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Isolation and Inhibitory Activity Testing of Alpha-Glucosidase Enzyme from Endophytic Bacteria in Kumis Kucing (*Orthosiphon aristatus* (Blume) Miq.) Leaves

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Abstract: *Orthosiphon aristatus* (Blume) Miq., commonly known as kumis kucing, is frequently used in traditional Indonesian medicine to treat diabetes, hypertension, rheumatism, gout, and as a diuretic. However, large-scale production of medicinal products from natural sources requires significant raw material and land, creating a need for alternative methods. This study explores the potential of isolating endophytic bacteria from kumis kucing leaves to serve as α-glucosidase enzyme inhibitors, offering a more sustainable approach. Eight bacterial endophytes were isolated and tested using bioautography assays and IC_{50} measurements to assess their inhibitory activity. Four isolates—KK 1, KK 3, KK 4, and KK 5—demonstrated significant α-glucosidase inhibition, with IC_{50} values of 41.35 µg/mL, 57.04 µg/mL, 56.70 µg/mL, and 164.16 µg/mL, respectively. Molecular testing identified KK 1 as *Priestia aryabhattai*, while KK 3 and KK 5 were identified as *Priestia megaterium*. These findings suggest that endophytic bacteria isolated from *Orthosiphon aristatus* leaves have the potential to be developed as natural sources of α-glucosidase inhibitors, which are beneficial for diabetes management.

Keywords: α-Glucosidase enzyme; Endophytic bacteria; LC-MS; *Orthosiphon aristatus* (Blume) Miq.

Introduction

Diabetes is a chronic disease characterized by elevated blood glucose levels due to the inability of the pancreas to produce and use insulin effectively (Le et al., 2023; Ningrum et al., 2023; Panduwiguna et al., 2023). In diabetic patients, the body cannot efficiently regulate blood glucose levels, leading to serious complications (Faridah et al., 2022; Nguyen et al., 2024; Sasongko et al., 2024). Various antidiabetic drugs, both modern and traditional, have been widely recognized in the community (Mahata et al., 2023; Najim et al., 2024; Prasetyastuti & Ghozali, 2021). One important mechanism of such drugs is the inhibition of alpha-glucosidase enzyme, an enzyme that plays a role in the breakdown of carbohydrates into glucose in the digestive tract (Ariyanti et al., 2022; Hati et al., 2023; Narasukma et al., 2021). Inhibition of this enzyme can help control blood sugar levels in patients with type 2 diabetes mellitus (Julianus et al., 2023; Nova & Virginia, 2023; Sahila et al., 2023).

In the effort to develop new drugs, natural ingredients have a very important role (Danimayostu et al., 2023; Ikhsan et al., 2023; Meriyani et al., 2023). However, drug production from natural sources is often limited by the availability of raw materials, which requires large tracts of land for cultivation of native plants (Grechana et al., 2023; Hawari et al., 2023; Riswanto et al., 2023). One of the more effective

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approaches is through the isolation of endophytic bacteria, microorganisms that live inside plant tissues without harming their host (Atikana et al., 2023; Putri et al., 2023; Sari & Widyasari, 2023). These bacteria can produce bioactive compounds with therapeutic potential, including as alpha-glucosidase enzyme inhibitors (Amalia et al., 2024; Arifin et al., 2024; Harimurti et al., 2024). Endophytic bacteria have the advantage of a relatively short life cycle and do not require large areas for growth, making them a more efficient alternative in the production of natural compounds (Mahyuni & Harahap, 2024; Nastiti et al., 2024; Orbayinah et al., 2024).

Previous studies have successfully isolated several types of endophytic bacteria from cat's whisker (*Orthosiphon aristatus* (Blume) Miq.) leaves, including Acinetobacter schindleri, Pantoea agglomerans, and Pseudomonas lurida. Cat whisker itself is an herbal plant that is often used in traditional medicine to treat various conditions such as hypertension, gout, and diabetes. Cat whisker leaves contain active compounds such as polyphenols and flavonoids, which show potential as antidiabetic agents. Several in vitro studies with cat's whisker leaf extract have proven its alpha-glucosidase enzyme inhibitory activity, providing a strong basis for its use in the management of type 2 diabetes.

