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Antioxidant activity of various solvent extracts from endophytic bacteria isolated from girang (*Leea indica*) leaves

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Abstract. *Arivo D, Mubarik NR, Rusmana I, Batubara I. 2023. Antioxidant activity of various solvent extracts from endophytic bacteria isolated from girang (*Leea indica*) leaves. Biodiversitas 24: 415-422.* Girang (*Leea indica* (Burm.F) Merr.) is one of the medicinal plants with various bioactive compounds that act as an antioxidant. However, isolating these bioactive compounds need a significant amount of biomass. Endophytic bacteria can be a good solution to produce the same bioactive compounds as their host. This study determined whether the potential isolate produces high antioxidant activity in various solvents, phytochemical screening, determination of total flavonoid and phenolic contents, and identification of the isolate. The BT4 isolate was isolated from *L. indica* leaves. The supernatant of BT4 was extracted in fractionated extraction. The antioxidant activity was determined using a DPPH assay. Folin-Ciocalteu and aluminum chloride colorimetric methods analyzed the total flavonoid and phenolic content. The isolate was identified using the 16S rRNA method. The best IC_{50} was obtained from *n*-butanol extract with a value of 59.021±0.541 µg/mL. The flavonoid and phenolic contents from *n*-butanol extract have the value of 4.686±0.476 mgQE/g extract and 31.406±0.716 mgGAE/g extract, respectively. The BT4 isolate contained flavonoids, saponin, tannins, terpenoids, and steroids and was identical to *Bacillus tequilensis* with 99% similarity. This study revealed that endophytic bacteria from *L. indica* leaves could be a potential source of the novel natural antioxidant compound.

Keywords: Antioxidant activity, *Bacillus tequilensis*, DPPH assay, endophytic bacteria, girang, *Leea indica*

INTRODUCTION

Leea indica (Burm. F.) Merr.) is one of the medicinal plants from Southeast Asia and grows spread in Indonesia (Setyawati et al. 2015). It is generally known as bandicoot berry and have local name e.g, *girang, mali-mali, silungkar, sibo* (Indonesia), *memali hantu* (Malaysia), *chhatri* (Sanskrit), *huo thong shu* (China), *hastipalash* and *kukurjiwah* (Hindi) (Kekuda et al. 2018). *Leea indica* has many properties, e.g., antitumor, sedative, and anxiolytic (Raihan et al. 2012), anti-inflammatory (Sakib et al. 2021), antidiabetic (Patel et al. 2016), hepatoprotective (Mishra et al. 2014), antifungal, radical scavenging activity (Ramesh et al. 2015), antihyperglycemic and hypolipidemic (Dalu et al. 2014), cytotoxic, antioxidant and anticancer (Emran et al. 2012; Reddy et al. 2014; Bogucka-kocka et al. 2016; Ghagane et al. 2017; Chen et al. 2019). Previous studies reported that many bioactive compounds are found in *L. indica* leaves, i.e., alkaloids, carotenoids, coumarins, dihydrochalcones, fatty acids, fatty alcohols, polyphenols, flavonoids, tannins, steroids, glycosides, terpenoids, and saponins (Rahman et al. 2012; Harun et al. 2016; Swamy et al. 2019; Khuniad et al. 2022). The content of these various bioactive compounds causes *L. indica* to have potential as antioxidants (Swamy et al. 2019; Baharom et al. 2020). However, isolating these bioactive compounds requires a significant amount of biomass and long plant growth. The use of endophytic bacteria can be an alternative to overcome this problem.

