ORIGINAL ARTICLE

Antibacterial Activity Test of Green Betel Leaf Extract (*Piper betle* L.) Against *Methicillin-Sensitive Staphylococcus aureus*

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ABSTRACT

Background: Asia is one of the regions with the highest prevalence rates of *Staphylococcus aureus* infection. Green betel leaves (*Piper betle* L.) have been proven to have antibacterial potential with their active compound content, namely essential oils, phenols, chavicol, flavonoids, alkaloids, saponins, tannins, and steroids so it is necessary to investigate whether betel leaf extract has an effect on *Methicillin-Sensitive Staphylococcus aureus* (MSSA). The aim of this study is to analyze the effect of green betel leaf extract on the growth of MSSA.

Methods: This research is a descriptive experimental study regarding the antibacterial activity test of green betel leaf extract against MSSA. The MSSA bacteria used were bacterial preparations in the Microbiology Laboratory of Faculty of Medicine Airlangga University. Green betel leaf extract was obtained from Batu City in 2023. The antibacterial activity test technique used was the dilution test.

Results: In the dilution test, for the growth of MSSA, in tubes with concentrations of 800 mg/mL, 600 mg/mL, 400 mg/mL, 200 mg/mL, and 100 mg/mL appear clear indicates that there is no growth of germs. At a concentration of 50 mg/mL it appears cloudy which indicates the growth of germs. The results obtained for the growth of MSSA on agar plates were at concentrations of 800 mg/mL, 600 mg/mL, 400 mg/mL, and 200 mg/mL, there was no growth of germs. Meanwhile, at concentrations of 100 mg/mL and 50 mg/mL, the growth of MSSA germs was found.

Conclusions: The conclusion of this research is that the minimum inhibitory concentration (MIC) of green betel leaf extract against MSSA is 100 mg/mL and the minimum bactericidal concentration (MBC) of green betel leaf extract against MSSA is 200 mg/mL. Further research is needed on the antibacterial activity of green betel leaf extract against other bacterial species that are resistant to many drugs.

Introduction

Asia is one of the regions with the highest prevalence rates of *Staphylococcus aureus* infection, with estimated proportions ranging from 28% in Indonesia to less than 70% in Korea for all *Staphylococcus aureus* isolates in early 2010.[1] In Java and Bali, S. *aureus* isolated from skin and soft tissue infections wounds in 257 (45.3%) patients, eight (3.1%) of whom were MRSA.[2] *Staphylococcus aureus* is a human pathogenic bacteria that can cause dangerous diseases. This bacterium is an agent that causes infections in humans including skin and wound infections, pneumonia, meningitis, toxic shock syndrome, scalded skin syndrome, bacteremia, and bone infections.[3,4] *Staphylococcus aureus* can become resistant to oxacillin and related β-lactams by acquiring new penicillin-binding proteins that are enzymatically active but not bound and inactivated by the antibiotics.[5]

Treatment of *Staphylococcus aureus* infection really depends on the type of infection and whether or not there are drug-resistant strains. In general, first-line parenteral antibiotics for MSSA are nafcillin, oxacillin, and first-generation cephalosporins. This therapy has side effects in the form of neutropenia, interstitial nephritis, hepatitis, allergic reactions include anaphylactic shock, urticaria, fever, joint swelling, and skin rashes.[6] This therapy also requires expensive costs, so an alternative treatment using natural ingredients is needed, one of which is using green betel leaves (*Piper betle* L.) which is known for its properties, is easy to obtain, and has an affordable price.

Green betel leaves (*Piper betle* L.) have been used as an ingredient for traditional medicine by Indonesians and Asian peoples since a long time ago. Green betel leaves (*Piper betle* L.) are commonly used by people to treat and prevent various diseases such as itching, coughs, colds and toothache.[7] Green betel leaves (*Piper betle* L.) have potential as an antibacterial with their active compounds, namely essential oils, phenols, chavicol, flavonoids, alkaloids, saponins, tannins and steroids.[8]

Previous research stated that green betel leaf extract (*Piper betle* L.) from Makassar and Denpasar could inhibit the growth of *Staphylococcus aureus* using the good method and the active substances flavonoids, tannins and phenols were found in the green betel leaf extract (*Piper betle* L.).[9,10] However, no one has studied the antibacterial activity of green betel leaves grown in the Batu area against MSSA. Based on the problems above, it is necessary to investigate whether green betel leaf extract specifically planted in the Batu area has an effect on MSSA. The objective of this research is to analyze the effect of green betel leaf extract, specifically planted in the Batu area, on the growth of *Methicillin-Sensitive Staphylococcus aureus*.

