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The Potential of Tapak Dara (*Catharanthus roseus*) Leaves Endophytic Bacteria BETD5 as Antioxidant and Anticancer Against T47D Breast Cancer Cells

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ABSTRACT

BETD5 is an endophytic bacteria from Tapak Dara (*Catharanthus roseus)* leaves that have the potential as an antioxidant and anticancer. Therefore, this study aimed to measure antioxidant activity, characterize biochemical properties, determine anticancer activity againstT47D cells line, and identify the secondary metabolites of endophytic bacteria Tapak Dara of BETD5 by Gas Chromatography-Mass Spectrometry (GCMS). The method used was experimental and the parameters observed were antioxidant and anticancer activities, consisting of IC_{50} (Inhibition Concentration) and biochemical properties. Antioxidant activity was measured using various sample extract concentrations, namely 5ppm, 10ppm, 25ppm, 50ppm, and 100ppm. Biochemical tests were carried out by Triple Sugar Iron Agar (TSIA), Simmons Citrate (SC), motility, indole, catalase, and Methyl Red (MR) tests. Cytotoxicity test on cells line T47D was performed by MTT assay to evaluate anticancer activity using concentrations of 3.125, 6.25, 12.5, 25, and 50 ppm. The results showed that antioxidant activity of BETD5 was strong because IC_{50} value obtained was 66.27 ppm. Furthermore, biochemical tests revealed that BETD5 belonged to the genus *Staphylococcus,* while cytotoxicity activity obtained an IC_{50} value of 14.28 ppm. This indicated that endophytic bacteria extract of BETD5 had moderate cytotoxicity against T47D breast cancer cells line. The highest secondary metabolite in endophytic bacteria isolate BETD5 was cis-Ocimene (24.31%) based on GCMS analysis.

Keywords: Antioxidant, Endophytic Bacteria, T47D Cells Line, Biochemical Properties, Cytotoxicity.

INTRODUCTION

Cancer is the second leading cause of death in the world, with approximately 18.1 million cases reported in 2020 (WHO, 2022). The most common types with the highest number of cases are breast, lung, colorectal, prostate, and stomach cancer (World Cancer Research Fund International, 2022). Indonesia is one of the countries with the highest number of cancer cases. The country has recorded approximately 946,000 cases in the last five years, where breast cancer ranks first with 69,000 cases (Global Cancer Observatory, 2020). One of the causes of breast cancer is the occurrence of a mutation of the p53 gene, which is often used for anticancer testing and is known as T47D cells (Safitri, 2020).

Chemotherapy is a common treatment for cancer that involves the use of drugs to destroy cancer cells or slow their growth. The discovery and development of chemotherapeutic agents is very important in the treatment of cancer. This is because the therapies used today are considered to be less effective and have serious side effects (Chu and Davita, 2019). According to Hridoy *et al*. (2022), natural compounds from endophytic bacteria can produce anticancer compounds safe for health, and biocompatible, with fewer toxicity problems. These endophytic bacteria are non-pathogenic bacteria in plant tissues that exhibit anticancer properties and can be used to control various types of cancer. Khiralla *et al*. (2015) also stated that endophytic bacteria produced chemical

compounds with biological activity and secondary metabolites such as antioxidant compounds.

The preliminary study conducted by Fauziah *et al*. (2022) showed the toxicity activity of endophytic bacteria using Brine Shrimp Lethality Test (BSLT) method. The results indicated that endophytic bacteria supernatant of BETD5 had the highest toxicity activity with a Lethal Concentration of 50% (LC_{50}) of 413,590 ppm, where the LC_{50} value was <1000 ppm. This suggested that the secondary metabolite of BETD5 had toxic properties and the potential to be developed as an anticancer agent. Furthermore, the secondary metabolites produced by BETD5 supernatant were alkaloids and saponins.

There is a need to further investigate anticancer potential possessed by secondary metabolites of endophytic bacteria *Staphylococcus* sp. BETD5. Therefore, this study aims to examine antioxidant activity, biochemical properties, and anticancer activity of BETD5 against T47D line cells using Microtetrazolium (MTT) assay method and secondary metabolite profiles through Gas Chromatography-Mass spectrometry (GCMS) analysis of supernatant endophytic bacteria BETD5.