Based on this background, this study aimed to isolate endophytic bacteria from cat's whisker leaves and evaluate their activity in inhibiting alpha-glucosidase enzyme. By identifying endophytic bacteria capable of producing enzyme-inhibiting compounds, it is hoped that this study can contribute to the development of more efficient and renewable natural resource-based antidiabetic therapies.

Method

Preparation of Samples

Kumis kucing leaves (*Orthosiphon stamineus*) were obtained from the Medicinal Plant Garden (KTO) of Andalas University, and plant identification was conducted at the Herbarium of Andalas University under number 29/K-ID/ANDA/I/2024. The materials used in this research included aquadest, Nutrient Agar (Merck®), Nutrient Broth, sodium hypochlorite, NaCl 0.9%, ethanol 96%, ethyl acetate, methanol p.a (Merck®), $BaCl_2$, concentrated sulfuric acid, silica gel 60, C18, toluene, formic acid, sodium acetate, acetic acid, aquabidest, α-Glucosidase (Sigma®), 2-naphthyl-α-D-glucopyranoside (Sigma®), 4-Nitro-Phenol-α-D-glucopyranoside (Sigma®), Fast Blue B Salt, Dimethyl Sulfoxide (DMSO), acarbose (Sigma®).

Isolation of Endophytic Bacteria

Kumis kucing leaf samples (*Orthosiphon aristatus* (Blume) Miq.) were thoroughly washed with running water and then dried. Subsequently, the sample surfaces were sterilized using 70% alcohol for 1 minute, 1% sodium hypochlorite (NaOCl) for 1 minute, and rinsed three times with sterile distilled water. The final rinse water from the leaves was inoculated onto Nutrient Agar (NA) media and incubated at 37°C for 48 hours in an incubator. After surface sterilization, the samples were cut into 1 x 1 cm pieces using sterile scissors. The sample pieces were placed on Petri dishes containing NA (Nutrient Agar) media and incubated for 24-48 hours at 37°C, and the growing colonies were observed (Anjum & Chandra, 2015).

Purification of Endophytic Bacterial Isolates

The bacteria that grew were purified individually by transferring different bacterial isolates from old NA media to new NA media in Petri dishes. If there were still colonies that differed macroscopically on the media, further separation was necessary to obtain pure isolates (Rustini et al., 2023).

Preparation of Endophytic Bacterial Isolate Suspensions

2-3 loops of endophytic bacterial isolates were taken using an inoculating needle and suspended in a tube containing 10 mL of sterile 0.85% NaCl solution. The suspension was then vortexed until homogeneous, and its turbidity was compared to McFarland 0.5.

Fermentation of Endophytic Bacteria

A total of 20 mL of bacterial inoculum previously prepared was transferred into a 1 L Erlenmeyer flask, then 400 mL of Nutrient Broth (NB) media was added. The bacterial culture was incubated using an incubator shaker at 37°C and 200 rpm for the time required to measure the optical density results.

Extraction of Endophytic Bacteria Fermentation Results

The fermentation results of the bacteria were extracted using ethyl acetate solvent in a 1:1 (v/v) ratio and macerated for 24 hours. The resulting extract was then separated using a separating funnel and concentrated using a rotary evaporator.

TLC Bioautography

A total of 5 mg of the extract in 1 ml of solvent was prepared to achieve a concentration of 5000 µg/ml for application on Thin Layer Chromatography (TLC) plates. Subsequently, elution was performed using eluent G (toluene: ethyl acetate: formic acid (70:25:5)). After elution, the TLC plate was dried and sprayed with an enzyme solution, followed by incubation at room temperature for 60 minutes. Subsequently, the incubated plate was sprayed with a mixture of substrate and Fast Blue B Salt, and observed for 2-5 minutes until a clear zone appeared against a dark background.

