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In vitro Antimicrobial Activity Test of *Zingiber officinale* var. *rubrum* Rhizome Extract against Methicillin-Sensitive *Staphylococcus aureus* (MSSA) and Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Introduction: Red ginger (*Zingiber officinale* var. *rubrum*) is a traditional herb that is believed to possess antibacterial properties. Throughout the years, *Staphylococcus aureus* has developed resistance to a broad range of antibiotics, including beta-lactams, particularly in the form of Methicillin-Resistant *Staphylococcus aureus* (MRSA). As treatment options dwindle, it is urgent to formulate novel antibiotics. This study aimed to examine the antibacterial activity of the ginger rhizome ethanol extract against Methicillin-Sensitive *Staphylococcus aureus* (MSSA) and MRSA.

Methods: This study was performed according to the post-test-only control group design. Through a good diffusion assay, the anti-MSSA and anti-MRSA activity of the red ginger extract concentrations (100%, 50%, 25%, 12.5%, and 6.25%) was observed by measuring the diameter of the clear inhibition zones. Dimethyl sulfoxide (DMSO) and an antibiotic disc were added as control groups.

Results: The red ginger extracts produced inhibition zones on both MSSA and MRSA. However, the antibacterial activity was considered weak (<12 mm). The concentration of the extract appeared to linearly affect its antibacterial activity against MSSA and MRSA. On MSSA, the 12.5% extract results differed significantly from those of the 100% and 50% extracts. Meanwhile, on MRSA, the extracts seemed to yield significantly different outcomes when compared to each other, except for the comparisons between 50%-25% and 12.5%-6.25%.

Conclusion: *Zingiber officinale* var. *rubrum* rhizome extracts showed weak antibacterial activity against MSSA and MRSA.

Highlights:
1. *Staphylococcus aureus*, particularly MRSA, had developed rapid resistance against antibiotics like beta-lactams.
2. Red ginger is believed to be antibacterial against MSSA and MRSA in vitro.
3. Red ginger rhizome extracts displayed weak activity against MSSA and MRSA.

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Introduction

Staphylococcus aureus, a spherical-cell gram-positive bacterium that can be naturally found in the skin and nose area, can induce various diseases, including osteomyelitis, meningitis, food poisoning, and toxic shock syndrome.[1] More localized forms of *S. aureus* infection usually manifest as pimples, abscesses, folliculitis, and other skin and soft tissue infections. In 2019, *S. aureus* had been estimated to cause 1,050,000 mortalities worldwide.[2] This establishes *S. aureus* as the leading cause of death for bacterial infection cases among 135 countries and the leading cause of mortality for individuals above 15 years old with bacterial infections. Around 45.3% of 567 patients in Indonesia suffering from skin and soft tissue infections scattered within Surabaya, Malang, and Bali hospitals were clinically diagnosed with *S. aureus* infection.[3] Around eight people were infected with methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is an *S. aureus* variant that has grown resistant to commonly used anti-staphylococcal drugs, including beta-lactams such as penicillin, cephalosporins, nafcillin, and oxacillin.[4]

The resistance of MRSA was induced by the mecA gene that expresses penicillin-binding protein 2a (PBP2a), a type of transpeptidase responsible for cell wall synthesis.[5] Different from ordinary penicillin-binding proteins (PBPs) that can be found within methicillin-sensitive *Staphylococcus aureus* (MSSA), a variant of *S. aureus* that is still susceptible to antibiotics resisted by MRSA, PBP2a is resistant to beta-lactams and numerous other antibiotics due to its low binding affinity. This protein then remains active and synthesizes cell wall components, thus inhibiting cell lysis.[5] Different from MSSA infection cases that typically resolve with beta-lactam administrations, MRSA infections usually require stronger antibiotics such as vancomycin (a first-line empirical antibiotic against MRSA). This has become an issue due to the emergence of vancomycin resistance in MRSA isolates.[6]

The growing antibiotic resistance of MRSA and *S. aureus* raised serious public health concerns over the future availability of antibiotics capable of fighting *S. aureus* infections. Unfortunately, the development of novel antibiotic agents that are effective against *S. aureus*, particularly MRSA, is considerably slow and cannot match the rapidly growing antibiotic resistance.[7] Regarding this issue, several studies utilized traditional herbs with high phenols and flavonoids (natural bioactive antimicrobial compounds) as potential new antibiotics.[8] *Zingiber officinale* var. *rubrum*, more commonly known as red ginger, is believed to be a suitable candidate due to the plant's long history of usage for nausea, coughing, inflammation, and the common cold.

