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Single Clove Garlic (*Allium sativum*) Essential Oil as an Inhibitor of *Staphylococcus aureus* Bacteria

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Abstract

Staphylococcus aureus is gram-positive bacteria that often infect the skin. *S. aureus* has been experiencing resistance to several antibiotics. One of the solutions to overcome the resistance is by using garlic that is believed by the society can overcome bacterial infection. The study aimed to find out the influence of the variation in concentration of local single clove garlic essential oil used on the inhibition and damage of morphological structure of *S. aureus* bacteria. The inhibition test was conducted using disc diffusion method. The experiment groups consisted of 1% DMSO as negative control, vancomycin 30µg/ml as positive control and single clove garlic essential oil (25 mg/ml, 50 mg/ml, 75 mg/ml and100 mg/ml) as treatment groups. The diameter of inhibition zone was measured using calipers. The morphological damage of the bacterial cells can be seen using Scanning Electron Microscopy (SEM) with magnification of 25000x. The result of Kruskal-Wallis test analysis indicated that the extract of local garlic essential oil has inhibitory activities against *S. aureus* bacteria ($P<0.05$). The damage to the morphological structure of bacterial cells with the administration of 30 µg/ml vancomycin was equal to 100 mg/ml single clove garlic essential oil extract. Single clove garlic essential oil can be used as an alternative treatment for skin infection diseases by inhibiting *S. aureus* growth.

How to Cite

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INTRODUCTION

Skin infection disease caused by bacteria is a health problem among the societies. Bacteria infecting the skin are usually from *Staphylococcus aureus* species. They are gram-positive bacteria with round shape and diameter of 0.7-1.2µm. The bacteria are arranged in irregular group like grapes and grow optimally at an temperature of 30-37^{0}C (Kadariya, Smith, & Thapaliya, 2014). Gram-positive bacteria contain 35-60% polysaccharide and only 0-2% lipid (Eja et al., 2007).

S. aureus is bacteria which has high influence in skin disease. *S. aureus* infected approximately 30% of the human population (Tong, Davis, Eichenberger, Holland, & Fowler, 2015). The number of patients of skin disease infected by *S. aureus* was also dramatically higher between 2006-2009 than in 1998-2001 (Ray, Suaya, & Baxter, 2012). The increasing of the number of bacterial-infected patients is caused by the bacterial mutation and antibiotic resistance (Setiawati, 2015). *S. aureus* bacteria show a resistance of 100% to *Trimethoprim+ Sulfamethoxazole* (TMP-SMX) and 89.4% to *Penicillin* (Kassim, Omuse, Premji, & Revathi, 2016)

Methicillin Resistant Staphylococcus aureus (MRSA) is a *S. aureus* bacteria strain which is resistant to beta-lactam antibiotics such as penicillin (Fair & Tor, 2014). MRSA occurs due to a genetic change caused by antibiotic exposure (Brooks, 2005). Long term antibiotics utilization can cause bacterial resistance to several antibiotics. Natural ingredients can be used as an alternative medicine which potentially act as an antibacterial. Single clove garlic (*Allium sativum* L.) is one of the herbal medicines that is widely used as an antibacterial.

Single clove garlic is a garlic consists of only one clove because it grows in an inappropriate environment (Untari, 2010). Single garlic contains active compounds as much as 5-6 cloves of regular garlic. The active compounds in single clove garlic relatively high compared to regular garlic caused by all substances were gathered in one clove. Thus, single clove garlic could be more efficacious compared with regular garlic (Utami & Mardiana, 2013).

Active compounds contained in single clove garlic which has antibacterial activity for example are allicin, ajoene, diallyl sulfide (DAS), diallyl disulfide (DADS) dan diallyl trisulfide (DATS). Allicin and ajoene have ability as an antibacterial by totally inhibit the RNA synthesis and partially inhibit the DNA and protein synthesis. This mechanism causes inhibition in bacterial growth that lead to the death of the bacterial cells (Eltaweel, 2014; Rehman & Mairaj, 2013). Diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) are reported to have hydrophobic property that could damage phospholipid in bacterial membrane that increase the permeability of bacterial cell membrane which lead to the penetration of cell cytoplasm and other cell substances with low molecular weight from the cell to the cell membranes thus causing the death of the bacteria (Mnayer et al., 2014). This study was intended to find out the effect of administration as well as concentration variation used of essential oil from single clove garlic (*Allium sativum* L.) to the inhibition and damage to cell structure of *S. aureus* bacteria.

