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Antibacterial and Antioxidant Activity of Endophytic Bacteria Isolated from *Hibiscus tilaceus* Leaves

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Abstract

Antibacterial is a compound that inhibits or kills bacteria, especially infectious pathogenic bacteria. Antioxidants are compounds to inhibit the activity of free radicals in the body. The leaf extract of the waru plant (*Hibiscus tiliaceus*) is reported to have antibacterial, antioxidant, and anti-inflammatory activity. Bioactive compounds obtained from plants generally require a lot of plant availability and large areas for growth and take a long time. One solution to solve this problem is to isolate endophytic bacteria from plants. Endophytic bacteria are bacteria that live symbiotically in the host tissue so that they can produce the same bioactive compounds as the host. In the study, several stages were carried out, including isolation of endophytic bacteria from hibiscus leaves, gram-staining of bacteria, secondary metabolites production, antibacterial activity analysis by disk method, antioxidant activity analysis by DPPH free radical scavenger method, phytochemical screening, and identification of genotypic endophytic bacteria. Isolates of endophytic bacteria from *Hibiscus tiliaceus* leaves were obtained in cocci. They formed gram-positive bacteria with the closest relationship with *Staphylococcus warneri* strain AW 25 and *Staphylococcus pasteuri* strain ATCC 51129. Endophytic bacteria from the *Hibiscus tiliaceus* leaves produce secondary metabolites containing alkaloids and saponins that can inhibit the growth of *Staphylococcus aureus* and *Salmonella typhi* and can be an antioxidant agent.

Keywords: Antibacterial activity, antioxidants, endophytic bacteria, *Hibiscus tiliaceus*, secondary metabolites.

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1. INTRODUCTION

Endophytic bacteria are microorganisms that host plants and can utilize their hosts without harming the host. Endophytic bacteria can produce new secondary metabolites with significant bioactivity potential (Biswas *et al.*, 2020). Endophytic bacteria were utilized to produce bioactive compounds because their host has advantages, including a faster life cycle, being produced in large quantities without large land, and obtaining new bioactive compounds by providing different conditions (Tanaka *et al.*, 1999).

Waru plant leaves contain alkaloids, carbohydrates, sesquiterpenes, quinone sesquiterpenes, steroids, triterpenes, and several types of acids such as amino acids, organic acids, and fatty acids (Bandaranayake, 2002) with tannin as the primarily compounds. Tannins are natural water-soluble brown pigments (Mulyani *et al.*, 2020). Besides that, in the waru tree, there are saponins in the leaves and roots. Samsudin *et al.*, 2019 reported that waru leaf extract is antibacterial and antioxidant. Ramposhad *et al.*, 2012 also reported that waru leaf extract could inhibit the growth of 3 strains: *Escherichia coli*, *Salmonella parathyphi*, and *Staphylococcus aureus*.

Secondary metabolite compounds can also be obtained from endophytic bacteria. Endophytic bacteria are microorganisms found in the entire tissue of a living plant species without harm to the host plant. Endophytic bacteria produce bioactive compounds with similar properties to the host plant due to the genetic transfer from the host plant to endophytic bacteria (Tan & Zou, 2001). Endophytic bacteria produce secondary metabolites with similar abilities to the host

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plant. Therefore, it can be applied to produce secondary metabolite compounds from endophytic bacteria (Radji, 2005).

This work aims to study the endophytic bacteria isolates that are symbiotic with waru leaves (*Hibiscus tiliaceus*) using Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) as growth media. Then, the isolate was analyzed for phytochemical screening, secondary metabolites extraction, antibacterial activity, antioxidant activity, and identification of endophytic bacteria.

2. MATERIALS AND METHODS

Materials and Instrumentation

The materials used in this study are Tryptic Soy Agar (Merck), Tryptic Soy Broth (Merck), Lysozyme (geneaid), demineralization water, Mg powder (Merck), 2,2-diphenyl-1-picrylhydrazyl (DPPH) p.a (Merck), Na_2CO_3 (Merck), Folin-Ciocalteu reagent (Merck), NaOH (Merck), crystalline violet, iodine solution, safranin, chloroform (Merck), $FeCl_3$ (Merck), H_2SO_4 concentrated (Merck), anhydride acetate (Merck), HCl (Merck), NaClO (Merck), ethanol (Merck), Dragendorff reagents (Merck), Mayer reagents (Merck). The instruments used are a set of laboratory glassware, laminar airflow, microscopes, analytical balances, UV-Visible spectrophotometer (T60U Spectrometer), polymerase chain reaction system 2400 (multigen optimax), Nanodrop 2000 spectrophotometer (Thermo Scientific), and UV transilluminator (Cambridge).