Research on red ginger's antibacterial activity against *S. aureus*, particularly those that differentiate *S. aureus* into MSSA and MRSA, is still relatively rare despite the plant's versatility and possible antibiotic properties.[9–11] The lack of references hinders the advancement of red ginger extract as a possible antibacterial agent, along with its correlation, effect, and application to MSSA and MRSA. Therefore, this in vitro study aimed to investigate the antibacterial activity of red ginger extract when exposed to MSSA and MRSA.

Methods

The extract's antibacterial capacity was determined using the agar-well diffusion method. Numerous wells were created perpendicular to the agar medium using a 6–8 mm cork borer with aseptic techniques to avoid contamination.[12] The extracts and dimethyl sulfoxide (DMSO) were placed in these wells using a micropipette with the same volume. This study discussed the effect of red ginger extract as the independent variable on MSSA and MRSA cultures (the dependent variable). Several factors, including the inoculation method, incubation period, research instruments, and agar conditions, were kept in the same condition across replications to minimize outside interventions and variables. The population of this experiment was all of the MSSA and MRSA clinical isolates stocked within the Laboratory of Clinical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya (7°15'53.7"S 112°45'34.4"E). From these isolates, four different isolates were chosen by simple random sampling. This study was conducted according to the post-test-only control group design. The experiment results were evaluated after the interventions, with no previous testing on the samples to determine any baselines.[13] The experiment was commenced in February until May 2023.

Rhizome Preparation and Extraction

The rhizomes were identified, harvested, and processed into powder by the Technical Implementation Unit (UPT) Balai Materia Medica (7°52'03.3"S 112°31'10.2"E) in Malang, Indonesia. The rhizomes were then extracted by maceration with 1,875 ml of ethanol 80% as a solvent for 120 hours. After 5 days, the ginger pulp was flushed and drained with 625 ml of ethanol at 80% until the end rhizome-solvent ratio of 1:10 was obtained. Maceration, a cold extraction procedure, was preferred due to the unstable nature of gingerol compounds at high temperatures.[14]

Extract Preparation

The crude extract was diluted into 50%, 25%, 12.5%, and 6.25% concentrations with 5% DMSO. After dilution, five extract concentrations were prepared into separate tubes (100%, 50%, 25%, 12.5%, and 6.25%).

S. aureus Antibiotic Resistance Confirmation Test

The MSSA and MRSA isolates underwent a confirmation test regarding their antibiotic resistance status using cefoxitin 30 microgram discs. Following an incubation period of 24 hours (37°C), the emerging inhibitory zones were measured for their diameters. The findings were then interpreted according to the Clinical & Laboratory Standards Institute (CLSI) guidelines.[15]

Bacteria Cultures

From the four clinical isolates of MSSA and MRSA previously chosen, one isolate was picked each for MSSA

and MRSA based on the antibiotic resistance confirmation test. Bacterial suspensions of 0.5 McFarland were prepared by visually matching the turbidity to a standardized suspension tube, aided by visual lines and double confirmation by laboratory analysts.