METHODS

Single Garlic Essential Oil Extraction

Single garlic extraction was using soxhletation method and N-Hexane as a solvent. The results were diluted into 25, 50, and 75 mg/ml in concentration.

Bacterial Gram Staining Test

The bacteria were taken aseptically using inoculating needle and put above the distilled water drop, slowly flattened and waited until it dried. Staining solution used was crystal violet. Lugol was added for 1 minute and then washed under tap water. Destaining solution used was 96% alcohol for 1 minute then washed with tap water. Safranin was added for 45 seconds and washed with tap water. Samples were air-dried carefully used blotting paper. The examination was conducted using microscope, gram-positive bacteria were indicated with purple color in bacterial cells (Dash & J Payyappilli, 2016)

Catalase and Coagulase Test

Catalase test was conducted using 3% hydrogen peroxide (H_2O_2) (Dewi, 2014). Positive result was indicated by the formation of air bubbles. Coagulase test was used slide method assay (clumping factor) (Dewi, 2014). Coagulase assay determined the coagulase bond. Positive result was indicated by granular precipitate formed.

Antimicrobial Activity and Inhibition Zone Diameter (IZD)

The inoculums preparation was done by subculture of bacteria in Nutrient Broth (NB) agar, incubated at room temperature for 1x24 hour. The turbidity of bacterial suspension

was adjusted before the antibacterial test using spectrophotometer at wavelength of 625 nm until Optical Density (OD) of 0.1 equal to 10^8 CFU/ml (Shafiei, Shuhairi, Md Fazly Shah Yap, Harry Sibungkil, & Latip, 2012).

The *in vitro* test of antimicrobial activity conducted by disc diffusion method using Muller-Hinton Agar (MHA) (Jobim et al., 2014). Six mm paper disc was dropped into each concentration of single clove garlic extract. Sterile swab was inserted into the suspension of 1x24 hours *S. aureus* bacteria and then the swab evenly engraved on the surface of Muller Hinton agar. Furthermore, the paper discs which loaded with different garlic oil concentration were put on inoculated MHA and incubated for 24 hours at 37°C. Vancomycin antibiotics (30µg/ml) was used as positive control while 1% DMSO as negative control. The diameter of inhibition zone was measured using calipers (Weinstein, 2018).

Analysis of Bacterial Morphology using Scanning Electron Microscopy (SEM)

The incubated bacteria which had been given a treatment were centrifuged at 3500 rpm for 15 minutes. Supernatant was disposed and pellet were washed using phosphate buffer saline (PBS) and re-centrifuged. Two percent glutaraldehyde (pH 7.3) was added into pellet and incubated for 1-2 hours. The pellet was added with 2% tannin acid and incubated for 1-2 hours. Buffer cacodylate was added to the pellet and incubated for 20 minutes. One percent osmium tetroxide was added, incubated for 1 hour and then 50% alcohol was added to the pellet and incubated for 20 minutes. The 70%, 80%, and 95% alcohol was added respectively, incubated for 10 minutes and then absolute alcohol was added and incubated for 20 minutes. After the incubation, the sample was centrifuged at 3500 rpm for 10 minutes. The supernatant was removed and t-butanol was added and incubated for 20 minutes. Suspense was made in the butanol as well as thin smear of the suspense on the frozen cover slip. The cover slip was air dried and then sample was read using electron microscope with 25000X in magnification (Goldbeck et al., 2014).

Statistical Analysis

The data of the IZD were analyzed using Kruskal-Wallis and *Mann-Whitney* as post hoc test. P value < 0.05 indicated significantly difference.

RESULT AND DISCUSSION

Based on bacterial identification test, the bacteria used in this research was *S. aureus* bacteria. The results of bacterial identification test are shown in Figure 1.

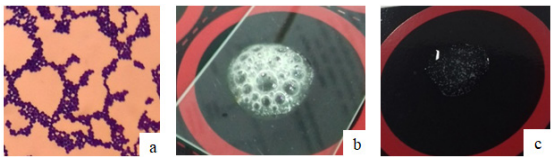

Figure 1 The Result of Bacterial Identification Test. (a) Gram staining sample showing Gram-positive (purple), (b) Catalase test showing air bubbles formed, (c) Coagulase test showing Granular precipitate.