Determination of % Inhibition and IC_{50} of Extracts Against α-Glucosidase Enzyme

Endophytic bacterial extracts exhibiting α-glucosidase inhibitor activity from TLC bioautography screening were prepared to determine the IC_{50} value. Acarbose was used as a reference with a stock solution concentration of 200 µg/ml. The enzyme was dissolved in phosphate buffer (pH 6.8) to achieve a concentration of 0.26 U/ml. Fifty microliters (µl) of the sample were mixed with 50 µl of enzyme and incubated for 10 minutes. After incubation, 100 µl of PNPG was added and further incubated for 20 minutes. Absorbance was then measured at a wavelength of 405 nm using a microplate reader (BioRad).

Molecular Biomolecular Identification of Endophytic Bacterial Isolates

Endophytic bacterial isolates were identified molecularly at the Biotechnology Laboratory, Faculty of Agriculture, Andalas University. The analysis was conducted using the PCR (Polymerase Chain Reaction) method. The process began with DNA isolation using a DNA isolation kit, followed by analysis using 16S rRNA. The DNA base sequences obtained were amplified via PCR and sequenced, then analyzed based on BLAST results in the NCBI database. The analysis revealed the species name of the endophytic bacteria from kumis kucing leaves (Handayani et al., 2023).

Result and Discussion

Isolation of Endophytic Bacteria from Kumis Kucing Leaves (Orthosiphon aristatus (Blume) Miq.)

Following purification, isolation of endophytic bacteria from kumis kucing leaves yielded 8 bacterial isolates: isolates 1 KK, 2 KK, 3 KK, 4 KK, 5 KK, 6 KK, 7 KK, and 8 KK. Macroscopic examination of endophytic bacteria in Figure 1. involved observing colony morphology and growth characteristics, including surface morphology, colony shape, color, and edge morphology. Upon examining these 8 isolates, variations in surface morphology, color, and colony edges were noted. Colony shapes ranged from round to irregular, even appearing as small dots. Colony colors included white and yellow hues. Colony edges exhibited uneven, wavy patterns, potentially indicating differences in bacterial species or types. In addition to macroscopic examination, microscopic characterization of the bacteria was performed. All 8 isolates of endophytic bacteria were identified as rod-shaped and gram-positive. The diversity of endophytic bacteria within a plant is influenced by the plant's growth conditions, particularly soil conditions. In some instances, plants of the same species may host distinct endophytic bacteria. Certain plants harbor specific and characteristic endophytic bacteria.

