values of the ethyl acetate extract from the endophytic bacterial isolate DR10 showed MIC values in the range of 1,000 < MIC < 5,000 ppm (Table 4). However, due to the absence of clear wells, the ethyl acetate extract of the endophytic bacterial isolate DR10 did not show MIC values for the test bacterium *P. aeruginosa*. This could be due to several factors, such as natural resistance or insufficient antibacterial concentrations [30, 31]. Additionally, the MBC value of the ethyl acetate extract from the endophytic bacterial isolate DR10 was higher than 625 ppm for *B. subtilis* and higher than 5,000 ppm for *S. aureus* and *E. coli*, indicating that a higher concentration is required to completely kill the test bacteria.

3.2. Molecular Identification and SEM Observation of DR10 Isolate

The molecular identification of the endophytic bacterial isolate DR10 was successfully performed by amplifying the 16S rRNA gene using PCR, and the amplicon was detected using a UV transilluminator (Figure 4). The 16S rRNA analysis results showed that the DR10 isolate had high similarity to *Bacillus* sp. TS8, with a high bit score and a low E-value (0), indicating a very high level of identity (100%) (Table 5). This suggests a high degree of homology between the sample sequences, and an E-value of zero indicates an identical match [32].

Table 4. MIC and MBC test on ethyl acetate extract of endophytic bacteria from *K. galanga* leaves

Code	Test bacteria							
	B. subtilis		*S. aureus*		*E. coli*		*P. aeruginosa*	
	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)
Control +	39.06	>78.12	39.06	>78.12	39.06	>78.12	39.06	>78.12
Control -	-	-	-	-	-	-	-	-
DR10	156.2	>625	5.000	>5.000	5.000	>5.000	-	-

Note: DR10: endophytic bacterial isolate from *K. galanga* leaves, MIC: Minimum Inhibitory Concentration, MBC: and Minimum Bactericidal Concentration, (control +): tetracycline 200 ppm and (control -): DMSO 100%.

Based on the phylogenetic tree, the DR10 isolate also showed the closest similarity to *Bacillus* sp. TS8 (Figure 5). Additionally, in accordance with the molecular analysis results, SEM analysis revealed that the DR10 isolate was rod-shaped (Figure 6). In the study by Mohanty and Kumar [33], this bacterium demonstrated a superior ability to decolorize and detoxify the Indanthrene Blue RS dye, effectively processing waste containing certain dyes in an aerobic environment. However, no studies have linked *Bacillus* sp. TS8 to antibacterial activity.

Table 5. The identity of DR10 isolates according to 16S rRNA sequence

Isolate	Closest relative species	E-value	Query cover	similarity	Accession number
DR10	*Bacillus* sp. TS8	0.00	99%	100%	EU215516.1

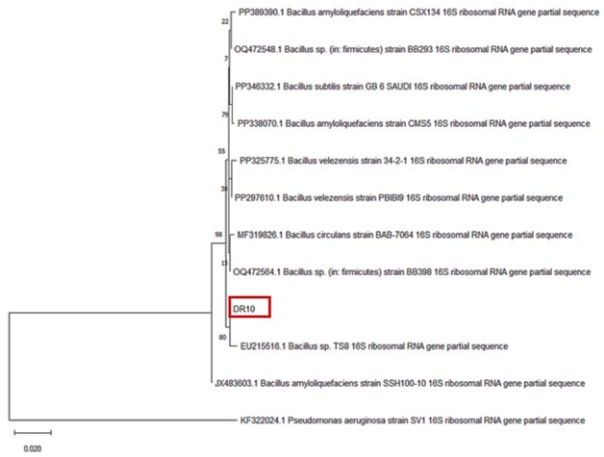

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences from endophytic bacterial isolates DR10

3.2.1. Active Compound Profile Analysis

There are 18 identified compounds, as shown in Table 6, and 51 compounds identified with an area percentage of less than 1% (Figure 7). These compounds have varying retention times. Fifteen of the eighteen compounds are known to have antibacterial, antifungal, anticancer, antioxidant, anti-inflammatory, and antiplasmodial activities. These compounds include pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-; cyclo(l-prolyl-l-valine); n-hydroxymethylacetamide; 2-piperidinone; pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-; 1,4-bis(trimethylsilyl)benzene; methyl glyoxal; butanoic acid, 2-methyl-; l-proline, n-valeryl-, decyl ester; 3-methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione; butanoic acid, 3-methyl-; cyclopentanol, acetate; and dimethyl sulfoxide.