MATERIALS AND METHODS

Cultivation of BETD5 Bacteria

The stored BETD5 isolate preparations were taken using a sterile Ose needle and inoculated in an NA medium by scratching. Subsequently, bacteria that had been scratched on the NA medium were incubated in an incubator at 37°C for 1x24 h.

Antioxidant Activity Test

BETD5 isolate was taken using an Ose needle and put into Nutrient Broth (NB) medium. The isolate was shaken at a speed of 170 rpm for 72 hours at a temperature of 37°C. Subsequently, the isolate was centrifuged at a speed of 4000 rpm, at a temperature of 4°C for 15 minutes, and the pellet of this culture was filtered. To prepare the test sample solution, a 100 ppm stock solution was made by taking 5 mL of the supernatant extract of endophytic bacteria and adding 50 mL of ethanol. The stock solution was diluted to concentrations of 5 ppm, 10 ppm, 25 ppm, 50 ppm, and 100 ppm, with 5 ml added to each test tube. This was followed by the preparation of the main solution of 100 ppm, by dissolving 2.5 mg of 1,1-diphenyl-2-2picrylhydrazyl (DPPH) into 50 mL of 95% ethanol. A 2 ml of each test sample solution was prepared and added with 2 ml of DPPH solution. The mixture was homogenized using a vortex and incubated for 30 minutes at 37°C, which caused a color change from DPPH activity. A control solution was prepared by adding 2 ml of 95% ethanol with 2 ml of 100 ppm DPPH solution. The absorption value was analyzed using UV-Vis Spectrophotometer maximum wavelength of 517 nm. The ability to reduce DPPH free radicals (inhibition) was determined from the absorption value using the equation below:

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

S = Sample absorbance; C = Absorbance without sample

Biochemical Test

Biochemical tests consisted of TSIA, MR, indole, motility, catalase, and Simmons Citrate (SC) tests.

Triple Sugar Iron Agar (TSIA) Test

One colony of the bacteria isolate was inoculated on TSIA medium by stabbing vertically on the butt and scratching zig-zag at the slant agar. The culture was incubated at 37°C for 24 hours and the color change of the medium was observed. When the slant was red and the butt was yellow, it indicated that the bacteria only fermented glucose. Meanwhile, when the slant and butt were yellow, the bacteria fermented lactose and sucrose.

Indole Test

One colony of bacteria isolates was inoculated into Sulfide Indole Motility (SIM) medium and incubated for 24 hours at 37°C. Indole test results were observed by adding 10 drops of Kovac's reagent. A positive result was indicated by the formation of a red layer on top of the culture.

Motility Test

One colony of bacteria isolates was put into SIM medium and incubated for 48 hours at 37°C. A positive result was indicated by the growth of bacteria that spreads on the media.

Methyl Red (MR) Test

One colony of bacteria isolates was inoculated into MR-VP medium and incubated at 37°C for 48 hours. MR test observations were conducted by adding 3 drops of MR reagent into the medium. A positive result was indicated by a change of the medium color to red, showing the formation of acid.

Catalase Test

Bacteria isolates aged 24 hours were dripped with 2 drops of 3% H_2O_2 on an objective glass. A positive result was indicated by the formation of air bubbles. This showed that the bacteria produced catalase capable of converting H_2O_2 into H_2O and O_2.

SC Test

One colony of bacteria isolates was inoculated into SC agar medium and incubated at 37°C for 48 hours. A change in the color of the medium was observed and a positive result was indicated by the change of medium color into blue.

Cytotoxicity Test

T47D Breast Cancer Cells Line Culture

T47D Cancer line cells were grown in RPMI 1640 medium (Gibco, CA, USA), supplemented with 10% FBS (Sigma, MA, USA), Pen Strep 2% (Gibco, CA, USA), and Fungizone 0.5% (Gibco, USA) CA, USA).

