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Toxicity And Antioxidant Activities of Endophytic Bacteria from Butterfly Pea (*Clitoria ternatea* Linn.)

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Abstract. Endophytic bacteria are bacteria that live in healthy plant tissues without causing damage. Several studies have reported that endophytic bacteria can produce active compounds similar to those secreted by their host and which potentially have medicinal value. Butterfly pea (*Clitoria ternatea* L.) was noted to be able to produce antioxidants and have toxicity potential from its compounds. Therefore, endophytic bacteria from butterfly pea have great potential to have antioxidant activity along with evaluating the toxicity level of the selected bacteria. This study aimed to determine the number of isolates, to characterize, and test the toxicity and antioxidant activities of endophytic bacteria from butterfly pea. Toxicity level was tested using the *Brine Shrimp Lethality Test* (BSLT) method while levels of antioxidants were tested using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. A total of fifteen endophytic bacteria were obtained and successfully purified. Based on the morphological observations, Gram staining, and biochemical test results, isolate EBT13 was determined to belong to the genus *Bacillus*. Isolate EBT13 was categorized as highly toxic, with the highest toxicity value with other bacterial isolates with an LC_{50} of 84 ppm and antioxidant activity with an IC_{50} value of 44.32 ppm. Based on the phylogenetic tree of 16S rRNA gene analysis, EBT13 belongs to the genus *Bacillus*, it forms a sister group with *Bacillus pumilus* with a bootstrap value of 100%. This study advances our knowledge of plant-microbe interactions by identifying a highly toxic, antioxidant-producing strain of bacteria of butterfly pea. The results have significance for the development of cytotoxic chemicals and natural antioxidants, which could advance biotechnological research and boost therapeutic purpose.

Keywords: antioxidant; endophytic bacteria; *Bacillus pumilus*; *Clitoria ternatea* L.; toxicity

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INTRODUCTION

Butterfly pea (*Clitoria ternatea* L.) is a plant species widely recognized for its medicinal properties (Afrianto et al., 2020). Plant organs are a natural source of antioxidants, all parts of this plant are believed to be used to cure various diseases (Dillak et al., 2019). Butterfly pea flower petals exhibit several beneficial properties, including antioxidants, anticancer, anti-cataract, anti-inflammatory, anti-obesity, antibiotic, and anti-diabetic effects. The bioactive compounds present in butterfly pea include phenolics, flavonols, anthocyanins, glycosides, flavonoids, phenolic acids, terpenoids and alkaloid compounds (Nurcholis et al., 2023). According to research by Juswardi et al. (2023), butterfly pea showed antioxidant content in the blooming butterfly pea flower at 6.58 ± 0.80 ppm.

However, utilizing the benefits of butterfly pea requires a substantial amount of biomass. Therefore, an alternative approach for harnessing the compounds in butterfly pea is being explored through the use of endophytic bacteria. Endophytic bacteria are bacteria that live colonizing various parts of the host plant (Simarmata et al., 2020), including roots, fruits (Susilowati et al., 2019), leaves, stems, and flowers. Endophytic bacteria associated with their host plant tissues are known to have the potential to produce valuable secondary metabolites. This process occurs due to genetic transfer between endophytic bacteria and their hosts through coevolution. During this evolutionary process, genetic recombination between the bacteria and their hosts provides benefits such as enhanced

growth and biological protection against diseases (Anand et al., 2023).

Secondary metabolite compounds in endophytic bacteria have many benefits, one of which is their antioxidant activity. Antioxidants are substances that can protect the body from exposure to free radicals. Research conducted by Lakhsan et al. (2020) showed that higher antioxidant content correlates with greater potential to combat toxicity and cancer-related effects. The study also found that the three varieties of butterfly peas exhibit high antioxidant activity, suggesting their potential as natural herbal medicines with anticancer properties. Additionally, the research by Zhao et al. (2020) revealed that endophytic bacteria from *Dendrobium officinale* plants have cytotoxic activity against Hep3B2.1-7 cancer cells. According to Arivo et al. (2023) endophytic bacterial isolates producing antioxidant compounds include *Bacillus tequilensis* isolated from *Leea indica*. The importance of conducting the initial stage of toxicity testing is to evaluate the potential damage that may be caused by chemical compounds to the test organism. In this case, it is essential to perform toxicity testing on endophytic bacteria containing antioxidants, with the objective of assessing the extent to which these bacteria may cause toxic effects on organisms (Rahayu et al. 2019). Studies on the role and potential of antioxidant-producing endophytic bacteria are still limited, especially in butterfly peas, even though this plant has been recognized for its antioxidant-producing potential. Therefore, this study attempts to close this gap in the research by assessing the abilities of endophytic bacteria from butterfly peas as antioxidants and their toxicity.