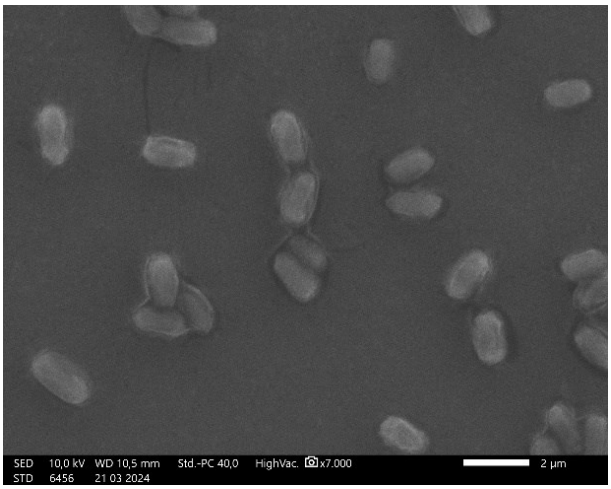

Figure 6. Microscopy observation of DR10 isolate using SEM analysis at 7000× magnification

Table 6. Compounds in endophytic bacterial extract DR10 and their bioactivity

No.	Compounds	Molecular formula	Area%	Retention time (minute)	Bioactivity	Reference
1	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	13.26	19.450	Antibacterial, Anticancer	[34]
2	Uracil, 1,3-dimethyl-6-hydrazino-	$C_6H_{10}N_4O_2$	8.43	21.731	-	-
3	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	6.28	23.496	Antifungal	[31, 32]
4	Cyclo(L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$	6.23	18.253	Antibacterial, antifungal, and antioxidant	[35, 36]
5	N-Hydroxymethylacetamide	$C_3H_7NO_2$	4.64	6.696	Antioxidant	[37]
6	2-Piperidinone	C_5H_9NO	4.36	10,275	Antibacterial, antioxidant, anti-inflammatory, and anticancer	[38, 39, 40]
7	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-	$C_{11}H_{18}N_2O_2$	3.92	17.648	Antibacterial, antioxidant, and anticancer	[41, 42, 43]
8	1,4-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	2.84	17.787	Antibacterial, antioxidant, anticancer, and anti-plasmodial	[44]
9	Methyl glyoxal	$C_3H_4O_2$	2.79	6.809	Antibacterial, antifungal, and antibiofilm	[45]
10	Butanoic acid, 2-methyl-	$C_5H_{10}O_2$	1.98	5.310	Antibacterial, antioxidant, and anticancer	[46, 47, 48]
11	L-Proline, N-valeryl-, decyl ester	$C_{20}H_{37}NO_3$	1.88	18.505	Antibacterial, antifungal, and anticancer	[49]
12	3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	$C_8H_{12}N_2O_2$	1.86	17.056	Antibacterial and antifungal	[50]
13	Butanoic acid, 3-methyl-	$C_5H_{10}O_2$	1.65	5.083	Antibacterial and antioxidant	[47]
14	Cyclo(alanylleucyl)	$C_{12}H_{22}N_2O_2$	1.63	17.396	-	-
15	Ethanol, 2-butoxy-	$C_6H_{14}O_2$	1.46	5.864	Antibacterial and antioxidant	[51, 52, 53]
16	Cyclopentanol, acetate	$C_7H_{12}O_2$	1.24	16.299	Antibacterial	[54]
17	N-Isobutyl-sec-butylamine	$C_8H_{19}N$	1.13	12.077	-	-
18	Dimethyl Sulfoxide	C_2H_6OS	1.05	4.919	Antibacterial and anti-inflammatory	[55]

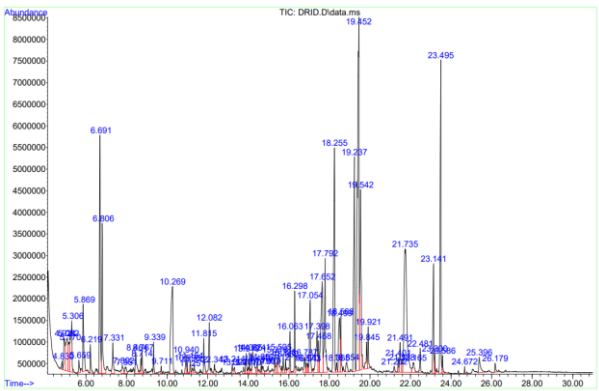

Figure 7. GC-MS chromatogram of endophytic bacterial extract DR10

4. Conclusion

The antibacterial activity of endophytic bacteria from *K. galanga* leaves was demonstrated by the endophytic bacterial isolate coded DR10, which inhibited the growth of four test bacteria (*B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*). Molecular identification based on 16S rRNA sequence analysis of the endophytic bacterial isolate DR10 from *K. galanga* leaves revealed similarities with *Bacillus* sp. TS8. Based on GC-MS results, the active compound suspected to have antibacterial properties in the endophytic bacteria from *K. galanga* leaves is pyrrolo[1,2-a] pyrazine-1,4-dione, which was found in the highest abundance.

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