

## Effectiveness and mechanism of *Zingiber officinale* var. *rubrum* (red ginger) ethanol extract as an inhibitor of *Escherichia coli* and *Staphylococcus aureus*

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### Abstract

*Escherichia coli* and *Staphylococcus aureus* are bacteria responsible for infectious diseases worldwide. With increasing cases of antibiotic resistance due to current medical developments, there is a growing need to explore alternative substances with bactericidal or bacteriostatic properties, including those derived from natural sources. *Zingiber officinale* var. *rubrum* (red ginger) is known for its medicinal properties, particularly its antibacterial effects. This study aimed to evaluate the ability of red ginger to inhibit the growth of *E. coli* and *S. aureus*. Phytochemical tests were done to identify the active compounds in the extract, while antibacterial activity was assessed using the minimum inhibitory concentration (MIC). The mechanism of antibacterial action was investigated with a spectrophotometer and scanning electron microscope (SEM). The results revealed that the red ginger extract contains active compounds such as alkaloids, flavonoids, saponins, tannins, and terpenoids. The minimum inhibitory concentrations were 125 µg/mL for *E. coli* and 500 µg/mL for *S. aureus*, respectively. The addition of red ginger ethanol extract at MIC 1 and MIC 2, measured at an absorbance of 260 nm and 280 nm, significantly affected cell leakage compared to the control ( $p < 0.01$ ). SEM analysis showed that bacterial cells treated with red ginger extract appeared damaged and vacuolated. Therefore, it can be concluded that red ginger extract has an inhibitory effect on the growth of *E. coli* and *S. aureus*, and may be recommended as an alternative to natural antibiotics for treating infectious diseases.

## 1. Introduction

The discovery of new compounds from plant secondary metabolism is one way to develop new drugs (Gorlenko *et al.*, 2020). Red ginger (*Zingiber officinale* var. *rubrum*) is one of the plants that is well known to society and used as a natural medicine. Red ginger is a plant of the Zingiberaceae family that has been used as a medicine for generations since it has the highest volatile (essential oil) and non-volatile (oleoresin) components compared to other species of ginger (Assegaf *et al.*, 2020). Red ginger rhizome is commonly used as an antioxidant, antitussive, analgesic, antipyretic, anti-inflammatory (Indrawati *et al.*, 2017) and antibacterial (Juariah *et al.*, 2023b). Therefore, red ginger can be used as a natural antibacterial that does not cause resistance to infection and even organ damage and immune

hypersensitivity (Azkiya *et al.*, 2017).

*Zingiber officinale* var. *rubrum* is widely used in traditional medicine in Asia. Unlike other gingers, red ginger is not used as a spice in cooking. To date, a total of 169 chemical constituents have been reported from red ginger. These include vanilloids, monoterpenes, sesquiterpenes, diterpenes, flavonoids, amino acids, and other compounds. Red ginger has many therapeutic roles in various diseases, including inflammatory diseases, vomiting, rubella, atherosclerosis, tuberculosis, growth disorders, and cancer. Scientific studies showed that red ginger extract has the potential as an antibacterial (Handayani *et al.*, 2018; Juariah *et al.*, 2023b), antifungal, antihypertensive, antihyperlipidemic, antihyperuricemic, anti-inflammatory (Juariah *et al.*,

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2024), antioxidant (Ghasemzadeh *et al.*, 2016), antiemetic and antidiabetic (Supu *et al.*, 2018). This biological activity is the underlying cause of the therapeutic benefits of red ginger. However, there are several reports of adverse side effects of red ginger (Zhang *et al.*, 2022).

Nosocomial infections, also known as hospital-acquired infections, are infections that occur during or after a hospital stay. These infections typically appear within three days of hospitalization. Nosocomial infections are prevalent worldwide, with the highest incidence in low-income and developing countries, where infectious diseases remain a leading cause of morbidity and mortality. According to the World Health Organization (WHO) in 2006, approximately 8.7% of 55 hospitals across 14 countries in Europe, the Middle East, Southeast Asia, and the Pacific reported cases of nosocomial infections, with the highest incidence in Southeast Asia at about 10% (Sikora and Zahra, 2023). Red ginger is known for its medicinal properties including antibacterial activity and the ability to inhibit bacterial growth of *Salmonella enterica* serovar Typhi, *Staphylococcus epidermidis*, and *Streptococcus mutans* (Juariah *et al.*, 2023b). Additionally, a combination of red ginger (*Z. officinale* var. *rubrum*) and black turmeric (*Curcuma caesia*) can inhibit the growth of *Klebsiella pneumoniae*, a bacterium responsible for pneumonia infections (Juariah *et al.*, 2023). Therefore, it is important to investigate whether red ginger (*Z. officinale* var. *rubrum*) can also inhibit the growth of gram-negative and gram-positive nosocomial bacteria.

