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Antibacterial Activity and Inhibition Mechanism of Red Ginger (*Zingiber officinale* var. *rubrum*) Ethanol Extract Against Pathogenic Bacteria

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ABSTRACT

Pathogenic bacteria can interfere with the body health by producing toxic substances, damaging body tissues, multiplying and killing healthy body cells. Therefore, it is crucial to explore safe alternative treatment strategies, such as developing natural bactericidal agents to eradicate infections. Red ginger (*Zingiber officinale* var. rubrum) has potential as an antibacterial, so it is important to study its activity in inhibiting pathogenic bacteria. The aim of this study was to assess the antibacterial activity and inhibition mechanism of red ginger extract as an inhibitor of pathogenic bacterial infection. Methods such as dilution, Kirby-Bauer disc diffusion, and an inhibition test mechanism using a spectrophotometer and SEM were conducted in this study. Red ginger ethanol extract can inhibit the growth of *Salmonela thypi, Staphylococcus epidermidis*, and *Streptococcus mutans* at a concentration of 500 µg/mL, while *Pseudomonas aeruginosa* at a concentration of 250 µg/mL. Further observation of bacterial cell leakage showed that the higher the red ginger ethanol extract concentration, the higher the bacterial cell leakage. Based on SEM results, the quantity of *S. thypi, S. epidermidis*, and *S. mutans* after treatment with red ginger ethanol extract decreased, and the cell wall became wrinkled and destroyed. The ethanol extract of the red ginger rhizome can be recommended as a future antibacterial agent, especially for infections caused by pathogenic bacteria.

1. Introduction

Infectious diseases result from a combination of agent (pathogen), host, and environmental factors [1]. Infectious agents can be live parasites, bacteria, fungi, or non-living viruses or prions [2]. Environmental factors determine whether the host will be exposed to any infectious agents. If disease occurs, the pathogen has infected (invaded and settled within) the host tissue [3]. Bacterial

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infections signal a severe risk to public health [4]. Based on statistical data (2019), there were 4.95 million (3.62-6.57%) deaths associated with bacterial resistance in the world [5].

The increase in infections due to multidrug-resistant (MDR) bacterial species significantly reduces the efficiency of antibiotic therapy [6]. The cause of the increase in drug-resistant bacteria is the irrational use of antibiotics [7]. Therefore, it is crucial to explore safe alternative treatment strategies, such as developing natural bactericidal agents to eradicate infections [8].

Alternative medicines using natural ingredients need to be developed to overcome the decline in antibiotic effectiveness caused by increased resistance. Traditional medicinal plants can prove the importance of natural materials for various human treatment processes. In recent years, many researchers have sought to source basic medicinal ingredients from natural materials as natural biological compounds as an alternative treatment that is safe and has no harmful side effects [9].

Ginger (Zingiberaceae) is already known and used by the community as a medicinal plant. Ginger contains bioactive compounds such as flavonoids, phenols, terpenoids, and essential oils. Ginger has activity as antibacterial, antiallergic, antidiabetic, anti-inflammatory and antiobesity, and treat cardiovascular diseases and respiratory disorders [10-16].

One species of ginger that is useful as an antibacterial is red ginger (*Zingiber officinale* var *rubrum*) [17]. Red ginger ethanol extract was shown to have the highest inhibition area on *Staphylococcus aureus* and *Escherichia coli* [18]. Ethanol extract from red ginger inhibits the growth of bacteria that cause acne [19]. Red ginger extract has antibacterial activity against *Streptococcus pyogenes*, a bacterium that causes pharyngitis in children and adults [20]. Ethyl acetate extract of red ginger rhizome was shown to be most influential in inhibiting the growth of Gram-positive bacteria compared to inhibition against Gram-negative [21]. However, there is no information on the mechanism of red ginger in inhibiting pathogenic bacteria and comparing the antibacterial activity of several pathogens that cause infection.

