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The Potential of Single Garlic Oil in Inhibiting The Growth and Damaging The Membrane of *Pseudomonas aeruginosa* Bacteria

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ABSTRACT

Nosocomial infection caused by *Pseudomonas aeruginosa* bacteria is hard to be treated since the infection transmission is fast and it is resistance to the antibiotic. Antibiotic resistance in the bacteria can be treated with various natural ingredients from plants and one of the plants is single garlic. Single garlic contains organosulfur compounds in form of alliin, allicin, and ajoene. This research aimed to analyze the potential of single garlic oil (SGO) in inhibiting the growth and the damage of membrane of *P. aeruginosa* bacteria in in vitro with disc diffusion method by giving treatments of essential oil extract in concentration of 25 mg/ml, 50 mg/ml, 75 mg/ml and 100 mg/ml, and 1% dimethylsulfoxide (DMSO) as the negative control and Ceftazidime of 30 µg/ml as the positive control. The damage of the bacterial cell membrane was observed with *Scanning Electron Microscopy* (SEM) with a magnification of 25000X. The result of the measurement of the zone of inhibition was analyzed using one-way ANOVA. The research results indicate that SGO could inhibit the growth of *P. aeruginosa* bacteria with strong criteria, which was in a concentration of 100 mg/ml.

1. Introduction

Infection is a big issue in Indonesia (Priyanto, 2008). Infectious diseases are diseases caused by biological agents, such as virus, fungus, bacteria or parasite and not because physical (such as burns) or chemical (such as poisoning) factors. Based on a health survey by the Department of Health in 2014, one of the main causes of death is infectious diseases of 28.1% (Department of Health, 2014).

One of the causes of infectious diseases is a nosocomial infection (Inweregbu, 2005), which is an infection occurred in the hospital that sourced from health facilities. Gram-negative bacteria causing a nosocomial infection that often found is *Pseudomonas aeruginosa* (Refdanita, et al., 2004). A research by Khan et al. (2015) using 315 sample of patients in a hospital in China indicates that there were 24.9% of all the patient were experiencing nosocomial infection caused by *Pseudomonas aeruginosa*. Nosocomial infection incident in developing countries has a fairly high prevalence including in Indonesia, which is about 6-16%.

Infection caused by *Pseudomonas aeruginosa* bacteria is hard to be treated since the infection transmission is fast and it is resistance to the antibiotic. This resistance to an antibiotic is a threat to the treatment of infectious diseases in the world. Therefore, in order to overcome it, the development of a new and efficacious alternative treatment is needed that acts as an antibacterial that derives from natural ingredients of plants.

One of the natural ingredients that can be used as antibacterial is single garlic. Single garlic is garlic consisted of one bulb since it grows in an inappropriate environment (Untari, 2010). Compounds in single garlic are mostly containing sulfur responsible for taste, aroma, and pharmacological properties (Ellmore and Feldberg, 1994). One of the most important organosulfur compounds in single garlic is essential oil that contains allicin, alliin, and

ajoene. Allicin is a sulfur component having antibacterial activities that work through a mechanism of inhibiting the formation of the bacterial cell membrane (Dusica, et al. 2011). Damage in bacterial cell membrane due to the interaction of antibacterial compounds can be observed with a Scanning Electron Microscope (Burt & Reinders, 2003). There is limited information used single garlic as an antimicrobial.

2. Materials and Methods

2.1. Materials

Pseudomonas aeruginosa was obtained from the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University. Single garlic extract was obtained by Soxhlet extraction using n-hexane solvent. The supporting substances used in the study were Mac Conkey Agar (Oxoid), Nutrient Agar (Oxoid), Nutrient Broth (Oxoid), Muller Hinton Agar (Oxoid), NaCl 0.9% (Merck), oxidase strips (Oxoid), gram stain, paper disk (Oxoid), Ceftazidime (Merck), Dimethyl Sulfoxide (DMSO), Sodium Cacodylate Trihydrate (Electron Microscopy Sciences), Phosphate Buffer Saline, Glutaraldehyde (Sigma), Osmium Tetraoxide (Electron Microscopy Sciences), 50% alcohol, 70% alcohol, 80% alcohol, 95% alcohol, absolute alcohol (Merck), t-Butanol (Merck).

