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ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT OF RED FRANGIPANI FLOWERS (*Plumeria rubra L.*) ON THE GROWTH OF THE BACTERIA *Methicillin-resistant Staphylococcus aureus* (MRSA)

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ABSTRACT

Introduction: MRSA is an antibacterial resistance problem that requires serious treatment. The limitations of antibacterials make it difficult to treat infections. The development of alternative antibacterial is needed.
Objectives: This study shows the antibacterial effect of ethanol extract of *Plumeria rubra L.* flowers against MRSA bacteria.**Methods:** This research is a true experimental research with the only post-test control group design. The Kirby-Bauer test method was used to evaluate the antibacterial activity of ethanol extract of *Plumeria rubra L.* flowers with concentrations of 20%, 40%, and 60% in triplicate. The test bacteria used MRSA ATCC 33591 bacteria. Antibacterial activity was assessed from the diameter of the inhibition zone formed in bacterial cultures on agar media.**Results:** The inhibition zone were not formed after ethanolic extract of *Plumeria rubra L.* flowers at various concentrations. These were not antibacterial activity shown from *Plumeria rubra L.* flowers extract at concentrations of 20%, 40%, and 60%.**Conclusions:** The ethanolic extract of *Plumeria rubra L.* flowers does not show antibacterial activity against MRSA bacteria.

Keywords: Red frangipani flowers (*Plumeria rubra L.*), antibacterial activity, MRSA.

ABSTRAK

Pendahuluan: MRSA merupakan masalah resistensi antibakteri yang memerlukan penanganan serius. Keterbatasan antibakteri menyebabkan pengobatan infeksi menjadi sulit. Pengembangan antibakteri alternatif sangat diperlukan.**Tujuan:** Penelitian ini menunjukkan efek antibakteri ekstrak etanol bunga *Plumeria rubra L.* terhadap bakteri MRSA.**Metode:** Penelitian ini merupakan penelitian eksperimen murni dengan *the only post-test control group design*. Metode uji *Kirby-Bauer* digunakan untuk mengevaluasi aktivitas antibakteri ekstrak etanol bunga *Plumeria rubra L.* dengan konsentrasi 20%, 40%, dan 60% dalam rangkap tiga. Bakteri uji menggunakan bakteri MRSA ATCC 33591. Aktivitas antibakteri dinilai dari diameter zona hambat yang terbentuk pada kultur bakteri yang ditumbuhkan pada media agar.**Hasil:** Tidak terbentuk zona hambat pada pemberian ekstrak etanol bunga *Plumeria rubra L.* pada berbagai konsentrasi. Tidak terdapat aktivitas antibakteri ekstrak bunga *Plumeria rubra L.* pada konsentrasi 20%, 40%, dan 60%.**Simpulan:** Ekstrak etanol bunga *Plumeria rubra L.* tidak menunjukkan aktivitas antibakteri terhadap bakteri MRSA.
Kata kunci: Bunga kamboja merah (*Plumeria rubra L.*), aktivitas antibakteri, MRSA.

INTRODUCTION

Antibacterial resistance is a severe danger to global health.[1] Antibacterial resistance significantly influences the frequency of infections, the severity of infectious illnesses, and the expense of health care.[2,3] According to data obtained from an analysis of antibacterial resistance cases in 204 countries in 2019, 4.95 million people died as a result of antibacterial resistance, the majority of which were caused by *Methicillin-resistant Staphylococcus aureus* (MRSA) infections.[4,5]

MRSA is a bacteria that causes nosocomial infections and is often found worldwide.[6] MRSA strains provide a health risk due to their genetic flexibility, which can render these bacteria resistant to several antibacterials. It affects the complexity of treating MRSA bacterial infections.[7]

This challenge undoubtedly necessitates investigating and developing novel antibacterial methods.[8] One method is using natural substances to generate novel antibacterial modalities.[9] Natural components that can be employed as antibacterial agents include phenolic compounds (flavonoids and tannins) and saponins.[10]

Secondary metabolites with antibacterial activity each have their mode of action. Flavonoid's significant antibacterial action is to interact with cell membranes.[11] As an antibacterial agent, tannin inhibits the production of cell walls, cell membranes, and fatty acid biosynthesis pathways.[12] Saponins, which have detergent-like qualities, operate as antibacterial agents by acting on cell walls and enhancing bacterial cell membrane permeability.[13]

Red frangipani flowers (*Plumeria rubra L.*) are one of the natural substances with the above secondary metabolite. *Plumeria rubra L.* flowers ethanol extract has been shown to exhibit antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.[14] However, there is no scientific proof that *Plumeria rubra L.* flowers ethanolic extract is efficient in preventing the development of MRSA bacteria. As a result, we intended to investigate the antibacterial activity of an ethanolic extract of red frangipani flowers (*Plumeria rubra L.*) on MRSA bacteria.