The purpose of this study was to investigate the toxicity and antioxidant activities of endophytic bacteria isolated from butterfly pea (*Clitoria ternatea* L.) (Suraweera et al., 2020). This research method includes isolating, characterizing, and identifying bacterial strains with potential cytotoxic and antioxidant properties to evaluate their applicability in scientific and medical fields. The research contributes valuable data on the biochemical properties of endophytic bacteria in butterfly pea, especially insights into floral organs. For society, it paves the way for more effective utilization of natural sources of antioxidants and cytotoxic agents, which can be useful in developing novel therapies, particularly for cancer and oxidative stress-related diseases. This kind of exploration of the toxicity and antioxidant activity of endophytic bacteria from butterfly pea has not yet been conducted, according to the literature currently in publication.

METHODS

Sampling and sterilization of surfaces

Samples of butterfly pea were collected from the field, placed in plastic clips, and brought to the laboratory. The collected samples were first washed with cleaned purified running water. In the next step, the samples were washed again with running water and then air-dried. Surface sterilization was then performed, where the butterfly pea samples were immersed in 70% alcohol for 30 seconds, followed by soaking in 5.25% sodium hypochlorite for 30 seconds, and finally immersed again in 70% alcohol for 30 seconds. The final step involved rinsing the samples with sterile distilled water.

Isolation of butterfly pea endophytic bacteria

Surface-sterilized butterfly pea samples were crushed using a mortar and pestle until the floral extract was released. A total of 0.1 mL aliquot of the extract was pipetted using a micropipette and transferred to a petri dish containing sterile NA medium. The extract was then spread evenly using a spreader bar. This process was repeated twice. As a control, 0.1 mL of the sterile water from the final rinse was also pipetted into a petri dish containing NA medium. The plates were incubated for 24 hours. Bacterial colonies that developed on the media were observed to characterize colony morphology, and the isolates were then transferred to fresh plates to obtain pure cultures. A single loopful of each isolate was taken for purification, followed by further incubation.

Biochemical test

Biochemical testing of the selected isolates was conducted, including the indole test, Simmons Citrate (SC) test, TSIA test, motility test, catalase test, Methyl Red (MR) test, and Voges-Proskauer (VP) test.

Preparation Stage of the *Brine Shrimp Lethality Test* (BSLT)

Bacterial isolates were collected from agar media using a 5 mm diameter cork borer, with five samples taken and placed into a 250 mL Erlenmeyer flask containing 150 mL of Nutrient Broth (NB) liquid medium. The samples were then incubated on a shaker incubator at 125 rpm for 2 days at room temperature. Cell biomass was

separated using filter paper and centrifuged at 11.000 rpm for 15 minutes. The resulting supernatant was collected as the test solution. The supernatant was then further diluted to obtain solutions with concentrations of 250 ppm, 500 ppm, 750 ppm, and 1000 ppm for each isolate (Fauziah et al., 2022). These four concentrations were subsequently tested on *Artemia salina* shrimp larvae.

***Brine Shrimp Lethality Test* (BSLT)**

Each tube was added with seawater and 10 *A. salina* larvae until the total volume reached 5 mL. Subsequently, the extract solution was added at concentrations of 250 ppm, 500 ppm, 750 ppm, and 1000 ppm. Each test sample was then incubated for 24 hours. After the incubation period, observations were made by counting the number of *A. salina* larvae that remained alive and those that had died. Observation for each sample was repeated three times using the *A. salina* larval mortality test. The data obtained were analyzed to determine the LC_{50}. According to Islam et al. (2023), the percentage of larval mortality is calculated using the following formula:

$$\% \text{ Larvae mortality} = \frac{\text{Number of dead larvae}}{\text{Total Larvae}} x\ 100\%$$

Furthermore, based on the results obtained from the mortality of *A. salina* larvae, the probit number was calculated, and a line equation was derived: Y = Bx + A, where Y represents the log concentration, and X represents the probit number. The LC_{50} was determined by substituting the probit value corresponding to 50% mortality into the equation. If the control group exhibited dead larvae, the percentage mortality was calculated using Abbott's formula as described by (Meyer et al., with modifications Burci et al., 2019).

$$\% \text{ Larvae mortality} = \frac{T-K}{10} x\ 100\%$$

Description:
T = Number of dead test larvae
K = Number of dead control larvae
10 = Number of larvae