Antimicrobial Assay

Mueller-Hinton agar (CLSI-recommended medium for *S. aureus* cultures) was prepared into six cell-culture plates.[15] In each plate, 7.77 mm wells were made using a cork borer until six wells were formed in each plate. Extracts in 100% (E1), 50% (E2), 25% (E3), 12.5% (E4), and 6.25% (E5) concentrations were inserted into the wells along with 5% DMSO as the negative control group (K(-)). The DMSO was made in Germany by Merck KGaA (49°53'43.7"N 8°39'05.1"E) with a catalog number 1.02952.2500. After all the wells were filled, the antibiotic disc erythromycin 15 microgram (for MSSA) or vancomycin 30 microgram (for MRSA) was put on the center of the agar as the positive control group (K(+)). The 231290 BD BBL™ Sensi-Disc™ Erythromycin Discs were made in USA by Becton, Dickinson, and Company (41°00'57.1"N 74°12'34.2"W). The Oxoid™ Antimicrobial Susceptibility Test (AST) Vancomycin Discs were manufactured in UK by Thermo Fisher Scientific Inc. (42°23'34.8"N 71°15'41.8"W) with a catalog number CT0058B. After overnight incubation at 37°C, the zones were measured using a vernier caliper with 0.01 mm precision. According to a sample calculation formula for pre-clinical group comparison studies, this experiment required at least 3 replications for MSSA and MRSA separately.[16]

Data Analysis

The data was analyzed with the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) application for macOS, version 29, with a confidence level of 95%.[17] First, the normality was determined using the Shapiro-Wilk test. The Kruskal-Wallis's test was enacted to compare the overall significance of the treatment groups. The Mann-Whitney U test was conducted to identify which groups exhibit significant statistical differences when compared to each other.

Results

Results of *S. aureus* Antibiotic Resistance Confirmation Test

According to CLSI guidelines, the existence of mecA-mediated resistance in *S. aureus* isolates can be determined based on the inhibition zone diameter caused by the cefoxitin disc.[15] A clinical isolate is deemed sensitive or susceptible if the zone diameter is ≥22 mm and is considered antibiotic-resistant if the diameter is ≤21 mm. After a 24-hour incubation period, both of the MSSA-labelled isolates were confirmed to be sensitive. Cefoxitin produced zones with diameters of 24.29 mm and 26.71 mm on MSSA isolate 1 and MSSA isolate 2, respectively. Meanwhile, the MRSA-labelled isolates were also confirmed to be resistant. Cefoxitin produced inhibition zone diameters of 20.92 mm and 12.40 mm on MRSA isolate 1 and MRSA isolate 2, respectively. MSSA isolate 2 and MRSA isolate 2 were selected to be tested against the extract because they displayed the antibiotic resistance characteristics of MSSA and MRSA better than their counterparts. In other words, MSSA isolate 2 was chosen because of its larger zone diameter than MSSA isolate 1, as opposed to MRSA isolate 2, which was chosen because its zone diameter was smaller than that of MRSA isolate 1.

Results of the red ginger antibacterial activity test on MSSA and MRSA

The antibacterial capacity of the extracts was assessed by measuring the clear inhibition zones generated around the wells using a vernier caliper. The extracts exhibited inhibition zones on both MSSA and MRSA, as shown in Figure 1, Table 1, and Table 2. On MSSA, the 100% extract (E1) emanated the greatest inhibition zone diameter average out of all the extract concentrations. The overall diameter average decreased linearly with the extract concentration, making 6.25% extract (E5) the lowest extract concentration to produce inhibition zones. Although the antibacterial activity of the E1 extract was the strongest out of all the extracts, the antibacterial strength of erythromycin (K(+)) was still much stronger than the extracts, as evidenced by the greater diameter average compared to the extracts and DMSO (K(-)).

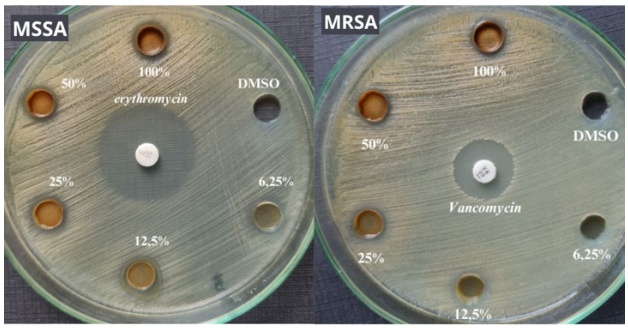

Figure 1. The effect of ginger extract and control groups on MSSA isolate 2 (left), and MRSA isolates 2 (right).

On MRSA, similar findings can be observed. Table 2 depicts the measurement results of the inhibition zones that developed in the treatment groups. The 100% extract (E'1) exhibited the largest diameter average compared to the other concentrations. The average diameter of the zones could also be seen declining proportionally according to the extract concentrations.