This study used several pathogenic bacteria that most often attack the human body, including *Pseudomonas aeruginosa, Salmonella typhii, Staphylococcus epidermidis,* and *Streptococcus mutans*. These bacteria are pathogens in the human body that are very easy to spread through water, air or food. Hence, this research aims to examine the antibacterial effectiveness of red ginger rhizome along with its inhibitory mechanism.

2. Methodology

2.1 Extraction

Sample extraction method was done according to Yadufashije *et al.,* [22] and Bhunu *et al.,* [23]. Red ginger rhizomes were washed, peeled, sliced, and dried, then ground to a fine powder. About 90 grams of ginger powder was soaked for 72 hours in ethanol solvent (450 ml), and the crude extract was filtered with filter paper. The extract was concentrated using a rotary evaporator at 40°C. The extract containing the solvent was then poured and filtered. To produce the crude ethanol extract, the filtrates from each extraction were combined, and the remaining solvent was evaporated using a rotary evaporator.

2.2 Phytochemical Screening

Red ginger samples were dissolved in different solvents to extract secondary metabolites. The extracts were then used to test for flavonoid and phenol secondary metabolites. The flavonoid assay was conducted according to the method described by Tanko *et al.,* [24]. The sample extract was mixed with 5 g of magnesium (Mg) powder, and five drops of concentrated hydrochloric acid (HCl)

were added until it changed colour. A positive test is indicated by a yellow colour change. The Phenol testing with FeCl3 test method [24]. The extract was mixed with 2% FeCl3 (2 mL) solution. A positive test is indicated by a blue-green or black colour change.

2.3 Preparation of Inoculum

This study used strains of pathogenic bacteria *Salmonella thypi, Pseudomonas aeruginosa, Staphylococcus epidermidis,* and *Streptococcus mutans* from the Microbiology and Parasitology laboratory of Abdurrab University. Bacterial strains were subcultured at 37^{o}C for 24 hours on MacConkey Media and then stored in a refrigerator (4^{o}C) to maintain stock cultures. One inoculating loops of the grown bacterial culture was inoculated in ¾ of a 10 mL nutrient broth (NB) tube at 37^{o}C in an incubator for 16-18 hours. The inoculum size of each bacterial strain was standardized by adjusting the optical density of the NB to turbidity corresponding to 0.5 at 620 nm using a spectrophotometer equivalent to 106 cfu/mL [25].

2.4 Disc Diffusion

The antibacterial activity was evaluated using the disc diffusion method by inoculating agar plates with culture and allowing substances that have antimicrobial potential to diffuse into the agar medium. Discs containing antibacterial substances are placed on the surface of agar plates containing the organism being tested. Antibacterial effectiveness is indicated by the inhibition zone, whose diameter is measured with a calliper with units of millimetres (mm) [26].

2.5 Determination Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC values were calculated using the liquid dilution of the Kirby-Bauer method, which was modified using nutrient broth (NB) liquid media and measured absorbance with a UV-Vis spectrophotometer before and after incubation to see the growth of test bacteria [27]. A total of 5 ml of sterile NB media was put into a test tube, and 0.5 ml of the extract with four concentrations was added. Red ginger concentration was made with 62.5 mg/mL mother solution, which was diluted at concentrations of 1000 µL/mL, 500 µL /mL, 250 µL /mL, and 125 µL /mL.

Furthermore, the media was added with 0.5 ml of a bacterial suspension at 106 CFU/mL, which has been adjusted to the 0.5 McFarland standard. The test tubes were then measured for absorbance (Optical Density = OD) of bacteria using a spectrophotometer (λ = 480 nm). Then the tubes were incubated at 37°C for 18-24 hours. After incubation, the absorbance of bacteria was measured again using a spectrophotometer (λ = 480 nm). MIC was determined by comparing the absorbance after incubation treatment minus the absorbance before treatment. If there is the lowest concentration that inhibits bacterial growth, indicated by the absence of turbidity (bacterial OD is ≤ 0), the MIC value is obtained. To MBC value, further tests were carried out on all tubes used in the MIC that did not show any turbidity against bacteria. A total of 0.2 mL of the suspension showing MIC was added into a test tube containing 5 mL of NB medium. The test tubes were incubated for 12-18 hours at 37°C in an incubator, and then absorbance (OD) was measured with a UV-Vis spectrophotometer (λ = 480 nm). If the measurement results show that the lowest concentration of the extract has an OD of 0 (no turbidity), the MBC value was obtained [28].