Antioxidant activity test

Endophytic bacterial isolates were inoculated using an Ose and transferred to Nutrient Broth (NB) medium. The cultures were shaken at 170 rpm for 72 hours at 37°C. Following incubation, the isolates were centrifuged at 4000 rpm and 4°C for 15 minutes, and the pellet was collected from the bottom of the tube. The supernatant from the culture extract was diluted by mixing with ethanol to achieve concentrations of 5 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm. Stock solution was prepared at a concentration of 100 ppm by dissolving 2.5 mg of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 50 mL of 95% ethanol. For the assay, 2 mL of each test sample solution was mixed with 2 mL of the DPPH solution, and the mixture was homogenized using a vortex. The solution was incubated for 30 minutes at 37°C and observed for a color change indicating DPPH activity. A control solution was prepared by mixing 2 mL of 95% ethanol with 2 mL of 100 ppm DPPH solution. The level of DPPH free radical scavenging activity (inhibition) was then measured using a UV-Vis spectrophotometer at a wavelength of 517 nm (Fariska et al., 2024). The DPPH inhibition percentage was calculated using the following equation:

$$\% \text{ inhibition} = \frac{(C-S)}{C}\ x\ 100\%$$

Description:
S = Sample Absorbance Constant
C = Absorbance value not containing sample

Molecular identification of 16S rRNA

The process of molecular identification of the 16S rRNA gene goes through several stages, namely: isolation of genomic DNA of selected endophytic bacteria using the Wizard Genome Purification Kit (Promega, USA), Measurement of DNA Concentration using NanoDrop, Amplification of 16S rRNA gene, Gel electrophoresis, and Sequencing of 16S rRNA gene.
Primer(5'-AGAGTTTGATCATGGCTCAG-3') and UniB 1492R(5'GGTTACCTTGTTACGACTT-3').

Phylogenetic tree construction analysis

Phylogenetic tree analysis was carried out in two stages. The first concentrated on primary sequences from Bact 27F and UniB 142R. Sequences showing similarities were aligned with those of the test isolates using MEGA X software. The alignment results were then used to construct a phylogenetic tree using MEGA X, employing the Neighbor-Joining method with a bootstrap value of 1000 replicates.

RESULTS AND DISCUSSION

Morphological characterization of endophytic bacteria

A total of fifteen endophytic bacteria were successfully obtained from butterfly pea flowers. Boonman et al. (2023) managed get 11 isolates of endophytic bacteria from flowers of *Ageratum*

conyzoides L. This difference was thought to occur due to different flower families and also different flower morphologies. Mitter et al. (2017) stated that flower morphology has a significant effect on the number of microbes contained in it, such as the morphology of the cell shape in the flower, the pigments produced by the petals, the texture of the petals, and nectar in parts such as floral adornment. Another factor that affects the presence of microbes in the petals is radiation exposure (Frank et al., 2017).

The fifteen isolates of endophytic bacteria obtained from butterfly pea were found to have different morphological characteristics (Table 1). The observed morphological characterization consists of shape, color, elevation, cell arrangement, and different edge colonies. The isolates also varied in shape, ten endophytic bacterial colonies were round, two isolates were spreading, two isolates were small and round, and one isolate was elliptical. The color of endophytic bacterial colonies was found to vary, six isolates were white, six isolates were beige, one isolate was yellow, and two isolates were orange. Eleven endophytic bacterial isolates had smooth edges, two isolates had filamentous edges, and two isolates had serrated edges. Elevations of eleven isolates had convex elevation, while four isolates had flat elevation.

The morphological diversity of endophytic bacterial colonies obtained in this study was thought to be due to the presence of sufficient nutrients in the host plant. Zhang et al. (2019) stated that the diversity of endophytic bacteria is influenced by the nutrient content and nutritional stability of the host plant. According to Nadarajah & Rahman (2021), there are several other factors that influence the interaction of microbes with their host plants such as genetic factors, geographical conditions, and plant tissue structure. Therefore, changes that occur in the environment can provide a diversity of microbial species, which will in turn affect the morphological and physiological properties of microbes.

The result from the Gram stain characterization (Table 1, and Figure 1), identified eleven isolates belonging to Gram-positive and four isolates belonging to Gram-negative, indicating the dominance of Gram-positive bacteria over Gram-negative bacteria (Figure 1). The process of plant adaptation to the environment is crucial to the survival of Gram-positive endophytic bacteria in specific environments, including in certain plant tissues. This adaption includes the ability to cope with changing environmental conditions, overcome the plant's natural defenses, or adapt to the specific physical and chemical properties of the plant tissue. Some Gram-positive endophytic bacteria are able to develop protective mechanisms from adverse external factors or environmental stress, so they can survive in plants even in less favorable environments (Ek-Ramos et al., 2019). These Gram-positive bacterial isolates have a variety of acid resistance systems that can help the bacteria overcome the threats posed by environments with low pH conditions and are also known to have high metal uptake capacity.