TAXONOMY OF *Plumeria rubra L.*

Based on data summarized by Manisha and An, the following is the taxonomy of *Plumeria rubra L.* :[15]

Kingdom	: *Plantae*
Division	: *Magnoliophyta*
Class	: *Magnoliopsida*
Ordo	: *Gentianales*
Family	: *Apocynaceaea*

MORPHOLOGY OF *Plumeria rubra L.*

Plumeria rubra L. grows 7-8 m (15-25 ft). This plant bears fragrant flowers in various colors (red, white, red, and three colors), 5-7 cm in diameter, five petals that create a spiral, and thin, gray-green bark with a smooth, somewhat glossy surface. The leaves are dark green, 15-40 cm long, grow in bunches at the end of each twig, and range from lanceolate to oval.[16]

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SECONDARY METABOLITES OF *Plumeria rubra L.*

The secondary metabolite concentration of each *Plumeria rubra L.* plant might vary depending on the climate and geographical circumstances in which the plant grows.[17] The secondary metabolites found in *Plumeria rubra L.* are flavonoids, tannins, saponins, carbohydrates, amino acids, glycosides, and monoglycerides. Flavonoids, tannins, and saponins are secondary metabolite contents with antibacterial activity.[10]

***Methicillin-resistant Staphylococcus aureus* (MRSA)**

Staphylococcus aureus is a natural human flora commonly found in the nose, skin, throat, and mouth. MRSA is a strain of this bacteria that is resistant to beta-lactam antibacterials.[18] MRSA is a non-motile, non-sporulating gram-positive bacteria in the form of cocci. These bacteria become resistant to antibacterials other than beta-lactams over time, including glycopeptides, aminoglycosides, and quinolones.[19]

MECHANISMS OF MRSA RESISTANCE TO BETA-LACTAMS

MRSA resistance to beta-lactams is caused by acquiring the *Staphylococcus Cassette Chromosome mec* (*SCCmec*) gene, which contains the *mec*A determinant.[20] This gene causes methicillin resistance, whereas resistance to penicillin is caused by the *bla*Z gene, which codes for the beta-lactamase enzyme.[21] The beta-lactamase enzyme is an extracellular enzyme produced in response to beta-lactam antimicrobial exposure. This enzyme hydrolyzes the beta-lactam ring, lowering penicillin's therapeutic efficacy.[22]

MECHANISMS OF MRSA RESISTANCE TO GLYCOPEPTIDES

MRSA glycopeptide resistance was initially documented in the late 1980s.[23] Resistance develops as a result of the acquisition of genes encoding particular enzymes that interfere with the manufacture of low-affinity peptidoglycan precursors, such as *D-Ala-D-Lactate* or *D-Ala-D-Serine*, while eliminating high-affinity precursors, such as *D-Ala-D-Ala*.[24] The *Van*A gene has a role in this resistance. These gene produces complicated physiological and morphological changes in cell wall synthesis (particularly cell wall thickening), reduced autolysis, and techoic acid content alterations.[25]

MECHANISMS OF MRSA RESISTANCE TO AMINOGLYCOSIDES

Aminoglycosides are antibacterials with a broad spectrum of action that function by blocking protein synthesis. Aminoglycosides decrease protein synthesis by attaching to the A-site on the 16S ribosome or disrupting the elongation process of the 30S ribosome.[26] Aminoglycoside resistance arises through various pathways, including in vitro mutations in ribosomal subunits, enzymatic changes, chromosomal mutation-induced target alterations, and efflux.[27]

MECHANISMS OF MRSA RESISTANCE TO QUINOLONES

Quinolones are broad-spectrum antibiotics used to treat resistant bacterial infections since 1980. Quinolones impede bacterial regulation of supercoiling within cells and target DNA gyrase and topoisomerase IV with different efficiency in each bacterium, resulting in reduced DNA replication at low doses and cell death at high concentrations.[28] Mutations in genes that control DNA gyrase (*gyr*A and *gyr*B) and topoisomerase (*Par*C and *Par*E) cause resistance to quinolone antibacterials.[29]