Endophytic bacteria live in plant tissues without causing damage or disease to the plant (Tripathy et al. 2022). Almost all plants worldwide have endophytic bacteria in their tissues (Pandey et al. 2017). The utilization of endophytic bacteria has advantages. They help host plants' growth, protect against pathogens and environmental stresses, and regulate the synthesis of secondary metabolites in plants; endophytic bacteria obtain nutrients from the host, get a safe niche from extreme environmental conditions, and compete with other microbes in the environment (Wu et al. 2021). Many endophytic bacteria are symbiotic mutualism with medicinal plants, causing they can be able to produce the same bioactive compounds as their host plants. For example, endophytic actinobacterial isolated from *Eleutherine palmifolia* bulbs. produce antioxidants like their host plant (Shabira et al. 2022). Curcumin which can act as an antioxidant produced by *Curcuma longa* (Jakubczyk et al. 2020), and endophytic bacteria from the

stem of *C. longa* also produce antioxidant compounds (Kumar et al. 2017). *Pseudomonas aeruginosa* isolated from *Achyranthes aspera* leaves can produce carotene and linoleic acid, which are also produced by their host plant (Devi et al. 2017). Endophytic microbes isolated from grape leaves (*Vitis vinifera*) can produce t-resveratrol and α-finiferin, bioactive compounds in grape plants (Roat and Saraf 2020). Based on previous studies, it is possible for endophytic bacteria isolated from *L. indica* leaves to be able to produce bioactive compounds that act as antioxidants like their host plants.

Study on antioxidant activity (IC_{50}) of endophytic bacteria isolated from *L. indica* leaves have never been reported yet, and the source of antioxidants from crude extracts are still limited. So, this study is importantly conducted to obtain endophytic bacteria isolated from *L. indica* leaves which can produce strong antioxidant activity. This study can be an element of novelty that increase the knowledge regarding the diversity of endophytic bacteria in *L. indica* leaves that have strong antioxidant activity and its opportunity as a source of new antioxidants that can be used in various industries (cosmetics, pharmaceuticals, food). A preliminary study has covered isolated endophytic bacteria from *L. indica* leaves and screened potential isolates with antioxidant ability (inhibition percentage) (Arivo et al. 2021). Thus, this study will continue with determining potential isolate that produces strong antioxidant activity in various solvent extracts, qualitative phytochemical screening, quantitative determination of total flavonoid and phenolic contents, and identifying potential isolate using 16S rRNA.

MATERIALS AND METHODS

Procedures

Culture stock preparation

We have selected endophytic bacteria from a previous study. BT4 is the selected endophytic bacteria isolated from *L. indica*, characterized by the previous study (Arivo et al. 2021). Production of bioactive extracellular compounds of BT4 was carried out according to Prastya et al. (2021) with some modifications. First, a loop of BT4 isolate was inoculated into 30 mL of tryptic soy broth (TSB) medium and incubated for 24 hours at room temperature. About 1% bacterial culture was put into 1,000 mL TSB in a rotary shaker for 3 x 24 hours at 120 rpm at room temperature. Finally, the supernatant was obtained by separating the culture from the bacterial cells using centrifugation at 6,000 rpm for 20 minutes.

Extraction of endophytic bacterial extracellular bioactive compounds using fractionated extraction method

The extraction of bioactive extracellular compounds of BT4 isolate was carried out by the fractionated extraction method. First, the bacterial supernatant was sequentially extracted using nonpolar-semipolar-polar solvents, i.e., *n*-hexane, ethyl acetate, and *n*-butanol, consecutively. The bacterial supernatant was extracted by adding 500 mL of *n*-hexane (ratio1:1 v/v) to the separatory funnel before shaking for two hours at 80 rpm in an orbital shaker, and then the solution was then filtered. The filtrate was collected as the BT4 *n*-hexane extract. The residue was then extracted further using 500 mL of ethyl acetate (ratio 1:1 v/v) and shaking for two hours at 80 rpm. The solution was filtered and collected as the BT4 ethyl acetate extract. The residue was then extracted using 500 mL of *n*-butanol (ratio 1:1 v/v) and shaking for two hours at 80 rpm. The filtrate was collected as the BT4 *n*-butanol extract. Each filtrate was concentrated under a rotary evaporator and kept at -4°C. The yield percentage was calculated by comparing the weight of the crude extract (g) with the volume (mL) of the supernatant (Sulmartiwi et al. 2018).