The aim of gram staining test was to distinguish the gram-positive or gram-negative bacteria. Moreover, this test were observed cell morphology and purity of the bacterial cells (Tshikhudo, Nnzeru, Ntushelo, & Mudau, 2013). This test showed that bacterial isolates were gram-positive caused by purple color as a result. The differences between the gram properties of bacteria were influenced by the content in cell walls, in which gram-positive bacteria has more peptidoglycan in their cell walls compared with gram-negative (Reith & Mayer, 2011). The morphology of bacterial isolates was coccus in shape and was arranged in an irregular group (similar to grapes).

Results of catalase test showed that the isolates bacteria were Staphylococcus. Catalase test on coccus bacteria was aimed to differentiate between *staphylococcus* and *streptococcus*. Staphylococcus has catalase enzyme as defense mechanism (Mustafa, 2014).

The result of coagulase test in the research indicated that the isolates bacteria has positive coagulase properties. Coagulase test was aimed to find out the ability of bacteria to produce coagulase enzyme. Positive coagulase reaction can be used to differentiate *S. aureus* to other *staphylococcus* species (Török & Day, 2005).

The result from of inhibition zone test of *S. aureus* bacterial growth using disc diffusion method was indicated that treatments using single clove garlic essential oil extract has antibacterial activity. The average of IZD are shown in Table 1.

Table 1. The Average of Inhibition Zone Diameter of *S. aureus* Bacteria Culture

Treatment Group (mg/ml)	Average of IZD (mm)
K -	17.40[d]
K +	6.00[a]
P 25	9.04[b]
P 50	10.24[b]
P 75	11.56[b]
P 100	14.15[bc]

K -, DMSO 1%; K +: Vancomycin 30 µg/ml; P 25, Single garlic essential oil extract concentration of 25 mg/ml; P50, Single garlic essential oil extract concentration of 50 mg/ml; P75, Single garlic essential oil extract concentration of 75 mg/ml; P100: Single garlic essential oil extract concentration of 100 mg/ml. [a,b] indicated significantly difference

Based on the Table 1, the IZD of *S. aureus* bacteria are different between groups. The IZD test showed that negative control group has no IZD and it indicated that DMSO has no antibacterial activity. This result informed that DMSO has a function only as a solvent. DMSO is used as a solvent due to its ability to dissolve both nonpolar and polar organic compounds and also plays role as an emulsifier (Carey & Sundberg, 2007; Singh et al., 2008).

The result of IZD test showed that treatment with 30µg/ml vancomycin antibiotics can inhibit *S. aureus* growth. Rahmaniar (2017) reported that vancomycin antibiotics are able to inhibit the growth of bacteria, especially gram-positive bacteria by inhibiting the synthesis of cell walls by binding the D-Ala-D-Ala in the peptide chains. The weakness on bacterial cells has an impact on increasing of sensitiveness to the osmotic pressure and causes lysis in the bacteria (Bugg, Braddick, Dowson, & Roper, 2011)

Based on statistical analysis, the administration of single clove garlic essential oil extract in IZD test showed significantly different result. The IZD in treatment groups with single clove garlic essential oil extract significantly larger than negative control group. Therefore, there was no significant difference in IZD between the various concentrations of the sample with single clove garlic essential oil. Variation in concentration used produced different inhibition zone diameter. The inhibition zone produced from the various concentrations is due to the presence of active compounds such as *allicin*, *ajoene*, *diallyl sulfide* (DAS), *diallyl disulfide* (DADS), and *diallyl trisulfide* (DATS).

Raw single clove garlic contained gamma-glutamyl-S-alk (en) il-L-sistein compound, which is the main sulfur compound that can be hydrolyzed and oxidized to produce *S-alkil (en)* yl-L-cysteine sulfoxide (alliin). When garlic is cut or chopped, the alliin changes into allicin by alliinase enzyme (Gao, Jiang, Wang, Zhao, & Wang, 2013). Allicin can pass through additional reaction to form other derivatives, for example, by forming various oil-soluble compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and ajoene (Omar & Al-Wabel, 2010). Thiol group in allicin will change the reaction of thiol compounds at enzymes thus causing disturbance in bacterial metabolism and bacterial growth (Borlinghaus, Albrecht, Gruhlke, Nwachukwu, & Slusarenko, 2014; Rehman & Mairaj, 2013)& Slusarenko, 2014; Rehman & Mairaj, 2013. Allicin and ajoene are also reported to have the ability as an antibacterial by totally inhibit the synthesis of RNA and partially inhibit the synthesis of DNA and protein thus it can inhibit bacterial cells and cause cells death (Eltaweel, 2014; Rehman & Mairaj, 2013).