2.6 Testing Antibacterial Mechanism Action Using Spectrophotometer

Measurement of cell metabolite release was carried out based on the research of Jenie *et al.,* [29] using an ultraviolet-visible spectrophotometer (UVS) at a wavelength of 260 nm for nucleic acids and 280 nm for proteins. A total of 1 mL of extract was added into 5 mL of MHB with 1 mL of bacterial inoculum. The active fraction and bacterial suspension were controls. Then the sample was incubated at 35^{o}C for 24 hours (aerobic). Next, the sample was centrifuged at 3500 rpm for 30 minutes, and then the liquid and residue were separated. The absorbance of the liquid was directly measured with a spectrophotometer.

2.7 Time-Kill Test

Time-kill examination was carried out using the method of Appiah *et al.,* [30] with modifications. Ethanol extracts were prepared at concentrations equal to the 1st and 2nd MIC values. Each concentration of 2 mL was transferred into a culture tube. A total of 2 mL of 0.5 McFarland inoculum was added to each culture tube and incubated at 37°C in a shaker. Samples were examined at 0, 2, 4, 6, 8, 10, 12, and 24 hours to determine the number of colony-forming units (CFU).

2.8 Testing of Antibacterial Mechanism Action Based on Scanning Electron Microscope (SEM)

Changes in the external morphology of bacterial cells were carried out according to the procedure of Su *et al.,* [31]. One colony of pure bacterial culture was inoculated into 5 mL of MHB and incubated for 18 hours at 35±2^{o}C. Afterward, 0.1 mL of culture suspension (1.5x108 cfu/mL) was re-inoculated into 5 mL of MHB and incubated at the same temperature for 12 hours. Cell pellets were soaked with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1.5 hours and then centrifuged for 30 min at 8000 rpm. Then the cell pellet was immersed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for one hour at 4°C. The samples were dried with 30%, 50%, 70%, 90%, and 96% ethanol. The dried samples were coated with a layer of gold and tested using a JEOL-JSM-6510LA SEM instrument [32].

3. Results

3.1 Red Ginger Phytochemicals

Ethanol extract of red ginger rhizome (*Zingiber officinale* var. *rubrum*) was shown to contain phenolic compounds and flavonoids that are thought to have inhibitory abilities against test bacteria (Figure 1). Red ginger has been shown to contain essential oils (EO) and oleoresins with antibacterial properties [33]. Oleoresins are a combination of essential oils and resins. Oleoresin has antimicrobial activity by denaturing proteins and damaging the cytoplasmic membrane of bacteria [20].

The active compounds in red ginger ethanol extract are proven to inhibit the growth of pathogenic bacteria *Salmonella thypi, Pseudomonas aeruginosa, Staphylococcus epidermidis*, and *Streptococcus mutans.* Flavonoid compounds inhibit bacterial DNA synthesis [34]. The flavonoid content is effective as an antibacterial by forming complex compounds against extracellular proteins that disrupt the integrity of bacterial cell membranes, disrupt microorganism cell function, and inhibit the microbial cell cycle [35-37].

