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The Effect of Ethanol Extract of Rhizophora mucronate Leaves on The Growth of Staphylococcus aureus Bacteria

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ABSTRAK

Sebagian besar infeksi kulit di Indonesia disebabkan oleh bakteri Staphylococcus aureus yang bersifat patogen oportunistik dan bertanggung jawab atas permasalahan resistensi antibiotik Metisilin dan Vankomisin terhadap S.aureus. Daun bakau R. mucronata mengandung senyawa metabolit sekunder seperti fenol, flavonoid, saponin, tanin, dan terpenoid yang memiliki aktivitas antibakteri. Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak etanol 96% daun bakau R. mucronata terhadap pertumbuhan bakteri Staphylococcus aureus. Penelitian dilakukan secara eksperimental laboratoris dengan sampel biakan murni Staphylococcus aureus murni, diambil dengan teknik random sampling, dicampur dengan aquades dan kekeruhannya setara dengan standarisasi 0,5 Mc Farland, ditanam di agar Mueller Hinton. Daun Bakau R. mucronata diekstraksi dengan metode maserasi dan dibuat 4 seri konsentrasi (25%, 50%, 75%, dan 100%). Uji antibakteri menggunakan metode difusi cakram dengan kontrol positif Siprofloksasin 5 mcg dan kontrol negatif akuades steril. Semua cawan petri dimasukkan kedalam inkubator selama 24 jam pada suhu 37°C, lalu diukur diameternya. Data dianalisis secara statistik dengan uji Nonparametrik Kruskal Wallis dan dilanjutkan uji posthoc Mann Whitney-U.Hasil penelitian menunjukkan peningkatan diameter zona hambat setiap konsentrasi. Rata-rata diameter zona hambat kontrol positif 25,4 mm; konsentrasi 100% 11,7 mm; konsentrasi 75% 9,9 mm; konsentrasi 50% 7,3 mm; konsentrasi 25% dan kontrol negatif 6,00 mm. Simpulan dalam penelitisn ini bahwa ekstrak etanol 96% daun bakau R. mucronata dapat menghambat pertumbuhan bakteri S.aureus secara in vitro.

ABSTRACT

Staphylococcus aureus bacteria which are opportunistic pathogens and are responsible for the problem of Methicillin and Vancomycin antibiotic resistance against S.aureus. R.mucronata mangrove leaves contain secondary metabolite compounds such as phenols, flavonoids, saponins, tannins, and terpenoids, which have antibacterial activity. The purpose of this study was to determine the effect of 96% ethanol extract of mangrove leaves R. mucronata on the growth of Staphylococcus aureus bacteria. The study was conducted experimentally in a laboratory with Staphylococcus aureus as a sample. Staphylococcus aureus used random sampling techniques, mixed with distilled water and its turbidity equivalent to 0.5 Mc Farland standardization, planted on Mueller Hinton agar. Mangrove leaves R. mucronata were extracted using the maceration method and 4 series of concentrations were made (25%, 50%, 75%, and 100%). The antibacterial test used the disc diffusion method with a positive control of 5 mcg Ciprofloxacin and a negative control of sterile distilled water. All petri dishes were put into an incubator for 24 hours at 37°C, then their diameters were measured. Data were analyzed statistically using the Kruskal Wallis Nonparametric test and continued with the Mann Whitney-U posthoc test. The results show an increase in the diameter of the inhibition zone for each concentration. The average diameter of the positive control inhibition zone was 25.4 mm; 100% concentration 11.7 mm; 75% concentration 9.9 mm; 50% concentration 7.3 mm. The conclusion in this study is that 96% ethanol extract of R. mucronata mangrove leaves can inhibit the growth of S. aureus bacteria in vitro

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1. INTRODUCTION

Skin diseases are one of the common diseases in Indonesia. This is due to its tropical and subtropical climate. The high prevalence of skin diseases is a special concern. A total of 900 million skin diseases affect human health worldwide (WHO, 2018). Indonesia has 4.60% - 12.95% of skin diseases as well as masculinity in third place out of the 10 most common diseases in Indonesia (Rahayu *et al*., 2023). Statistical data in 2020, skin diseases ranked 6th with a total of 4.53% in Surabaya (Health Office., 2024).