Isolation of Endophytic Bacteria from *Hibiscus tiliaceus* Leaf Tissue

Waru plant (*Hibiscus tiliaceus*) leaves were obtained from Lapangan Tembak Diponegoro Semarang, Indonesia. The isolation process refers to Oktavia & Pujiyanto's (2018) method with some modifications. Waru leaves were washed, cut, and sterilized aseptically by soaking with ethanol 70% and sodium hypochlorite. Then, the leaves were rinsed gradually using sterile water three times and ground until smooth. Next, the last rinse water was used as a negative control, and the obtained waru leaf extract was inserted into a centrifuge tube. Both were spread on TSA media that had solidified in a petri dish using a spreader and then incubated for 24 hours at room temperature. The growing bacteria were separated by their morphological appearance and refreshed on TSA media to obtain isolate stocks.

Gram Staining

The gram staining test was carried out following standard staining procedures and techniques. Microscopic observation was carried out by adding immersion oil to the bacterial sample so that the morphology of the bacterial colony was more clearly observed on the microscope.

Secondary Metabolites Production

Endophytic bacteria isolates were inoculated on liquid media and incubated in an incubator shaker for 3 hours at room temperature as a starter. Then re-inoculated in 200 mL of liquid media and incubated in an incubator shaker at 125 rpm for 20, 22, and 24 hours at room temperature. The 20^{th} hour is the final stationary phase, and the 22^{nd} and 24^{th} hours are the final phase of death obtained based on the growth curve of waru leaf endophytic bacteria. The culture obtained was centrifuged for 9 minutes at 6000 rpm. The obtained supernatant was freeze drying for 7 days to obtain a secondary metabolite extract.

Antibacterial Activity Test with Disc Diffusion Method

30 µL of test bacteria have been standardized at 0.5 McFarland was inoculated on a solid medium of petri dishes. Subsequently, disc paper with a diameter of 5 mm soaked in a solution of secondary metabolites of various concentrations (10.000, 21.000, 23.000, 27.000, and 30.000 ppm) was placed in the medium and incubated for 24 hours. A clear zone around the disc's perimeter indicates the absence of bacterial growth as measured by a ruler.

Antioxidant Activity Test with DPPH Reagent

Metabolite samples were dissolved using ethanol with varying concentrations; 1 mL metabolite of each concentration variation was dissolved with 3 mL DPPH 0.1 mM and was then homogenized and incubated for 30 minutes. The absorbance of the solution was measured using UV-Vis spectrophotometry at 517 nm. The absorbance value was used to calculate the IC_{50} value with the equation 1:

$$\%\ inhibiton = \frac{A\ control - A_{test\ sample}}{A\ control} \times 100\% \text{ .....(1)}$$

The regression equation is intercepted between sample concentration on the x-axis and the value of %inhibition on the y-axis. The IC_{50} value was calculated when the %inhibition was 50%.

Phytochemical Screening

Phytochemical screening of flavonoids, alkaloids, saponins, tannins, and quinones is performed based on (Usman *et al.*, 2009) method.

Genotypic Identification

DNA Isolation and Purification

DNA isolation is carried out by the Promega method. Bacteria cultured overnight were put into a micro-tube, centrifuged for 2 minutes at 16.000 rpm, and discarded. The obtained cell pellets were added lysozyme and then incubated for 30 minutes and centrifuged. The cell pellets were added to nuclei lysis solution and Protein Precipitation solution gradually and, in each treatment, incubated at room temperature for 10 minutes, after which it was centrifuged. DNA purification was performed by adding isopropanol to the obtained supernatant from the previous step and centrifuging for 3 min at 13.000 rpm. The obtained DNA pellets were added with 70% ethanol and centrifuged. The obtained supernatant was removed, and then the micro-tube was aerated for 15 minutes. A rehydration solution was added and stored at -20 °C to prevent the destruction of the isolated DNA. The DNA obtained was then quantitatively tested for concentration and purity using the NanoDrop 2000 spectrophotometer.

Amplification of 16S rRNA Gen using PCR

The obtained DNA samples were amplified in vitro by the PCR method. 16S rRNA gene fragments were amplified using reverse primers 1492 R (5' TAC GGY TAC CTT GTT ACG ACT T 3') and forward 27 F (5' AGA GTT TGA TCM TGG CTC AG 3') (Leonita *et al.,* 2016). A total of 2 µL of DNA samples were added 2 µL of 27F primer, 2 µL of 1492R primer, 25 µL of Taq polymerase, and 19 µL of distilled water in a microtube. The sample entered in the PCR machine is amplified for 30 cycles.

Electrophoresis

Electrophoresis was performed using 1% agarose gel. Agarose gel was added TAE 1x until all its surfaces were submerged. 5 µL fragments of the amplified 16S rRNA gene were inserted into the agarose gel well. Electrophoresis was performed within 45 minutes at 100 volts. Observation of agarose gel after the electrophoresis process is completed under a UV transilluminator to observe DNA bands. The obtained DNA bands show fragments of chromosome DNA implicated at the PCR. Then the nucleotide sequence was read by the sequencing method.

Sequencing

PCR results of the 16S rRNA gene were sequenced at the Sequencing Service. The sequencing analysis results are forward and reserve DNA sequences. Then the DNA sequences are combined using Baser DNA counting results (DNA sequence output). Then it was compared to DNA sequences from GenBank data using the BLAST program contained in NCBI (National Centre of Biotechnological Information).

