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Toxicity test of rose periwinkle (*Catharanthus roseus*) leaves endophytic bacteria using Brine Shrimp Lethality Test (BSLT) method

FAUZIAH FAUZIAH[1,♥], MAULINASARI[2], ESSY HARNELLY[1], YULIA SARI ISMAIL[1], LENNI FITRI[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala. Jl. Syech Abdurrauf No. 3, Kopelma Darussalam, Syiah Kuala, Banda Aceh 23111, Aceh, Indonesia. Tel.: +62-651-7410248, Fax.: +62-651-755138, ♥email: fauziah@unsyiah.ac.id

[2]Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala. Jl. Syech Abdurrauf No. 3, Kopelma Darussalam, Syiah Kuala, Banda Aceh 23111, Aceh, Indonesia

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Abstract. *Fauziah F, Maulinasari, Harnelly E, Ismail YS, Fitri L. 2021. Toxicity test of rose periwinkle (*Catharanthus roseus*) leaves endophytic bacteria using Brine Shrimp Lethality Test (BSLT) method. Biodiversitas 23: 171-177.* Endophytic bacteria can be obtained from plants and may have the same ability as their host to produce secondary metabolites. This study aimed to determine the toxicity of endophytic bacterial extract from rose periwinkle (*Catharanthus roseus*) leaves using the Brine Shrimp Lethality Test (BSLT) method to determine their secondary metabolite contents. A toxicity test was carried out using *Artemia salina* shrimp larvae. The effect of the extract was determined based on the shrimp larvae mortality percentage using four concentration levels of 1000 ppm, 750 ppm, 500 ppm, and 250 ppm. Furthermore, the LC_{50} value of the extract against shrimp larvae was analyzed using SPSS software to obtain eight isolates of endophytic bacteria from *Catharanthus roseus* leaves. Macroscopic observations showed different characters, and five isolates were Gram-positive bacteria, while three were Gram-negative bacteria. The BETD5 isolate had the lowest LC_{50} value of 413,590 ppm, and the secondary metabolites of BETD5 extract were alkaloid and saponin. The molecular identification indicated that BETD5 had the highest identity percentage of 99.38% with *Staphylococcus arlettae* strain NR_036903, *Staphylococcus gallinarum* strain LMG 19119, *Staphylococcus* sp. strain Fop 222, *Staphylococcus gallinarum* strain E2 and *Staphylococcus* sp. strain MFC2.

Keywords: *Artemia salina* Leach, BSLT, endophytic bacteria, LC_{50}, rose periwinkle

INTRODUCTION

Endophytic bacteria live in plant tissues without causing disease symptoms to the host. Several types had been reported to have antibacterial, antifungal, and anticancer activities (Purwanto et al. 2014). Klimova et al. (2017) explained that the symbiosis between plants and endophytes benefits both parties. The advantage for endophytes is to obtain nutrients from plants, while plants get protection from pathogens. There have been many studies on endophytic bacteria aimed at finding new bioactive compounds. These bacteria can obtain the active content of a plant without many samples. Also, it can be isolated from various types of medicinal plants, such as the rose periwinkle (*Catharanthus roseus*).

According to Kharwar et al. (2008), rose periwinkle is a herbal plant that can grow wild or be cultivated as an ornamental plant. In a study conducted by Das and Sharangi (2017), rose periwinkle was used as a medicine for diabetes (vindolineindolinee and vindolicine), hypoglycemic (ursolic acid), and cancer (vincristine, vinblastine, catharanthine). Furthermore, Mekky et al. (2017) found that the vinblastine and vincristine active substance in rose periwinkle can act as an anticancer. Vincristine is a bisindole alkaloid group that works by inhibiting cell division, and the content may also be produced by endophytic bacteria living in rose periwinkle. Oktavia and Pujiyanto (2018) stated that endophytic bacteria can be isolated from plant body tissues, such as flowers, leaves, stems, and roots.