Antioxidant activity using DPPH free radical scavenging assay

The free radical scavenging 1.1-diphenyl-2 measured the antioxidant activity of crude extracts- picrylhydrazyl (DPPH), according to a previous study by Batubara et al. (2009). The BT4 crude extract solution at various concentrations (0, 20, 40, 80, 160, 320, and 500 µg/mL) was added into 96 microwell-plate and mixed with 100 µL of 125 mM DPPH solution. The mixture was incubated at room temperature in a dark condition. The absorbance was measured on a spectrophotometer ELISA reader at 514 nm. Ascorbic acid was used as a positive control. The results of the absorption wavelength are used to calculate the percentage of inhibition by the following formula.

$$\%\ inhibition = 1 - \frac{(sample\ absorbance - control\ absorbance)}{(blank\ absorbance - control\ absorbance)} \text{x} 100\%$$

The sample absorbance was a mixture of BT4 crude extract and DPPH, the control absorbance was methanol, and the blanko absorbance was a mixture of methanol and DPPH. The linear regression analysis determined the IC_{50} value.

Phytochemical screening

A total of 0.1 gram BT4 *n*-butanol crude extracts was dissolved in 50 mL methanol and subjected to preliminary phytochemical screening tests, including flavonoids, saponins, tannins, alkaloids, quinones terpenoids, and steroids (Shaik and Patil 2020). The alkaloid test was carried out by adding the sample with 25% NH_4OH and $CHCl_3$. The filtrate was extracted with concentrated HCl. The acid layer was then added with two drops of Dragendorff's reagent. The formation of a brick-red precipitate indicates the presence of alkaloids. The phenolic test was carried out by adding three drops of 1% $FeCl_3$ in ethanol. The formation of green, red, purple, or black colors strongly indicates the presence of phenolics. Tests for flavonoids, quinones, saponins, and tannins were carried out by boiling the sample in hot water for 5 minutes and then divided into 4 test tubes. The first tube was added with magnesium powder, concentrated HCl, and amyl alcohol and shaken vigorously for about 30 seconds. The presence of flavonoids was indicated by the appearance of red, yellow, or orange on the alcohol layer. The second tube was added with 1N NaOH. The appearance of a red

color indicated the presence of quinones. The third tube is shaken vigorously. The presence of foam that does not disappear after being left for 10 minutes indicates the content of saponins. The fourth tube is added with 1% $FeCl_3$ solution, and the appearance of green or blue indicates the presence of tannins. The terpenoid and steroid tests were carried out using a sample on a drip plate. The sample was then added with two drops of acetic anhydride and shaken slowly. The sample was then added with a drop of 96% H_2S. The presence of terpenoids was shown in purple or orange, while steroids were shown in green.

Total Phenolic and Flavonoid Contents (TPC and TFC)

Total phenolic content (TPC) was determined using the *Folin-Ciocalteu* reagent (FCR) method. Each 0.1 gram of BT4 crude extracts was dissolved in 1 mL of methanol pro analysis. As much as 20 µL of the BT4 crude extract was mixed with 110 µL *Folin-Ciocalteu* reagent 10% and 70 µL sodium carbonate 7.5%. The mixture was homogenized and incubated for 30 minutes at a dark room temperature. Gallic acid with variant concentrations (0, 3.125, 6.25, 12.5, 25, 50 100 ug/mL in methanol) was used as a standard curve. The absorbance was then measured at wavelength 765 nm. TPC was expressed as milligrams of gallic acid equivalents (mg GAE)/g extract. Each assay was performed in triplicate (Musdalipah et al. 2021).