Phylogeny Studies

The sequencing results were then phylogeny studied by aligning using the MEGA 7 program. The 16S rRNA gene nucleotide sequence from the sample with comparison bacteria from BLAST (Basic Local Alignment Search Tool Nucleotide) found in NCBI (National Centre of Biotechnological Information) was aligned using the Alignment by Clustal W menu, and the output data was obtained in file.mega. Next, construct a phylogeny tree from the align results using the Mega 7 program, click the Phylogenetic Analysis menu, then enter the alignment results of Alignment by Clustal W as an input data. After that, select Phylogenetic Analysis construct/Test Neighbor-Joining Tree with the phylogeny test using Bootstrap methods to obtain a phylogeny tree construction.

3. RESULTS AND DISCUSSION

Isolation of endophytic bacteria

Table 1 shows that isolates of endophytic bacteria are cocci-shaped and classified as Gram-positive bacteria. The acquired bacterial isolates are inoculated on the medium to be oblique for a single bacterial isolate stock.

Table 1. Characteristics of waru leaf endophytic bacteria (*Hibiscus tiliaceus*)

Isolates	Color	Colony Form	Edge Shapes	Cell Shape	Elevation	Gram staining
Endophytic bacteria	Yellowish white	Round	Soft	Cocus	Convex	positive

Bacterial Growth Curve

The observation of the bacterial growth curve aims to determine the phases of bacterial growth. In this study, observations aim to determine the time of production of isolated secondary metabolites of endophytic bacteria. **Figure 1** shows the end of the bacterial death phase at the 24th hour. The final phase of bacterial death is one of the phases where bacteria compete with each other for nutrients because the availability of nutrients has run out. Therefore, some cells will die, and live bacteria carry out metabolic processes to produce secondary metabolites used to survive while fighting for nutrients. The following research stage is the production of secondary metabolites of endophytic bacteria.

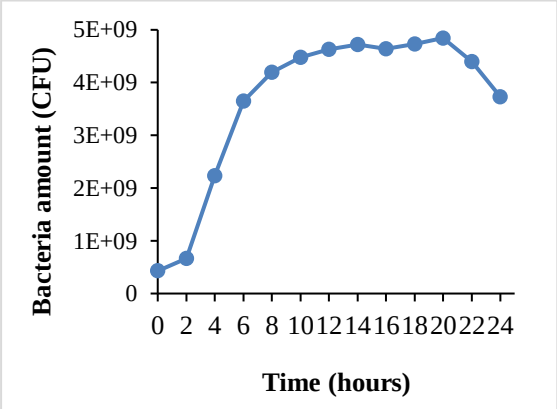

Figure 1. Growth curve of endophytic bacteria

Production of Secondary Metabolites

Based on the growth curve of endophytic bacteria in **Figure 1**, secondary metabolites are produced three times, namely at the 20th hour, which is the final stationary phase. The final stationary phase has a condition where the nutrients have begun to decrease, and there is a buildup of metabolites resulting from metabolic activity. The production of secondary metabolites is then carried out at the 22nd and 24th hour is the death phase, where in this phase, the number of live bacteria will decrease, and the number of cells formed is less than that of dead cells. The more prolonged the incubation of nutrients contained in the medium, the less it will be, causing all cells to eventually die if they are not transferred to a new medium (Sharah *et al.*, 2015). It can be seen in the chart decreasing at the 22nd and 24th hour. The obtained secondary metabolites are concentrated using the freeze-drying method. The obtained metabolite is brown, gaining a mass of 14.08 g at the 24th hour. The viscous extract of secondary metabolites was tested for phytochemicals. **Table 2** shows the results of the phytochemical screening of secondary metabolites.

Table 2 shows that the extract of the endophytic bacteria and waru leaves contain the same secondary metabolite compounds, saponins, and alkaloids. Tan & Zou (2001) explained that it could happen because endophytic bacteria that host plants can produce bioactive compounds and have the potential to produce bioactive compounds similar to or the same as the host plant. Kusari *et al.* (2012) reported that plants and endophytic microbes could sometimes produce similar bioactive compounds. Brader *et al.* (2014) explained that some metabolites are not only produced by a single organism but by plants in combination with microorganisms or related bacteria. The metabolites produced include endophytic microbes that interact with plants, bilateral synthesis, and plants due to induction from endophytic microbes. This interaction between the endophyte and its host can induce the synthesis of secondary metabolite compounds. In the extract of secondary metabolites of endophytic bacteria of waru leaves, no flavonoids, tannins, or quinones were identified. It happens because of several possibilities, namely the difference between the environment of bacterial media and plants.