## 2. Materials and methods

### 2.1 Materials

The materials used in this study were red ginger (*Z. officinale* var. *rubrum*), ethanol, disc antibiotic (chloramphenicol), nutrient broth (NB), aqueous and sodium chloride (NaCl).

### 2.2 Data collection procedures

#### 2.2.1 Collection of plant material

Red ginger (*Z. officinale* var. *rubrum*) was collected from community gardens in Riau Province. The collected plant material was identified and validated by the Biology Laboratory of the University of Riau with specimen number rab052.

#### 2.2.2 Extract preparation

The rhizome of red ginger (*Z. officinale* var. *rubrum*) was cleaned and sun-dried. Once dried, the sample was ground in a blender and sifted through an 80-mesh sieve to obtain a fine powder. This red ginger powder was then extracted with 70% ethanol over a period of five days,

forming a suspension. The resulting liquid extract was concentrated by evaporation using a rotary vacuum evaporator at 70°C until a thick extract was obtained (Akbari *et al.*, 2017).

#### 2.2.3 Inoculum preparation

The bacterial strains used for antibacterial screening were *Staphylococcus aureus* and *Escherichia coli*, obtained from the Laboratory of Abdurrah University, Pekanbaru, Indonesia. These strains were maintained on nutrient agar for gram-positive bacteria and MacConkey agar for gram-negative bacteria, and were stored in the refrigerator for future use. To prepare the cultures, a full loop of bacteria grown for 12 hrs was inoculated into 10 mL of nutrient broth and incubated at 37°C on a rotary shaker for 16-18 hrs. The inoculum size for each bacterial strain was standardized by adjusting the optical density of the nutrient broth to turbidity corresponding to 0.5 at 620 nm using a spectrophotometer, which is equivalent to approximately 10<sup>8</sup> CFU/mL (Pandey and Gupta, 2014).

#### 2.2.4 Phytochemical test

The extract was used to test for different secondary metabolites, such as flavonoids, alkaloids, saponins, phenols, and terpenoids (Pramiastuti and Joharoh 2020).

#### 2.2.5 Antibacterial activity testing

The minimum inhibitory concentration (MIC) was determined using a modified Kirby-Bauer liquid dilution method (Fatisa *et al.*, 2013). The absorbance of nutrient broth (NB) was measured with a UV-Vis spectrophotometer before and after incubation to assess bacterial growth. A total of 4 mL of sterile NB was added to each test tube, followed by 0.5 mL of the extract at concentrations of 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL. The test was conducted in triplicate. Additionally, 0.5 ml of a bacterial suspension at 10<sup>6</sup> CFU/mL, adjusted to a 0.5 McFarland standard, was added to the media. Chloramphenicol was used as the positive control, and ethanol solution served as the negative control.

#### 2.2.6 Antibacterial mechanism of action testing

The measurement of cell metabolite release was conducted according to the method described by Jenie *et al.* (2008) using a UV-visible spectrophotometer. Absorbance was measured at wavelengths of 260 nm (for nucleic acids) and 280 nm (for proteins) to assess the antibacterial mechanism. In each test, 5 mL of Mueller-Hinton broth (MHB) was combined with 1 mL of bacterial inoculum and 1 mL of either the extract, active fraction, or a bacterial suspension (as a control). The

samples were incubated at 35±2°C for 24 hrs under aerobic conditions. After incubation, the samples were centrifuged at 3500 rpm for 30 min to separate the liquid from the precipitate. The absorbance of the supernatant was then measured using the spectrophotometer.