Skin diseases are caused by bacteria, viruses, fungi, the environment, climate, and unhealthy lifestyle habits, allergies and others (Primadiamanti *et al*., 2021). Human skin generally has a wide variety of microbes that live on its surface as well as in hair follicles that play an important role in skin homeostasis. *Staphylococcus aureus* is an opportunistic pathogen and its development is responsible for most infections of the skin (Parlet *et al*., 2019). Skin infections occur regardless of a person's age, if the type is mild, it can be treated with self-medication (Primadiamanti *et al*., 2021).

The Staphylococcus *group of bacteria* has resistance to several antibiotics. S*. aureus* bacteria are resistant to methicillin and vancomycin which are named *Methicillin Resistant Staphylococcus aureus* (MRSA) and *Vancomycin Resistant Staphylococcus aureus* (VRSA). The emergence of new strains of *S. aureus* bacteria results in disorders with different characteristics as well as a higher degree of resistance to antibiotics (Dewa *et al*., 2019). This problem is one of the main problems in the treatment of infection against *S. aureus*, so the existence of other bioactive materials is urgently needed for the development of antibiotics in the future.

The discovery of new, effective antibiotics is urgently needed to combat infections that were once deadly, especially with increasing resistance to existing antibiotics. (Rollando, 2019). Rhizophora mucronata *mangrove leaves* have active antibacterial compounds, namely phenols, flavonoids, tannins, saponins, and terpenoids (Rajivgandhi *et al*., 2024). Alkaloids and Flavonoids can actively fight pathogenic bacteria including *S. aureus* (Manilal *et al*., 2016). There are differences in the concentration of *S. aureus leaf content* which is influenced by climatic conditions, air, humidity, temperature, soil, light, and water in each region (Kusmana, 2010). Java Island, one of which is in the Eastern Region of Surabaya City, has 3 locations of mangrove areas, namely the Gunung Anyar Mangrove Area, Medokan Sawah and Wonorejo. This Mangrove Botanical Garden has a collection of 57 types of mangroves with a total area of 34 hectares in all three locations. This plant collection includes mangrove types *Rhizophora mucronata*, *Avicennia lanata,* and *Rhizophora mangle*. Researchers are interested in conducting research on the effect of inhibition of *Staphylococcus aureus* bacteria by ethanol extract of mangrove leaves *Rhizophora mucronata* from Surabaya, East Java.

2. METHOD

Contains how data is collected, data sources and ways of data analysis. The method in this study used quantitative in vitro experimental laboratory research with 6 treatment groups and each treatment group required 4 samples, so that the number of samples to be studied was 24 with the post-test only control group design method. The sample used was a pure culture of Staphylococcus auireiuis grown on Muilleir Hinton agar media. Bacterial culture was carried out at the Microbiology Laboratory of the Faculty of Medicine, Hang Tuiah University. The sampling technique used simple random sampling.

Mangrove R.mucronata was collected at Jl. Wisata Mangrove Gunung Anyar Tambak, Kec. Gunung Anyar, Surabaya City, East Java. The process of making mangrove R.mucronata extract was carried out at the Pharmacy Lab, Hang Tuah University, Surabaya. The research was conducted at the Microbiology Lab, Faculty of Medicine, Hang Tuah University, Surabaya. The research group consisted of 4 treatment groups with concentrations (25%, 50%, 75%, 100%) and 2 control groups, namely positive control (Ciprofloxacin) and negative control (sterile aquadest). Each treatment was repeated four times.

Tools and Materials

The tools used in this study consisted of test tubes and racks, petri dishes, micropipettes, cotton swabs, McFarland 0.5 bacterial turbidity standards, tweezers, disc paper, matches, and Bunsen burners, digital scales, digital calipers, vortex mixers, incubators, and autoclaves.

The materials used consisted of R.mucronata mangrove leaves, Staphylococcus aureus bacteria, Mueller Hinton agar media, sterile distilled water, 5 mcg Ciprofloxacin disc, 96% ethanol.

