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INTRODUCTION Staphylococcus aureus is a clinically relevant pathogen due to antibiotic resistance and the increasing use of medical devices1. Staphylococcus aureus is non-motile, non-sporing, facultatively anaerobic, catalase-positive, and oxidase-negative. Staphylococcus aureus thrives at a temperature of 6.5-46ºC with a pH of 4.2-9.32. Symptoms of infection with this bacterium are stomach cramps, vomiting, and severe diarrhea. Staphylococcus aureus can cause various diseases, ranging from minor skin infections, poisoning, and systemic infections3. A previous study reported that S. aureus infection had a mortality rate of 25%4. Currently, S.

aureus is a serious health problem, which has increased the bacteria's resistance to various antibiotics (multi-drug resistance). Inappropriate administration of antibiotics can cause bacterial resistance, in which S. aureus can adapt to its environment and cause resistance to antibiotics5. Staphylococcus aureus resistance to the penicillin class of antibiotics has reached 80%6. Methicillin and vancomycin antibiotics have caused resistance to S. aureus, such as Methicillin Resistance S. aureus (MRSA) and Vancomycin Resistance S. aureus (VRSA)7,8. Patients with these infections are often given antibiotic therapy such as cloxacillin, dicloxacillin, and erythromycin in its management9.

One of the factors that complicate the treatment of S. aureus infection is the ability of these bacteria to form biofilms10. Biofilm is a collection of microbial cells irreversibly attached to a surface and encased in a self-produced matrix of extracellular polymer substance (EPS), accompanied by an increase in the number of phenotypes such as climate change and gene transcription from planktonic cells or free cells11. Biofilm is one of the severe health problems related to infection prevention.

Biofilms are part of the defense of microorganisms and are relatively difficult to eradicate with antibiotics; therefore, pathogenic organisms in the form of biofilms can endanger human health12,13. Various efforts have been made to fight antibiotic resistance due to biofilm formation, one of which is the use of herbal ingredients as the basis of treatment14. Along with public awareness of the negative impact of synthetic drugs, the use of traditional medicinal plants continues to increase, so that people begin to switch from synthetic drugs to traditional medicines from natural ingredients.

The application of traditional medicine has many advantages and very practical to make at home, as an alternative medicine for the community, including in Indonesia, which has abundant natural wealth in medicinal plants15. One of the plants from Indonesia that can be processed into herbal medicine to treat infections is calincing or Oxalis corniculata L. Oxalis corniculata is reported to have antibacterial activity, especially from the leaves16-18. Empirically in Kalimantan, people use the leaves as a medicine for stomach aches, canker sores, and coughs16. Several previous reports found that the

ethanolic extract of the O. corniculata herb could inhibit Escherichia coli and S. aureus[19,20].

However, no studies have reported the profound antibiofilm activity of this plant. Therefore, this study aimed to determine the activity of O. corniculata leaves extract, which can inhibit the growth of S. aureus biofilms. MATERIALS AND METHODS Materials The material used in this study was O. corniculata leaves (Figure 1), collected from the National Park of Mount Merapi, Yogyakarta. The plant was determined at the Faculty of Biology, Universitas Gadjah Mada. Other materials were standard biofilm-forming S. aureus isolate (ATCC 25923), chloramphenicol, 1% DMSO, NaCl, McFarland standard 0.5, sterile distilled water, Brain Heart Infusion (BHI) media, phosphate buffer saline (PBS) solution, and crystals violet 1%.

The instrument used in this study were Laminar Air Flow, incubator (IF-2B) (Sakura, Japan), micropipette Pipetman (Gilson, France), multichannel micropipette (Socorex, Switzerland), microplate flat-bottom polystyrene 96 well (Iwaki, Japan), microtiter plate reader (Optic Ivymen System 2100-C, Spain), spectrophotometer UV Genesys 10 UV Scanning, 335903 (Thermo Scientific Spectronic, US), autoclave (Sakura, Japan), and analytical balance (AB204-5, Switzerland). / Figure 1. Oxalis corniculata L. plant Methods Bacterial strains Staphylococcus aureus was grown within 24 hours at 37°C in BHI media. The optical density (OD) 600 of the microbial culture was adjusted to 0.1 (equivalent to the McFarland standard 0.5 - 1.5

x 108 CFU/ml) and then diluted in a new growth medium to 0.01 OD600. Antibacterial test An antibacterial test was carried out using the microdilution method. The test was carried out on microtiter plate flat-bottom polystyrene 96 wells with a series of levels of test compounds: 1, 0.5, 0.25, and 0.125% w/v. The control used was chloramphenicol 1% w/v. Growth control in the form of a microbial suspension and solvent control adjusted to the solvent of the test compound. The microplate wells were inserted BHI media and bacterial suspension, then incubated at 37°C for 24 hours.