Table 1. Macroscopic and microscopic characteristics of butterfly pea endophytic bacteria isolates

No.	Isolate	Characteristics					
		Colony Characteristics				Cell Characteristics	
		Form	Color	Elevation	Edge	Cell arrangement	Staining Gram
1.	EBT1	Circular	Yellow	Convex	Entire	Tetracoccus	Negative
2.	EBT2	Irregular	Cream	Flat	Lobate	Bacilli	Positive
3.	EBT3	Circular	White	Convex	Entire	Streptococcus	Positive
4.	EBT4	Circular	Cream	Flat	Lobate	Bacilli	Positive
5.	EBT5	Elips	White	Convex	Entire	Diplococcus	Negative
6.	EBT6	Circular	Cream	Convex	Entire	Coccus	Positive
7.	EBT7	Circular	White	Convex	Entire	Diplobasil	Positive
8.	EBT8	Circular	Orange	Convex	Entire	Tetracoccus	Positive
9.	EBT9	Circular	Orange	Convex	Entire	Tricoccus	Positive
10.	EBT10	Punctiform	Cream	Convex	Entire	Diplococcus	Positive
11.	EBT11	Punctiform	White	Convex	Entire	Diplobacili	Negative
12.	EBT12	Irregular	White	Flat	Filamentous	Bacilli	Positive
13.	EBT13	Circular	White	Convex	Entire	Streptobasil	Positive
14.	EBT14	Circular	Cream	Flat	Filamentous	Diplobacili	Positive
15.	EBT15	Circular	Cream	Convex	Entire	Coccus	Negative

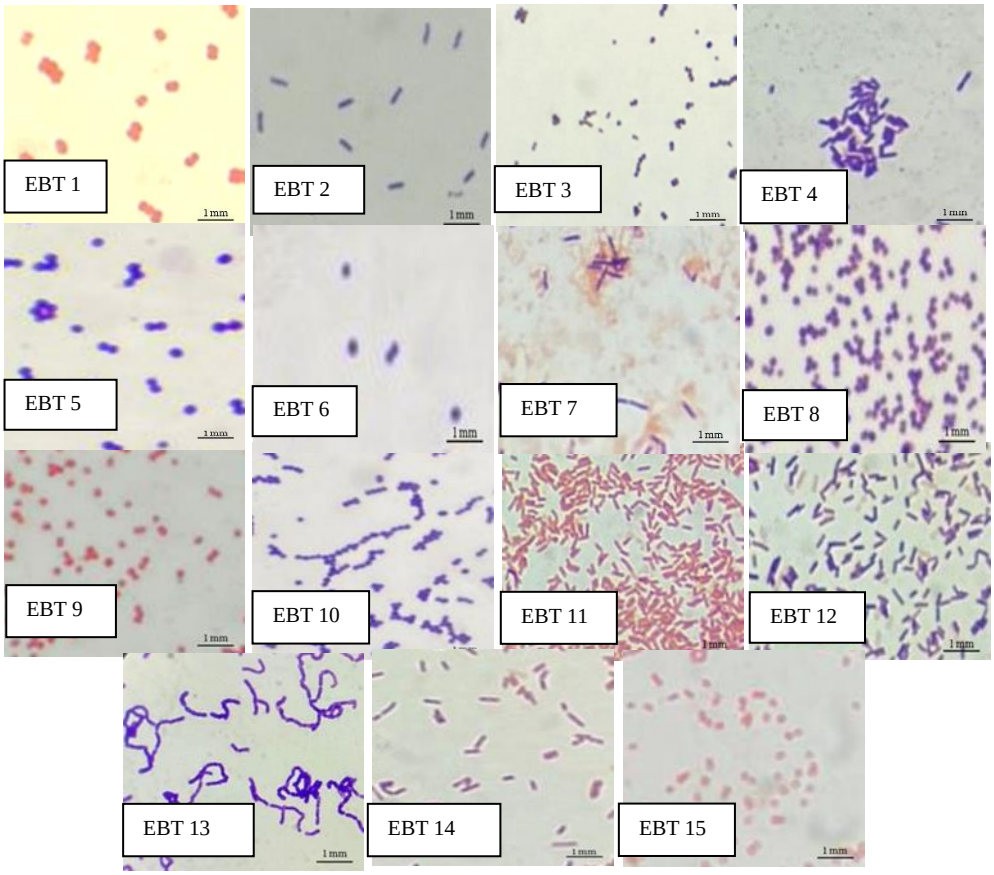

Figure 1. Cell morphology of butterfly pea endophytic bacteria (100x magnification)