Figure 1. Rhizophora mucronata mangrove leaves (Bei, 2021)

The Course of Research

1. Making extracts

The extract was made using the maceration method with 96% ethanol solvent. Making R.mucronata mangrove leaf extract requires 5 kg. The collected R.mucronata mangrove leaves will be processed by wet sorting to remove impurities attached to the sample and cleaned first using clean water, dried in an oven at a temperature of 40-60 ° C, then left to dry. Blend until smooth and in the form of powder called simple powder and then sieved with a 60 mesh sieve to filter to obtain R.mucronata leaf powder.

The simplicia powder is soaked in 96% ethanol solvent with a solvent sample ratio of 1:10. Optimize the pH of the extract using 0.5M NaOH so that the pH becomes 8 to 13. Soak for 72 hours in a container tightly closed with aluminum foil to avoid direct light. The results of the R. mucronata mangrove leaf extract are sorted with filter paper to become filtrate. Where the filtrate obtained is evaporated through a 40°C rotary evaporator to remove 96% ethanol mixed in the liquid extract (Nasri et al., 2022).

2. Preparation of a series of concentrations of mangrove leaf extract solutions of R. mucronata

The extract will be diluted with sterile aquades solvent to obtain concentrations of 25%, 50%, 75% and 100%. Dilution of R.mucronata leaf extract for 100% concentration by taking 1 mg of R. mucronata leaf extract with a pipette and then dissolving it in 1 ml of sterile distilled water, 75% concentration by taking 0.75 mg of R. mucronata leaf extract with a pipette and then dissolving it in 0.25 ml of sterile distilled water (add up to 1 ml), 50% concentration by taking 0.50 mg of R. mucronata leaf extract with a pipette and then dissolving it in 0.50 ml of sterile distilled water (add up to 1 ml), and 25% concentration by taking 0.25 mg of R. mucronata leaf extract with a pipette and then dissolving it in 0.75 ml of sterile distilled water (add up to 1 ml).

3. Preparation of bacterial suspension

Before being cultured, the bacteria will be made younger so that they are not resistant first in the incubator for 24 hours. Cultivation of Staphylococcus aureus bacteria is carried out by inoculating 1 loop of pure culture of Staphylococcus aureus bacteria into Mueller Hinton Agar media, then incubated at 37°C for 24 hours in the incubator. The cultured bacteria will be equalized for their density with the McFarland standard of 0.5. The McFarland standard is a test standard to compare the density of bacteria with the same density results with a number of 1.5 x 108cfui / ml (Nasri et al., 2022).

4. Antibacterial test

The prepared Mueller Hinton solution was put into 6 petri dishes, each dish filled with 10 mL. Scratch the S.aureus bacterial suspension evenly across the petri dishes using sterile tweezers in vertical, horizontal, and diagonal directions. Place the disc paper then drip the R.mucronata mangrove leaf extract in concentrations of 25%, 50%, 75%, and 100% using a micropipette with a dose of 20 micrograms. Ciprofloxacin 500 mcg acts as a positive control and sterile aquadest as a negative control. The petri dishes containing the S.aureus suspension and disc paper were placed in an incubator for 24 hours at 37°C. Measurement of the diameter of the inhibition zone using a caliper in vertical, horizontal, and diagonal positions to assess antibacterial activity. The diameter of the disc paper used was 6.00 mm.

Data analysis

The normality test was first performed using the Shapiro-Wilk test because the sample size was 50. Significance was seen from the p-value or significance, data <0.05, then the data was not normally distributed. The Kruskal Wallis test was performed because it did not meet the parametric requirements to assess whether or not there was a difference. Assessing which treatment group had a significant difference using the Post-hoc Mann-Whitney U test.