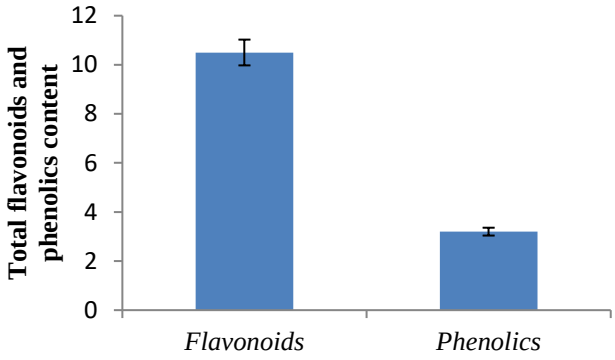

Fig. 1. Total Flavonoids (mgQE/100g) and phenolics (mgGAE/100g) of Red Ginger extracts

Phenol is a bioactive compound that is polar and acts as an antibacterial. The mechanism of action of phenol compounds in killing bacterial cells is denaturing bacterial cell proteins. As a result of denatured bacterial cell proteins, all metabolic activities of bacterial cells catalyzed by proteins are stopped. At high concentrations, phenol content penetrates, disrupts the bacterial cell wall, and precipitates proteins in bacterial cells. In lower concentrations, phenols activate important enzyme systems in bacterial cells [38].

3.2 Antibacterial Effectiveness of Red Ginger

Red ginger extract has the potential as an antibacterial [17]. Gram-positive bacterial species that can cause infection are *S. mutans, S. epidermidis* which are resistant to gentamicin antibiotics, while for negative bacteria *S. thypi* and *P. aeruginosa* are resistant to kanamycin, erythromycin, and clindamycin antibiotics [39]. This research has found natural antibiotics as phytotherapy for infectious diseases.

Ethanol extract from red ginger was tested against several species of pathogenic bacteria using the diffusion method (Table 1) and dilution method (Figure 3). The diameter of the inhibition zone produced in the diffusion antibacterial test is presented in Figure 2.

Table 1
Disc diffusion test results of red ginger ethanol extract against several pathogenic bacteria

Extract	Zona of growth inhibition				
	Concentration	*S. thypi*	*P. aeruginosa*	*S. epidermidis*	*S. mutans*
Rhizome	20%	6.3 ± 0.33	6.1 ± 0.00	6.1 ± 0.05	11.33 + 0.33
	40%	7.0 ± 0.00	8.4 ± 0.29	6.1 ± 0.06	10 ± 0.58
	60%	na	9.4 ± 0.33	6.5 ± 0.06	10 ± 0.50
	80%	na	10.2 ± 0.12	6.4 ± 0.08	11.16 + 0.73
	Control (+)	38.3 ± 0.67	21.1 ± 0.55	32.7 ± 1.20	29.16 + 0.60
	Control (-)	na	na	na	na

na = no activity

Determination of the antibacterial activity of the diffusion method showed a significant effect on bacterial growth after giving the concentration of red ginger extract and chloramphenicol control ($p<0.05$). The results of LSD post hoc analysis showed a significant difference in bacterial growth

between concentration groups and positive control groups. Overall, there was a significant difference between the response of each bacterium after the administration of red ginger ethanol extract ($p< 0.05$). Red ginger had antibacterial activity against *S. aureus* with the average inhibition zone diameter formed at concentration 75% (11.75 mm) and 100% (13.17 mm) [40]. The ethanolic extract of red ginger with a concentration of 12.5% producing a clear zone against *Staphylococcus* sp. (25 mm) and *Propionibacterium* sp. (17.5 mm) [19].

(a) (b) (c) (d)

Fig. 2. Inhibition zone diameter (a) *Salmonella thypi* (b) *Pseudomonas aeruginosa* (c) *Staphylococus epidermidis* (d) *Streptococus mutans*

3.3 Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Value

Determination of antibacterial activity by dilution, red ginger extract and positive control have a significant difference with the negative control, where the negative control uses bacterial suspension, and there are no obstacles so that bacteria can grow well with a significant increase in absorbance value. The ANOVA statistical test results showed a significant effect on bacterial growth after giving the concentration of red ginger extract and chloramphenicol control ($p<0.05$). The results of LSD post hoc analysis showed a significant difference in bacterial growth between concentration groups and positive control groups (Figure 3).