Total flavonoid content (TFC) was determined using a colorimetric method with some modifications. Each 0.1 gram of BT4 crude extract was dissolved in 1 mL of methanol pro analysis, mixed with 20 µL of 10% aluminum chloride, 20 µL of 1 mol/L sodium acetate, 300 µL of methanol, and 560 µL of distilled water, and then incubated for 30 minutes at room temperature. The absorbance was then measured at a wavelength of 415 nm. Quercetin was used as the standard curve at various concentrations (0, 2, 4, 6, 8, 10, 20, and 40 g/mL in methanol). Total flavonoid content (TFC) was expressed as milligrams of quercetin equivalents (mg QE)/g extract. Each assay was performed in triplicate (Septiana et al. 2019).

Molecular characterization of endophytic bacteria using 16S rRNA gene sequencing

Molecular characterization of endophytic bacteria began with DNA extraction using the previous study (Alvionita et al. 2020) with some modifications. First, the BT4 genome DNA was extracted using Presto Mini gDNA Bacterial Kit (Geneaid, Taiwan), following the manufacturer's guidelines. The concentration and purity of DNA were then measured using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Next, the 16S rRNA gene was amplified using the primer set 63F (5′-CAG-GCC-TAA-CAC-ATG-CAA-GTC-3') and 1387R (5′-GGG-CGG-WGT-GTA-CAA-GGC-3') on Polymerase Chain Reaction (PCR) machine with the following condition: pre-denaturation at 94ºC for 5 minutes, followed with 35 cycles comprising denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and elongation of 72ºC for 1 minute 39 seconds. The PCR process was ended by post-elongation at 72ºC for 10 minutes and cooling at 4ºC for 7 minutes. The PCR product was visualized in 1% (v/w) agarose gel and sequenced by PT Genetika Science (Jakarta, Indonesia). The DNA sequences were then aligned to the National Center of Biological Information (NCBI) dataset using Basic Local Alignment Search Tool X (BLAST X). Finally, a phylogenetic tree was created using the maximum likelihood method and the Hasegawa-Kishino-Yano model with 500x bootstraps.

Data analysis

All the experiments were performed in triplicates (n = 3). Statistical analysis results were presented as experiments' mean ± standard deviation (SD). The data were analyzed on the resulting means standard deviation by a one-way ANOVA and SPSS 15.0; the Duncan test was used to assess the differences between means. The result was considered statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Endophytic bacteria bioactive compound extraction

The BT4 isolate was grown on a TSA plate for further tests. The fractionated extraction directly affects the physical characteristics of BT4 crude extracts (yields, colors, and shapes). For example, the highest yield was found in BT4 *n*-butanol crude extract, and the lowest was in BT4 *n*-hexane crude extract (Table 1). In addition, the color of the crude extract varies from dark green, yellowish, or brownish, while the physical was a paste or powder (Figure 1).

Antioxidant activity (IC_{50})

We performed the DPPH free radical to asses the BT4 isolate crude extracts' antioxidant activities. The antioxidant activity was expressed in IC_{50}. The IC_{50} value indicates the activity of the extract to stabilize or degrade 50% of the DPPH free radical. Therefore, if the IC_{50} value is lower, the extract's antioxidant activity is higher. We compared the BT4 crude extracts in various solvents (*n*-hexane, ethyl acetate, and *n*-butanol). Based on the results, the lowest IC_{50} was found in BT4 *n*-butanol crude extract with a value of 59.021 ug/mL. Therefore, it is categorized as having a high antioxidant activity (>50 ug/mL). However, the activity value is still smaller than the positive control. The IC_{50} of BT4 *n*-butanol crude extract was eight times lower than ascorbic acid (Table 2).

Phytochemical screening

Phytochemical screening was carried out to provide an overview of the compounds in various solvents contained in each crude extract. We performed seven tests (Flavonoids, tannins, saponins, alkaloids, hydroquinones, triterpenoids, and steroids) to determine the compound groups that can act as antioxidants. Based on the result, the BT4 *n*-butanol crude extract was the most capable of extracting many groups of compounds, namely flavonoids, saponins, tannins, and steroids, compared to the other BT4 crude extracts (Table 3).