MTT Test

Cells Harvesting

The state of cells was observed with an inverted microscope. Cells were harvested when they were 80% confluent and the medium was discharged into the flask. PBS was poured into the flask, which was closed and shaken to wash cells from the waste medium. Subsequently, PBS was removed and added with Trypsin EDTA (Gibco, CA, USA) 0.25%, 0.5-1 ml, and incubated for 4 minutes in a CO_2 incubator. The flask was removed from the incubator and agitated to release cells from the artificial matrix of the flask. A 10 ml of complete medium was added to inactivate Trypsin EDTA 0.25%. The flask walls were rinsed to remove any adhering cells, resuspended, transferred to a 15 ml sterile conical tube, centrifuged at 2500 rpm for 5 minutes, and the supernatant was discarded. A 1 ml of complete medium was added and resuspended until homogeneous. This was followed by the collection of 10 µl of cells suspension, which was transferred to a hemocytometer, and observed under a microscope. Cells counts were performed using a counter.

Cells Cultivation in a 96-well plate (Nunc, MA, USA)

A 96-well plate was prepared and 100 µl of cells suspension was added to each well with a cells count of 2.5 x 10^4 and incubated for 24 hours. Subsequently, cells were incubated for 24 hours and treated with bacteria supernatant according to serial concentrations of 3.125, 6.25, 12.5, 25, and 50 ppm.

MTT Assay 96-well plate

The media in each well was discarded and washed using PBS. MTT reagent (Biobasic, NY, USA) with a concentration of 0.5 mg/ml was prepared, 100 MTT 0.5 mg/ml was added to each well, and incubated for 4 hours. The reaction was stopped with DMSO (Merck KGaA, Darmstadt, Germany) 100 ul/well. The absorbance was read using a Tecan Spark® (Tecan Trading AG, Switzerland) at a wavelength of 570 nm. The data was analyzed using the formula below:

Cells viability calculation formula

$$\%\ Viability = \frac{OD_{Treatment} - OD_{Medium\ Control}}{OD_{Cells\ Control} - OD_{Medium\ Control}} \times 100\%$$

GCMS Test

GCMS analysis was analyzed using Shimadzu GCMS-QP2010 Ultra under the following conditions= Carrier gas : Helium; Column : Rxi-1MS; Column Oven Temp : 310°C; Injection Temp : 270.00°C; Injection Mode : Splitless; Sampling Time : 1.00 min; Flow Control Mode : Linear Velocity; Pressure : 66.6 kPa; Total Flow : 50.3mL/min; Column Flow : 1.30 mL/min; Linear Velocity : 40.9 cm/sec; Purge Flow : 10.0 mL/min; Split Ratio : 30.0

Data Analysis

Data analysis of biochemical tests was carried out descriptively using Bergey's Manual of Determinative Bacteriology. To analyze antioxidant and anticancer activities, the average value of cells viability and the 50% Inhibition Concentration (IC_{50}) with BETD5 supernatant treatment were calculated using Microsoft Excel 2022 and non-linear regression analysis. One Way ANOVA was performed using GraphPad Prism 7 software (GraphPad Software, CA, USA) with the following expression model.

$$y = Ainf + \frac{A0 - Ainf}{1 + 10^{(\log 10x - \log 10IC50)Hill\ slope}}$$

A0 is the minimum value that can be obtained at a concentration of 0. Ainf is the maximum value that can be obtained at an infinite concentration, IC_{50} is a concentration at a maximum value of 50%, hill slope is r a slope factor,

y is a percentage of inhibition, and x is an inhibitor concentration. Data analysis of GCMS results is carried out descriptively and presented in the form of numbers and tables.

RESULTS AND DISCUSSION

Antioxidant Activity

This study used extracts of endophytic bacteria from Tapak Dara leaves (BETD5) as a source of antioxidant. The curve (Figure 1) of the relationship between log concentration and inhibition of antioxidant activity of BETD5.