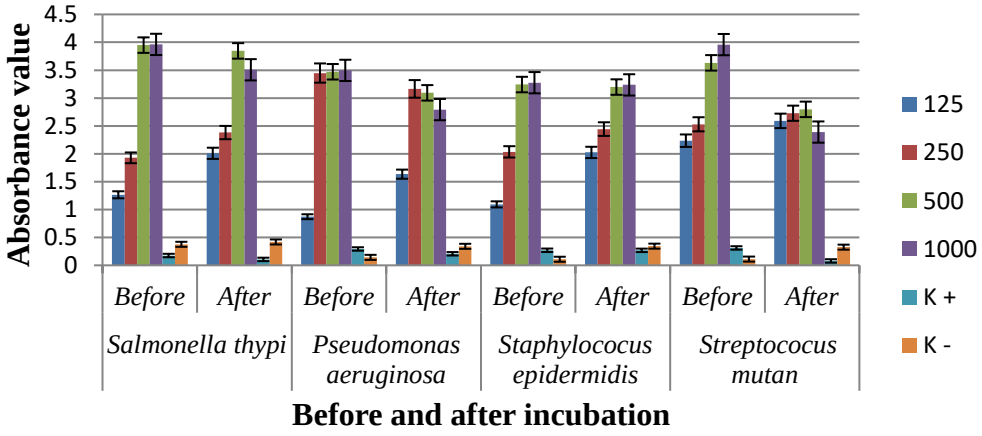

Fig. 3. MIC and MBC value

At 125 and 250 ug/mL concentrations, the absorbance value after incubation of S. *thypi, S. epidermidis* and *S. mutans* still increased. This indicates that the secondary metabolite compounds contained in the ethanol extract of red ginger have not worked against bacterial growth, so the bacteria can still grow well. While in *P. aeruginosa* bacteria, at a concentration of 250 ug/mL, the absorbance value has decreased. The decrease in absorbance value in *S. thypi, S. epidermidis* and *S. mutans* occurred at a concentration of 500 ug/mL. So that the minimum inhibitory concentration

(MIC) in red ginger ethanol extract against the growth of *S. thypi, S. epidermidis* and *S. mutans* bacteria occurs at a concentration of 500 ug/mL, while in *P. aeruginosa* bacteria at a concentration of 250 ug/mL.

Red ginger ethanol extract at a concentration inhibits *S. epidermidis* growth at 125 µg/mL (60%), 250 µg/mL (45%), and 500µg/mL (65%) [41]. Antibacterial activity of red ginger ethyl acetate extract against pathogen bacteria using microdilution method showed that could inhibit *S. aureus* growth by MIC (6.3%) and MBC (25%) and inhibit *E. coli* with MIC 25% and MBC 50% [21].

The Paired T-Test showed that the difference in average absorbance before and after incubation of all groups of bacteria after being treatment with red ginger extract did not show a significant difference ($p > 0.05$).

The ability of antibacterials to inhibit bacterial growth can occur through several mechanisms, including leakage in bacterial cells, which disrupts the bacterial membrane due to antibacterial activity [42]. Bacterial cell leakage activity can be seen by measuring the release of amino acids and protein using a spectrophotometer at wavelengths 260 and 280 nm (Figure 4). This analysis is done by observing the increase in absorbance value at a wavelength of 260 nm (nucleic acids) and 280 nm (proteins). The release of nucleic acids and proteins indicates that the cell is leaking due to cell wall damage or changes in cell membrane permeability, causing the bacteria to die.