Total Phenolic and Flavonoid Contents (TPC and TFC)

Based on the result of phytochemical screening, the BT4 *n*-butanol crude extract was the best extract capable of obtaining many chemical compounds. Thus, we only tested the total phenolic content (TPC) and total flavonoid content (TFC) in the BT4 *n*-butanol crude extract. The TPC was determined using folin-Ciocalteu's reagent and is expressed based on the amount of milligram gallic acid equivalent/gram extract (mg of GAE/g of extract, standard curve equation; y= 0.0064x + 0.021, R2 = 0.99). The TFC was determined using the calorimetric method and quantified based on the amount of milligram equivalent of quercetin (QE)/gram extract (mg of QE/g of extract, standard curve equation; y = 0.0021 - 0.0032, R2 = 0.9949). The standard curves of quercetin and gallic acid for TFC and TPC were plotted, and all the findings were compared to quercetin and gallic acid. The result showed that BT4 isolate extract in *n*-butanol solvent had TPC and TFC of 31.406 ± 0.716 mg GAE/g and 4.686 ± 0.476 mg QE/g extract, respectively (Figure 2).

Identification of endophytic bacteria using 16S rRNA gene

BT4 isolated from *L. indica* leaves was identified using the 16S rRNA gene. Genomic DNA was extracted and examined for purity using a Nanodrop 2000 spectrophotometer (Table 4). The gene successfully amplified was indicated by forming a single band with a sequence length of ± 1300 bp (Figure 3). The amplification of the 16S rRNA gene from BT4 isolate showed high similarity with *Bacillus tequilensis* strain 10b 1465 (NR 104919) (similarity: 99.45%; E-value 0.00; Query cover: 99.53%) (Figure 4).

Table 2. Antioxidant activity of endophytic bacteria BT4 isolate expressed in IC_{50}

Sample	IC_{50} (ug/mL) ± SD
Positive control (ascorbic acid)	7.299 ± 0.020^{a}
n-butanol extract	59.021 ± 0.540^{b}
ethyl acetate extract	196.236 ± 1.410^{d}
n-hexane extract	78.938 ± 1.931^{c}

The values listed above are means and standard deviations in three replicates. One-way ANOVA determined statistically significant differences following the Duncan test (p-value <0.05). Value with the different superscript letters is significantly different.

Table 3. Phytochemical qualitative tests were conducted to determine the content of compound groups in endophytic bacteria crude extracts

Phytochemical test	Solvents		
	n-Butanol	Ethyl acetate	*n*-*H*exane
Flavonoids	+	+	+
Saponins	+	-	-
Tannins	+	-	-
Terpenoids	-	-	-
Steroids	+	+	-
Alkaloids	-	+	+
Hydroquinones	-	-	-

Note: +: means present, and -: means absent

Table 1. Crude extract yield percentage from endophytic bacteria BT4 isolate in various solvents

Solvent	Volume (mL)	Color	Shape	Weight (g) ± SD	Yield (%) ± SD
n-Butanol	2000	Brownish-green	powder	1.335 ± 0.465	0.0668 ± 0.023
Ethyl acetate	2000	Yellowish-green	paste	0.1816 ± 0.062	0.0062 ± 0.006
n-Hexane	2000	Dark-green	paste	0.1396 ± 0.062	0.0081 ± 0.003