2.2.7 Antibacterial working mechanism testing based on scanning electron microscope

Changes in the outer morphology of bacterial cells were assessed following the method described by Borges et al. (2016). One colony of pure bacterial culture was inoculated into 5 mL of MHB and incubated for 18 hrs at 35±2°C. Subsequently, 0.1 mL of the culture suspension (1.5×10<sup>8</sup> CFU/mL) was transferred into 5 mL of MHB containing 0.1 mL of each extract and active fraction, and the mixture was incubated at 35±2°C for 12 hrs. Cell pellets were then collected by fixation with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 1.5 hrs, followed by centrifugation at 8000 rpm for 30 mins. The pellets were further fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 hrs. The samples were dehydrated using graded ethanol concentrations (30%, 50%, 70%, 90%, and 96%) at room temperature. After dehydration, the samples were coated with gold and examined using a JEOL JSM-6510LA scanning electron microscope (SEM). Chemical fixatives such as aldehydes and osmium tetroxide were used to preserve cell morphology and enhance the visibility of differences in cell shape. Dehydration with ethanol and acetone was carried out in stages to replace water in the samples (Hrubanova et al., 2018).

2.2.8 Statistical analysis

The determination was done in triplicate and the data were analysed using one-way analysis of variance (ANOVA) with the Statistical Package for the Social Sciences (SPSS version 16.0, IBM Corp., USA).

3. Results and discussion

The results of the phytochemical test of red ginger extract obtained alkaloid compounds, flavonoids, saponins, tannins, and terpenoids (Table 1, Figure 1). However, the alkaloid test using Mayer's reagent was negative. The flavonoid test was categorized as positive if an orange colour was formed. Meanwhile, the saponin test was categorized as positive if the foam was formed, and the tannin test was categorized as positive if a black/blackish-green colour was formed. The test results are categorized as positive for the terpenoid test if a black/purple ring is formed.

Phytochemical tests showed that red ginger has antimicrobial properties. All filtered constituents were

present in the red ginger ethanol extract (Akintobi et al., 2013). The antibacterial activity test of the red ginger ethanol extract was performed on several types of Gram-positive and Gram-negative bacteria (Figure 2). Chloramphenicol was used as a positive control because it is known as one of the broad-spectrum antibiotics used for treatment. A previous study showed that gram-negative bacteria are more difficult to inhibit compared to gram-positive bacteria (Juariah et al., 2023a).

Table 1. Phytochemical test results of red ginger extract (*Zingiber officinale* var. *rubrum*)

Secondary metabolites	Reagent	Extract	Reaction
Alkaloids	Mayer	-	No white precipitate is formed.
	Burchard	+	A brown precipitate is formed.
	Wagner	+	A brown precipitate is formed.
	Dragondorf	+	An orange precipitate is formed.
Flavonoids		+	Formation of orange colour.
Saponins		+	Foam formed.
Tanins		+	Formed in black/blackish green.
Terpenoids		+	Formation of a black/purple ring.

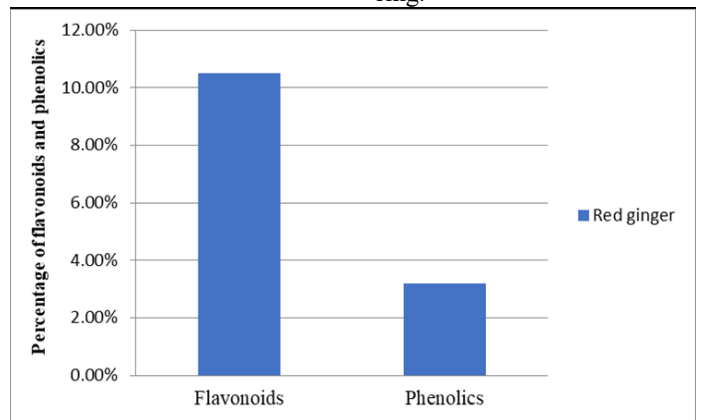


Figure 1. Comparison of flavonoid and phenolic content of red ginger ethanol extract.