One of the methods often used to test the early stages of anticancer compounds is a toxicity test using the BSLT (Brine Shrimp Lethality Test) method to determine a secondary metabolite compound's 50% Lethal Concentration (LC50) value. According to Hodgson and Levi (2000), LC_{50} estimates the concentration of a substance that can kill 50% of the total tested animals. Therefore, the BSLT method is fast, easy, inexpensive, and straightforward in the early stages of cytotoxic testing and screening for anticancer drugs. This method used *Artemia salina* Leach shrimp larvae to determine the $LC5_0$ value. Subsequently, this study was conducted to obtain endophytic bacteria derived from leaves extract of *Catharanthus roseus*, which has a toxic effect against *Artemia salina* Leach shrimp larvae.

MATERIALS AND METHODS

Isolation and purification of endophytic bacteria

Isolation of endophytic bacteria from periwinkle leaves was initiated by sterilizing the plant surface following the modified Fisher et al. (1993) method. The leaves are washed with running water to remove dust and dirt in the first stage before air-drying the sample. Subsequently, each leaf part was immersed in 70% alcohol for 2 minutes, 5.25% sodium hypochlorite for 2 minutes, and 70% alcohol for 1 minute. The samples were then rinsed four times with

sterile distilled water and air-dried for about 15 minutes before being ground with a sterile mortar to remove the liquid from the leaves. A micropipette placed 0.1 1 of the resulting liquid in a petri dish containing sterile NA media. Furthermore, the petri dishes were labeled and incubated at 37°C for 24-48 hours.

The endophytic bacteria were purified by inoculating one colony into new media, and the purification was carried out to obtain a single isolate (Handayani et al. 2015). Afterward, morphological characterization was conducted by observing macroscopically and microscopically.

Extraction of endophytic bacteria obtained from leaves of rose periwinkle

Bacterial isolates from the NA medium were taken and then separated using a cork borer with a diameter of approximately 5 mm. A total of 5 isolates were put into an Erlenmeyer containing 150 mL of Nutrient Broth (NB) medium, then incubated in a shaker incubator at a speed of 150 rpm for one day at room temperature. Cell biomass was separated using filter paper and then centrifuged at 11,000 rpm for 15 minutes and filtered using filter paper. Afterward, the supernatant was used as a test solution. Each dilution carried out with different concentrations was labeled 1,000, 750, 500, 250 ppm and control to have five types of concentration tested on *A. salina* shrimp larvae in the BSLT test (Sandrawati et al. 2019).

Preparation of *Artemia salina* Leach shrimp larvae

Preparation of shrimp larvae was conducted by taking 1 gram of *A. salina* eggs. Subsequently, the hatching was completed by immersing the eggs in 2 L artificial seawater with the lighting of 40-60 watt incandescent lamps and aerated for 48 hours (Frengki et al. 2014).

Brine Shrimp Lethality Test (BSLT)

A total of 1 mL extract solution was taken and put into a test tube, then seawater and ten larvae of *A. salina* were added until the volume reached 5 mL. As a control, seawater containing ten larvae of *A. salina* was put into a test tube until the volume reached 5 mL. After being left for 24 hours, the live and dead *A. salina* larvae were counted, and each sample was tested for mortality three times. The data obtained were entered into the observation sheet and analyzed to determine the LC_{50} value. The percentage of death/mortality of the test animals was used to analyze toxicity data. Artemia salina Leach larvae mortality for 24 hours after treatment served as an indicator of the toxic level. The following formula was used to calculate the percentage of mortality (Meyer et al. 1982).

$$\% \text{ of death larvae} = \frac{\text{Number of death larvae}}{\text{Total number of initial larvae}}$$

LC_{50} for testing the toxicity effect on *A. salina* Leach was determined using probit analysis. As a result, the concentration that resulted in 50% mortality in *A. salina* Leach larvae was discovered.

Secondary metabolite content test

The secondary metabolite test was carried out on bacterial isolates with the lowest LC_{50} value. The tests consisted of flavonoid, alkaloid, steroid/terpenoid, saponin, and phenol/tannin test.

Flavonoid test

Flavonoid test was done using HCL solution, and then about 2-4 and 2 pieces of Mg metal were added to the extract solution. The indicated flavonoid was discovered through color changes from dark yellow to orange (Harborne 1987).