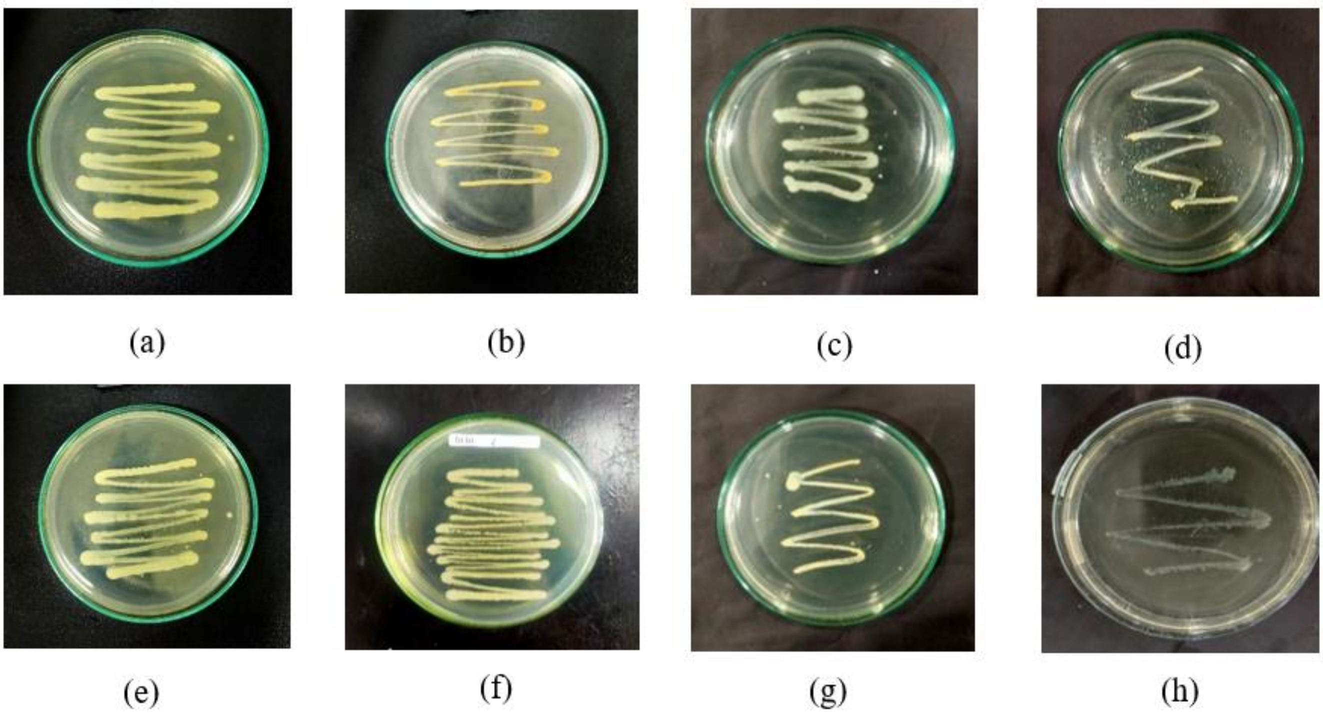


Figure 1. Isolates of endophytic bacteria from Kumis Kucing leaves (*Orthosiphon aristatus* (Blume) Miq). KK 1 (a), KK 2 (b), KK 3 (c), KK 4 (d), KK 5 (e), KK 6 (f), KK 7 (g), KK 8 (h)

Thin Layer Chromatography (TLC)-Bioautography Test

The TLC-bioautography test was conducted to identify points with α-glucosidase enzyme inhibitor activity. The extract to be tested was applied to a TLC plate and eluted using an eluent of toluene: ethyl acetate: formic acid (70:25:5). After the plate was dried and observed (under UV light and with ANS reagent), the patterns formed by each spot indicated separation of the respective extracts, as shown in Figure 2.

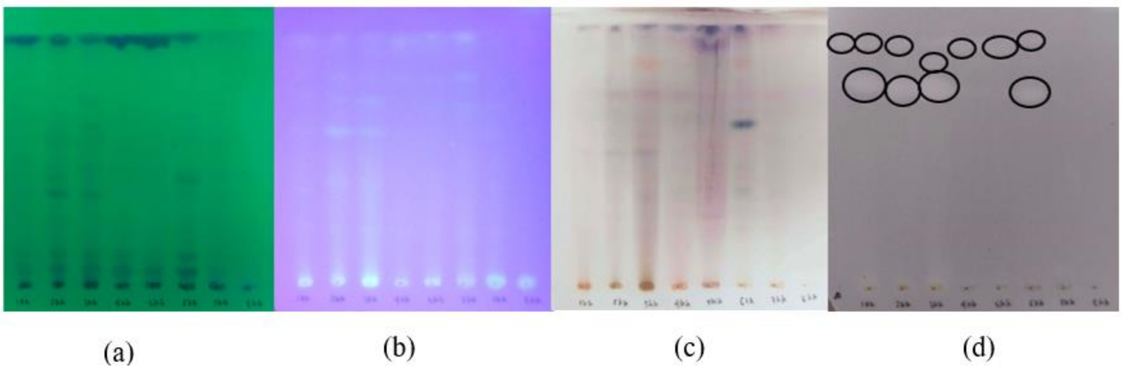


Figure 2. Testing of α-glucosidase enzyme inhibitor activity of ethyl acetate extract from kumis kucing leaf bacteria using TLC-bioautography: (a) TLC plate under UV 254 nm; (b) TLC plate under UV 360 nm; (c) TLC plate with ANS stain visualization; (d) TLC plate reacted with α-glucosidase enzyme, substrate, and Fast Blue B Salt stain visualization

The above figure shows the results of TLC on ethyl acetate extracts from kumis kucing leaf bacteria. There are 8 isolates on each TLC plate tested, sequentially from left to right: isolates 1 KK, 2 KK, 3 KK, 4 KK, 5 KK, 6 KK, 7 KK, and 8 KK. In Figure 2 (d), the reaction after adding the reagent with secondary metabolites resulted in a color change, while zones without color change (white spots) indicate the presence of active compounds as α-glucosidase enzyme inhibitors (Nostro et al., 2000). This observed reaction is due to the use of specific substrate type and concentration, temperature, and incubation time, as well as appropriate pH of the buffer solution.