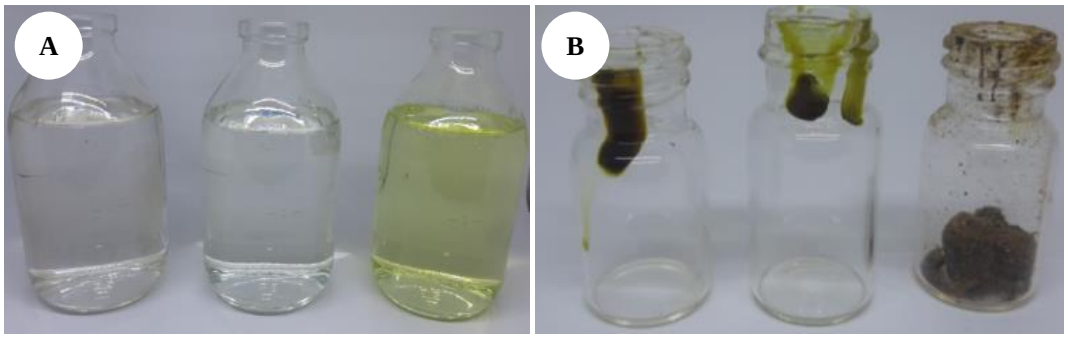

Figure 1. Extraction of secondary metabolites from the bacterial supernatants BT4 isolates in various solvents before evaporation (A) and after evaporation using fractionated extraction (B). *n*-hexane extract (1), ethyl acetate extract (2), and *n*-butanol extract (3)

Discussion

Medicinal plants are known to be a potential source of endophytic microbes that can produce bioactive chemical compounds (Singh et al. 2017). However, this study is the first endophytic bacteria isolated from *L. indica* leaves reported to produce strong antioxidants. Previous studies reported that *L. indica* leaves produce various bioactive compounds that act as antioxidants (Ghagane et al. 2017; Khuniad et al. 2022). *Bacillus tequilensis*, which we isolated, has the same ability as its host plant to produce antioxidants.

Extraction was performed using polar-semipolar-nonpolar solvents, i.e., *n*-butanol, ethyl acetate, and *n*-hexane, consecutively to obtain various bioactive compounds. That is done because the targeted bioactive compound of the crude extracts with high antioxidant activity was yet to be known. Thus, we used fractionated extraction using various solvents to obtain optimal bioactive compound yield extract. The optimal extraction affects the qualitative detection of phytochemical tests, IC_{50}, and quantitative detection of total phenolic and flavonoid contents. For example, it can be shown from the *n*-butanol crude extract with the highest yield, the highest antioxidant activity, and a more diverse group of compounds than the others. The same study was reported by Minsas et al. (2020), which used fractionated extraction to get optimal bioactive compound yields.

The crude extract from various solvents ranged from 0.0062 % to 0.0668 % in various solvents. The BT4 *n*-butanol crude extract, extraction time of two hours, and ratio sample: solvent (1:1) gave the highest yield (Table 1). It indicates that the BT4 *n*-butanol crude extract contained polar compounds rather than nonpolar compounds. Therefore, the content of the extract will be directly correlated with the activity. This study was similar to Elamin (2020), which reported that the *n*-butanol crude extract of *Tamarix gallica* has a higher percent yield compared to other extracts. Extraction of bioactive compounds from microbes is more effective than host plants because it does not require a long time in the production process and does not threaten the biodiversity preservation of host plants (Bogucka-Kocka et al. 2016).

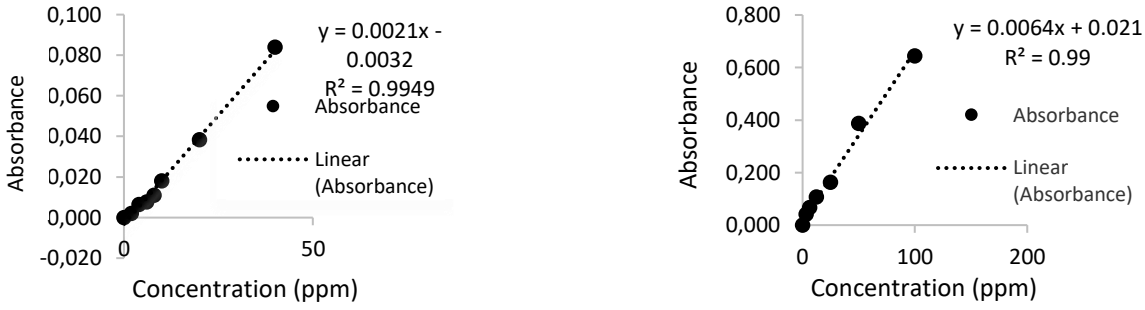

Figure 2. The standard curve of quercetin for quantification of total flavonoid content (A) and gallic acid as a standard for total phenolic content quantifications (B)