Alkaloid test

A 2 mL test extract solution was evaporated on a porcelain plate until residue was produced. After that, the residue was dissolved in 5 mL of HCl 2 N, and three test tubes were filled with the solution. The first, which serves as a blank, was filled with 2 N HCl. Three drops of Dragendorff reagent were added to the second tube, and three drops of Mayer reagent were supplemented to the third. Subsequently, alkaloids were visible in the production of orange deposits in the second tube and white to yellowish deposits in the third tube (Harborne 1987)

Steroid/terpenoid test

A Liebermann-Burchard reagent was used to test the extract solution. First, a chloroform solution dissolved in acetic anhydride was dripped onto the solution, and the concentrated sulfuric acid was then squeezed. A change indicates the presence of terpenoids in color from orange to purple. Finally, when it becomes blue, it indicates the presence of steroids (Harborne 1987).

Saponin test

The saponin test was performed using the fourth method, which involved placing 2 mL of the sample into the test tube, adding 10 mL of distilled water, shaking it for 30 seconds, and observing the results. Saponin was present when a solid foam was created in less than 30 seconds (Harborne 1987).

Phenol/tannin test

The test extract solution was reacted with a 10% iron (III) chloride solution to detect phenol and tannin compounds when the color was dark blue, blackish-blue, or greenish-black (Harborne 1987).

Molecular identification based on 16S rRNA Gene

The identification of the selected isolates was carried out based on the 16S rRNA gene sequence. First, extraction of DNA isolates was carried out using Genomic DNA Mini Kit (Geneaid) followed by amplification of 16S rRNA gene using Primers 20F (5'GATTTTGATCCTGGCTCAG3') and 1500R (5'GTTACCTTGTTAC-GACTT3').

The amplification reaction was conducted using a Thermal cycler (model 480 Perkin-elmer, USA), with the following cycles: 5 minutes of initial denaturation at 94°C, 45 seconds of denaturation at 94°C, and 45 seconds of primary annealing at 57°C, 1 minute extension at 72°C and

7 minutes of final extension at 72°C, a total of 30 cycles. The amplification results were obtained using 1% agarose gel at 70 volts for 45 minutes. The electrophoretic staining was conducted using ethidium bromide for 15 minutes and visualized using a transilluminator UV lamp. The DNA bands that appeared were documented using Gel Doc (Handoyo and Rudiretna 2001).

The PCR results were sequenced using sequencing service MacroGen, Korea. DNA sequence analysis was performed using the Bioedit program, and BLAST analysis was performed on the NCBI data library gene bank. Phylogenetic analysis or kinship between endophytic bacteria was conducted using a phylogenetic tree using the neighbor-joining method and MEGA 6.0 software (Tamura et al. 2013). The topology of phylogenetic tree construction was evaluated using bootstrap analysis with 1000 replications.

Data analysis

The data obtained from morphological characterization are shown in the figures and tables. Also, the data from the toxicity test were re-analyzed by probit analysis using SPSS Statistics 22.0 application to determine LC_{50} value (Chusniasih and Tutik 2020).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria

The results obtained eight isolates of endophytic bacteria from the leaves of *Catharanthus roseus* L. The umber of isolates obtained was different from study conducted by Oktavia and Pujiyanto (2018), which did not find endophytic bacteria from *Catharanthus roseus* L leaves. The leaves samples were from Diponegoro University, Semarang, while the leaves were from the Alue Naga coast. Furthermore, the difference in the isolation results obtained was due to the different sampling locations. According to Wulandari et al. (2012) and Afzal et al. (2019), one factor influencing the diversity of endophytic bacteria and the process of successful colonization in plants was the geographical condition of the environment, age of the host plant, climate, and soil conditions.

Macroscopic and microscopic morphological characteristics of endophytic bacteria obtained from rose periwinkle leaves

Macroscopic characterization of endophytic bacteria in rose periwinkle leaves can be seen in Figure 1.

BETD was assigned to each endophytic bacteria isolated from Rose periwinkle leaves (Endophytic Bacteria of Rose periwinkle). According to Parija (2012), macroscopic characterization of bacterial morphology can be accomplished by observing the colony's shape, elevation, margin, and color as shown in Table 1.

Based on macroscopic observations, endophytic bacterial isolates obtained had different colony shapes, colors, margins, and elevations. Colony shapes varied from circular and irregular, while colors varied from cream and milky white. The colony margin obtained was entire, and the elevation varied from convex, raised, and flat.