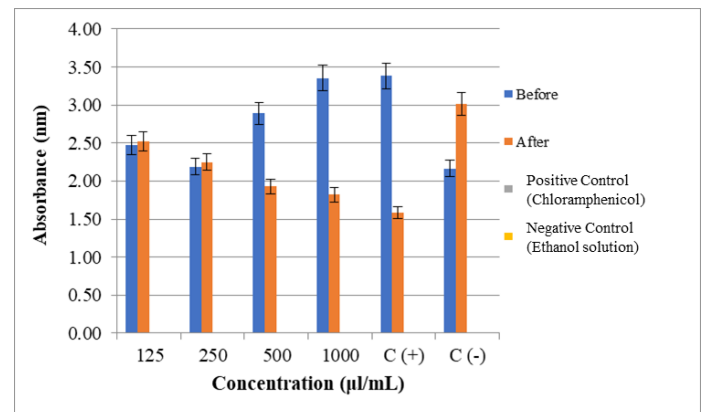


Figure 2. Comparison diagram of the average absorbance value of the growth of *S. aureus* bacteria before and after incubation with ethanol extract of red ginger

The decrease in the absorbance value only occurred at a concentration of 500  $\mu\text{L}/\text{mL}$ , which indicated that the inhibition of the growth of *Staphylococcus aureus* bacteria by red ginger ethanol extract only occurred at that concentration. The decrease continued at a concentration of 1000  $\mu\text{L}/\text{mL}$ , and the control was positive (chloramphenicol). In the negative control, there was an increase in the absorbance value, which showed that there was no inhibition of the growth of *S. aureus* bacteria by ethanol. Thus, ethanol used as a solvent for the extract had no inhibitory effect on the tested bacteria.

All concentrations and positive controls showed a decrease in the absorbance value (Figure 3). This decrease in absorbance value indicates that at the lowest concentration, the red ginger ethanol extract inhibited the growth of *E. coli* bacteria. Antibacterial activity of red ginger ethanol extract increased with increasing concentration used.

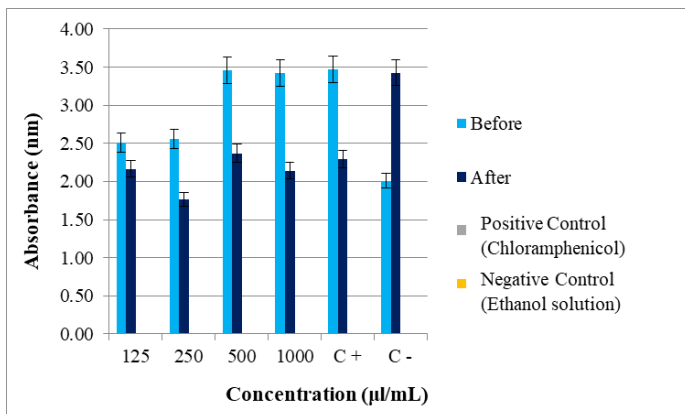


Figure 3. Diagram of the comparison of the average absorbance value of the growth of *E. coli* bacteria before and after incubation with ethanol extract of red ginger.

The negative control using ethanol solution showed no inhibition of bacterial growth, marked by an increase in the absorbance value. This finding proves that the inhibition that occurs in *S. aureus* and *E. coli* bacteria is caused by secondary metabolic compounds in red ginger ethanolic extract. The minimum inhibitory concentration of red ginger ethanol extract on *S. aureus* was 500  $\mu\text{L}/\text{mL}$ . Meanwhile, the minimum inhibitory concentration of red ginger ethanol extract on *E. coli* bacteria was 125  $\mu\text{L}/\text{mL}$ . The results of the T-paired statistical test also proved a significant difference in the concentration of red ginger ethanol extract on the growth of *S. aureus* and *E. coli* bacteria before and after incubation compared to the negative control ( $p < 0.05$ ).

Previous research conducted by Siroj et al. (2021) showed that red ginger ethanol extract has the ability to inhibit the growth of *E. coli* at different concentrations. In this study, they reported that the antibacterial effect

increased as the extract concentration increased. Furthermore, ginger have strong antibacterial activity against gram-negative bacteria such as *E. coli*, while the mechanism of action of these compounds involves damage to the bacterial cell membrane, which results in leakage of cell contents and death of the bacteria. Wang et al. (2021) reported that ginger extract contains bioactive compounds such as gingerol and shogaol which have a strong ability to inhibit the growth of gram-positive bacteria such as *S. aureus*. This study shows that the antibacterial activity of red ginger involves damage to the bacterial cell wall and inhibition of bacterial protein synthesis, which causes bacterial cell death. Apart from that, the active antibacterial compound in the form of gingerol, which is the main compound in red ginger, has a mechanism of action that can cause damage to bacterial cell membranes, which leads to leakage of intracellular components and inhibition of growth (Wang et al., 2022). Other research states that gingerol can cause damage to the structure of bacterial cell walls through oxidative mechanisms, thereby causing bacterial death (Hasan et al., 2023).

