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ORIGINAL ARTICLE

Antibacterial test of secondary metabolites of Rhizosphere bacterial isolates of cat whisker against *Escherichia coli* and *Staphylococcus aureus*

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Abstract

The rhizosphere is the zone between the root surface and soil. Rhizosphere bacteria can produce metabolites that are antibacterial because they produce phytotherapeutic compounds that have the same properties as those produced by the plant. The purpose of this study was to isolate secondary metabolite compounds from the rhizosphere bacteria of cats' whisker plants (*Orthosiphon stamineus*) as antibacterial agents against *Escherichia coli* and *Staphylococcus aureus*. This study began with the isolation of rhizosphere bacteria, purification, bacterial characterization, bacterial fermentation, and antibacterial tests. The results of the isolation of *Orthosiphon stamineus* rhizosphere bacteria obtained as many as six isolates, with each isolate coded as RKK-1, RKK-2, RKK-3, RKK-4, RKK-5, and RKK-6. The results showed that RKK-5 had an inhibition zone against *Escherichia coli* with a diameter of 8.5 mm. Based on these results, it can be concluded that RKK-5 has a moderate response to *Escherichia coli.*

Keywords: rhizosphere bacteria, cat's whisker plant, antibacterial, *Escherichia coli*, *Staphylococcus aureus*

Introduction

Herbal plants have been widely used in traditional medicine for centuries around the world. One example is the cat's whisker plant, which has the Latin name *Orthosiphon stamineus*. This cat whisker plant has long been used in traditional medicine in East India, Southeat Asia and tropical Australia.[1] Based on pharmacological studies of cat whisker plant in the medical field as antioxidant, antidiabetic, antihypertensive, anti-inflammatory, antipyretic, antiobesity, antibacterial and antifugal.[2] The use of the cat's whisker plant as an antibacterial has been widely proven. Surahmaida et al.[3] demonstrated that the ethyl acetate extract of *Orthosiphon stamineus* (cat whisker) leaves possesses inhibitory activity against the growth of pathogenic bacteria, including *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Staphylococcus aureus*. This antibacterial effect is attributed to the presence of secondary metabolites within the leaves, such as flavonoids, alkaloids, saponins, steroids, and terpenoids.[4] The leaves of the cat whisker plant (*Orthosiphon stamineus*) have demonstrated pharmacological activities, including antioxidant, antibacterial, anti-inflammatory, and antihypertensive properties.[5]

Studies have shown that cat whiskers possess antibacterial properties. Nisak's research demonstrated that cat whisker extract exhibits an inhibition zone of 20.71 mm against Staphylococcus saprophyticus.[6] The antibacterial efficacy of these extracts is likely influenced by several factors, as reported by Nabila & Advinda.[7] These factors include the mechanism of action of the extract, its concentration, the structure of the bacterial cell wall, and the composition of the peptidoglycan layer. Gram-positive bacteria, such as *Staphylococcus aureus*, have a distinct cell wall structure compared to gram-negative bacteria like *Escherichia coli*. *Staphylococcus aureus* cell wall comprises an inner layer of

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cytoplasm, a middle layer of peptidoglycan containing lipids and polysaccharides, and an outer layer rich in teichoic acid. In contrast, *Escherichia coli* cell wall is tripartite structure with an inner layer of lipoproteins, a middle layer of lipopolysaccharides and phospholipids, and an outer layer of thin peptidoglycan.[7] Pangow et al.[7] further corroborated the antibacterial activity of cat whisker extracts against both *Escherichia coli* and *Staphylococcus aureus*. Their study suggests that a larger inhibition zone corresponds to a greater potency of the compounds within the cat whiskers to inhibit bacterial growth.

Rhizosphere, the zone of soil surrounding plant roots, harbors a unique bacterial community known as rhizobacteria.[8] These bacteria exhibit promising potential for inhibiting the growth of pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumoniae*. This inhibitory effect is attributed to the production of metabolites by rhizobacteria that possess similar properties to those produced by the host plant. Fatmawati's[9] research demonstrated the antibacterial properties of specific rhizobacteria, including *Azospirillum* sp., *Azobacter* sp., *Actinomycetes* sp., and *Enterobacter* sp. This study isolated and evaluated the antibacterial activity of bacteria from the rhizosphere of cat's whisker against *Escherichia coli* and *Staphylococcus aureus*. Notably, the medicinal properties of cat's whisker are primarily attributed to its leaves. The absence of prior research investigating the antibacterial potential of cat's whisker rhizosphere bacteria underscores the significance of this study.