3. RESULT AND DISCUSSION

Result

The results of the antibacterial activity test of 96% ethanol extract of R.mucronata mangrove leaves against S.aureus bacteria using the disc diffusion method showed significant results. The inhibitory activity of bacterial growth can be seen from the presence of a clear zone around the disc paper

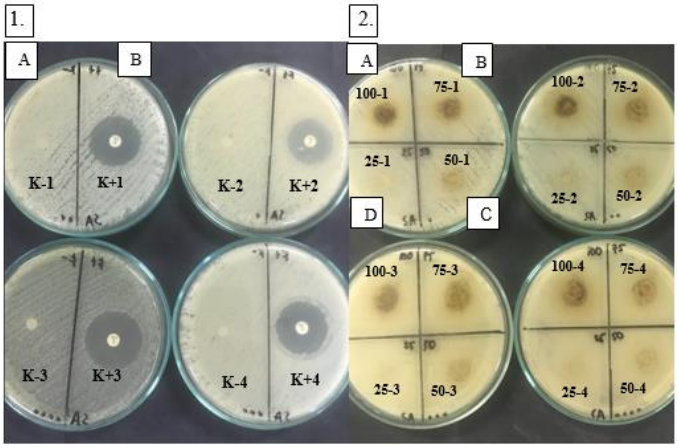

Figure 2. Inhibition zone of S.aureus bacteria

Control test (1) positive control (A) and negative control (B) and repeated 4 times (K+1 to 4; K-1 to 4). Mangrove leaf extract test R. mucronata (2) concentration 100% (A); concentration 75% (B); concentration 50% (C); and concentration 25% (D) repeated 4 times (Concentration 100% to 1 to 4; 75% to 1 to 4; 50% to 1 to 4; and 25% to 1 to 4).

Table 1. Results of Inhibition Zone Diameter Measurement

Test Group	Inhibition Zone Diameter (mm)				
	Repetition I	Repetition II	Repetition III	Repetition IV	Mean
K (-)sterile Aquades	6,00	6,00	6,00	6,00	25,4350
K (+) Ciprofloxacin 5 mcg	24,45	25,94	25,90	25,45	11,7275
Concentration 100%	12,52	11,81	11,6	10,98	9,8550

Concentration 75%	10,81	9,49	9,83	9,29	7,3625
Concentration 50%	7,54	6,76	7,70	7,45	6,0000
Concentration 25%	6,00	6,00	6,00	6,00	6,0000

Discussion

The concentration of S.aureus bacterial inhibition increased along with the increasing concentration of the extract. The group with the highest inhibition value was the positive control with the largest average diameter of 25.4 and the extract concentration of 100%, the largest average diameter of 11.7 mm. The treatment group that gave the smallest diameter was the 50% concentration with an average diameter of 7.4 mm. The factors that influence the formation of the inhibition zone diameter are antibacterial secondary metabolites, types of bacteria, types of solvents, solvent concentrations, extract concentrations, extraction processes and diffusion power (Egra et al., 2019). The activity of compound content secondary metabolites of R. mucronata leaves are phenols, flavonoids, saponins, tannins, and terpenoids. Phenol works by penetrating the bacterial cell wall, destroying the membrane, deinaturing the protein which then reduces the membrane permeability and destroys the cell wall. Phenol can deactivate the work of bacterial lysozyme enzymes which causes changes in membrane permeability which is the main factor in antimicrobial mechanisms (Kuilla et al., 2023). Flavonoids by inhibiting bacterial motility. Saponins work by attracting water (hydrophilic) and can also dissolve fat (lipophilic), this makes it able to inhibit the cleavage of bacterial cells, pategon in the cell membrane, disrupt transmembrane proteins and reduce membrane permeability of the cell. Saponins can modulate bacterial geniculate material, disrupt bacterial transmembrane proteins (porins) and cause bacterial cell damage by reducing the surface tension of the cell, as a result, it can cause the destruction of bacterial structures (Kuilla et al., 2023). Tannins work by interfering with enzymes such as reiveirsei transcriptase and even DNA topoisomerase, so that the process of DNA replication and transcription of bacterial cells is disrupted. Tannins target polypeptides in the bacterial cell wall layer, this causes the formation of the cell wall to be imperfect, as a result, the bacterial cell undergoes lysis due to osmotic or physical forces that cannot be overcome, and results in the death of the bacterial cell (Kuilla et al., 2023). Teirpeinoids inhibit protein synthesis and changes in the structure of cell components, so this results in inhibiting the growth of S. aurieriuis bacteria (Akasia et al., 2021). The yield results affect the concentration of secondary metabolite compounds, the high yield is thought to be the result of high production of secondary metabolite compounds. The yield formed in this study was 0.08% by dividing the weight of 500 grams of simplex with the weight of 40 grams of extract multiplied by 100%. The factor that affects the yield value is temperature, if the temperature increases, it will cause the viscosity of the solution to decrease so that the mass transfer resistance will be smaller (Muttaqin, 2018). High concentrations will provide higher antibacterial compound content so that they can build larger inhibition zones (Egra et al., 2019). The type of solvent affects the polarity of the extracted compound so that it has different abilities when diffusing. The solvent used in the extraction is ethanol, in this case ethanol is included in one of the strong categories which is assessed based on its antibacterial activity in the extract (Hafizah et al., 2024).