Methods

This is a true experimental research, with a posttest-only control group design. This research used a dilution test on *Methicillin-Sensitive Staphylococcus aureus* by administering betel leaf extract (*Piper betle* L.) in various concentrations in vitro. According to Federer's formula, four repetitions were carried out on the sample for each treatment. Samples were taken by random sampling. This research has received ethical clearance from the Health Research Ethics Committee, Faculty of Medicine, Airlangga University, Surabaya.

The materials used in this research were green betel leaves (*Piper betle* L.) obtained from Batu City in 2023 which has been determined, MSSA bacteria obtained from the Department of Microbiology, Faculty of Medicine, Airlangga University, Surabaya. The research was carried out at the Microbiology Laboratory, Faculty of Medicine, Airlangga University, Surabaya. The research was carried out within 11 months (July 2023-May 2024). The green betel leaves were then determined at the Batu Herbal Materia Medica Laboratory UPT. This aims to ensure the correctness of the plants to be used.

Making betel leaf extract using the maceration method. The solvent used is 96% ethanol. Green betel leaves are washed clean and then dried in an oven at 40°C. Then knead and grind until it becomes powder. 500 grams of betel leaf powder was weighed, then soaked in 3.5 liters of 96% ethanol solvent for 5x24 hours and the filtrate was taken for filtration. Stirring was done 12 times for 15 minutes. After 5 days, the betel leaf extract is filtered and squeezed using filter paper and a funnel to separate the filtrate from the dregs. The filtering results are then stored in a new container that is tightly closed. The remaining dregs are macerated with ethanol for two days and stirred occasionally. The extract was filtered after two days, then the filtered results were mixed with the previous filtrate and the solvent was evaporated using a rotary evaporator at a temperature of 55°C. Then concentrate in a water bath at a temperature of 55°C. Obtained 12 grams of thick extract free from solvents and extracts used in subsequent tests. MSSA cultures that were 24 hours old were taken as 0.1 mL of 0.5 Mc Farland (1.5 X 10^8 CFU/mL). Then placed in a test tube containing a

liquid medium and incubated for 24 hours at 37°C in an O_2 incubator.

The antibacterial activity test against MSSA used the dilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of betel leaf extract (*Piper betle* L.). MIC is the smallest concentration of an antibiotic substance that is still able to inhibit the growth of germs, while MBC is the smallest concentration of an antibiotic substance that is capable of killing germs. In this research dilution test, 9 test tubes were needed, namely 3 control tubes and 6 test tubes. The first and second control tubes are negative controls and the third control tube is positive controls. The first control tube was filled with 2 mL of sterile liquid medium, the second control tube was filled with 1 mL of sterile liquid medium and 1 mL of green betel leaf extract (*Piper betle* L.), and the third tube was filled with 1 mL of sterile liquid medium plus 1 mL of MSSA bacterial colonies.

The first test tubes were filled with 1.6 mL of green betel leaf extract and 0.4 distilled water (800 mg/mL). The second tubes were filled with 0.6 mL of green betel leaf extract and 0.4 distilled water (600 mg/mL). The third to sixth tubes were filled with 1 mL sterile liquid medium. Then 1 mL of the solution from the first test tube is taken and added to the third tube, and so on until the sixth tube. 1 mL of the sixth tube solution was discarded so that the volume of solution for each test tube was 1 mL with different concentrations, namely 800 mg/mL, 600 mg/mL, 400 mg/mL, 200 mg/mL, 100 mg/mL and 50 mg/mL. Then, in the first to sixth test tubes, 1 mL of a colony of *Staphylococcus aureus* bacteria was added so that the final volume of all tubes was 2 mL. MIC is determined by looking at the smallest concentration of green betel leaf extract (*Piper betle* L.) in the tube that is still able to inhibit bacterial growth. The clear tubes after the MIC tube has their specimens taken are then cultured on an Agar plate so that the MBC can be determined.