Method

This study began with sampling in the morning by taking the rhizosphere of the cat's whisker plant. This type of research includes experimental research using disk diffusion method. This study was conducted from August to September 2023 at the Integrated Research Laboratory of Universitas Prima Indonesia. The equipment used were autoclave, petri dish,erlenmeyer, measuring cup, beakers glass, incubator (Labnet), Biological Safety Cabinet (Biobase), ose needle, spritus lamp, drop pipette, micro pipette, test tube rack, analytical balance (Shimadzu), hot plate stirrer. The materials used were Aquadest, Physiological Sodium Chloride (NaCl), *Escherichia coli, Staphylococcus aureus*, 70% ethanol, iodine solution, safranin solution, Nutrient Agar (NA), Nutrient Broth (NB), amoxicillin 25 µg (Oxoid), and rhizosphere samples of cat's whisker plants.

Media preparation, NA media was weighed as much as 2.4 g. NA media that has been weighed is put into a glass Beaker, after which it is dissolved with 120 mL of distilled water. Then heated on a hot plate stirrer to boil. After boiling, it was sterilized using an autoclave for 15 minutes at 121°C.[10] Nutrient broth media was made as much as 10 mL in a test tube. Add a little ketoconazole 500 mg and vortex, then add rhizosphere samples and incubate for 24 hours. Isolates that have grown on NB media, then made a dilution by taking 1 mL of NB put into a test tube containing 9 mL of sterile physiological NaCl and then vortexed until homogeneous and obtained a dilution of 10^{-1}. Furthermore, 1 mL was taken from dilution 10^{-1} to do a multilevel dilution of 10^{-2}, 10^{-3} to dilution 10^{-5}. Taken 1 ml from dilution 10^{-3} to 10^{-5} and spread on sterile NA media. Repeated twice, then incubated for 24 hours.[11] Purification of bacterial isolates is carried out by taking bacteria that are different in morphology and color using an ose needle, then scraped using the quadrant line method and incubated for 24 hours.[12]

Characterization of bacterial purification was carried out by macroscopic and microscopic methods. Macroscopic observations were made by observing the shape of colonies, elevation, edges, color of colonies produced on NA media. Microscopic observations were made through gram staining, the glass object was dripped with distilled water three points and then the bacterial isolate was applied to the glass object using an ose needle. After evenly fixed on the bunsen fire. Then dripped with Crystal violet dye and left for 60 seconds, then rinsed with distilled water and dried. Drip dye lugol solution into the object glass let stand for 60 seconds, after which it is rinsed with 96% alcohol and then rinsed with distilled water and dried. The object glass is again dripped with safranin dye let stand for 30 seconds, after which it is rinsed with distilled water and dried. Then observed under a microscope. Gram-positive bacteria will be purple while negative bacteria will be pink.[13] Bacteria that grew in NA media were taken using an ose needle and transferred into sterile NaCl. Then measured the absorbance with OD (Optical Density) 0.1 with a wavelength of 600 nm using UV-Vis spectrophotometry.[14] Then the bacteria that have measured the absorbance are pipetted as much as 0.5 mL into an Erlenmeyer containing 9.5 mL of sterile NB media and placed in a shaker incubator for 72 hours at 37°C. After completion, each bacterial isolate was transferred

into a centrifuge tube and centrifuged at 5500 rpm for 10 minutes at 4°C. Then the solution resulting from centrifugation is filtered using a filter. The filter results obtained are referred to as supernatant taken for antibacterial testing.[15]

Bacterial rejuvenation was performed on pathogenic *Escherichia coli* and *Staphylococcus aureus*. Bacteria were grown on sterile NA media in petri dishes. After completion, it was incubated at 37°C for 24 hours.[16] Antibacterial testing was carried out by the agar diffusion method using paper disc. *Escherichia coli* and *Staphylococcus aureus* bacterial suspensions that have been measured uptake are taken using a cotton swab and then scraped onto sterile NA media. Then the supernatant was pipetted as much as 10 µL, dripped onto the paper disc. Then the paper disc was inserted into the NA media that had been scratched with pathogenic bacteria. After that, it was incubated for 24 hours at 37°C. Then measure the diameter of the inhibition zone in the clear area of the paper disc using a caliper.