Determination of % Inhibition and IC_{50} of Extracts Against a-Glucosidase Enzyme

A smaller IC_{50} value indicates stronger inhibitory activity of the extract against the enzyme. Among the 8 samples tested for % inhibition and IC_{50} against the α-glucosidase enzyme, 4 demonstrated favorable IC_{50} values: isolate 1 KK (41.35 µg/mL), 3 KK (57.04 µg/mL), 4 KK (56.70 µg/mL), and 5 KK (164.16 µg/mL). In Juliani et al. (2016) study, the IC_{50} value for the ethanol extract of kumis kucing leaves was determined to be 465.83 µg/mL. These results indicate that the bacterial extract from kumis kucing leaves exhibits better IC_{50} activity compared to the ethanol extract of kumis kucing leaves.

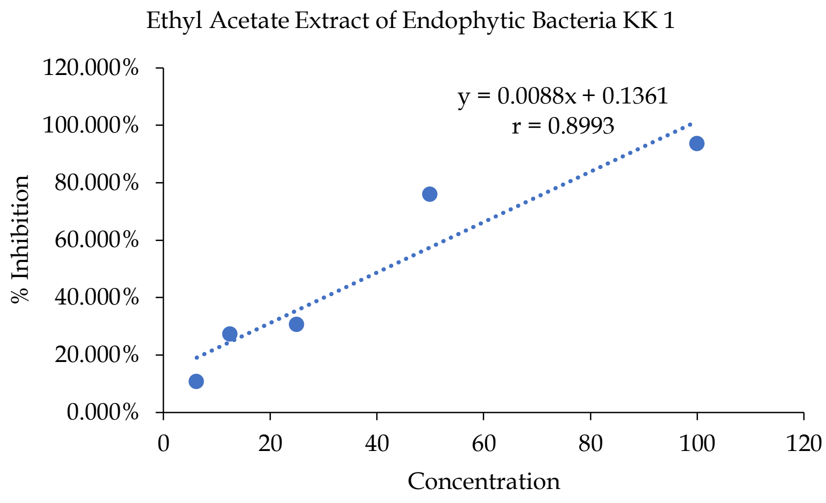


Figure 3. % inhibition of ethyl acetate extract of endophytic bacteria KK 1

The differences in solvent type and the secondary metabolites produced result in varied compound contents, which may account for the differences in biological activity between the endophytic bacterial extract of kumis kucing leaves and the ethanol extract of kumis kucing leaves (Juliani et al., 2016). Data on % inhibition and IC_{50} of extracts against the α-glucosidase enzyme are presented in the diagram below.