The microscopic observations based on Gram staining showed that the shape of endophytic bacterial cells from the leaves was cocci and bacilli (Figure 2). There were six isolates of coccus-shaped bacteria, while bacilli-shaped ones were two. The purpose of Gram staining was not only to determine the shape but the class of a bacterium or to classify bacteria based on the composition of the cell wall. The Gram staining results exhibited five isolates belonged to the group of Gram-positive, and three isolates belonged to the group of Gram-negative. According to Rahayu and Muhammad (2017), Gram staining can be regarded as one of the methods to identify a bacterium. This method is a useful differential staining and is the most widely used in the microbiology laboratory.

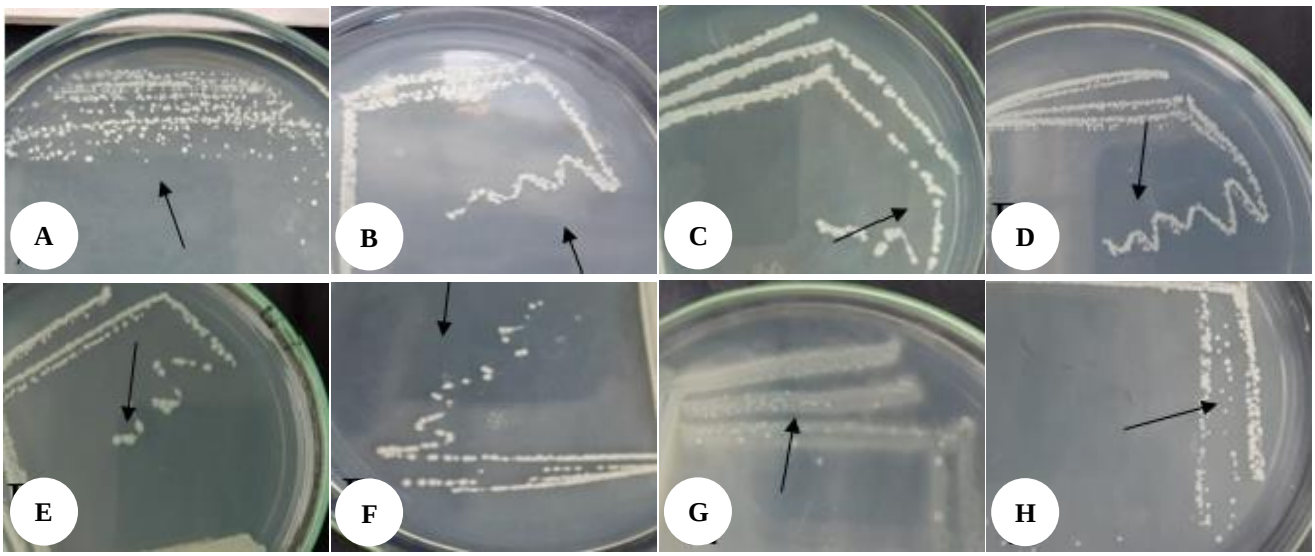

Figure 1. The results of macroscopic observations of one-day-old endophytic bacteria obtained from *Catharanthus roseus* leaves in NA media, (A) isolate BETD1, (B) isolate BETD2, (C) isolate BETD3, (D) isolate BETD4, (E) isolate BETD5, (F) isolate BETD6, (G) isolate BETD7, (H) isolate BETD8

Table 1. The morphological (macroscopic) character of endophytic bacterial isolates on rose periwinkle (*Catharanthus roseus*) leaves

Isolate	Macroscopic Morphology				Microscopic Morphology	
	Colony Shape	Colony Color	Margin	Elevation	Gram	Isolate Form
BETD1	Circular	White	Entire	Raised	Positive	Cocci
BETD2	Irregular	Cream	Entire	Convex	Negative	Cocci
BETD3	Circular	Cream	Entire	Raised	Positive	Cocci
BETD4	Circular	Milky white	Entire	Flat	Negative	Cocci
BETD5	Circular	Milky white	Entire	Raised	Positive	Cocci
BETD6	Circular	Cream	Entire	Raised	Positive	Baccili
BETD7	Irregular	Cream	Entire	Convex	Negative	Baccili
BETD8	Circular	Milky white	Entire	Convex	Positive	Cocci