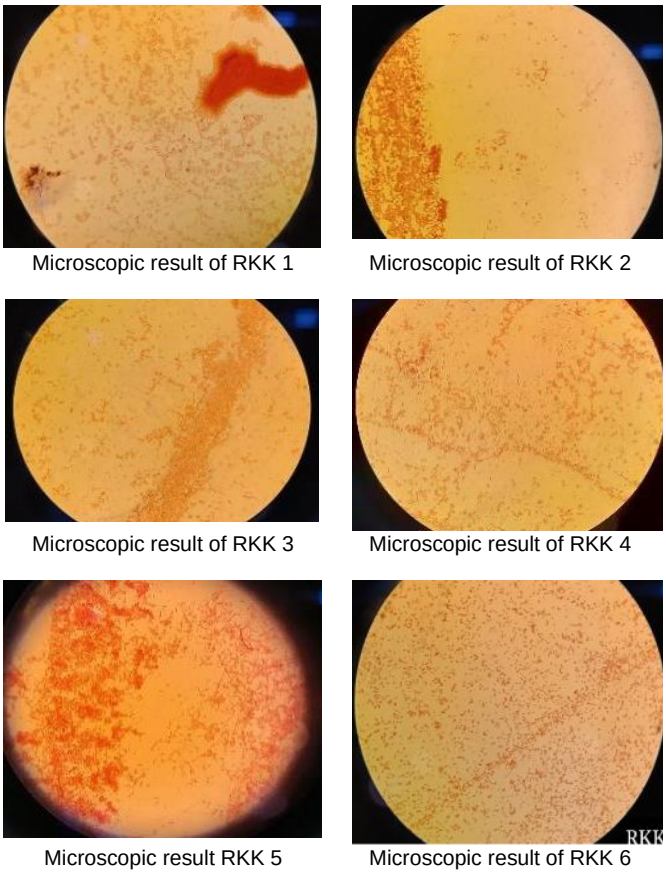
Results

After conducting bacterial isolation research from rhizosphere samples of cat whisker plants, the isolation results of 6 isolates of rhizosphere bacteria were obtained. The results of isolation of *O. stamineus* rhizosphere obtained 6 isolates, from each isolate coded RKK-1, RKK-2, RK-3, RKK-4, RKK-5, RKK-6 (Table 1).

Table 1. Results of microscopic observations of rhizosphere bacterial isolates of *O. stamineus*

No.	Isolate codes	Microscope		
		Color	Shape	Description
1	RKK-1	Pink	Diplococcus	Gram negative
2	RKK-2	Pink	Diplococcus	Gram negative
3	RKK-3	Pink	Coccus	Gram negative
4	RKK-4	Pink	Diplococcus	Gram negative
5	RKK-5	Pink	Diplococcus	Gram negative
6	RKK-6	Pink	Diplococcus	Gram negative

Description RKK: Rizosfer Kumis Kucing



Microscopic result of RKK 1 | Microscopic result of RKK 2
Microscopic result of RKK 3 | Microscopic result of RKK 4
Microscopic result RKK 5 | Microscopic result of RKK 6

Figure 1. Gram staining results of cat whisker rhizosphere bacteria

Discussion

Bacterial isolation is done to separate a type of bacteria from other microorganisms. Isolation begins with a multistage dilution method to reduce the number of microorganisms in the sample. Microbes that produce antimicrobial-producing microbes produce a substance that allows them to survive in the media in which they grow.[15] Colonies that are isolated are those that show clear area, indicating the presence of a substance that is able to defend its life from other bacterial colonies around it, so that other colonies cannot grow.[15] The resulting dilution was collected using a micropipette and poured into a petri dish containing sterile NA medium and incubated for 24 hours[15]. Bacteria are then grown on agar media until single-celled colonies are formed to be observed and tested further.[17] Characterization of rhizosphere bacteria with gram staining method aims to divide bacteria into two types, namely gram negative and gram positive bacteria. Six isolates were obtained which were included in the gram negative group, which were diplococcus. Isolation activities in this study only focused on bacterial groups. The purification process aims to obtain the desired pure culture without contamination by other bacteria.Purification serves to separate each each bacterial colony according to observing morphological differences in microscopically. Purification of bacterial isolates is done by moving bacteria using the quadrant line method which is then grown on NA media Based on the results of the conducted

research, 6 strains of rhizosphere bacteria isolated from the roots of cat whisker (*Orthosiphon stamineus*) have characteristics of bispherical shape and pink color.[18]