Table 2. The result of the biochemical test of endophytic bacteria

Isolate	Biochemical tests							
	Catalase	**Motility**	**Indole**	**TSIA**	**Citrate**	**MR**	**VP**	**Genus bacteria**
EBT1	+	Non-motile	-	y/r	-	-	-	*Xanthomonas*
EBT2	+	Motile	-	y/r	+	-	+	*Bacillus*
EBT3	-	Non-motile	-	y/r	-	+	-	*Enterococcus*
EBT4	+	Motile	-	r/r	+	-	+	*Bacillus*
EBT5	-	Motile	-	r/r	+	-	+	*Streptococcus*
EBT6	+	Motile	-	y/r	-	+	-	*Micrococcus*
EBT7	+	Motile	-	r/y	-	-	+	*Bacillus*
EBT8	+	Non-motile	-	y/r	-	+	-	*Staphylococcus*
EBT9	+	Non-motile	-	y/r	-	-	-	*Xanthomonas*
EBT10	+	Motile	-	y/y	-	+	-	*Micrococcus*
EBT11	+	Motile	-	y/r	+	-	+	*Pseudomonas*
EBT12	+	Motile	-	y/r	+	-	+	*Bacillus*
EBT13	+	Motile	-	y/r	+	-	+	*Bacillus*
EBT14	+	Non-motile	-	y/r	+	-	+	*Bacillus*
EBT15	+	Non-motile	-	y/r	-	+	-	*Veillonella*

Notes : +: positive -: negative y/y : *slant* yellow/*butt* yellow m/k : *slant* red/*butt* yellow m/m: *slant* red/*butt* red

Gram-positive endophytic bacteria can form mutualistic symbiotic relationships with their host plants. This interaction can provide multiple benefits to plants, such as increasing nutrient availability, producing growth hormones, and increasing tolerance to environmental stress. These Gram-positive bacteria have several abilities that support survival in plant tissues, including providing nutritional sources in the form of sugars, amino acids, or other organic compounds for the survival of the host plant. In addition, Gram-positive bacteria have an

important role as biocontrol agents that can control plant diseases (Kandasamy & Kathirvel, 2023).

Physiological characterization of endophytic bacteria

The results of biochemical tests of butterfly pea endophytic bacterial isolates are presented in Table 2. The identification process refers to Bergey's Manual of Determinative Bacteriology 8th Edition, a comprehensive and widely recognized reference for bacterial classification. This manual serves as a key resource in microbiology, providing descriptions of bacterial species, including their morphological, physiological, and biochemical traits. As a result of these criteria, the bacterial isolates obtained from the butterfly pea plants were accurately identified, allowing for a better understanding of their potential roles as endophytes in the plant. The results obtained from the biochemical tests help in distinguishing between different bacterial strains and offer insights into their possible potential.

Endophytic bacteria EBT1 and EBT9 shared similar characteristics with members of the genus *Xanthomonas*. They are coccus-shaped cells arranged in pairs and are Gram-negative. The biochemical tests show positive results for catalase and oxidase tests, but both strains are nonmotile and yield a negative result for the Indole test. EBT1 and EBT9 cultured on the red slant would turn the bud of the agar yellow, indicating that these bacteria can only ferment glucose. The negative citrate test result suggested that they cannot utilize citrate as a carbon source. Additionally, both strains showed negative results for the MR and VP tests, indicating that they do not produce significant amounts of acid from glucose fermentation.

Bacterial isolates EBT2, EBT4, EBT7, EBT12, EBT13, and EBT14 were identified as belonging to the genus *Bacillus*, with biochemical tests showing positive results for catalase and oxidase, and nonmotile properties. Indole test results were negative. Isolate EBT3 was classified as belonging to the genus *Enterococcus*, characterized by Gram-positive staining, catalase-negative properties (although some strains may be catalase-positive on blood agar), oxidase-negative status, and facultative anaerobic behavior, typically appearing singly, in pairs, or in chains. The bacteria from isolate EBT8 exhibit characteristics consistent with the genus *Staphylococcus*, including a positive catalase test, Gram-positive staining, and coccus-shaped cells. These findings align with those of Condessa et al. (2024), who identified several genera of endophytic bacteria isolated from *Acacia longifolia*, including *Staphylococcus* and *Bacillus*.

EBT10 was classified under the genus *Micrococcus*. These bacteria are typically spherical (cocci), with most cells occurring in pairs and some exhibiting irregular shapes. They generally do not form spores, are aerobic (though facultative anaerobes are rarely encountered), are Gram-positive, and are catalase-positive. EBT11 belongs to the genus *Pseudomonas*, which comprises Gram-negative bacteria with bacillus-shaped cell morphology. These bacteria are motile, exhibit a negative indole test, are catalase-positive, ferment only glucose, yield a positive citrate test, and are negative for both the MR and VP tests. The characteristics of isolate EBT11 suggest similarities with *Pseudomonas*.