Table 2. *Artemia salina* Leach mortality due to endophytic bacterial isolate extract

Concentration (ppm)	Mortality(number)							
	BETD 1	BETD 2	BETD 3	BETD 4	BETD 5	BETD 6	BETD 7	BETD 8
1000	28	29	29	23	29	25	26	29
750	26	26	24	24	25	24	27	23
500	8	9	7	8	20	6	8	7
250	3	8	4	3	5	2	7	3
0	0	1	2	1	1	0	1	1
Total Mortality	65	73	66	59	80	57	69	63

Brine shrimp lethality test (BSLT)

BSLT (Brine Shrimp Lethality Test) is a preliminary screening used to monitor bioactive compounds or compounds suspected of being efficacious as anticancer drugs. According to Mayang et al. (2021), a toxicity test was conducted to determine the toxic effect of giving a substance within 24 hours. The toxicity test results using the BSLT method revealed that the highest mortality value of larvae was found at concentrations of 1000 ppm, followed by 750, 500, and 0 ppm. Therefore, different concentration levels were used to investigate the relationship between the test solution and the mortality of *A. salina*. Table 2 shows the effect of different concentrations of *Catharanthus roseus* leaves extract on *A. salina* Leach larvae.

Table 2 showed that the highest total larval mortality was found on BETD5 extract with 80 *A. salina* larvae killed, while the lowest total mortality was found on BETD6 extract, which was 57 individuals. The observation results indicated that all concentrations of endophytic isolate extract caused the death of *A. salina* larvae.

The concentration of the endophytic bacterial extract was directly proportional to the mortality of *A. salina* larvae. This is because the higher the concentration used, the more active compound content can be produced. According to Setiawan et al. (2018), the toxicity test results on *A. salina* larvae indicated that the concentration of the extract was directly proportional to the mortality percentage of *A. salina* larvae. Therefore, the higher the concentration of the extract given, the higher the mortality percentage of *A. salina* larvae.

LC_{50} value was calculated from the mortality of *A. salina* larvae caused by administering the endophytic bacterial extract. The results of LC_{50} value were obtained using probit analysis with a logarithm value of 10, as seen in Table 3.

LC_{50} value indicated the estimated concentration of test solution that can kill 50% of the tested animal population (Hodgson and Levi 2000). The results of probit analysis in Table 3 showed that all LC_{50} values obtained were toxic, where the concentration obtained was <1000 ppm. This result was consistent with Meyer et al. (1982) study, where an extract will show toxic activity when it can cause the death of 50% of the test animals at a concentration of < 1,000 ppm. However, the extract is not toxic when it can cause the death of 50% of the tested animals at a concentration of > 1,000 ppm. According to Carballo et al. (2002), when the LC_{50} value using the BSLT method on bacterial extracts was toxic, it can be developed as an anticancer drug. The results also signified that the lowest LC_{50} value was obtained with BETD5 isolate extract, which was 413,590 ppm. Therefore, this isolate can be tested for secondary metabolite content and molecular identification.

Table 3. LC_{50} value of endophytic bacterial extract obtained from rose periwinkle

Isolate	LC_{50} (ppm)
BETD 1	610.788
BETD 2	639.978
BETD 3	643.842
BETD 4	648.558
BETD 5	413.590
BETD 6	654.254
BETD 7	647.426
BETD 8	642.847