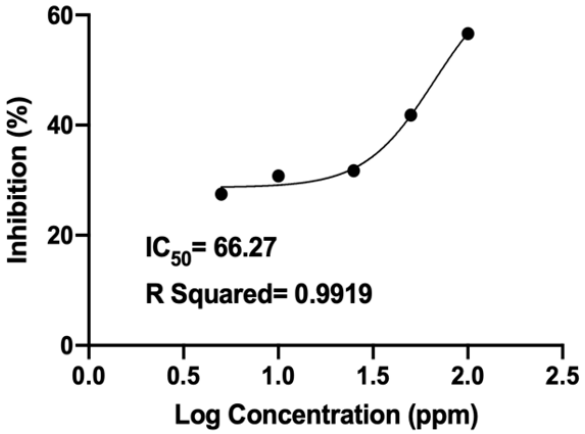

Figure 1. Relationship between Log Concentration and Inhibition

The percentage of inhibition increased at each concentration (Figure 1), has indicated that the test sample contained more antioxidant at a higher percentage of inhibition. Subsequently, the inhibition percentage was made into a nonlinear regression curve to obtain a Coefficient of Determination R^2 = 0.9919. This indicated that 99.19% of the inhibition was influenced by the concentration of BETD5 supernatant. Savic *et al*. (2022) stated that the R^2 value described linearity of the concentration to inhibition percent. An R^2 value close to +1 (positive value) indicated that the higher the concentration of the sample extract, the greater the antioxidant activity.

The nonlinear regression analysis of the four parameters logistic equation revealed that antioxidant activity test on BETD5 supernatant had an IC_{50} value of 66.27 ppm, indicating a strong antioxidant ability. Jun *et al*. (2003), categorized antioxidant ability of a compound as very strong when IC_{50} value was less than 50 ppm, strong between 50-100 ppm, medium between 100-150 ppm, and weak between 150 -200 ppm. Saha and Verma (2016) added that DPPH testing provided information about antioxidant activity in counteracting free radicals by measuring the amount of C_{50} of the test solution on its ability to reduce free radical activity by 50%, as indicated by IC_{50} value. Therefore, the smaller the IC_{50} value, the higher the reduction of free radical activity.

Biochemical Test

Physiological characterization of BETD5 was conducted through various biochemical tests, including TSIA, Indole, motility, catalase, MR, and SC tests. These tests were carried out to identify and differentiate the genus of each bacteria (Kosasi *et al*., 2019). The results of biochemical test of BETD5 (Table I).

Table I. BETD5 isolate biochemical test

No	Biochemical Test	BETD5
1.	TSIA Test	*y/r*
2.	SC Test	-
3.	Motility Test	-
4.	Indole Test	-
5.	MR Test	+
6.	Catalase Test	+

Annotation= + : There was a positive reaction; - : There was a negative reaction; *y/r* : Yellow slant/Red butt

The results of TSIA test showed that BETD5 isolate after being incubated for 24 hours had a yellow slant and red butt. According to Wibowo *et al* (2020), a yellow (alkaline) slant and a red (acidic) butt on TSIA medium indicated that bacteria partially fermented carbohydrates, namely lactose and sucrose.

The results of the SC test for BETD5 isolate were negative, as indicated by the absence of color change in the medium to blue. The test was conducted to assess BETD5 isolate using citrate as a carbon and energy source. The results of the observation on the motility test showed that BETD5 isolate was non-motile bacteria. This was indicated by the absence of bacteria growth around the Ose needle prick in the medium. According to Anjum and Chandra (2015), a negative result was observed when the growth of bacteria did not spread and only grew straight in the puncture area. Meanwhile, a positive result showed the growth of bacteria around the puncture area to the surface of the medium.

The results of the indole test on SIM medium that had been incubated for 24 hours and dripped with Kovac's reagent were negative, as shown by the absence of a red layer on the culture. MR test

observations showed a positive result, as indicated by the change in the color of the medium into red after being added with MR reagent. The catalase test showed positive results, indicated by the formation of air bubbles (O_2). Similarly, Kosasi *et al*. (2019) stated that positive results of the catalase test were marked by the formation of oxygen bubbles. This indicated that the microorganism produced catalase to convert hydrogen peroxide into water and oxygen. The results of biochemical tests showed that BETD5 belonged to the genus *Staphylococcus*. Fauziah *et al*. (2022) also found that BETD5 had a similarity of 99.38% with *Staphylococcus arlettae* strain NR 036903 based on the 16S rDNA test.