EBT15 bacteria belong to the genus *Veillonella*, these bacteria are anaerobic and non-motile. The colony morphology is small and round, and the bacterial cells are Gram-negative cocci. The bacteria are indole negative, MR test positive, citrate negative, and VP negative. They are classified as *Veillonella* sp., characterized by their round shape, Gram-negative bacteria, absence of endospores, positive catalase reaction, and positive oxidase reaction.

Mortality of *Artemia salina* larvae in the toxicity test

The results showed that the addition of endophytic bacterial supernatant led to the mortality of *Artemia salina* larvae. Larval mortality is believed to be caused by the penetration of toxic compounds through the cell membrane, which inhibits metabolic processes within *A. salina.* The mortality may be attributed to alterations in the concentration gradients between the intracellular and extracellular environments, resulting from the addition of secondary metabolite compounds from the endophytic bacteria of the butterfly pea flower. The results also showed that higher concentrations of endophytic bacterial supernatant were associated with increased larval mortality in *A. salina*. This finding is consistent with the statement of Rajabi et al. (2015), who reported that the mortality of shrimp larvae caused by toxic secondary metabolite compounds can occur through the respiratory process and diffusion process. In the respiratory process, the toxic compounds enter the body through the respiratory tract, while in diffusion, the compounds are absorbed through the thin skin of the larvae.

Table 3. Mortality of *Artemia salina* larvae and toxicity category of endophytic bacteria

Isolate	Concentration (ppm)				Mortality	LC_{50} (ppm)	Category*
	1000	750	500	250			
EBT1	25	24	20	14	83	210	Very toxic
EBT2	30	30	27	23	80	190	Very toxic
EBT3	30	27	24	22	103	212	Very toxic
EBT4	29	22	20	18	89	240	Very toxic
EBT5	30	22	22	16	90	295	Toxic
EBT6	21	17	14	7	59	581	Moderate toxic
EBT7	28	25	23	20	96	158	Very toxic
EBT8	15	8	6	2	31	284	Toxic
EBT9	30	23	18	14	85	332	Toxic
EBT10	30	23	21	16	90	298	Toxic
EBT11	26	23	17	14	80	322	Toxic
EBT12	30	30	29	27	116	113	Very toxic
EBT13	27	27	24	22	100	84	Very toxic
EBT14	30	30	26	24	110	184	Very toxic
EBT15	22	18	17	15	72	289	Toxic
Control	0	0	0	0	0	0	-

*according to Riris et al. (2020)

The chemical concentration that causes death in 50% of test animals is known as the Lethal Concentration 50 (LC_{50}) (Islam et al., 2021). The results presented in Table 3. indicate that all endophytic bacteria from the *Clitoria ternatea.* L flower exhibit an LC_{50} value < 1000 ppm. This shows that these endophytic bacteria have the potential to an anticancer. Among them, isolate EBT13 demonstrated the lowest LC_{50} value, with a toxicity of 84 ppm. This is by the statement of Riris et al. (2020) which states that an extract with an LC_{50} concentration ≤ 1000 ppm is toxic, whereas an LC_{50} value ≥ 1000 ppm is classified as non-toxic.

Antioxidant Activity of EBT13

Antioxidant activity was measured on the EBT13 isolate. The concentration of sample solution needed for inhibiting 50% of a biological process, such as free radicals, is known as the Inhibition Concentration 50 (IC_{50}) (Hasan et al., 2024). A metric often used to evaluate antioxidant activity or medication efficacy is IC_{50}. The EBT13 isolate was selected due to its classification as highly toxic and having the lowest LC_{50} value among the other tested isolates. Consequently, further analysis, specifically the measurement of antioxidant activity, was conducted on this bacterial isolate.

Table 4. Antioxidant result of EBT13

Concentration (ppm)	Absorbance Of control	Mean Absorbance	% Inhibition	IC_{50} (ppm)	Category*
EBT13					
5	0.41	0.58	14.08		
10	0.41	0.57	17.70		
25	0.41	0.47	40.23	44.32	Very active
50	0.41	0.38	61.95		
100	0.41	0.27	90.51		
Ascorbic Acid					
5	0.41	0.25	39.42		
10	0.41	0.18	55.91		
25	0.41	0.16	60.34	9.18	Very active
50	0.41	0.12	71.60		
100	0.41	0.04	91.31		

*according to Wang et al., 2022.