Microplate absorbance reading process using a microplate reader at a wavelength of 595 nm. Test of inhibition of biofilm formation mid-phase and maturation-phase using the microbroth dilution method A 96-well flat-bottom polystyrene microtiter plate was used to assess the effect of the test isolates on the formation of mono-species S. aureus biofilms[21]. About 100 L of media containing ethanol extract of O. corniculata leaves with a series of concentrations was added to each well.

A medium without microbial growth was used as a control medium, and a microbial suspension was used as a negative control. A microbial suspension was used as a

positive control, given 1% chloramphenicol w/v. The plates were then incubated at 37°C for 24 hours to form the mid-phase biofilm and 48 hours to form the maturation phase biofilm. Next, the plate was washed using distilled water three times and dried at room temperature for five minutes to remove the remaining water.

A total of 125 L of 1% crystal violet solution was added to each well to color the formed biofilm (both dead cells and live cells, which were also components of the biofilm), then incubated at room temperature. After incubation, the microplate was washed with running water three times to remove the remaining crystal violet, and 200 L of 96% ethanol was added to each well to dissolve the formed biofilm. The OD readings were carried out with a microplate reader at a wavelength of 595 nm. The OD value was then used to calculate the percent inhibition in Formula 1.

The sample level that could inhibit at least 50% biofilm formation was considered Minimal Biofilm Inhibition Concentration (MBIC50)21,22. % ????h?????????????? = (???? ???????????????? ?????????????? ?????????????? – ???? ???????? ???????????? ??????????????) ???? ???????????????? ?????????????? ?????????????? ×100... [1] Staphylococcus aureus biofilm eradication activity from O. corniculata leaves Tests for biofilm eradication (degradation) were almost similar to biofilm inhibition, but the processing time differed.

The biofilm degradation test takes five days, while the biofilm inhibition takes about 1-2 days, depending on the inhibition desired. The biofilm was inoculated with a microtiter plate. After incubation at 37°C for 48 hours, the plates were washed with 150 L of sterile distilled water three times to remove nonadherent cells. A total of 100 L of media containing ethanol extract of O. corniculata leaves with a series concentration was added to each well that had been washed, then re-incubated at 37°C for 48 hours. Chloramphenicol at a concentration of 1% w/v were used as positive controls.

After incubation, the plates were washed three times with 200 mL of sterile PBS to remove adhering cells. Biofilm eradication was quantified with 125 L 1% crystal violet solution into each well, then incubated at room temperature for 15 minutes. After incubation, the microplate was washed with PBS, and 200 L of 96% ethanol was added to each well to dissolve the formed biofilm. The OD readings were carried out with a microplate reader at a wavelength of 595 nm16. Statistical methods Statistical analysis was performed using ANOVA and normality test performed using the Shapiro–Wilk, with a p-value of 0.05 or less. The data were analyzed using the Statistical Package for the Social Sciences (SPSS).

RESULTS AND DISCUSSION Antibacterial activity of O. corniculata extract against S.

aureus The ethanolic extract of O. corniculata leaves produced antibacterial activity of 76.23±0.01% (*P <0.05) at a concentration of 1% w/v, and the control chloramphenicol was higher by 79.42±0.01% (*P <0.05), as shown in Figure 2. These results indicate that 50% ethanol extract of O. corniculata leaves could reduce the growth of S. aureus bacteria. This result was supported by previous research, which states that the inhibitory power of O. corniculata leaves was extreme to reduce bacterial growth with a concentration level of 40%17. / Figure 2.