Ethyl Acetate Extract of Endophytic Bacteria KK 3

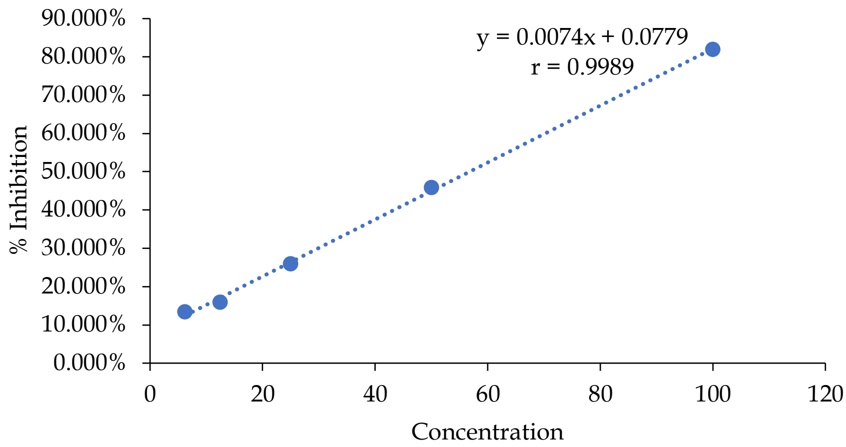


Figure 4. % inhibition of ethyl acetate extract of endophytic bacteria KK 3

Ethyl Acetate Extract of Endophytic Bacteria KK 4

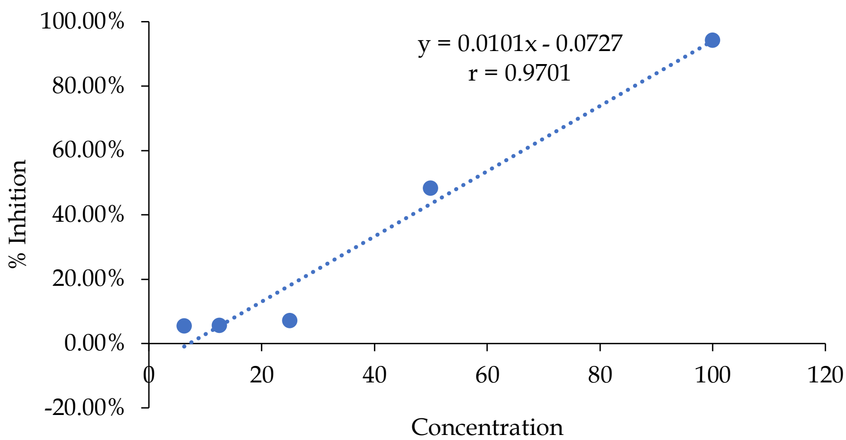


Figure 5. % inhibition of ethyl acetate extract of endophytic bacteria KK 4

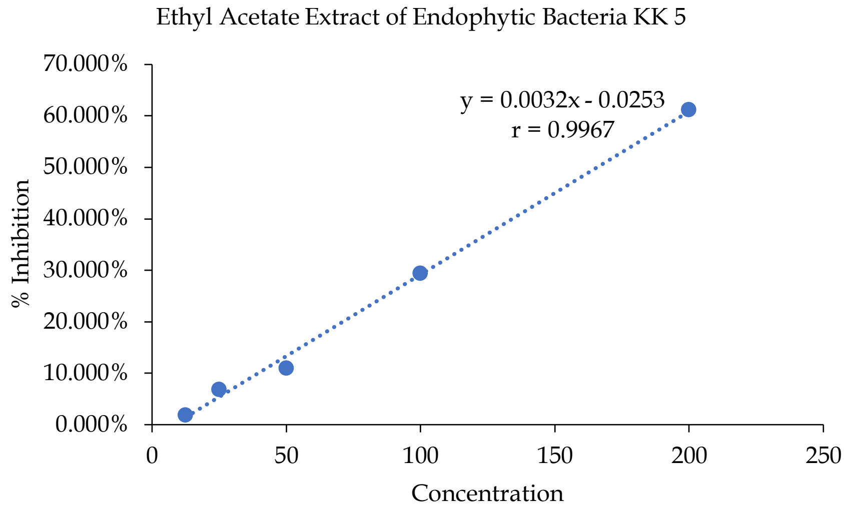
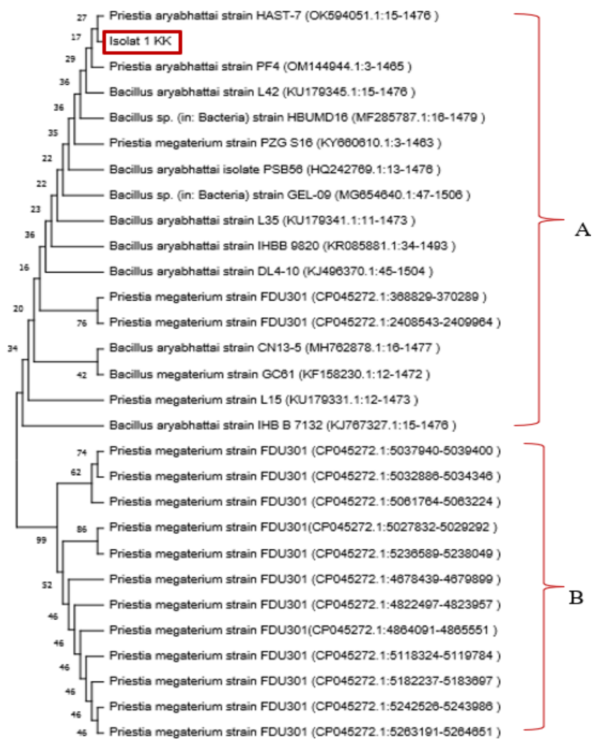
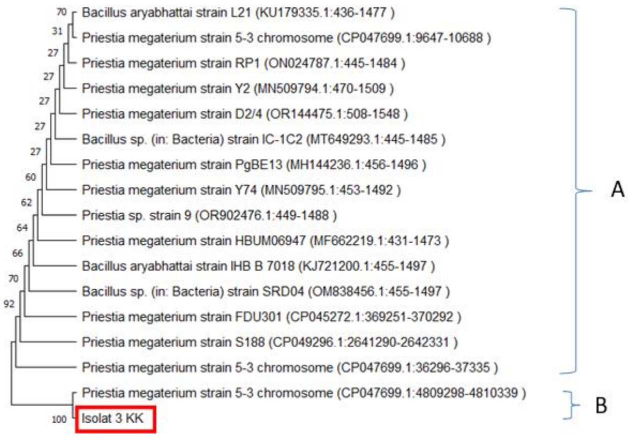