Note: ppm: Parts per million, LC_{50}: Lethal Concentration

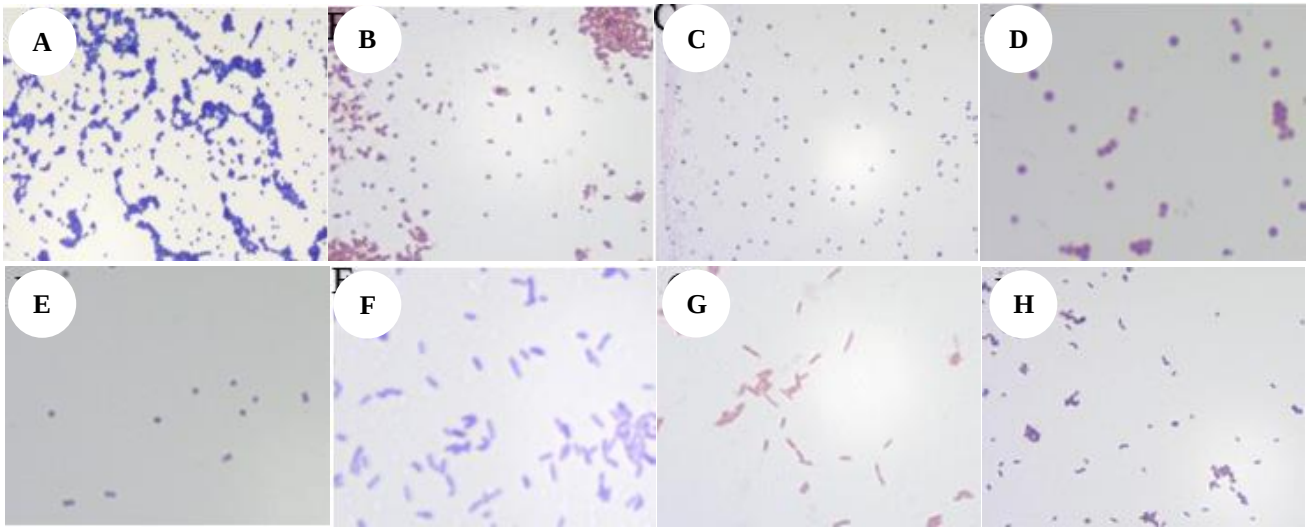

Figure 2. The results of Gram staining of endophytic bacteria from rose periwinkle leaves, (A) isolate BETD1, (B) isolate BETD2, (C) isolate BETD3, (D) isolate BETD4, (E) isolate BETD5, (F) isolate BETD6, (G) isolate BETD7, (H) BETD8 isolate (1000 x magnification)

The content of secondary metabolites from BETD5

The results of secondary metabolite testing of BETD5 extract are shown in Table 4. These qualitative data presented the secondary metabolite content of BETD5 extract. Subsequently, the test of secondary metabolite content was carried out to know the secondary metabolite compounds contained in BETD5.

The results of secondary metabolite tests showed that BETD5 isolate contained two types of compounds, namely alkaloids and saponins. The alkaloid content in the endophytic bacterial extract BETD5 can be seen from the addition of Mayer's reagent, which formed a white precipitate. Furthermore, Wagner's reagent formed a brown precipitate, while Dragendroff reagent formed a red precipitate. Saponin compounds were characterized by foam formation when the endophytic bacterial extract was added with distilled water and then shaken for ± 5 seconds (Table 4). The mechanism of alkaloids and saponins as anticancer compounds became poison for larvae. This was under Marzuki et al. (2019) statement, which revealed that alkaloid compounds can act as stomach poisons. When the compounds enter the body of *A. salina* larvae, their digestion will be disrupted.

Other compounds such as flavonoids, steroids, terpenoids, phenolics, and tannins showed negative results. This was presumably because the endophytic bacterial extract had low immersion. It contained a lot of water solvent that did not evaporate, thus allowing the secondary metabolite compounds in the bacterial extract to be insufficient when tested.

Identification of BETD5 Based on 16S rRNA

Molecular identification was performed on the isolate BETD5 with the lowest LC_{50} value. According to the BLAST results (Table 5), BETD5 had the highest percent identity of 99.38% with *Staphylococcus arlettae* strain NR 036903, *Staphylococcus gallinarum* strain LMG 19119, *Staphylococcus* sp. strain Fop 222, *Staphylococcus gallinarum* strain E2 and *Staphylococcus sp.* MFC2 strain. A study conducted by Haidar et al. (2018) discovered that *Staphylococcus arlettae* is an endophytic bacterium obtained from *Corchorus olitorius*. Recently, staphylococcus has been identified as beneficial plant-associated microbes. Adaptation with evolutionary changes have allowed some pathogenic strains like *Staphylococcus*, become endophytic bacteria.

A phylogenetic analysis is required to characterize the position and link between the findings and the sequences recorded in the NCBI Database. Figure 3 shows a phylogenetic tree of BETD5 endophytic bacterial isolates based on the 16S rRNA gene.