The addition of red ginger ethanol extract at MIC 1 and MIC 2 concentrations significantly affected cell leakage, as indicated by absorbances at 260 nm and 280 nm, compared to the control ( $p < 0.01$ ) (Figure 4). The addition of red ginger ethanol extract to *S. aureus* bacteria resulted in the release of nucleic acid (Figure 4) from the bacteria observed at an absorbance of 260 nm with a concentration of MIC 1 and MIC 2 of 0.7295 and 0.9471, respectively. In comparison, protein leakage from *S. aureus* bacterial cells was observed at an absorbance of 280 nm with a concentration of MIC1 and MIC 2 of 1.0372 and 1.0749, respectively.

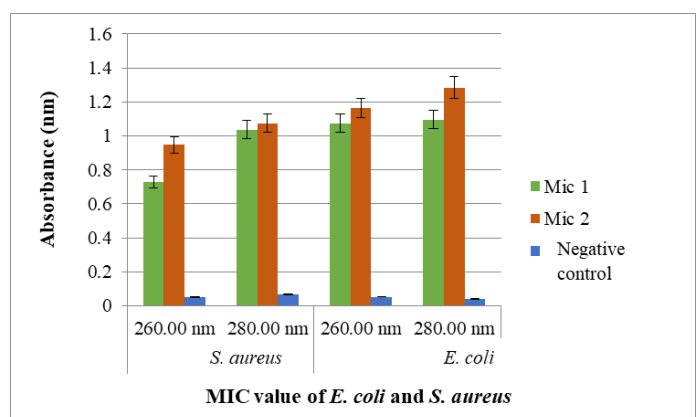


Figure 4. Comparison of leakage of bacterial cell contents of *S. aureus* and *E. coli* due to the addition of red ginger ethanol extract, which reads at wavelengths of 260 nm and 280 nm.

In *E. coli*, the ethanolic extract of red ginger resulted in the release of nucleic acids in the cells, which were observed at an absorbance of 260 nm with a concentration of MIC 1 and MIC 2 of 1.0751 and

1.1638, respectively. In addition, protein leakage from *E. coli* bacterial cells was observed at an absorbance of 280 nm with a concentration of MIC 1 and MIC 2 of 1.0952 and 1.2837, respectively. Meanwhile, protein leakage from control bacterial cells, namely *S. aureus*, observed at absorbances 260 and 280 were 0.0497 and 0.065, respectively. For the control of *E. coli*, protein leakage from bacterial cells observed at absorbances of 260 nm and 280 nm were 0.0536 and 0.0393, respectively.

Compounds such as caryophyllene oxide,  $\alpha$ -pinene,  $\alpha$ -terpineol, linalool, 1,8-cineol, and geraniol, are compounds that play a role in the antibacterial activity of the red ginger extract. The mechanism of bacterial inhibition of gingerol compounds consists of 2 ways: denaturation of proteins and destroying the cytoplasm bacterial membrane. The essential oil in red ginger can damage the structural component of the bacterial cell membrane. Other compounds in its essential oil also have antimicrobial activity, namely linalool, geraniol, and citral. Linalool and geraniol cause the denaturation of microbial proteins. Citral perform the function of antimicrobial compounds in two ways: making an alkylation process on nucleophilic groups and denaturation processes in microbial proteins. This mechanism triggers the inactivation of the enzymes inside bacteria (Assegaf *et al.*, 2020).

In addition, sesquiterpenoid and monoterpenoid compounds are determining factors for the effectiveness of a plant extract as an antibacterial. Essential oils containing aldehyde and phenol compounds have the highest inhibitory power against bacteria. These compounds damage the bacterial cell wall, which causes disturbances in the amino acid sequence of bacteria, causing disturbances in cell function (Dewi *et al.*, 2018).