The Potency of Single Clove Garlic Essential Oil to Damage the Morphological Structure of *S. aureus*

Analysis of the damage to the morphological structure of *S. aureus* bacterial cells was conducted using Scanning Electron Microscopy (SEM). The results of the analysis are shown in Figure 2.

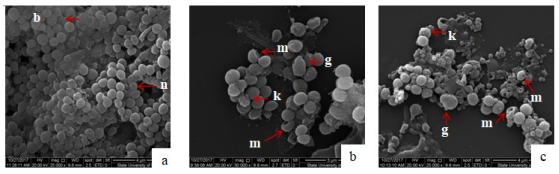

Figure 2. The Result of Observation of the Morphology of *S. aureus* bacteria using *Scanning Electron Microscopy* with magnification of 25000X (a) *S. aureus* bacteria without treatment, (b) *S. aureus* bacteria + *vancomycin* antibiotics, (c)*S. aureus* bacteria + single clove garlic essential oil extract. b: dividing, g: bulging, k: rough surface, m: shrinking, n: normal.

The morphological damage of *S. aureus* bacteria after treatment using 30µg/ml *vancomycin* was indicated by bacterial shrinking cells as well as bulging and damage on cell surface. It was caused by vancomycin that inhibit the bacterial cells synthesis by binding to the cell wall-forming precursors (Bugg et al., 2011).

The morphological structure of *S. aureus*

bacteria after the treatment with single clove garlic essential oil 100mg/ml showed the rough cell surface of the bacteria, small bulges, shrinking cells, and some of them were swelling. Allicin contained in garlic essential oil has ability to degrade the bacterial cell walls by weakening the peptidoglycan layer and modify the bacterial cells membrane, thus it caused shrinking and swelling of the bacterial cell; in addition, the compound could (Booyens, Labuschagne, & Thantsha, 2014). The damage of cell membrane can cause decreasing in cell membrane permeability. When there were many substances entering the cells, the cells would swell, meanwhile the excitation of cell substances can cause the cells to shrink. Moreover, this excitation could inhibit the metabolism by decreasing the ATP for the cell growth and finally caused cells death (Retnowati, Bialangi, & Posangi, 2011).

Another allicin derivative compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) have antibacterial activity. That compounds were hydrophobic compounds and they can cause phospholipid damaging. It can increase bacterial cell membrane permeability, so does the allicin. (Mnayer et al., 2014). In addition, the administration using single clove garlic essential oil also cause the cell surface of *S. aureus* bacteria became rough due to the bulges in the cell surface. The bulge was caused by damaging in peptidoglycan cell walls. Single clove garlic essential oil also contains Ajoene that caused the cell walls unable to resist a high intracellular pressure. Xue et al. (2018) stated that ajoene is able to penetrate components on bacterial cell surface and finally cause the thinning in cell walls. This state results in the release of cytoplasm and damage to organelles in cells. Another research showed that 100 mg/ml single clove garlic oil could inhibit the growth of *P. aeruginosa* bacteria with strong criteria (Lestari, Witjoro & Pujiani, 2018). The small bulges usually formed in the areas that weakening by the antibacterial compounds. The formation of the small bulges was indicated disturbing the biosynthesis process of cell wall (Miksusanti, Betty Sri, Bambang, & Gatot, 2008). Based on the results of this study, single clove garlic essential oil has antibacterial activities against *S. aureus* bacteria, thus it can be used as a treatment for skin infection disease.

CONCLUSION

The local single clove garlic (*Allium sativum* L.) essential oil has antibacterial activity against *S. aureus* bacteria. The higher the concentration of the essential oil, the larger the IZD formed. The damage on the morphological structure of bacterial cells caused by 30μg/ml Vancomycin was equal to 100 mg/ml single clove garlic essential oil, which was indicated by small bulges formed, shrinking cells, and swelling bacterial cell.

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