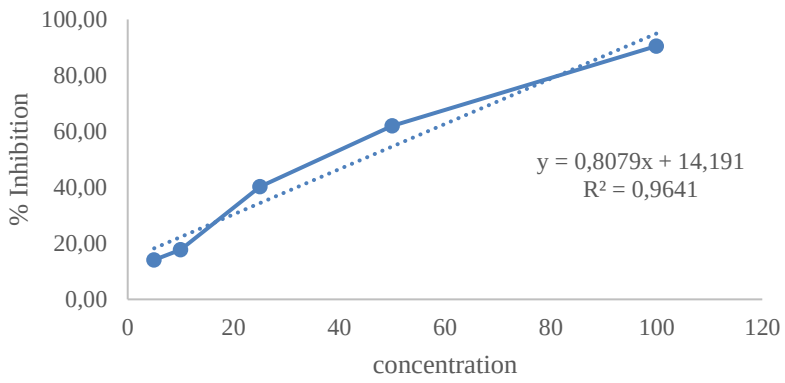

Figure 2. Regression linear graphic of inhibition percentage EBT13 to DPPH

Based on the inhibition percentage results (Table 4.), the concentration needed for blocking 50% of free radicals can be extrapolated from the IC_{50} value at each concentration. From these results, it is evident that the inhibition ability increases with the increasing of concentrations, which indicates a greater antioxidant content in the test sample solution. Based on the results, the IC_{50} value for the EBT13 isolate was 44.32 ppm, while the IC_{50} value for ascorbic acid was 9.18 ppm. According to Martinez et al. (2020), both of these results were categorized as very active. Ascorbic acid or vitamin C was used as a control to assess the potential of the test compound. The percentage of inhibition was plotted to produce a linear regression equation, yielding y = 0.8079x + 14.191. The R^2 value of 0.9641 indicates that 96.41% of the inhibition was influenced by the EBT13 bacterial isolate test extract.

In this model, the R^2 value states the linearity of the test concentration to the percentage of inhibition. The R^2 value which is close to 1, indicates that the higher the concentration of the test extract, the higher the antioxidant activity.

This relationship is depicted in the curve showing the correlation between test concentration and its role in DPPH inhibition, as illustrated in Figure 2. Chaudhary et al. (2023) concluded that antioxidant properties can directly scavenge free radicals and inhibit anticancer mechanisms by suppressing P450 isoenzymes responsible for procarcinogen production. According to Muchtadiri et al. (2024) natural and semi-synthetic antioxidant compounds have the ability to inhibit tumor growth and are even cytotoxic to cancer cells.

Molecular identification 16S rRNA EBT13

Molecular identification was carried out on isolate EBT13. The results showed that the PCR product containing the 16S rRNA gene could be amplified and visualized on agarose gel with the DNA size obtained from the PCR product showed the alignment with 1500 bp marker (Figure 3). As a result, the obtained sequence can be used to align with other sequence data in GenBank using the BLAST-N tool.

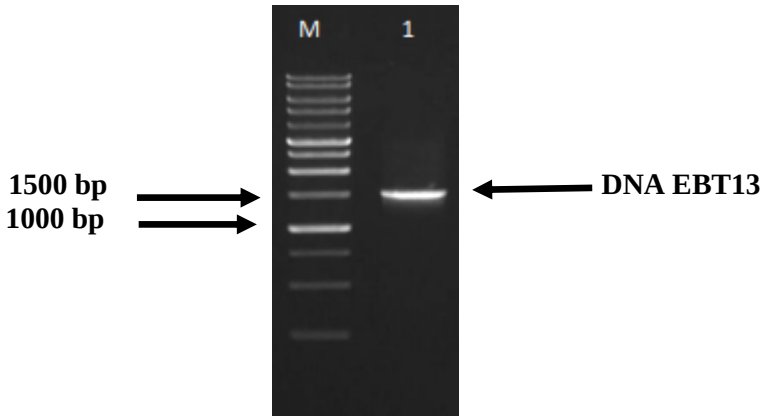

Figure 3. Visualization of EBT13 amplicon (bp= *base pairs* M: *marker* 1 kb *plus* ; 1= Isolate EBT13)

Table 5. BLAST Result EBT13 from NCBI

Description	Max score	Total score	Query cover (%)	E value	Ident (%)	Accession
Bacillus pumilus strain 2.2	2678	2678	100	0.0	100	KM265464.1
Bacillus pumilus strain CSH4	2678	2678	100	0.0	100	KU921592.1
Bacillus pumilus strain McSt7	2678	2678	100	0.0	100	KY078801.1
Bacillus pumilus strain KSU_12	2678	2678	100	0.0	100	MN208467.1
Bacillus pumilus strain PB4	2678	2678	100	0.0	100	MT367713.1