Table 1. Inhibition zone diameter measurements of MSSA isolate 2

Extract Concentration	Inhibition Zone Diameter (mm)			Means (mm)
	1st Replication	2nd Replication	3rd Replication	
E1 (100%)	10.30	9.12	9.85	9.75
E2 (50%)	10.03	8.85	9.77	9.55
E3 (25%)	9.98	8.72	8.95	9.21
E4 (12.5%)	8.87	0	8.51	5.79
E5 (6.25%)	9.32	0	0	3.10
K(-)	0	0	0	0
K(+)	26.55	23.96	25.20	25.23

Source: Research data, processed

The lowest extract concentration that could inhibit the growth of MRSA was 12.5% (E'4). The MRSA resisted the 6.25% extract (E'5), as evidenced by the lack of inhibition zones. Vancomycin (K'(+)) established the greatest diameter average compared to the extracts and DMSO (K'(-)). In both MSSA and MRSA, DMSO did not produce inhibition zones. This result confirmed that DMSO, as a diluent agent, does not possess intrinsic antibacterial properties and did not influence the outcomes of the experiment.

Table 2. Inhibition zone diameter measurements of MRSA isolate 2

Extract Concentration	Inhibition Zone Diameter (mm)			Means (mm)
	1st Replication	2nd Replication	3rd Replication	
E'1 (100%)	9.74	9.39	9.40	9.51
E'2 (50%)	8.56	8.96	8.72	8.74
E'3 (25%)	8.42	8.65	8.48	8.51
E'4 (12.5%)	0	0	8.39	2.79
E'5 (6.25%)	0	0	0	0
K'(-)	0	0	0	0
K'(+)	18.23	16.90	16.06	17.06

Source: Research data, processed

Statistical Analysis

The data obtained from the MSSA antimicrobial assay was not normally distributed (p-value < 0.05). The Kruskal-Wallis's test was then performed to determine whether the outcomes of the treatments were significantly different. The significance of the MSSA test group (p-value) was 0.014, indicating that there were significant differences in the overall obtained results (p-value < 0.05). Through the Mann-Whitney test, it was revealed that the 12.5% extract (E4) yielded significantly different outcomes compared to the 100% (E1) and 50% (E2) extracts. The erythromycin (K+) produced inhibition zones that were significantly different than those of the extracts and DMSO (K-). The complete Mann-Whitney analysis of the MSSA test results can be seen in Table 3.

Table 3. The interpretation of the Mann-Whitney U test of the results of MSSA isolate 2

Extract Concentration	Significance Comparisons (p-value)						
	E1	E2	E3	E4	E5	K(-)	K(+)
E1 (100%)	-	NS	NS	S	NS	S	S
E2 (50%)	NS	-	NS	S	NS	S	S
E3 (25%)	NS	NS	-	NS	NS	S	S
E4 (12.5%)	S	S	NS	-	NS	NS	S
E5 (6.25%)	NS	NS	NS	NS	-	NS	S
K(-)	S	S	S	NS	NS	-	S
K(+)	S	S	S	S	S	S	-

S = Significant; NS = Not significant
Source: Research data, processed

The outcomes of the MRSA antibacterial assay were not normally distributed (p-value < 0.05). Overall, there were significant differences between the MRSA results, as indicated by the Kruskal-Wallis's test result of 0.017 (p-value < 0.05).

Table 4. The interpretation of the Mann-Whitney U test of the results of MRSA isolate 2

Extract Concentration	Significance Comparisons (p-value)						
	E'1	E'2	E'3	E'4	E'5	K'(-)	K'(+)
E'1 (100%)	-	S	S	S	S	S	S
E'2 (50%)	S	-	NS	S	S	S	S
E'3 (25%)	S	NS	-	S	S	S	S
E'4 (12.5%)	S	S	S	-	NS	NS	S
E'5 (6.25%)	S	S	S	NS	-	NS	S
K'(-)	S	S	S	NS	NS	-	S
K'(+)	S	S	S	S	S	S	-

S = Significant; NS = Not significant.
Source: Research data, processed

Using the Mann-Whitney test, nearly all of the extract concentrations gave significantly different results compared to each other, with insignificances found between 50% (E'2) and 25% (E'3), 12.5% (E'4) and 6.25% (E'5), including 6.25% (E'5) and DMSO (K'(-)). Vancomycin (K('+)) results proved to be significantly different in contrast to the rest of the treatment groups. The complete Mann-Whitney analysis of the MRSA test results can be seen in Table 4.