Table 2. Phytochemical screening of secondary metabolites of endophytic bacteria

Compound Group	Secondary Metabolite Extract Test Results			
	20th hour	22nd hour	24th hour	Waru Leaf Ethanol Extract
Saponins	+	+	+	+
Flavonoids	-	-	-	+
Alkaloids	+	+	+	+
Quinones	-	-	-	+
Tannins	-	-	-	-

Antibacterial Activity

The method used for the antibacterial activity test is the disc diffusion method. The disc diffusion method can determine the effect of inhibition of extracts of secondary metabolites of waru leaf endophytic bacteria to *Salmonella typhi* test bacteria as gram-negative bacteria and *Staphylococcus aureus* as gram-positive bacteria. Thick extract of freeze-drying dissolved into several concentrations, namely 10.000, 21.000, 23.000, 25.000, 27.000, and 30.000µg/mL. The test bacteria measured their absorbance at $5x10^8$ CFU/mL or equivalent to a scale of 0.5 McFarland, which aims to condition the number of bacterial cells as standardized. The results of the antibacterial test is shown in **Figure 2-4**. **Figure 2** shows that the viscous extract of secondary metabolites of endophytic bacteria of waru leaves of the 20th hour has weak antibacterial activity against bacterial growth due to the non-formation of an inhibitory zone in the test media.

Figure 3 shows that the viscous extract of endophytic bacteria of the 22nd-hour waru leaves has antibacterial activity against the growth of test bacteria. **Figure 3** exhibits the antibacterial activity profile tends to be more potent against gram-negative Salmonella typhi than gram-positive Staphylococcus aureus. **Figure 3** shows that the higher the concentration of antibacterial metabolites, the higher the tendency to bacteriolytic.

Figure 4 shows that the viscous extract of secondary metabolites of endophytic bacteria of waru leaves of the 24th hour has more potent antibacterial activity against the growth of *Staphylococcus aureus* than *Salmonella typhi* because at a concentration of 21.000 ppm inhibition has occurred. **Figure 4** shows that the higher the concentration of antibacterial-producing metabolites, the higher the tendency to be bacteriolytic.

Viscous extracts of secondary metabolites of endophytic bacteria of waru leaves in the stationary phase of the 20th hour have no antibacterial activity. In contrast, it has antibacterial activity in the final stationary phase at the 22nd hour and the midwifery phase at the 24th hour. Secondary metabolites contained at the 20th hour could not inhibit the growth of test bacteria. Secondary metabolites are typically produced during the final growth phase of microbes. The resulting metabolites are antibiotics, antiparasitics, and others (Sanchez & Demain, 2011). Secondary metabolites usually accumulate in the final stationary phase. The compounds produced in this phase are secondary metabolites formed after the bacterial growth is completed (Raji *et al.*, 2019).

Secondary metabolite extracts in the final stationary phase at the 22nd hour can already inhibit both test bacteria at 10.000 ppm. In comparison, at the 24th hour, it can only inhibit the growth of *Staphylococcus aureus* bacteria at 21.000 ppm. Lakna (2017) explains that the differences in cell wall structure between gram-positive and gram-negative bacteria cause differences in bacterial responses to alkaloid extracts. Inhibition in gram-negative bacteria is high because the cell wall in gram-negative bacteria only consists of 11-22% lipids and 10% peptidoglycans. While gram-positive bacteria have a thicker cell wall arrangement which is composed of more than 50% peptidoglycans, polysaccharides (teichoic acid), and 1-4% lipids. The cell membrane of gram-negative bacteria is also more hydrophobic than gram-positive (Chitemerere & Mukanganyama, 2011).

Dong *et al.* (2020) reported that saponin works by diffusing through the outer membrane and cell wall. Moreover, a change in the permeability of the bacterial cell membrane damages the cell membrane. It causes the removal of essential components in bacterial cells, such as nucleic acids, nucleotides, and proteins.

The antibacterial activity test using the disc diffusion method showed that the secondary metabolites of endophytic bacteria waru leave has antibacterial activity for *Salmonella typhi* and *Staphylococcus aureus*. The inhibition zone formed is caused by the activity of alkaloids and saponins that diffuse into the agar medium and inhibits bacterial growth. Alkaloids and saponins are secondary metabolites of plants that have been shown to have strong pharmacological activity (Yan *et al.*, 2021; Dong *et al.*, 2020).