Anticancer Activity

A cytotoxicity test was carried out to determine anticancer effect of BETD5 supernatant against T47D breast cancer cells line with parameter IC_{50} parameter. The method used for cytotoxicity test was MTT assay method. Wozniak & Keely (2005) also used the MTT assay as a test system to measure the amount of yellow MTT salts reduction to purple formazan by tetrazolium succinate reductase, which was included in the mitochondrial respiration chain of living cells.

The test was carried out using several concentration levels of 3.125, 6.25, 12.5, 25, and 50ppm and measured at a wavelength of 570 nm. The results showed that BETD5 supernatant had anticancer activity against T47D cells line used in this study.

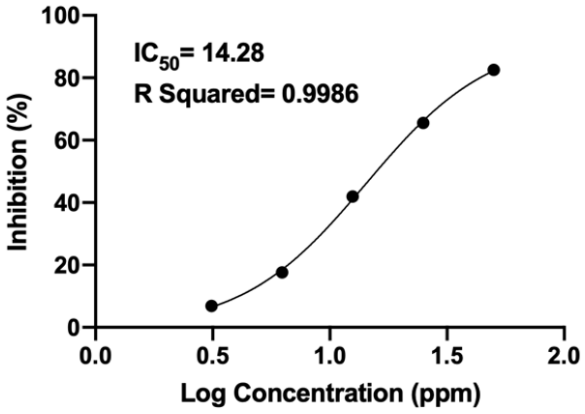

Figure 2. Relationship between Log Concentration and Inhibition

The relationship between the concentration log and the percentage of inhibition tested through nonlinear regression analysis of four logistic parameters using GraphPad Prism 9 software (Figure 2). The results of absorbance readings using Elisa Reader revealed that the higher the concentration used, the lower the absorbance value produced. This absorbance value was also related to the percentage of inhibition, where the higher the absorbance value, the lower the percentage of inhibition. The highest percentage of inhibition was at a concentration of 50 ppm at 82.52%, while the lowest was at 3.125 ppm at 6.86%.

The correlation between log concentration and percentage inhibition was shown by the R^2 values. In this study, the R^2 value (Coefficient of Determination) obtained was 0.9986, indicating that 99.86% of the percentage of inhibition was influenced by the supernatant concentration of *Staphylococcus* sp. BETD5. According to Puspitasari and Proyogo (2017), an R^2 value close to one indicated a relationship between log concentration and percentage value. Therefore, the higher the concentration of *Staphylococcus* sp. BETD5 supernatant, the greater the percentage of inhibition.

IC_{50} value of 50% supernatant *Staphylococcus* sp. BETD5 obtained from nonlinear regression analysis of four logistic parameters using GraphPad Prism 9 software was 14.28 ppm (µg/ml). The result showed that the supernatant *Staphylococcus* sp. BETD5 had strong cytotoxic properties against T47D breast cancer cells. The United State National Cancer Institute (NCI) classified toxic compounds into 4 categories based on their IC_{50}, namely $IC_{50} \leq 20$ µg/ml = strong, IC_{50} 21-200 µg/ml = medium, IC_{50} 201-500 µg/ml = weak and $IC_{50} > 501$ µg/ml = non-toxic (Sajjadi *et al*., 2015). Therefore, BETD5 supernatant had the potential to be used as an anticancer agent due to its highly toxic compounds. The results of the nonlinear regression analysis of the four parameters logistic obtained can be used for further comparison and validation purposes for future investigation. The nonlinear regression equation of four logistic parameters is as follows.