Discussion

The chemical compounds inside the rhizome prompted the antibacterial capacity of red ginger. Phytochemical studies of the red ginger rhizome extract suggested the existence of six main compounds, namely alkaloids, tannins, phenols, steroids, terpenoids, and flavonoids.[18,19] These components then inhibit bacterial growth through various mechanisms. Alkaloids can disrupt cell wall synthesis, destroying the cell membrane due to the pressure difference between the intracellular and extracellular compartments.[20] Tannins may induce cell membrane destruction, protein binding, enzyme inactivation, and toxic complex formation.[20] Terpenoids such as geraniol, linalool, limonene, and zingiberol can cause protein denaturation, cell permeability disruption, cell membrane destruction, and oxidative phosphorylation inhibition of the cell membrane.[9,21] Citral, another terpenoid, may decrease intracellular adenosine triphosphate (ATP), decrease cytoplasmic pH, destroy cell membranes, and inactivate enzymes.[9,22] The steroid component of the extract can inhibit *S. aureus* growth by destroying the cell membrane and forming steroid-extracellular protein complexes.[23]

Gingerol and shogaol are regarded as the most potent phenolic compounds in red ginger.[24] Gingerol inhibits *S. aureus* by disrupting the cell membrane and cellular proteins.[9] Another study also proved the great antimicrobial potency of gingerol, as its fractionated and purified [6]-Gingerol form can significantly impede the in vitro and in vivo growth of *M. tuberculosis.* The results can be explained by the ability of [6] Gingerol to modulate the immune system by enhancing the protective response of Th1 and Th17 and inducing specific anti-tuberculosis immune pathways in the infected mice host.[25] Shogaol and gingerenone A can specifically target *S. aureus* by inhibiting the function of the 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, which disrupts essential folic acid synthesis.[26] Flavonoids interrupt cell permeability, leading to cell lysis.[27] It may also act against MRSA through various mechanisms. Flavonoids in red ginger can be diversified into five compounds, catechin, epicatechin, kaempferol, quercetin, and rutin. Catechin can disintegrate cells and inhibit superoxide dismutase and catalase enzymes, prompting reactive oxygen species accumulation.[28] Kaempferol and quercetin displayed synergism with ciprofloxacin and quinolones, respectively, due to their target similarity. Ciprofloxacin and kaempferol inhibit the deoxyribonucleic acid (DNA) topoisomerase II enzyme, while quinolones and quercetin specifically inhibit the DNA topoisomerase IV enzyme. Rutin can also inhibit DNA topoisomerase IV, altering cell membrane permeability, influencing energy metabolism, and disrupting the nucleic acid synthesis of bacteria.[29] Despite the flavonoids' potential against bacteria, further studies that address the bioavailability and proper formulation of flavonoids are needed to develop flavonoids as potent antibacterial agents properly.

Based on the diameter average, it can be concluded that the diameters of the inhibition zones are linear to the extract concentration. These findings were also found in other similar experiments that discussed the antibacterial potential of red ginger rhizome extract.[11,30] This phenomenon can be explained by the solubility of the extract.[31] The high water solubility made it easier for the chemical compounds inside the extract to travel further within the agar matrix.[11] Low-solubility materials tend to diffuse less freely within an agar medium.[31] Extracts with higher concentrations contain greater amounts of antibacterial compounds, resulting in the 100% concentration extract emitting the largest inhibition zone diameter average. Based on the interpretation criteria used by Odilla, *et al.* (2022), antibacterial potency can be classified into weak (diameter <12 mm), moderate (diameter 12–20 mm), and strong (diameter 20 mm).[32] Abiding by this classification, all of the extract concentrations displayed weak antibiotic properties against both MSSA and MRSA, as evidenced by the average diameter of <12 mm.