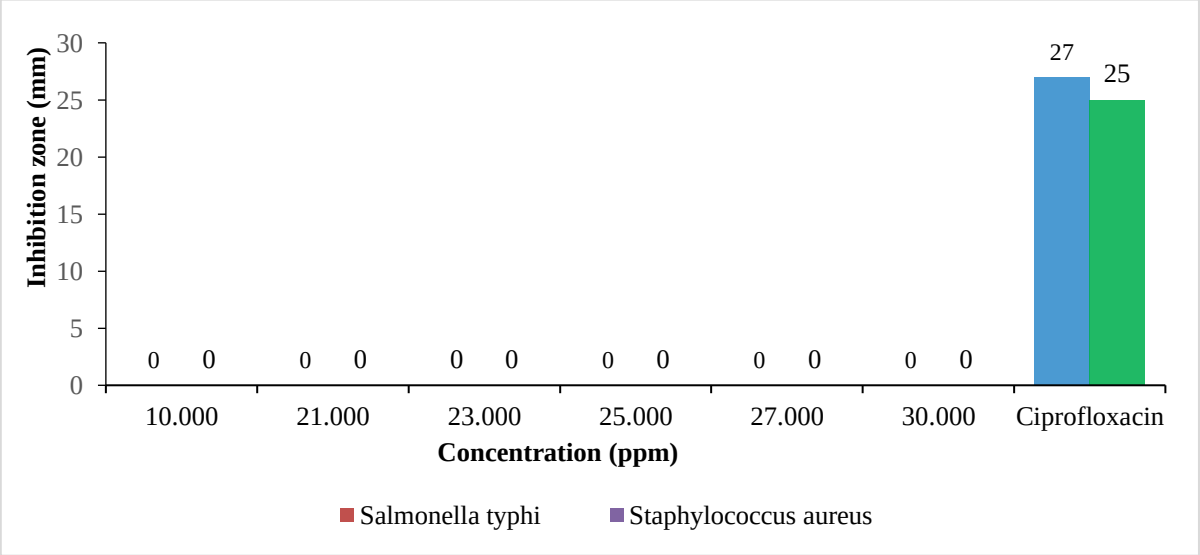

Figure 2. The antibacterial activity of secondary metabolites at the 20th hour

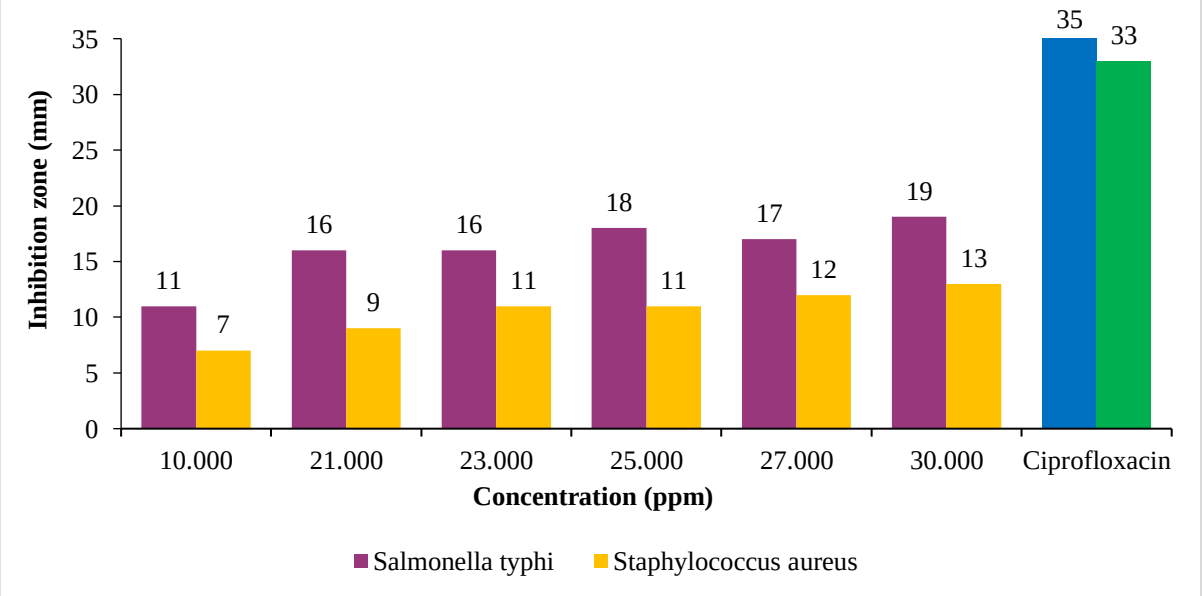

Figure 3. The antibacterial activity of secondary metabolites at the 22nd hour

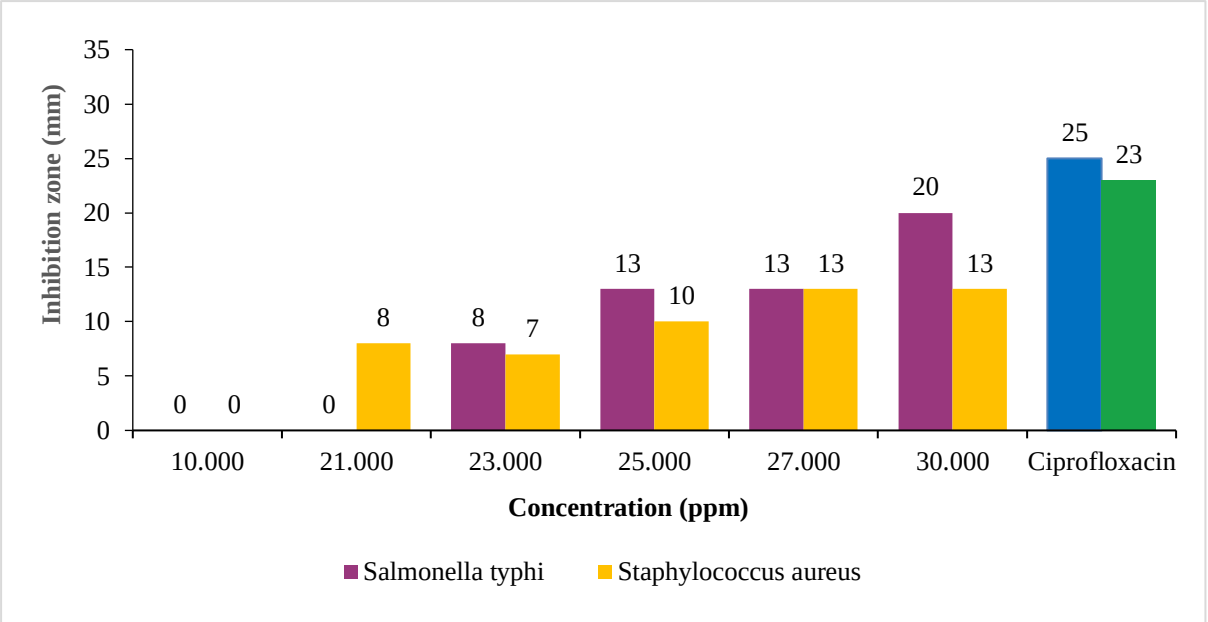

Figure 4. The antibacterial activity of secondary metabolites at the 24th hour

Alkaloids are alkaline and semi-polar compounds with nitrogen atoms in their basic structure and can react with amino acids on cell walls and bacterial DNA (Yan *et al.*, 2021). These reactions can change the structure and arrangement of amino acids and genetic balance in the DNA strand, which can cause the destruction of cell walls and bacterial DNA, which causes cell lysis so that bacteria die. Alkaloids can act as antibacterials by changing the constituent components of peptidoglycan in bacterial cells so that the cell wall layer is not formed intact and causes cell death (Amalia et al., 2017). Antibacterial activity may be caused by the presence of the hydroxyl (OH) group in alkaloids' structure that changes cell proteins' properties (denaturation). The alkaloid compound in waru leaf extract is 3-Piperidinol (Bata & Rahayu, 2017). It increases the permeability of cell membranes and causes the loss or leakage of the contents of a bacterial cell out or through the direct connecting membrane of bacterial cells, thus causing damage to the polar membrane of bacteria leading to the gradual death of bacteria (Gurrapu & Mamidala, 2017).

On the other hand, saponins can also be antibacterial compounds (Bernard *et al.*, 2022). Saponins are glycoside compounds with two parts. The glycon consists of monosaccharides such as fructose, glucose, and others. Another part of saponins is aglycon which is sapogenins. Saponins work by lowering the water surface tension and forming foam on the water surface after shaking. It is due to soap compounds in saponins that can damage hydrogen bonds in water. Surfactants have two different parts of polarity properties, hydrophilic and lipophilic groups (Nurzaman *et al.*, 2018). The hydrophilic group is the polar site that can bind to water molecules.