Results

Extraction of green betel leaves using ethanol produces 50 mL of liquid extract. The results obtained from the MIC test of green betel leaf extract against MSSA were that tubes with concentrations of 800 mg/mL, 600 mg/mL, 400 mg/mL, 200 mg/mL, and 100 mg/mL appeared clear, indicating that there was no germ growth. At a concentration of 50 mg/mL it appears cloudy which indicates the growth of germs (Figure 1). It can be concluded that the MIC value of green betel leaf extract against MSSA is 100 mg/mL (Table 1).

Figure 1. Test tube results in the MSSA bacterial dilution test after 24 hours incubation

After measuring the MIC, tube fluid with a concentration of 800 mg/mL to 50 mg/mL was planted on an agar plate and observations were made to measure the MIC after incubation for 24 hours. The results obtained for the growth of MSSA were at concentrations of 800 mg/mL, 600 mg/mL, 400 mg/mL, and 200 mg/mL, there was no growth of MSSA germs on the agar plates. Meanwhile, at concentrations of 100 mg/mL and 50 mg/mL, MSSA germ growth was found (Figure 2). The first, third and fourth repetitions gave MSSA MBC results of 200 mg/mL and the second repetition gave MSSA MBC results of 100 mg/mL. It can be concluded that the MBC value of green betel leaf extract against MSSA in this study was 200 mg/mL. (Table 2).

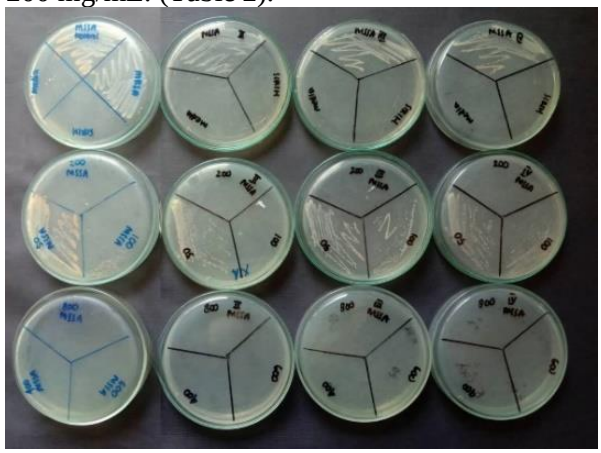

Figure 2. Agar culture results of the entire MSSA bacterial dilution test tube

Table 1. Results of Minimum Inhibitory Concentration (MIC) Test (mg/mL)

	800	600	400	200	100	50
I	-	-	-	-	-	+
II	-	-	-	-	-	+
III	-	-	-	-	-	+
IV	-	-	-	-	-	+

+= Cloudy tube (bacteria grew)
-= Clear tube (bacteria didn't grow)

Table 2. Results of minimum bactericidal concentration (MBC) test (mg/mL)

	800	600	400	200	100	50
I	-	-	-	-	**+**	+
II	-	-	-	-	-	+
III	-	-	-	-	+	+
IV	-	-	-	-	+	+

+= Agar plate was overgrown with bacteria (bacteria was not dead)
-= Agar plate was not overgrown with bacteria (bacteria was dead)

Discussion

In the green betel leaf extraction process, researchers used ethanol as a solvent because ethanol is an extraction solvent that is widely used for active ingredients that are antibacterial from inside cells. Ethanol is a universal solvent that can extract polar or semipolar compounds, is non-toxic, can mix with water, and the heat required for concentration is less so that the active ingredients used in this research can be extracted properly.[11]

Green betel leaves have antibacterial activity against MSSA bacteria because they contain various antibacterial active ingredients including alkaloids, phenols, flavonoids, essential oils, chavicol, saponins, tannins and steroids. These various compounds play a role in inhibiting cell wall synthesis, disrupting cell membrane permeability, disrupting cell metabolism, damaging nucleic acids, and inhibiting cell protein synthesis so that bacterial cells can die. These compounds have antimicrobial, antioxidant and anti-inflammatory effects.