The fermentation process is carried out in a *shaker incubator* which aims to obtain secondary metabolites from the isolation results in larger quantities. The purpose of shaking during the incubation process is to accelerate the production of secondary metabolites by microbes and to ensure the bacteria remain stationary and produce secondary metabolites during fermentation. To survive, microorganisms can build their own defenses by producing secondary metabolites that affect other microorganisms. Thus preventing other microorganisms from growing and multiplying.[19] The fermentation system used is a closed batch system, meaning that there is no addition of ingredients or taking of results during fermentation. This is because all the nutrients needed by microorganisms during growth and product formation are in one farmentor, so there is no addition of materials or retrieval. Then the fermentation results were centrifuged at 5500 rpm for 10 minutes to obtain the supernatant. The supernatant was continued in antibacterial testing.[15] Antibacterial test activity using the *KirbyBauer* agar diffusion method against *Escherichia coli* and *Staphylococcus aureus* test bacteria. The use of these microbes is based on its ability to fight microorganisms that contaminate the human body and have the potential to cause various kinds of disease. In a study conducted by Wahab[20],it was demonstrated that cat's whiskers extract can be a good bacterial inhibitor, especially against gram-negative bacteria such as *Escherichia coli*. According to research by Pangow et.al[7] cat's whisker leaf extract has quite effective antibacterial effects against *Escherichia coli*. These bacteria are used because they have described pathogenic microorganism that infect the human body and can cause various diseases. The positive control used the antibiotic Amoxicilin 25 µg/disk because it has a broad spectrum of action. The negative control used sterile distilled water as it is a solvent that does not have antibacterial activity and therefore does not produce a clear zone.[21]

Antibacterial activity can be known from the inhibition zone formed. The larger the inhibition zone formed, the more effective antibacterial compund.[20] The size of the inhibition zone formed in the antibacterial activity test depends on the type and active substance contained in the supernatant, as well as differences in the structure and composition of bacterial cell walls, which affect the sensitivity and speed of diffusion of active compounds from secondary metabolites into the media. The decrease in inhibition diameter occurs because the bacterial isolate has entered the death phase due to the lack of nutrients in the medium. The formed transparent zone was measured with a caliper due to the inhibition diameter of the antibacterial activity test.[20] Inhibition diameter is classified into four types based on its ability to inhibit test bacteria, namely very strong (>20 mm), strong (10-20 mm), moderate (5-10 mm) and weak (<5 mm).[22] Based on this classification, the results showed that 5 isolate showed a moderate response to *Escherichia coli* bacteria with a diameter of 8.5 mm. The positive control Amoxicillin gave a strong response to *Escherichia coli* bacteria with a diameter of 30.5 mm and for *Staphylococcus aureus* bacteria gave a strong response with a diameter of 38.5 mm. complete data on the diameter of the inhibition zone of the samples studied in Table 2. Based on the research conducted, isolates of *Orthoshiphon stamineus* rhizosphere bacteria are only able to inhibit the growth of *Escherichia coli* bacteria or called bacteriostatic antibacterial compounds and are not bacteriostatic bacteria.

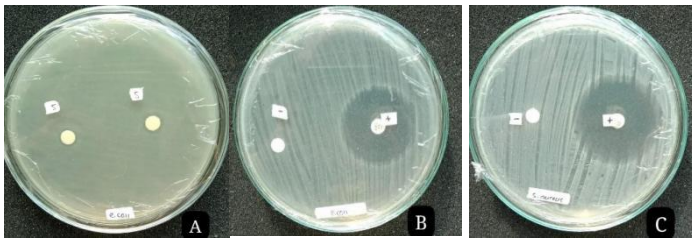


Figure 2. Antibacterial test result
(a) Activity of RKK isolate -5, (b) Positive control activity against *E.coli*, (c) Negative control activity against *S.aureus*.

Table 2. Results of antibacterial testing

Isolates	*Staphylococcus aureus*		*Eschericia coli*	
	Zone of inhibition	Description	Zone of inhibition	Description
RKK-1	-	-	-	-
RKK-2	-	-	-	-
RKK-3	-	-	-	-
RKK-4	-	-	-	-
RKK-5	-	-	8.5 mm	Medium
RKK-6	-	-	-	-
Amoxicillin	38.5 mm	Very Strong	30.5 mm	Very Strong
Aquadest	-	-	-	-

Conclusion

Based on the research findings, it was concluded that isolates of bacteria from the rhizosphere of the cat's whisker plant exhibited no antibacterial activity against Staphylococcus aureus. However, one isolate demonstrated antibacterial activity against Escherichia coli, with an inhibition zone of 8.5 mm.

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