Based on research conducted by Saptiani regarding the antibacterial test of R. mucronata from Probolinggo against S. aureus, it showed significant inhibition results with concentrations of 50%, 60%, 70%, 80%, 90%, and 100%. The concentration of 100% showed the highest inhibition with an average inhibition zone diameter of 15.99mm compared to other concentrations. A study by the Wibowo group with concentrations of 50% and 60% was not effective in inhibiting S. aureus bacteria, but a concentration of 70% was quite effective, and concentrations of 80%, 90%, 100% were effective in inhibiting S. aureus bacteria (Saptiani, 2011). Based on research conducted by Karundeng et al. from Aceh regarding the antibacterial test of R. mucronata leaf extract against S. aureus bacteria with ethyl acetate solvent, it showed the presence of an inhibition zone diameter (Karundeng et al., 2022). The difference in solvents used did not have a significant effect on antibacterial compounds because they were both polar, only ethanol was stronger in attracting secondary metabolite compounds (Susiloningrum & Indrawati, 2020).

Research from Karundeng et al. on the antibacterial test of R. mucronata leaf extract against S. aureus bacteria, showed that there was an inhibition zone diameter in the development of S. aureus by R. mucronata mangrove leaf extract (Karundeng et al., 2022). Research from Papatungan using R. mucronata mangrove leaf extract as an ointment on rabbit back wounds infected with S. aureus bacteria showed significant wound improvement as measured by the length of the rabbit's wound (Papatungan, 2014).

The concentration of the 25% treatment group did not show any inhibitory activity. Factors that inhibit antibacterial activity are the low content of secondary metabolite compounds at a concentration of 25% when mixed with sterile aquades solvent which has a ratio of extract and sterile aquades of 1:4, so that it is unable to inhibit the growth of microorganisms (Afifi & Erlin, 2017). S.aureus bacteria are gram-positive bacteria that have a cell wall containing teichoic acid and peptidoglycan as cell wall substances, making them thick and stiff, making them more difficult to penetrate (Kaseng et al., 2016). The length of incubation affects antibacterial activity, the shorter it is, the less optimal the diameter produced (Fransisca et al., 2020).

Research conducted by Kaseng et al. related to the antibacterial test of R. mucronata leaf extract from Makassar against S. aureus bacteria showed no effect of R. mucronata mangrove leaf extract on S. aureus (Kaseng et al., 2016). Several factors that can affect antibacterial failure are the process and duration of evaporation, disc paper, and geographical location. The duration of the extract evaporation process can affect the concentration of compounds, if evaporation exceeds or is less than the optimum limit, it can result in a decrease in the content of secondary metabolite compounds. An extract evaporated with a rotary evaporator will get concentrated results and the compounds contained in it are not damaged because they have a low boiling point below the solvent so that the solvent will evaporate and the extract will settle underneath. If the disc paper is too dry, it can cause the formation of a less than optimal inhibition zone and if it is too wet, it can cause the inhibition zone to widen (Arin, 2018).