In contrast, the lipophilic group is a part of saponins that have non-polar properties and can bind fats. Zahro & Rudiana (2013) reported that saponins could change the permeability of bacterial cell membranes, damage cell membranes, and eliminate cell components like nucleotides, proteins, and nucleic acids. The cytoplasmic membrane can preserve cell components and control electrons, mineral ions, amino acids, sugars, and other metabolites that pass through the membrane. Therefore if the membrane is damaged, it can inhibit cell growth or cause cell death. Saponins cause a decrease in the surface tension of the bacterial cell wall because saponins have an active site, aglycon, which is membranolytic or can form complexes with cholesterol on the cell membrane of protozoa. After the surface tension of the bacterial cell wall decreases, the saponins will fix the complex with sterols causing the formation of a single ion channel. A single ion channel causes membrane instability and inhibition of enzyme activities, especially enzymes that function as ion transport for bacterial survival. Bacterial growth will be inhibited if the ion transport is inhibited.

Antioxidant Activity

The antioxidant activity of metabolites can be seen from the color changes of DPPH after and before incubation from purple to yellow. This color change is affected by the reducing stable DPPH compound into 2,2-diphenylpicrylhydrazyl compound (Molyneux, 2004). From measurements with UV-Vis spectrophotometry, absorbance data were obtained, and the inhibition percentage was calculated. Inhibition percentage is the sample's ability to inhibit the activity of certain free radicals that the difference in the absorbance between the sample and the control can measure.