Figure 6. % inhibition of ethyl acetate extract of endophytic bacteria KK 5

Biomolecular Testing



(a)



(b)

(c)

Figure 7. Results of BLAST and phylogenetic construction of endophytic bacterial isolates from Kumis Kucing leaves (*Orthosiphon aristatus* (Blume) Miq.) KK 1 (a), KK 3 (b), and KK 5 (c)

In the biomolecular testing of bacterial isolates from the leaves of kumis kucing (*Orthosiphon aristatus* (Blume) Miq) based on sequencing results and bioinformatics analysis, two groups were identified from the phylogenetic tree construction shown in Figure 7 (a): group A and group B. Group A consists of 16 bacteria, while group B consists of 12 bacteria. Isolate 1 KK is placed in group A along with 3 *Priestia aryabhattai* bacteria, 5 *Priestia megaterium bacteria*, 6 *Bacillus aryabhattai bacteria*, 1 *Bacillus megaterium bacterium*, and 1 *Bacillus sp. bacterium*. Isolate 1 KK is located on the branch closest to *Priestia aryabhattai*.

In Figure 7 (b), based on the phylogenetic tree construction, there are two clusters: cluster A and cluster B. Cluster A consists of 15 bacteria, while cluster B consists of 2 bacteria. Isolate 3 KK is found in cluster B (highlighted in a red box). Members of cluster B include Isolate 3 KK along with *Priestia megaterium* strain 5-3 Chromosome. Based on the analysis of the 16S rRNA gene sequence fragments obtained, Isolate 3 KK is identified as *Priestia megaterium*.

For Isolate 5 KK in Figure 7 (c), cluster A consists of 10 bacteria, while cluster B consists of 5 bacteria. Isolate 5 KK is found in cluster B (highlighted in a red box). On the nearest branch, Isolate 5 KK is closely related to *Priestia megaterium*. Based on the analysis of the 16S rRNA gene sequence fragments obtained, Isolate 5 KK is identified as *Priestia megaterium*.

The endophytic bacterium *Priestia megaterium*, initially known as *Bacillus megaterium*, is a gram-positive bacterium. It is aerobic and forms spores in a wide range of environments, from plant host tissues to soil, paddy fields, dry foods, seawater, honey, humans, and blood samples. This bacterium is named for its large size, nearly 100 times that of *Escherichia coli*, and is used as a model organism for extensive research on sporulation, cell biology, biochemistry, and bacteriophages of gram-positive bacteria (Hwang et al., 2022).