Antibacterial activity with concentration variants within 24 hours and eradication activity of O. corniculata leaves extract against S. aureus biofilm, ANOVA with p <0.05. Red: Antibacterial activity phase; Blue: Degradation phase This inhibitory potential was found in the juice and decoction of O. corniculata leaves, and other studies have found the best concentration of ethanol extract of O. corniculata leaves to inhibit the growth of S. aureus with a concentration of 10% with an average inhibition zone diameter of 10.4 mm and the average inhibition zone diameter was 8.69 mm18. Besides that, previous studies reported that O.

corniculata leaves could suppress the development of several bacteria, including S. aureus, Salmonella typhi, Vibrio sp., and E. coli. This activity was due to the active chemical compound in diethyl ether extract23. Oxalis corniculata extract activity against S. aureus biofilm in the middle phase (24 hours) The ethanol extract of O. corniculata leaves 1% w/v gives antibiofilm activity of S. aureus in the middle phase (24 hours) of 71.32±0.05% (*P <0.05). In contrast, chloramphenicol was 69.32±0.05% (*P <0.05). In the middle phase of the biofilm, the activity of the ethanol extract of O. corniculata leaves decreased slightly compared to its antibacterial activity.

These results indicate that the decrease in antibacterial activity was caused by forming the S. aureus biofilm community with a solid and complex EPS matrix structure that synergizes. This process causes the activity of O. corniculata leaves to be unable to penetrate the EPS biofilm of cells S. aureus, resulting in reduced effectiveness as presented in Figure 3. Biofilms are composed of microbial cell chains and EPS, which make up 50 to 90% of the total organic carbon in biofilms24. Changes in bacterial phenotype from planktonic to biofilm form increase the production of S. aureus toxin25.

The EPS matrix in the biofilm can help cells survive longer by providing a protective layer against antibiotics26. / Figure 3. Oxalis corniculata leaves extract activity against S. aureus biofilm in the middle phase, with ANOVA p <0.05 Oxalis corniculata extract activity against S. aureus biofilm in the maturation phase (48 hours) The results provide evidence that 1% ethanol extract of O. corniculata leaves provides the antibiofilm activity of S. aureus at the 48-hour phase of 69.33% (*P<0.05). In contrast,

chloramphenicol was 67.21±0.05% (*P<0.05), as shown in Figure 4. These results showed decreased activity compared to its activity against antibacterial and antibiofilm in the middle phase.

In this phase, the biofilm-forming microbes are already attached to the substrate so that the ethanol compound of O. corniculata leaves is more difficult to kill the biofilm than in the middle phase. Microbes form a strong biofilm and matrix EPS defense system in the maturation phase and establish a cell communication mechanism called quorum sensing27. This result is by the statement of Hamzah et al.28, who said that it is difficult to kill and destroy bacteria that form biofilms in the maturation phase of antibiotics. / Figure 4. Oxalis corniculata leaves extract activity against S. aureus biofilm in the maturation phase, with ANOVA p <0.05 Eradication activity of O. corniculata leaves extract against S.

aureus biofilm The EPS matrix in the biofilm could help microorganism cells survive more extended than in planktonic conditions to help the microcortium of various species of microorganisms in the degradation process29. For S. aureus biofilm eradication activity, the general graphic pattern of antibiofilm activity decreased as the inhibitory concentration decreased. The best activity was produced at a concentration of 1% w/v, with an eradication activity of 64.1±0.05% (*P <0.05), and the lowest biofilm eradication at a concentration of 0.125% (w/v) with an eradication activity of 32.23±0.05% (*P <0.05). In contrast, chloramphenicol was 62.89±0.05% (*P <0.05), as presented in Figure 2.

At a concentration of 1% w/v, the ethanol extract had the highest eradication activity, but its activity was not the same as that of the antibacterial. This was since the EPS matrix produced by S. aureus was already very thick and abundant, so that the O. corniculata leaves extract was unable to kill the biofilm protected by the EPS matrix. In addition to the EPS matrix produced, the biofilm group in this phase was neatly structured, and nutritional adequacy was always maintained, causing the effectiveness of the O.

corniculata leaves extract to only penetrate the outside, and this also indicates that a very high dose was needed compared to the dose given to antibacterial24. The mechanism in this phase is by forming a network of highly structured cells and synergizing between microbes in forming biofilms to produce a complete and thick composition of EPS and nutrients30. The more EPS produced, the thicker and complex the defense is made so that the S. aureus biofilm that is formed is very difficult to penetrate by antibiotics. These findings indicate that O.

corniculata leaves extract ethanol can be developed as an antibiofilm agent. Further research can be conducted to determine the composition of the components of O. corniculata leaves and the mechanism of action of the ethanol extract of O. corniculata leaves. CONCLUSION Ethanol extract from O. corniculata leaves can inhibit the growth of S. aureus biofilm and may be developed as a candidate for new antibiofilm agents against S. aureus.

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