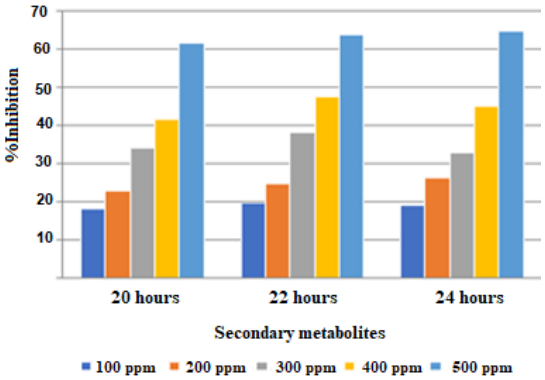

Figure 5. Antioxidant activity of secondary metabolites of endophytic bacteria

Figure 5 revealed that at 500 ppm, each extract has a high inhibition percentage value. The higher the concentration of the extract followed, the more excellent the inhibition percentage. The antioxidant activity of the extract is determined by an IC_{50} value calculated using a linear regression equation. The IC_{50} value of the extract was calculated using linear regression.

Table 3. The IC_{50} value of secondary metabolites extract of endophytic bacteria waru leaves

Sample	IC_{50} (ppm)	Information
Acid Gallic	62.91	Strong
20th hour	435. 09	Very weak
22nd hour	400.34	Very weak
24th hour	412.74	Very weak

An IC_{50} value is calculated to determine the amount of extract that can inhibit 50% free radicals. The smaller the IC_{50} value, the higher the antioxidant activity of the extract (Rivero-Cruz *et al.*, 2020). When the IC_{50} < 50 ppm, then the antioxidant activity of the sample is strong, the IC_{50} ranging from 100-150 is moderate, and the antioxidant activity of the sample is weak if the IC_{50} > 200 ppm (Munteanu & Apetrei 2021). **Table 3** shows that extracts from metabolites at the 20th, 22nd, and 24th hours have very weak antioxidant activity. It is because extracts from metabolites contain only alkaloid compounds and saponins, which are not as good as flavonoid compounds for reducing free radicals.

Alkaloid compounds include secondary metabolite compounds that are widely found in plants. It has alkaline properties composed of one or more nitrogen atoms and is in heterocyclic rings (Harborne, 1987). Fusaritricines A-I (1-9) is a new imidazole alkaloid compound known to have antioxidants in kiwi endophytic microorganisms (Ma *et al.*, 2022).

Saponins, also secondary metabolite compounds, are steroid glycosides or triterpenes that have aglycon (not glucose groups) in the form of sapogenins (Lim *et al.*, 2019). Brindhadevi *et al.* (2022) reported that saponin extracts from Curcuma angustifolia have excellent antioxidant activity. Chen *et al.* (2014) also reported that saponins could have antioxidant activity in vitro even though their levels are trimmed by inhibiting free radicals.

Genotypic Identified

DNA Isolation and Purification

Molecular biology identification is based on the nucleotide sequence of the 16S rRNA gene. Chromosomal DNA is obtained by isolation carried out by cell lysis. The purity of DNA isolation endophytic bacteria can be seen in **Table 4**. **Table 4** shows that the endophytic bacterial DNA concentration and purity test obtained a nucleic acid concentration of 70.5ng/µL. The purity measured at 260/280 nm is 2.08, and at 260/230 nm is 2.60 nm. Nucleotides, DNA, and RNA will absorb at 260 nm, while proteins and other contaminants absorb at 280 nm.

The success of DNA isolation is an important stage as a template for the PCR process. The quality of isolated DNA can be seen from the purity value and concentration of DNA, where DNA must have high purity. DNA purity is measured using an absorbance ratio of 260/280 nm. Isolation using the Promega method is the isolation that adds lysozyme. The function of lysozyme is to damage the cell wall of

bacteria. Adding lysozyme to gram-positive bacteria is significant because gram-positive bacteria contain thick peptidoglycans. When lysozyme successfully hydrolyzes the bacterial cell wall, it will be damaged and cause essential components like bacterial DNA in the cell to come out.

Table 4. Results of DNA nanodrop isolates of endophytic bacteria

Sample	Nucleic Acid Concentration (ng/µL)	A 260/280 (nm)	A 260/230 (nm)
Endophytic waru	70.5	2.08	2.60

Amplification of 16s rRNA Gene

The sequencing stage requires sufficient DNA, so it is necessary to amplify DNA. DNA amplification aims to double the number of sequences obtained through the Polymerase Chain Reaction (PCR) technique by using chromosomal DNA as a mold. **Figure 6** shows that DNA amplification showed positive results marked by amplicons from endophytic bacteria with the formation of DNA strands measuring 1500 bp.