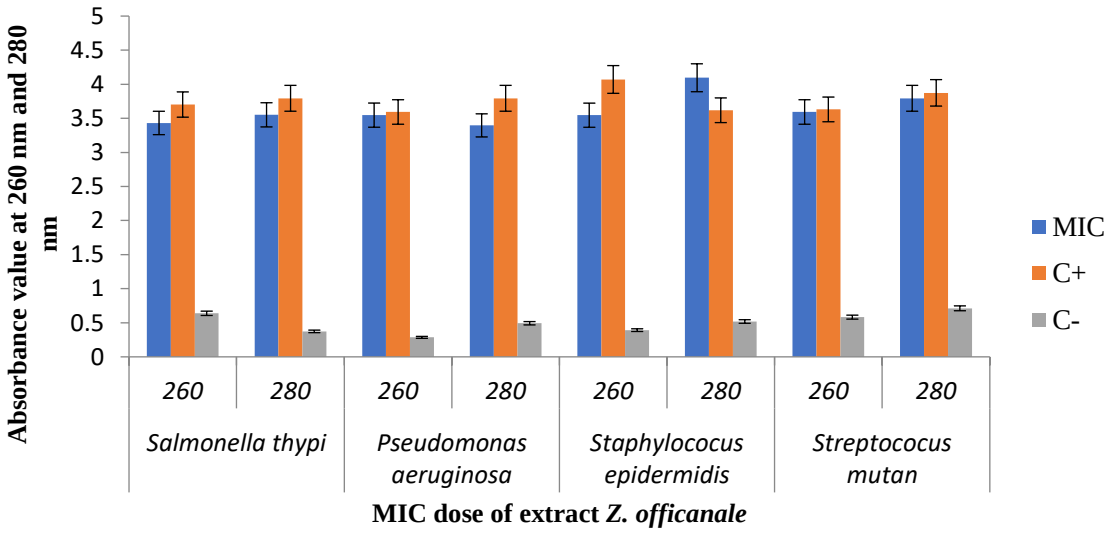

Fig. 4. Protein and nucleid acid leakage

Statistical analysis shows that the significant value of cell leakage at 260 nm and 280 nm wavelengths ($p<0.05$). This indicates that the treatment of the addition of ethanol extract from red ginger has a significant effect on cell leakage in pathogenic bacteria (*S. thypi, P. aeruginosa, S. epidermidis* and *S. mutans*). Statistical analysis also shows no significant difference in cell leakage (protein and nucleic acid) between one bacterium and another.

LSD analysis showed that adding red ginger ethanol extract with a concentration of 250 ug/mL and 500 ug/mL and positive control chloramphenicol gave a significant value of $p<0.05$ as compared to without the addition of the extract. This shows a significant difference in cell leakage at 260 nm and 280 nm with the addition of red ginger ethanol extract compared to without the addition of red ginger ethanol extract. Statistical analysis also showed no significant difference in cell leakage (protein and nucleic acid) between one bacterium and another.

Cell leakage at 260 nm wavelength was highest in *S. mutant* bacteria, and the lowest value in *S. thypi* bacteria, while at 280 nm wavelength was highest in *S. Epidermidis* and the lowest value in *P.*

aeruginosa bacteria. Cell leakage in Gram-positive bacteria such as *S. mutan* and *S. epidermidis* and Gram-negative bacteria such as *S. thypi* and *P. aeruginosa* is caused by the interaction between antibacterial metabolites of red ginger extract with components of the outer membrane of bacterial cells, such as phospholipid components that form pores in the bacterial cell membrane. If the membrane pores enlarge due to changes in phospholipids, larger molecules can leave the cell membrane or changes in membrane semi-permeability. Disruption of bacterial cell membrane permeability causes leakage of proteins and nucleic acids [43].

3.4 Time-Kill Analysis

The antibacterial activity of red ginger ethanol extract was further tested using the time-kill method. Time-kill is a method to determine the time to kill bacteria [44]. The inhibition ability of red ginger extract against several types of pathogenic bacteria requires different killing times (Figure 5).