The SEM images in Figure 5 demonstrate the impact of different treatments on *E. coli* cells. In the negative controls (A1, B1), the bacterial cells appear intact with smooth, undamaged surfaces, indicating that their cell walls are healthy and uncompromised. However, in the positive controls (A2, B2), which were treated with chloramphenicol, the bacterial cells exhibited significant morphological damage, including cell shrinkage and distortion. This damage is attributed to chloramphenicol known mechanism of action, where it inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit, ultimately leading to cell death due to the cessation of vital protein production (Mashhadi *et al.*, 2013).

The red ginger ethanol extract-treated cells (A3, B3) show visible damage, including irregular surfaces, cell wall disruption, and in some cases, complete lysis. The damage to the bacterial cells caused by red ginger extract

can be linked to the presence of bioactive compounds such as gingerol and shogaol, which are known to disrupt bacterial cell membranes. These compounds increase the permeability of the cell membrane, causing leakage of cellular contents, loss of membrane potential, and eventually cell death. Additionally, oxidative stress induced by the antioxidant properties of these compounds may further contribute to the damage observed, as reactive oxygen species can damage cellular components, including lipids, proteins, and DNA, leading to cellular dysfunction and death. This type of cell damage is consistent with findings from previous studies on ginger extracts, which have demonstrated similar antibacterial effects against various bacterial species (Mashhadi *et al.*, 2013; Mao *et al.*, 2019).

The results of this study had provided new data for the therapeutic potential of spices, particularly red ginger. Using spices as an adjunct or alternative treatment in developing countries such as Indonesia will reduce the clinical burden of developing drug resistance and the side effects and costs of treatment with allopathic drugs. The results of this study also emphasize the usefulness of red ginger as a source of medicinal ingredients. Therefore, the potential of red ginger needs to be explored and tested on an ongoing basis.

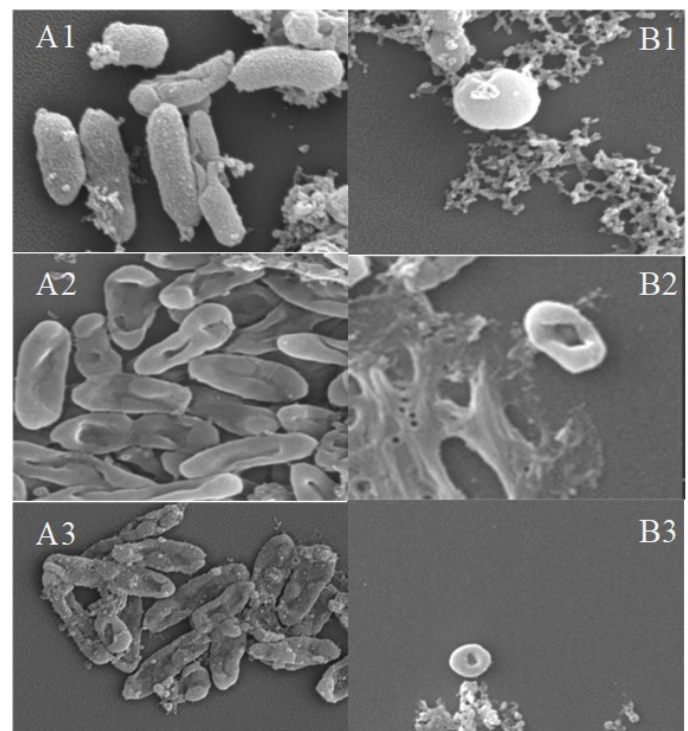


Figure 5. Scanning electron microscope photo. Morphological changes in *E. coli* cells: (A1) negative control, (A2) positive control (chloramphenicol), (A3) treatment with *S. aureus* red ginger ethanol extract, (B1) negative control of *E. coli*, (B2) positive control (chloramphenicol), (B3) treatment with red ginger ethanol extract.

#### 4. Conclusion

The extracts of red ginger evaluated in this study showed different levels of antibacterial activity. The MIC of red ginger ethanol extract on *S. aureus* bacteria was 500 µL/mL. Meanwhile, the MIC of red ginger ethanol extract on *E. coli* bacteria was 125 µL/mL. The inhibition that occurred in *S. aureus* and *E. coli* bacteria was caused by secondary metabolic compounds in red ginger ethanolic extract.

#### Conflict of interest

The authors declare no conflict of interest.

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