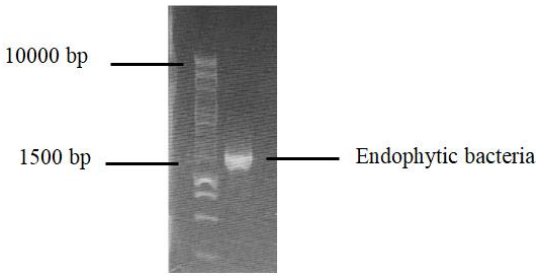

Figure 6. Results of electrophoresis of fragments of the gene 16S rRNA of endophytic bacteria

Endophytic Bacteria Amplicon Sequencing Results

Sequencing is used to determine the sequence of nucleotide bases of 16S rRNA gene fragments so that genotypic bacterial identity will be obtained. In the sequencing process, 27F and 1492R primers were used to elongate the 16S rRNA gene fragment. The extension of the 16S rRNA gene fragment chain begins and ends using primers that are complementary to specific regions of the nucleotide sequence of the molded DNA. In the sequencing process, four types of deoxinsinucleotide bases (DNA-forming units) and ddNTP or chain-stopping nucleotides with low concentrations were added. The sequencing process will produce two types of data: nucleotide sequence data (file.seq) and electrophoregam data (file.ab1). The nucleotide sequence's correctness can be validated by checking the electropherogram's quality and readjusting the sequence using the sequence scanner 2 programs. The two nucleotide sequence data were combined (contig) using a baser-assembled DNA program. Combining the two nucleotide sequences is the sequence data of 16S rRNA gene nucleotide measuring 1421 bp.

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AAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTA
CCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATATTGAAC
CGCATGGTTCAATAGTGAAAGGCGGCTTTGCTGTCACTTATAGATGGATCCGCGCCGT
ATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGAYTCCTACGGGAGGCAGCAGT
AGGGATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
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TACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT
TTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAA
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GCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACA
TCCTTTGACCGCTCTAGAGATAGAGTCTTCCCCTTTGGGGGACAAAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACA
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CACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCAAGCAAATCCCA
TAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCG
CTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCG
CCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAG
CTAGCC
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Figure 7. Result of nucleotide base sequence of 16S rRNA fragments

Phylogenetic Tree Study of Endophytic Bacteria

The construction of a phylogeny tree can reveal the kinship of an organism from its genotypic characteristics. The obtained sequence of nucleotide bases of gene fragments 16S rRNA isolates of endophytic bacteria of contig sequencing was used to determine the kinship relationship of endophytic bacterial isolates with comparison bacteria from the GenBank database using the BlastN program. The results of the nucleotide sequence data of nearby bacteria were carried out in the advanced stages of phylogeny tree construction using Alignment by Clustal W. Based on the blast results, 50 comparison bacteria from the genus Staphylococcus have been obtained with an average similarity rate of 98%-99%. The construction of the phylogeny tree of waru leaf endophytic bacteria is shown in **Figure 8**.

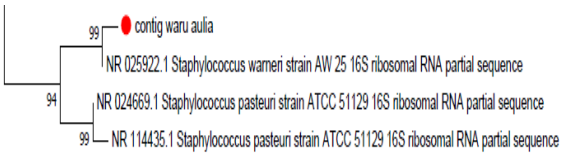

Figure 8. Endophytic bacterial phylogeny tree

Table 5. Comparison of characteristics of bacteria

Bacteria	Characteristic	Habitat
Isolates of Endophytic Bacteria	Cocci, gram-positive	*Waru leaves (Hibiscus tiliaceus)*
Staphylococcus warneri	Kokus, gram-positive (Dong *et al.* , 2017)	Apple fruits (Phukon *et al.*, 2013)
Staphylococcus pasteuri	Kokus, gram-positive (Chesneau *et al.* , 1993)	Plants of Arabidopsis, soybeans and ginger root (Jasim *et al.*, 2013), *cerrado* plant (Oliveira *et al*., 2012)

Based on the partial results of the phylogeny tree construction in **Figure 8**, it shows that the isolates of waru leaf endophytic bacteria have a kinship with Staphylococcus Warneri bacteria strain AW 25 and Staphylococcus pasteuri strain ATCC 51229. *Staphylococcus warneri* and *Staphylococcus pasteuri* have similarity rates of 99.71% and 99.29%, respectively, to isolates of endophytic bacteria. From the magnitude of the percent similarity, it can be seen that the isolates of endophytic bacteria are closer in kinship with *Staphylococcus warneri*. It corresponds to the construction of the phylogeny tree obtained. **Table 5** shows that endophytic bacteria isolates have a similar phenotypic character to *Staphylococcus warneri* and *Staphylococcus pasteuri* bacteria. The equation can be seen from the characteristics that all three bacteria are gram-positive and have a coccal shape. In addition, all three are also endophytic bacteria, even though they do not come from different parts of plants.

4. CONCLUSIONS

Isolates of endophytic bacteria symbiotic with the leaves of *Hibiscus tiliaceus* are classified as cocci-shaped gram-positive bacteria. Phytochemical screening indicates that the secondary metabolites of endophytic bacteria are alkaloids and saponin. These secondary metabolites can inhibit the growth of *Staphylococcus aureus* and *Salmonella typhi*. It also has antioxidant capabilities with IC_{50} at the 20^{th}, 22^{nd}, and 24^{th} hours are 435.09, 400.34, and 412.74 ppm, respectively. Waru leaf endophytic bacteria are related to *Staphylococcus Warneri* bacteria strain AW 25 and *Staphylococcus pasteuri* strain ATCC 51229.

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