MATERIALS AND METHODS

This research was done from November 2023 to December 2023 and was authorized by the R.S.U.P. Prof. Dr. I.G.N.G. Ngoerah Denpasar research ethics commission under 2068/UN14.2.2.VII.14/L.T./2023. This research uses an experimental with a true experimental design, the post-test-only control group design. The treatment groups included concentrations of ethanol extract of *Plumeria rubra L.* flowers of 20% (P1), 40% (P2), and 60% (P3). The control group included linezolid positive control (K+) and 96% ethanol negative control (K-). The antibacterial activity test used the disc diffusion method (Kirby-Bauer test).

PREPARATION OF ETHANOLIC EXTRACT OF *Plumeria rubra L.* FLOWERS

By picking fresh flowers, *Plumeria rubra L.* flowers were selected from plants blooming in Penatih Dangin Puri village, East Denpasar subdistrict, Denpasar city. The extract was prepared in the Udayana University's Faculty of Medicine's Pharmacology Laboratory. Fresh flowers weighing 1 kilogram are cleansed with clean water first, then dried in the sun in a container covered with a black cloth. The dried flowers were mixed until smooth before being extracted in a securely wrapped pan for three days using the maceration method with 96% ethanol solvent in a 1:10 ratio. The maceration products are then evaporated to produce a thick extract.

The thick extract was then diluted to achieve 20%, 40%, and 60% concentrations. Dilution was used to create a 4 ml mixture. A 20% concentration is generated by combining 0.8 grams of thick extract with 3.2 ml of 96% ethanol, a 40% concentration is formed by mixing 1.6 grams of thick extract with 2.4 ml of 96% ethanol, and a 60% concentration is made by combining 2.4 ml of 96% ethanol with 2.4 grams of thick extract.

PREPARATION OF MRSA SAMPLES

The test bacteria used MRSA ATCC 33591 bacteria**.** The MRSA bacteria samples utilized in this study were cultivated at Udayana University's Faculty of Medicine's Microbiology Laboratory. Bacteria grown will be turned into a suspension by dissolving them in NaCl solution until hazy, according to the Mc Farland standard (1.5 x 10^8 cells/ml). The bacterial suspension is then spread over the Mueller-Hinton agar medium and is ready for antibacterial activity testing. Under Laminar Air Flow (LAF), all phases of MRSA sample preparation were carried out with aseptic procedures.

ANTIBACTERIAL ACTIVITY TESTING

Ethanol extracts of *Plumeria rubra L.* flowers at 20%, 40%, and 60% concentrations, and negative control were put onto a disc using a 20 µl micropipette and soaked for one hour. The positive control was a linezolid disc obtained from the Microbiology Laboratory at Udayana University's Faculty of Medicine. The disc is then put on an agar medium with bacterial suspension and incubated for 24 hours at 35-37 degrees Celsius. The test was repeated three times. The clear zone generated after incubation is the bacterial inhibition zone, which will be measured in millimeters (mm) with a ruler.

DATA PROCESSING AND ANALYSIS

The collected inhibitory zone diameter data will be processed and evaluated with IBM SPSS Statistics 21, Microsoft Excel, and Microsoft Word software. The collected data will be examined in many steps, namely:

1) The Shapiro-Wilk test will be used to determine the normality of the data distribution.
2) Because the received data is not normally distributed, Levene's test will be used to assess if the variance in the data distribution is the same.
3) The Kruskal-Wallis test will be used to determine whether there is a statistically significant difference in the diameter of the inhibition zone between the treatment and control groups.
4) The statistics reveal a statistically significant difference in the diameter of the inhibitory zone; hence, the Mann-Whitney U test will be repeated.

RESULTS

EXTRACTION OF *Plumeria rubra L.* FLOWERS

Plumeria rubra L. flowers were extracted at the Pharmacology Laboratory, Faculty of Medicine, Udayana University. *Plumeria rubra L.* flowers weighing 1 kilogram are dried first, yielding dried flowers weighing 350 grams. The dried flowers were macerated to provide a thick extract weighing 5.25 grams. The yield produced using this extraction method was 0.525% **(Table 1)**.