Another thing to note is that the antibacterial activity of the extracts was discovered to be greater against MSSA than MRSA. Based on the diameter average in Table 1 and Table 2, the extracts appeared to exhibit more prominent inhibition zones on MSSA than MRSA. While neither the exact resistance factor of MRSA against red ginger extract nor its correlation with PBP2a is known, a molecular study stated that flavonoids may be capable of modifying PBP2a to be more susceptible to antibiotics.[33] Flavonoids can induce structural changes in PBP2a, making it bind with beta-lactams more effectively. Flavonoids, such as catechin and epicatechin, showed potential as anti-MRSA agents. Catechin, in its purified and fractionated form, has proven to inhibit MRSA growth in vitro.[28] A derivate of epicatechin, epicatechin gallate (ECG), also inhibits MRSA by disrupting genes responsible for cell wall repair.[34] Although more commonly associated with green tea leaves, more phytochemical studies that revolve around ECG quantity within red ginger rhizomes and its antibacterial mechanisms against MRSA are needed to assess the capacity of this compound as an anti-MRSA carefully. Further molecular studies that discuss the effect of PBP2a after its exposure to red ginger extract are also needed to assess the compound's antibacterial mechanisms against PBP2a properly and to determine whether there is another resistance factor that may impede the anti-MRSA activity of the red ginger extract.

The 6.25% extract (E5) produced the smallest diameter average on MSSA. Although its results are expected to be significantly different compared to the other extract concentrations, the extreme data (9.32 mm) prompted the Mann-Whitney test to declare that the result was insignificantly different (p-value > 0.05). This data could appear due to various reasons, such as a mere probability or the existence of other factors influencing the experiment. It is also important to consider that the Mann-Whitney test is a nonparametric test that utilizes the median and rank sum of a dataset instead of the average or means. The extreme data hindered the test because it directly influenced the rank sum when inserted into the Mann-Whitney formula with the other results.[35] Therefore, in

cases of extreme data emergence, the interpretation of the 6.25% extract and its significance relative to the other interventions should also consider the overall diameter average and not be based on statistic tests alone. In conclusion, the 6.25% extract yielded the smallest diameter average but was statistically insignificant compared to the other extract concentrations due to extreme data.

Strength and Limitations

This experiment provided additional perspective and clearer insights for future developments concerning red ginger's antibacterial potency against MSSA and MRSA. It is one of the first experiments to investigate the efficacy of red ginger rhizome extract against MRSA in Indonesia. With a lengthy explanation regarding the properties of chemical compounds within the rhizomes, this experiment opened opportunities for future improvement and refinement of red ginger as a potential anti-MSSA and anti-MRSA agent.

Differences in plant preparations such as harvest methods, harvest timing, soil condition, and other agroecology factors may influence the amount of chemical compounds within the rhizomes, which then may cause different results when compared to other studies.[11] Considering the rhizomes were harvested in the rainy season of March 2023, variables like temperature, humidity, and rain intensity may also influence the antibacterial activity of the extract.[36] Fractionation and purification of the compounds within the extract prior to the experiment may exhibit greater antimicrobial activities.

Conclusion

The antibacterial activity of the red ginger extract was concluded to be weak against MSSA and MRSA in vitro. The antibacterial property of the extract was noted to be linear to the concentrations, with 100% extract exhibiting the largest zone diameters on both MSSA and MRSA. Subsequent analyses of the results indicated that some of the extracts concentrations inhibited MSSA and MRSA growth by significantly different magnitudes. The antibacterial activity of the extracts was relatively weaker compared to the erythromycin and vancomycin discs on both MSSA and MRSA.

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Conflict of Interest

The authors declared there is no conflict of interest.

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Ethical Clearance

This study was ethically approved by the Health Research Ethics Committee of Universitas Airlangga, Surabaya (No. 195/EC/KEPK/FKUA/2022) on 27-10-2022.

Authors' Contributions

Study conception: ARJ. Proposal and experiment preparation: ARJ and MP. Data collection and analysis: ARJ, AM, and MP. Final version review and approval: AM, MP, and WR.

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