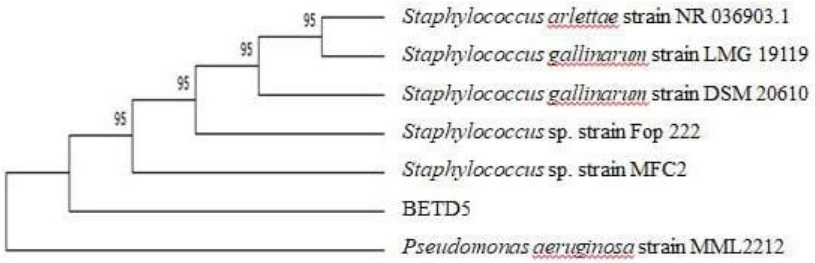

Figure 3. Reconstructed phylogenetic tree of BETD5 isolates based on the 16S rRNA

Table 4. The content of secondary metabolites in BETD5

Secondary metabolite	Reagent	BETD 5	Result
Alkaloid	Mayer	+	White precipitate is formed
	Wagner	+	Brown precipitate is formed
	Dragendroff	+	Red precipitate is formed
Flavonoid	HCl dan Logam Mg	-	Orange precipitate is not formed
Steroid	Liebermann-Buchard test	-	Green or blue precipitate is not formed
Terpenoid	Liebermann-Buchard test	-	Red or purple precipitate is not formed
Saponin	Aquadest	+	Foam Formed
Fenolik	$FeCl_3$	-	There is no green color
Tanin	$FeCl_3$	-	There is no green color

Annotation: (+) showed positive result, while (-) showed a negative result

Table 5. Alignment data of several BLAST organisms with BETD5 isolates

Description	Max score	Total score	Query cover	E value	Ident (%)	Accession
Staphylococcus arlettae strain NR 036903.1	2623	2623	99%	0.0	99.38%	MN851075.1
Staphylococcus gallinarum strain LMG 19119	2623	2623	99%	0.0	99.38%	MK015788.1
Staphylococcus gallinarum strain DSM 20610	2623	2623	99%	0.0	99.38%	MK015772.1
Staphylococcus sp. strain Fop 222	2623	2623	99%	0.0	99.38%	MF678881.1
Staphylococcus sp. strain MFC2	2623	2623	99%	0.0	99.38%	MH071157.1

The results of phylogenetic tree construction using the Neighbour-Joining algorithm with 1000x replication showed that BETD5 was resolved earlier than the Staphylococcus group. However, this sample formed a monophyletic group to the staphylococcus with a bootstrap value of 95 (Figure 3). This result indicated that this isolate belongs to the Staphylococcus genus, but 16S rRNA cannot identify the BETD5 sequence until species level. Therefore, further identification using taxonomic polyphasic should be conducted to determine the species of BETD5. The bootstrap value is listed in the branching of the phylogenetic tree, and it indicates the degree of branching accuracy in the phylogenetic tree. The level of topological confidence in the phylogenetic tree reconstruction is directly proportional to the bootstrap value (Yuliani et al. 2017). According to Hall (2001), a clade can be trusted with a bootstrap value of 90% but not 25%. Furthermore, Hillis and Bull (1993) stated that bootstrap analysis with 70% or higher values indicates a reliable phylogenetic group.

In conclusion, a total of 8 endophytic bacterial isolates were successfully obtained from *Catharanthus roseus* L leaves. The results of macroscopic morphological characters showed that the isolates had different characteristics. Meanwhile, the microscopic morphological characters demonstrated that six isolates were cocci, and two were bacilli. Five isolates were classified as Gram-positive bacteria, while three were Gram-negative bacteria. All endophytic bacterial extracts had an LC_{50} value < 1000, but the smallest was BETD 5, with an LC_{50} value of 413,590 ppm. Therefore, the isolate can be further tested to see its ability as an anticancer. The results of molecular identification confirmed that BETD5 had the highest percent identity with the *Staphylococcus arlettae* strain NR_036903, *Staphylococcus gallinarum* strain LMG 19119, *Staphylococcus* sp. strain Fop 222, *Staphylococcus gallinarum* strain E2 and *Staphylococcus* sp. MFC2 strain that is equal to 99.38%.

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