Other research shows that green betel leaf extract is able to inhibit MSSA bacteria in various concentrations. Research by Tagrida using ethanol extract showed that the MIC of green betel leaves on MSSA was 3.12 mg/mL.[12] Research by Lao shows that the MIC of green betel leaves on MSSA is 2.5 mg/mL.[13] Research by Ali shows that the MIC of green betel leaves on MSSA is 2 mg/mL.[14] Research by Visutthi showed that the MIC of green betel leaves on MSSA was 1.25 mg/mL.[15] Research by Leesombun shows that the MIC of green betel leaves on MSSA is 1.024 mg/mL.[16] Research by Saeloh shows that the MIC of green betel leaves on MSSA is 0.62 mg/mL.[17] Research by Rivera showed that the MIC of green betel leaves on MSSA was 0.312 mg/mL.[18] Research by Sungkatavat shows that the MIC of green betel leaves on MSSA is 0.25 mg/mL.[19]

Green betel leaf extract can kill MSSA bacteria in various concentrations. Research conducted by Research by Lao shows that the MBC results of green betel leaves on MSSA are 5 mg/mL.[13] Research by Tagrida shows that the MBC results of green betel leaves on MSSA are 3.12 mg/mL.[12] Research by Visutthi showed that the MBC of green betel leaves on MSSA was 1.25 mg/mL.[15] Research by Saeloh shows that the MBC results of green betel leaves on MSSA are 0.62 mg/mL.[17] Research by Rivera showed that the MBC of green betel leaves on MSSA was 0.312 mg/mL.[18] Research by Sungkatavat shows that the MBC of green betel leaves on MSSA is 0.25 mg/mL.[19]

Based on the results of this study, it shows that the MIC of MSSA is 100 mg/mL, and the MBC of MSSA is 200 mg/mL with a typical local plant of green betel leaf which is specifically planted in the Batu area. There are differences in results from several other research results mentioned previously which could be caused by green betel leaf plant factors. This could be caused by the composition of the active ingredients in green betel leaves being influenced by several things. The quality of the active ingredients contained in the green betel leaf medicinal plant can be influenced by external and internal factors. External factors include the growing conditions of green betel plants such as land conditions, temperature, climate, altitude where they grow, environmental pollution, pests, high ultraviolet intensity, heavy metal contamination, and humidity where the plants grow. Internal factors include genetic quality and

plant age. The higher the height at which some plants grow which contain essential oils or the lower the temperature of the environment where they grow, the more the levels of essential oils in these plants will increase.[20]

The difference in antibacterial sensitivity from previous research could be caused by several factors, such as the choice of solution, storage time of green betel leaf extract, method of making the extract, and the bacterial isolates used in the research. The choice of solution in the extraction process requires an appropriate solution because the active compound taken is following the solution used during extraction. Betel leaf extract which has a longer storage time may experience a decrease in quality due to reduced antibacterial compounds. The method of the extraction process can influence the yield value which is related to the success in extracting the amount of active compound. The bacterial isolates and strains used may differ due to differences in research locations, which can influence the results of existing research. The limitation of this research is that betel leaves have various varieties, but this research only examined green betel leaves. Based on the results obtained in this study, a concentration of 100 mg/mL of green betel leaf extract from Batu City inhibits the growth of both MSSA and MRSA bacteria. Additionally, a concentration of 200 mg/mL of green betel leaf extract kills both MSSA and MRSA bacteria. Therefore, this concentration can be developed as a new alternative treatment as an effective antibiotic for killing the growth of MSSA and MRSA bacteria.

Conclusion

Based on this research, it is concluded that green betel leaf extract (*Piper betle* L.) specifically planted in the Batu area has antibacterial activity against MSSA with a minimum inhibitory concentration of 100 mg/mL and a minimum killing concentration of 200 mg/mL. Green betel leaf extract (*Piper betle* L.) specifically planted in the Batu area with a concentration of 200 mg/mL can be developed as a new alternative treatment as an antibiotic that is effective in killing the growth of MSSA bacteria.

Conflict of Interest

No conflict of interest in this study

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