The results of this study indicate the successful isolation of endophytic bacteria from the leaves of *Orthosiphon aristatus* (Blume) Miq. by obtaining eight bacterial isolates that have significant morphological diversity and macroscopic characteristics. Variations in colony color, shape, and edge indicated the presence of various species of endophytic bacteria in the cat's whisker leaves (Faharuddin et al., 2023; Madonna et al., 2022; Sumarto et al., 2023). The presence of gram-positive bacteria as the dominant result in this study demonstrates the unique potential of cat's whisker leaves in supporting the life of certain endophytic bacteria, which may be influenced by environmental conditions and plant tissue type (Antara et al., 2023; Nurjanah et al., 2023; Octriany & Ratnawulan, 2023).

Thin Layer Chromatography (KLT) bioautography test showed that some endophytic bacterial isolates had α-glucosidase enzyme inhibitory activity, as evidenced by a white zone indicating inhibitory activity (Hawari et al., 2021; Hidayah et al., 2023; Rismayanti et al., 2024). This study successfully identified isolates with the highest activity, such as isolate 1 KK, which showed an IC_{50} value of 41.35 μg/mL. This is a significant result because it is lower than the IC_{50} of the previously reported ethanol extract of cat's whisker leaves (465.83 μg/mL). The low IC_{50} value indicates that the secondary metabolites produced by endophytic bacteria have stronger α-glucosidase enzyme inhibitory potential than the direct leaf extract (Pratiwi et al., 2023; Sukmawati et al., 2022; Syamsuri et al., 2023).

These findings are important as they demonstrate that endophytic bacterial isolates, such as *Priestia aryabhattai* and *Priestia megaterium*, identified through biomolecular analysis, possess significant biological activity (Hadi et al., 2023; Jacinda et al., 2024; Setyowati & Agustin, 2022). The potential inhibition of α-glucosidase enzyme exhibited by these endophytic bacteria supports the idea that they could be a useful source of bioactive compounds in the development of antidiabetic agents (Mubarak et al., 2023; Paujiah et al., 2024; Riska et al., 2023).

Compared to the results of a previous study that found gram-negative bacteria of the species *Acinetobacter schindleri*, *Pantoea agglomerans*, and *Pseudomonas lurida*, this study emphasized the presence of different gram-positive bacteria, such as *Priestia megaterium* and *Priestia aryabhattai*. The presence of gram-positive bacteria in plant tissues has the potential to increase plant resistance to environmental conditions and pathogens. Gram-positive bacteria are also known to have the ability to produce diverse secondary metabolites, which may explain their potent biological activities, including inhibition of α-glucosidase enzymes.

The results of this study strengthen the understanding that endophytic bacteria from cat's whisker leaves play an important role in producing bioactive compounds with significant pharmacological potential. These results also open up opportunities for further research to identify and develop specific compounds from secondary metabolites of endophytic bacteria as potential therapeutic agents, especially for the treatment of diabetes through the mechanism of α-glucosidase enzyme inhibition.

Conclusion

This study showed that from 8 ethyl acetate extracts of endophytic bacterial isolates of cat's whisker (*Orthosiphon aristatus* (Blume) Miq.) leaves, 4 isolates (KK 1, KK 3, KK 4, and KK 5) had significant α-glucosidase enzyme inhibitory activity, with the best IC_{50} in isolate KK 1 (41.35 μg/mL). Biomolecular testing identified isolate KK 1 as *Priestia aryabhattai*, and isolates KK 3 and KK 5 as *Priestia megaterium*. These results indicate the potential of endophytic bacteria from cat's whisker leaves as a source of bioactive compounds that are effective for the development of diabetes therapy through the inhibition of α-glucosidase enzyme.

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Author Contributions
I.T.H.P., R., and F.I. conceived of and designed the study. I.T.H.P., V.F.S., and N.A. performed data analysis. I.T.H.P., F.I., and R. interpreted the result and revised the paper. I.T.H.P., and R. supervised the manuscript. All authors have read and approved the final manuscript.

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No conflict of interest.

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