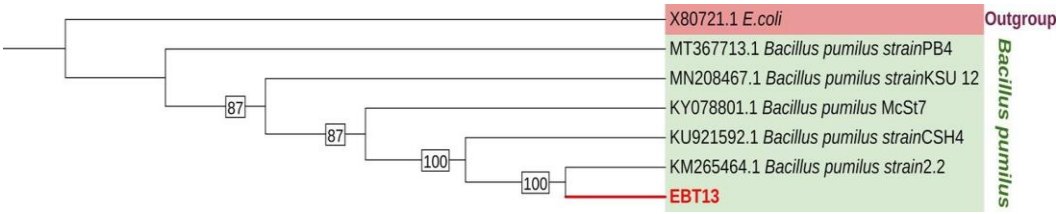

Figure 4. Phylogenetic tree reconstruction results from EBT13

The results of BLAST-N (*Basic Alignment Search Tool Nucleotide*) analysis using GenBank on NCBI show that isolate EBT13 shares 100% similarity with *Bacillus pumilus* in Table 5. Fatwa et al. (2021) state that isolates with homology similarities exceeding 97% can be considered representatives of the same species. Homology similarity within the 93-97% range can reflect identity at the genus level.

The results from Figure 4 indicated that the phylogenetic tree of EBT13 formed is monophyletic, meaning all members in the phylogenetic tree belong to the same genus (except the out-group), namely *Escherichia coli*. According to Basith et al. (2021), the phylogenetic tree resulting from the analysis is monophyletic, transmitting genetic, morphological, and biochemical characteristics to all common descendants. The results of the phylogenetic tree construction showed that the EBT13 isolate forms a sister group with *Bacillus pumilus*, with a bootstrap value of 100%, as shown in Figure 4. According to Lestari et al. (2018) bootstrap value categories are classified as high (>85%), moderate (70-85%), weak (50-69%), or very weak (<50%).

Based on the results of morphological and physiological identification, isolate EBT13 was confirmed to belong to the genus *Bacillus*. The results of molecular identification results of analysis using the 16S rRNA gene stated that isolate EBT13 had a similarity with the species *Bacillus pumilus*. This identification was further supported by the multi-sequence analysis similarity index. Based on research by Mohammed et al. (2022) *Bacillus pumilus* BY has morphological characteristics identified as being cream-yellow, flat, rod-shaped, and having elevated edges. A 1500 bp segment of the 16S rRNA gene was obtained through molecular characterization using PCR analysis. Antioxidant activity has been reported for *Bacillus* species in research by Liu et al. (2020) which found that *Bacillus pumilus* LZP02 promotes rice root growth by increasing carbohydrate metabolism and phenylpropanoid biosynthesis. According to research by Kumar et al. (2021), *Bacillus pumilus* is able to increase the activity of soil enzymes, such as alkaline phosphatase, acid phosphatase, urease, and β-glucosidase. Moreover, Zhao et al. (2018) also revealed that the *Bacillus* genus has potential as an anticancer agent and effectively inhibits the viability of K562 leukemia cells at a concentration of 100 μM, with an IC_{50} value of 65.76 μM. This research is the initial information that endophytic bacteria have been identified from butterfly pea, these endophytic bacteria are also known to have the potential to produce antioxidant activity and have toxicity characteristics but further research is needed to apply directly to target cells.

CONCLUSION

A total of fifteen endophytic bacteria were successfully isolated from butterfly pea flowers with different characteristics. This study

successfully proved that the potential antioxidant properties and toxicity characteristics exhibited by the host plant was also present in the endophytic bacteria isolated from it. The endophytic bacterial isolates from butterfly pea showed the capability to produce both toxic and antioxidant compounds. Based on the results of morphological and biochemical characterization, fifteen bacterial isolates successfully isolated from butterfly pea were belonging to several genera, namely: *Xanthomonas, Bacillus, Enterococcus, Streptococcus, Staphylococcus, Micrococcus, Pseudomonas,* and *Veillonella*. Among them, isolate EBT13 exhibited the highest toxicity, classified as very toxic with an LC_{50} value of 84 ppm, and demonstrated strong antioxidant activity with an IC_{50} value of 44.32 ppm. Analysis using 16S rRNA gene sequencing confirmed that this isolate was *Bacillus pumilus*. Further research is still needed to test the efficacy of these properties by applying the isolates to target cells. Isolate EBT13 has very strong antioxidant activity, therefore further testing such as the MTT (3-(4,5 dimethythiazol-2-yl)-2,5-diphenyltetra-zolium bromide) needs to be done on isolate EBT13 in order to determine its potential as an anticancer.

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