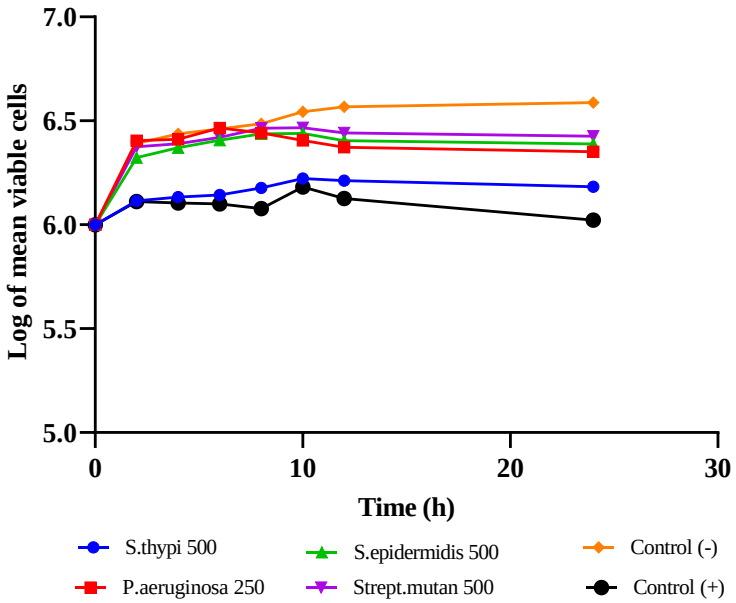

Fig. 5. Time Kill graph of red ginger extract against *S. thypi, P. aeruginosa, S. epidermidis,* and *S. mutans*

The results of the time-kill test of red ginger ethanol extract carried out on *S. thypi, P. aeruginosa, S. epidermidis,* and *S. mutans* showed that at hours 0, 2, 4, 6, and 8 the bacteria were still alive. This is because the ethanol extract of red ginger against bacteria has not been maximized due to the short time. However, the curve decreased at 10, 12, and 24 hours, indicating a decrease in antibacterial activity. This is because the ethanol extract of red ginger as an antibacterial has worked well and can kill bacteria *S. thypi, P. aeruginosa, S. epidermidis* and *S. mutans*. Thus, the longer red ginger ethanol extract contacts with bacteria, the more bacteria die because extracts take relatively longer than isolates or single compounds to eradicate bacteria as a whole because they contain a mixture of compounds that can affect activity [45,46].

3.5 Antibacterial Mechanism Action

The number of *S. thypi, P. aeruginosa, S. epidermidis,* and *S. mutans* after treatment with ethanol extract of red ginger decreased, cell walls became wrinkled, and cell shape and size were also damaged (Figure 6). The cell wall experienced changes in surface roughness and the appearance of grooves in the cell wall. Incubation with higher doses caused cell wall leakage. The bacterial cell wall protects the cytoplasmic membrane, maintains cell shape, and prevents lysis due to osmosis pressure. If the cell wall does not function, the cell will undergo lysis because the surrounding fluid, which is hypoosmotic, diffuses into the cell, causing swelling. The presence of active compounds in the extract causes bacterial cell leakage [47,48].