Geographical location greatly affects mangrove growth in terms of coastal topography, temperature, climate, salinity, tides, waves, currents, and nutrients, these are included in the physical factors of mangroves. Makassar has an altitude ranging from 0.5-10 meters above sea level (MDPL) and an average air temperature ranging from 34.1 ° C - 31.8 ° C (Malino et al., 2021). The mangrove plants used in our test were taken from Gunung Anyar, Surabaya, East Java, which is part of East Surabaya. East Surabaya has an average temperature of 24.57 - 36.39 ° C (Jatayu & Susetyo, 2017). Makassar has a higher minimum temperature standard than Surabaya. Rainfall will tend to be lower the further east you go, while rainfall has benefits in the content of inorganic nutrients in mangrove leaves (Malino et al., 2021). Research on Sonnetaria alba mangrove as an antibacterial for S.aureus showed no bacterial inhibition when using methanol and hexane solvents, but there was bacterial inhibition when using ethyl acetate solvent. This difference is likely due to the presence of salt levels in the leaves of Sonnetaria alba mangrove which are hygroscopic so that they can destroy the compound levels in the Sonnetaria alba leaf extract (Manuhuttu & Saimima, 2021).

The use of R.mucronata mangrove as an antibacterial has been scientifically tested for several other bacteria. Research from Egra et al., namely the antibacterial test of R.mucronata leaf extract against Ralstonia solanacearum bacteria which are classified as Gram-negative bacteria, was carried out with an antibacterial test using the well method which showed bacterial inhibition results at concentrations of 10,000 ppm and 20,000 ppm (Egra et al., 2019). Research on the antibacterial test of R.mucronata leaf extract against Aeromonas salmonicida and Vibrio harveyi bacteria, showed no antibacterial activity in A.salmonicida bacteria but antibacterial activity in V. harveyi bacteria (Suciati et al., 2012). Antibacterial test research of R. mucronata leaf extract against Helicobacter pylori bacteria showed the results of measuring the diameter of the inhibition zone in the weak category (Pertiwi et al., 2024).

Several research results show the benefits of mangrove plants from various parts of the mangrove, including leaves, roots, skin, and fruit, especially in their activity as antibacterials. Research on the antibacterial test of mangrove root endophytic fungus Rhizophora stylosa extract on the growth of S.aureus and E.coli bacteria, showed that endophytic fungi in mangrove roots produce the same antibacterial compounds as their hosts, resulting in an inhibition zone diameter

(Sumampouw et al., 2014). Research on the antibacterial test of mangrove leaf endophytic fungus R.apiculata against P.aeruginosa and S.aureus bacteria, obtained two isolates of black and white fungi, black fungi proved to be greater antibacterial (Santoso et al., 2015). Research on the antibacterial test of mangrove Avicenia marina against the growth of S.aureus showed significant bacterial inhibition results (Johanes, 2017).

Table 2. Results of the Mann-Whitney U post-hoc test

	Negative Control	Consen-Tration 25%	Consen-Tration 50%	Consen-Tration 75%	Consen-Tration 100%	Positive Control
Negative Control	-	1.000	0,14*	0,14*	0,14*	0,14*
Consen-tration 25%	-	-	0,14*	0,14*	0,14*	0,14*
Consen-tration 50%	-	-	-	0,21*	0,21*	0,21*
Consen-tration 75%	-	-	-	-	0,21*	0,21*
Consen-trationi 100%	-	-	-	-	-	0,21*
Positive Control	-	-	-	-	-	-

The results of the Mann Whitney test showed that each treatment showed significant differences except for the group with a concentration of 25% compared to the negative control. The difference in the extract treatment with the negative control and a concentration of 25% showed that the ethanol extract of R. mucronata mangrove leaves had the ability to inhibit the growth of S. aureus bacteria while sterile aquadest and a concentration of 25% did not have the ability to inhibit bacterial growth, however, the ability of the ethanol extract of R. mucronata could not match the ability of the positive control ciprofloxacin.

4. CONCLUSION

R.mucronata leaf extract has a significant effect on the development of S.aureus bacteria at certain concentrations, namely 50%, 75%, and 100% but not at 25% concentration. Several limitations in this study that can affect research results so that it is necessary to conduct in-depth research on secondary metabolite compounds that are bacteriostatic and bactericidal from R.mucronata leaves and more specific research is needed on one of the secondary metabolite compounds to test its activity as an antibacterial agent. The latest findings on the antibacterial activity of R.mucronata mangrove leaves can be scientific information for complementary therapy in infections caused by S.aureus and can be used as a natural alternative therapy or therapeutic treatment.

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