$$y = 92.87 + \frac{-0.6608 - 92.87}{1 + 10^{(\log 10x - \log 10IC50)\ 1.640}}$$

Table II. GCMS Analysis of BETD5 supernatant

Peak#	Name	R. Time	Area	Area%
1	.alpha.-Thujene	5.953	35477687	3.08
2	cis-Ocimene	6.091	279690674	24.31
3	l-Phellandrene	6.662	134334479	11.67
4	2-.BETA.-PINENE	6.729	160873209	13.98
5	.beta.-Myrcene	6.893	41779604	3.63
6	1-PHELLANDRENE	7.108	18112972	1.57
7	.DELTA.3-Carene	7.234	14320656	1.24
8	.alpha.-Terpinene	7.316	82469753	7.17
9	dl-Limonene	7.518	89194534	7.75
10	.gamma.-Terpinene	7.963	80274931	6.98
11	trans Sabinene hydrate	8.020	1045912	0.09
12	.ALPHA.-TERPINOLENE	8.412	30454448	2.65
13	endo-Borneol	8.490	3138196	0.27
14	3-Cyclohexen-1-ol, 4methyl-1-(1-methylet	9.700	47955651	4.17
15	.alpha.-Terpineol	9.847	12781145	1.11
16	Safrole	11.151	11170420	0.97
17	ENDOBORNYL ACETATE	11.230	1493720	0.13
18	Benzene, 1-methoxy-4-pentyl- (CAS) P-N-	11.505	4566319	0.40
19	Eugenol	12.060	1596437	0.14
20	Benzene, 1,2dimethoxy-4-(2propenyl)- (C	12.604	2894222	0.25
21	.alpha.-Copaene	12.694	1746602	0.15
22	trans-Isoeugenol	13.267	3670049	0.32
23	1,3-Benzodioxole, 4methoxy-6-(2propeny	14.194	89279342	7.76
24	Benzene, 1,2,3trimethoxy-5-(2propenyl)-	14.484	2341403	0.20
			1150662365	100.00

GCMS Analysis

GCMS analysis was performed on BETD5 supernatants to identify the compounds contained in the supernatant, which were displayed based on compound peaks, retention times, and area percentages. The number of compounds contained in BETD5 supernatant was indicated by peaks on the chromatogram, while the name/type of compounds present was interpreted based on spectrum data from each peak using the library approach method in GCMS database (Table II).

Based on Table 2, the secondary metabolite profile results from BETD5 supernatant GCMS analysis showed that there were 24 compound peaks. There were 20 compounds classified as terpenoids, 3 as phenols, and 1 as alkaloids. Furthermore, approximately 83.3% of the compounds in BETD5 supernatant were terpenoids. According to Marianna *et al*. (2014), terpenoids are dehydrogenated and oxygenated derivatives of terpenes. Terpenoids were also called isoprenoids because they had the same carbon skeleton the same as the isoprene compound (C5H8). Furthermore, terpenoids were chemical compounds of natural materials consisting of several isoprene units. Most of these compounds had varying structures that are biologically active and widely used in the treatment of various diseases, such as cancer.

The highest compound component in BETD5 supernatant was cis-ocimene, located at peak 2 with an area percentage of 24.31%, followed by 2-Beta pinene at peak 4 with an area percentage of 13.98%, and 1-Phellandrene at peak 3 with an area percentage of 11.67%. Ocimene belonged to the acyclic terpenoids and had been reported to have in vitro cytotoxicity to human breast tumor cells MDA-MB-231 (Ali *et al*., 2014). Beta pinene belonged to unsaturated bicyclic terpenoids and had been found to have an inhibitory effect on breast cancer and leukemia (Salehi *et al*., 2019). Meanwhile, Phellandrene belonged to cyclic

terpenoids and according to Basholli-Salihu *et al*. (2017), the compound had the potential to be the main component of anticancer drug development, where it showed cytotoxicity activity against colon cancer cells.

CONCLUSION

This study showed that BETD5 had strong antioxidant activity, with an IC_{50} of 66.27 ppm. Biochemical characterization of BETD5 bacteria isolate showed that BETD5 belonged to the genus *Staphylococcus*. Anticancer activity evaluation of BETD5 supernatant obtained an IC_{50} value of 14.28 ppm. This indicated that BETD5 supernatant had strong cytotoxicity against T47D breast cancer cells line. GCMS analysis showed that BETD5 supernatant had the highest secondary metabolite content, namely cis-Ocimene at 24.31%.

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