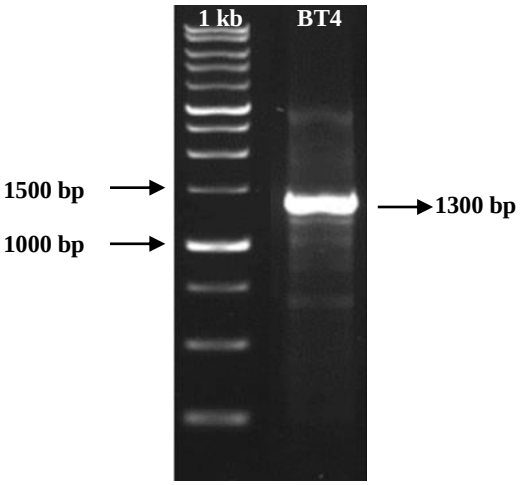

Figure 3. Electrophotogram of 16S rRNA gene amplification with a size of ±1300 bp. Marker: 1 kb ladder

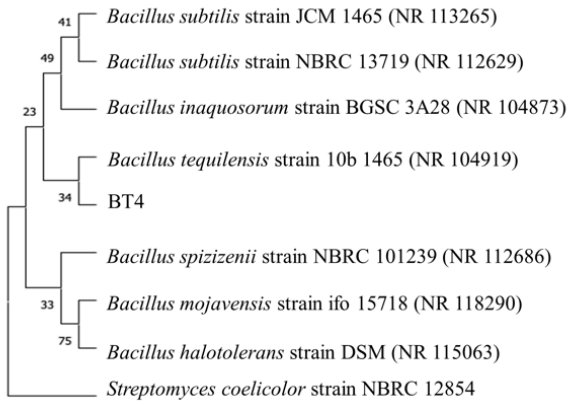

Figure 4. Phylogenetic tree of endophytic bacteria BT4 isolate and its related species constructed by a neighbor-joining model with 500x bootstraps

Solvents are key in obtaining target constituents of desired quality and quantity. Solvent selection is based on chemical properties, i.e., the polarity and target compound hydrophobicity (Joshi and Adhikari 2019). Polar solvents could attract more bioactive compounds than semipolar and nonpolar solvents (Nawaz et al. 2020). N-butanol is the shortest linear alcohol; when mixed with water is capable of forming separate phases under ambient conditions. Thus, it is very suitable for liquid-liquid extraction from water so that it can be able to extract polar compounds from aqueous solutions, which produces a high yield (Reetz and König 2021).

Moreover, we assessed the antioxidant activity against the DPPH scavenging assay to analyze its biological activity. Molyneux (2004) categorizes the antioxidant activity of an active compound based on the IC_{50} values, i.e., very strong (<50 µg/mL), strong (50-100 µg/mL), moderate (100-150 µg/mL), weak (150-200 µg/mL) and very weak (>200 µg/mL). Based on the results, *n*-butanol and *n*-hexane extracts had strong antioxidant activity classification. In contrast, ethyl acetate extract had weak antioxidant activity (Table 2). the *n*-butanol extract had the strongest DPPH antioxidant activity with an IC_{50} value of 59,021 ± 1,931 µg/mL. This study was similar to Hassane et al. (2022), which reported that the *n*-butanol crude extract of endophytic microbe isolated from black seed had the highest antioxidant activity than other solvents. Lahneche et al. (2019) reported that the *n*-butanol extract of *Centaurea sphaerocephala* had IC_{50} with a value of 16.67 ± 0.11 µg/mL. However, this value was still higher than the positive control of ascorbic acid, which had an IC_{50} value of 7.299 ± 0.020 g/mL. These results indicate that *n*-butanol extract has eight times weaker DPPH antioxidant activity than ascorbic acid.