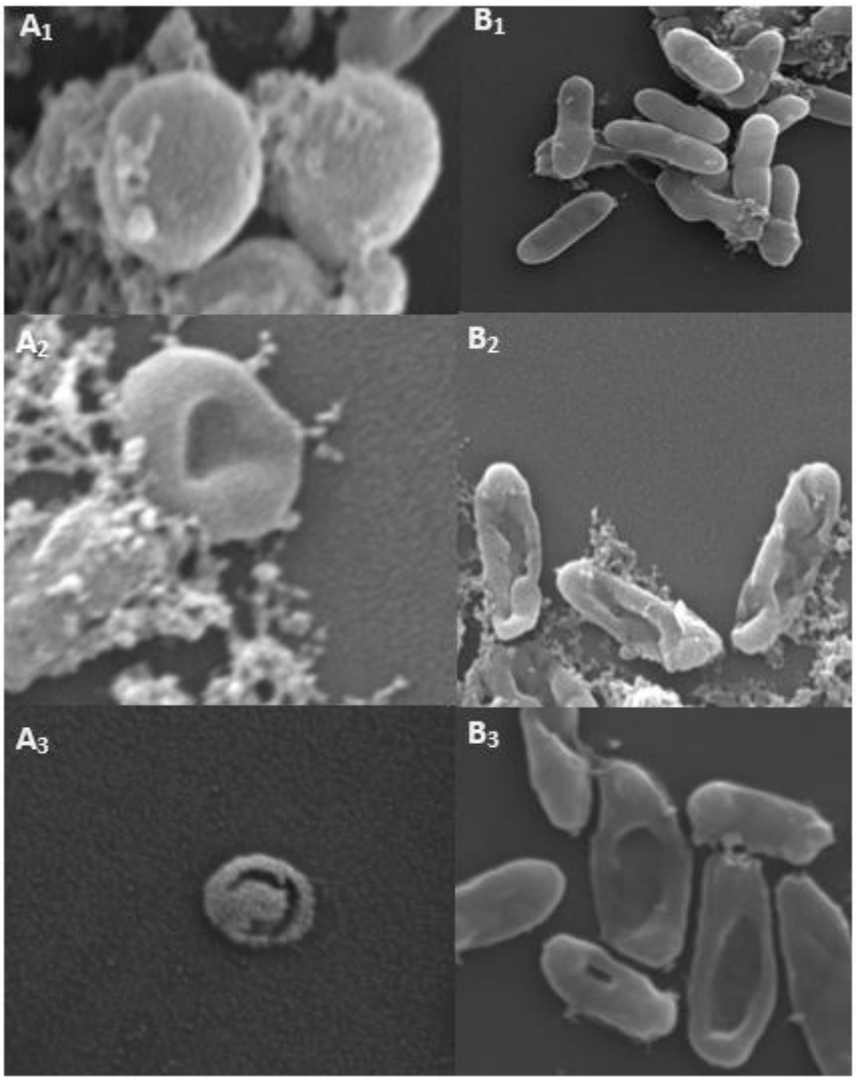

Fig. 6. Scanning electron microscope photo. Morphological changes in gram positive and gram negative bacterial cells. (A1) Negative control (A2) Positive control (Chloramphenicol) (A3) treatment with red ginger ethanol extract (B1) negative control (B2) positive control (Chloramphenicol) (B3) treatment with red ginger ethanol extract

This study found the same results of changes in bacterial morphology after red ginger extract treatment as other studies. One of the metabolites that show antibacterial activity is essential oil. The ginger essential oil extract is proven to increase the permeability of cell membranes by causing

leakage of intracellular substances, causing the cell shape to become irregularly concave and shriveled [49]. The inhibitory mechanism of this essential oil is by adhering to and disrupting the integrity of the cell wall surface and membrane structure, thus causing cell lysis. The essential oil concentration (100-500 µL/mL) effectively affects the growth of pathogenic bacteria [50].

The results showed that red ginger extract is an antibacterial against bacteria that cause infectious diseases. The ethanol extract of the red ginger rhizome can be recommended as a future antibacterial agent, especially for the phytotherapy of infections caused by pathogenic bacteria. Phytotherapy is an important aspect of modern therapy, encouraging scientists to deepen their research on familiar medicinal plants to discover new treatments. In this case, the solution may be more efficiently commercializing country-specific native flora. However, there are still shortcomings in this study, namely that crude extracts are still used, and specific compounds that inhibit the growth of test bacteria cannot be identified.

4. Conclusion

Red ginger ethanol extract can inhibit the growth of *S. thypi, S. epidermidis*, and *S. mutans* bacteria at a concentration of 500 ug/mL, while in *P. aeruginosa* bacteria at a concentration of 250 ug/mL with a significant decrease in absorbance value before and after incubation. Further observation of bacterial cell leakage showed that the higher the red ginger ethanol extract concentration, the higher the bacterial cell leakage. Based on SEM results, the quantity of *S. thypi, S. epidermidis*, and *S. mutans* after treatment with red ginger ethanol extract decreased, and the cell wall became wrinkled and destroyed. From this study, red ginger extract could be used as a natural antibacterial in inhibiting the growth of bacteria that cause infectious diseases.

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