Table 1. Obtained Extract Yield Results

Wet Weight (g)	Dry Weight (g)	Thick Extract Weight (g)	Yield (%)
1,000	350	5.25	0.525

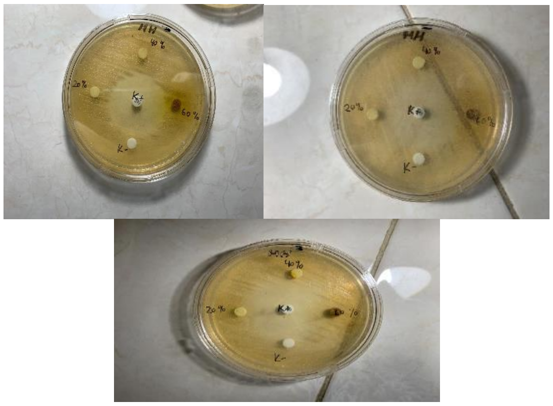
ANTIBACTERIAL ACTIVITY TESTING

The disc diffusion method (Kirby-Bauer test) evaluated the extract's antibacterial activity thrice. After 24 hours of incubation, the diameter of the inhibitory zone was measured in millimeters (mm) using a ruler. The findings of evaluating the diameter of the inhibition zone on the development of MRSA bacteria are shown in **Table 2** and **Figure 2**.

MRSA bacterial cultures treated with ethanol extract of *Plumeria rubra L.* flowers at concentrations of 20%, 40%, and 60% did not have an inhibitory zone for MRSA bacteria development, according to the findings of measuring the width of the inhibitory zone. The positive control treatment, linezolid, demonstrated antibacterial efficacy with a median inhibitory zone diameter of 41 mm, but the negative control, 96% ethanol, revealed no inhibition zone.

Table 2. Results of Measuring the Diameter of the Zone of Inhibition on the Growth of MRSA Bacteria

Treatment Groups	Median (mm) (Min-Max)
P1 (Extract concentration 20%)	0(0-0)
P2 (Extract concentration 40%)	0(0-0)
P3 (Extract concentration 60%)	0(0-0)
K+ (Positive control linezolid)	41(41-44)
K- (Negative control ethanol 96%)	0(0-0)



Picture 2. Antibacterial Activity Test Results of *Plumeria rubra L.* Ethanol Extract

The width of the inhibitory zone on MRSA bacteria growth was then examined using IBM SPSS Statistics 21. First, the Shapiro-Wilk test was used to evaluate the normality of the data distribution on the diameter of the inhibitory zone on MRSA bacteria growth. The test findings demonstrate that the data is not normally distributed, with a p-value = <0.001.

A homogeneity test was also performed on the inhibition zone diameter data to see if there was the same variation in the distribution of the inhibition zone diameter data on MRSA bacteria growth. Because the data is not normally distributed, the Levene test is performed to determine homogeneity. The test findings suggest that the data is not homogenous, with a p-value = <0.001.

The non-parametric Kruskal-Wallis test will be used since the data is not regularly distributed. The non-parametric Kruskal-Wallis test demonstrated that the width of the bacterial inhibitory zone differed between the test concentration, positive control, and negative control. The significance value of the test findings is p-value = 0.008, indicating that at least some of the treatment groups in this study exhibited significant variations in the width of the inhibition zone.

A Mann-Whitney U test will be performed since the Kruskal-Wallis test findings demonstrate a significant difference in the inhibitory zone diameter data between the test concentration, positive control, and negative control. The test findings demonstrate no variation in diameter between practically all treatment groups. The test findings revealed that, except for the positive control with extract concentrations of 20%, 40%, and 60%, and the negative control, there was no significant difference in the diameter of the inhibitory zone with a p-value = 4.500. This demonstrates that only the positive control substantially differs in inhibitory zone diameter **(Table 3)**.