The results of the phytochemical test showed that *n*-butanol crude extract contained the largest group of compounds, i.e., flavonoids, saponins, tannins, and steroids (Table 3). This result indicates that *n*-butanol extract is thought to have antioxidant activity due to the presence of various compounds. These compounds are known to be a group that reported having a dominant antioxidant activity (Banjarnahor and Artanti 2014). Furthermore, Nawaz et al. (2020) reported that polar solvents could attract various bioactive compounds, including flavonoids, terpenoids, tannins, saponins, polypeptides, steroids, alkaloids, and anthocyanins.

The bacterial extract obtained was then calculated as the number of chemical compounds, including total phenols and flavonoids. The results showed that the BT4 *n*-butanol crude extract had a high total phenol content. There are still no reports on the analysis of chemical compounds, including total phenols and flavonoids from bacterial extracts associated with *L. indica* leaves. Therefore, we analyzed the relationship between the high content of chemical compounds and antioxidant activity. Previous studies reported that *L. indica* has abundant biological and pharmacological activities in leaves. *L. indica* leaves had high phenolic compounds and strong antioxidant activity (Singh et al. 2019). Sulistyaningsih et al. (2017) reported that the total phenols and flavonoids of *L. indica* leaf methanol extract were 267.56±2.01 mg GAE/g extract and 101.90±3.24 mg QE/g extract, respectively. The antioxidant activity correlated with total phenols and flavonoids (Musdalipah et al. 2021). It indicates that phenol and flavonoid compounds are dominant contributors to the antioxidant activity of *L. indica* leaf (Reddy et al. 2012). Therefore, we analyzed the relationship between high chemical compound content and antioxidant activity.

Molecular identification of BT4 isolates using the 16S rRNA gene. The 16S rRNA gene was chosen because it has the ideal size of ±1500 bp. This size is statistically significant enough for informational purposes compared to the 5S rRNA gene and 23S rRNA gene with a more extended size. In addition, it is present in all cells. It has the same function in every cell and does not change quickly. If a change occurs, it can be well conserved, so it is quite accurate to show the evolutionary distance (Ibal et al. 2019). The sequencing result showed that BT4 had 99.34% similarity with *Bacillus tequilensis* strain 10b 1465 (NR 104919). Isolates with 16S rRNA similarity of more than 97% could represent the same species, while sequence similarities between 93% and 97% could represent the identity of bacteria at the genus level (Salih et al. 2019). This result is similar to previous studies that reported *B. tequilensis* as endophytic bacteria isolated from *Angelica dahurica* (Li et al. 2018) and *Solanum lycopersicum* (Bhattacharya et al. 2019). *Phaenibacillus* and *Bacillus* are endophytic bacteria found in the medicinal plant *Eucalyptus pellita* (Susanti et al. 2021). Pudjas et al. (2022) reported *Bacillus* as endophytic bacteria derived from *Hoya multiflora* Blume. produced antioxidant activity. *Bacillus* isolated from *Berberis lycium* produced antioxidant activity (Nisa et al. 2022).

In conclusion, *B. tequilensis* is endophytic bacteria that was first reported to produce antioxidants from *L. indica* leaves. The crude extract *n*-butanol of this bacteria had the strongest antioxidant activity correlated with total phenolic and flavonoid contents. This extract contains flavonoids, saponins, tannins, and steroids. Thus, this endophytic bacteria could be a potential source of novel antioxidant compounds. This study will be continued with the search for the active fraction of compounds that act as strong antioxidants, which will then be analyzed using LCMS. In addition, the diversity of endophytic bacteria in leaves will be analyzed using metagenome analysis.

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