Table 3. Mann-Whitney U Test Results Data on the Diameter of the Inhibition Zone on the Growth of MRSA Bacteria

Treatment Groups		p-value
Extract concentration 20%	Extract concentration 40%	4.500
	Extract concentration 60%	4.500
	Positive control linezolid	<0.001*
	Negative control ethanol 96%	4.500
Extract concentration 40%	Extract concentration 60%	4.500
	Positive control linezolid	<0.001*
	Negative control ethanol 96%	4.500
Extract concentration 60%	Positive control linezolid	<0.001*
	Negative control ethanol 96%	4.500
Positive control linezolid	Negative control ethanol 96%	<0.001*

* There is a statistically significant difference in the diameter of the inhibition zone

DISCUSSION

The maceration approach was chosen in this work to extract *Plumeria rubra L.* flowers since it is the simplest and most optimum method for extracting thermolabile chemicals. Maryam et al. conducted a study demonstrating that the maceration technique extracts the greatest average total flavonoid concentration of 59.13 mg/g compared to the percolation, soxhletation, and reflux procedures.[30] The solvent used in this study is 96% ethanol because it dissolves almost all secondary metabolites, both polar and nonpolar, and has a low boiling point, making it easier to evaporate and not damaging thermolabile secondary metabolites.[31]

A clear zone that emerges around the disc placed on agar media demonstrates the antibacterial activity of the disc diffusion technique.[32] This investigation revealed that ethanol extracts of *Plumeria rubra L.* flowers at 20%, 40%, and 60% concentrations did not exhibit antibacterial activity against the development of MRSA bacteria because no clear zone appeared around the disc on which the extract was dripped. Sari et al. found that an ethanolic extract of *Plumeria rubra L.* flowers had antibacterial activity at a concentration of 20% against *Escherichia coli* with an inhibitory zone diameter of 4.14 mm and *Staphylococcus aureus* with an inhibitory zone diameter of 7.30mm.[14]

MRSA also has genes that can cause complicated physiological and morphological alterations in cell wall production, particularly cell wall thickening and modifications in cell membrane components.[34] This influences the extract's secondary metabolites' antibacterial susceptibility because secondary metabolites in *Plumeria rubra L.* ethanol extract exhibit antibacterial activity via interacting with the bacterial cell wall and cell membrane.[11–13]

Aside from the factors indicated above, the discrepancy in antibacterial test findings between Sari et al. and this study might be due to the natural extracts employed.[14] The extracts employed were created in separate facilities, and the raw materials for the extracts were sourced from various locations, resulting in varying outcomes. Chemical parameters such as the kind and amount of antibacterial chemicals in the extract can also impact its quality.[33]

Extract efficiency might vary based on biological differences and secondary metabolite levels in the plants employed.[33] Even if they are from the same species, these variances might emerge due to changes in the conditions in which the plants are used. Secondary metabolites in plants can fight against bacteria that cause illness.[35] Secondary metabolite content is the primary element influencing plant adaptation to biotic stimuli, with plants producing greater secondary metabolite content under more dangerous environmental situations.[9] Abiotic variables such as light intensity, temperature, water, and soil fertility all impact plant growth and development, including the plant's capacity to manufacture secondary metabolites.[36]

Zhang et al. discovered that increasing the intensity of ultraviolet B (UV-B) light exposure significantly increased total flavonoids.[37] Because UV-B is a damaging light, plants will manufacture more flavonoids to defend themselves from UV-B exposure.[38] The effect of temperature on plants is inversely proportional to this. Cawood et al. discovered that total flavonoid levels drop as temperature rises.[39] This is because flavonoids are thermolabile chemicals that deteriorate when exposed to high temperatures.[30]

Previous research employed *Escherichia coli* and *Staphylococcus aureus* microorganisms to investigate antibacterial activity. The outcomes also varied because these two species differ from the MRSA species utilized in this investigation. Each bacterial species has unique cell wall components and genetic material, resulting in various antibacterial defense mechanisms.[33]

The difference in antibacterial activity test results with prior studies may be due to resistance in the microorganisms utilized as test samples. Bacterial resistance must be considered because the bacteria utilized as test samples is MRSA. Several strains of this bacteria are resistant to antibacterials.[21]

CONCLUSIONS AND RECOMMENDATIONS

This study found that an ethanolic extract of red frangipani flowers (*Plumeria rubra L.*) has no antibacterial activity against the development of MRSA bacteria. Some

recommendations that we can provide through this research are:

1) A plant identification test will be conducted to determine that the extract's natural constituents are *Plumeria rubra L.* flowers.
2) Innovation and renewal of extraction techniques for secondary metabolite content with antibacterial activity.
3) Screening and quantitative secondary metabolite testing to guarantee the extract includes antimicrobial secondary metabolites.

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