2.2. Methods

2.2.1. Bacterial Identification

The identification of bacteria was conducted based on the method of Chaskes et al. (2015). The preparation of sterile water and bacterial colonies were put in object glass and air dried. The preparations were fixed above a Bunsen light and stained with violet crystal and safranin (the modification of Chakes et al., 2015). The positive result of observation with a magnification of 100x for *Pseudomonas aeruginosa* bacteria was red in color and in a rod shape.

2.2.2. Oxidase Test

Oxidase test was conducted by preparing the oxidase test strip by taking one *ose* (inoculating loop) of *Pseudomonas aeruginosa* bacteria and engraving it on the oxidase test strip. A positive result was indicated by oxidase strip that has a color of purple-blue.

2.2.3. Biochemical Test

The biochemical test was conducted through the oxidase test using Microbact System. One colony of bacteria that had been incubated for 24 hours at a temperature of 37^0C was taken aseptically and put into 5 ml NaCl 0.9% and vortexed so the suspense was homogenous. The bacterial suspense of 100ml was piped and put into plate well, for lysine, ornithine and H_2S wells, 1-2 drops of mineral oil were added. The pate was incubated at a temperature of 37^0C for 18-24 hours. The reagent of Nitrate A and B of 2 drops were dropped into well 7; 2 drops of Indol Kovach reagent was dropped into well 8; 2 drops of VP I and VP II reagents were dropped into well 10, and 1 drop of TDA reagent was dropped into well 12. Carbohydrate fermentation test was conducted on plate 12B without any addition of reagents. The positive result of fermentation was indicated by yellow color and it was negative if there was no change occurred in the color, which was it remained blue

2.2.4. The Preparation of Test Bacterial Culture

The culture of test bacteria was conducted by taking one *ose* of bacteria from Nutrient Agar (Oxoid) and inoculated in a liquid medium of Nutrient Broth (Oxoid), and vortexed to be homogenous. The culture was incubated at a temperature of 37 °C for 24 hour. The suspense of the test bacteria in a liquid medium of Nutrient Broth that had been incubated was measured using spectrophotometer in the wavelength of 625 nm until Optical Density (OD) of 0.1 equal to 10^8 CFU/ml was known (Murray, et al., 1999).

2.2.5. The Activity Test of Extract of Single Garlic Essential Oil on *Pseudomonas aeruginosa*

The in vitro test of bacterial inhibition was conducted using disc diffusion method. The dosage of SGO used for testing was 25mg/ml, 50mg/ml, 75mg/ml, and 100mg/ml respectively. The cultivation of test bacterial culture was conducted aseptically using sterile cotton swab containing suspense of *Pseudomonas aeruginosa* bacteria and scrapped it softly on the surface evenly. Paper discs dropped with each concentration of single garlic essential oil were put on the surface of the agar. Test cups were incubated in a reversed position in an incubator at a temperature of 37^0C for 1x24 hour. The marking of the inhibition zone diameter was conducted using calipers. The effectiveness of active materials was determined by comparing the inhibition zone diameter to the standard value. The activities were grouped into two categories: strong (10-20 mm), very strong (>20-30 mm) (Greenwood, 1995).

2.2.6. Test on the Damage of Bacterial Membrane using Scanning Electron Microscope (SEM)

Method used for SEM observation was Bazzolla and Russel (1999) by which bacteria at the age of 1x24 hour and

had been given a treatment were centrifuged in velocity of 3500 rpm for 15 minutes. Supernatant formed was disposed and sediment was washed with phosphate buffer. Sediment was added with 2% glutaraldehyde at pH of 7.3 and kept for 1 -2 hours. Next, the sediment was added with tannin acid of 2% and kept for 1-2 hours. Buffer cocodylate was added to the sediment and kept for 20 minutes. 1% osmium tetraoxide was added and kept for 1 hour and then 50% alcohol was added to the sediment and kept for 20 minutes. Respectively, 70%, 80%, and 95% alcohol were added and kept for 10 minutes and then absolute alcohol was added and kept for 20 minutes. Centrifuge was conducted at a velocity of 3500 rpm for 10 minutes. t-butanol was added to the sediment and kept for 20 minutes. The process was conducted two times. 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 magnification of 25,000x.

2.3. Analysis

Data of antibacterial effect analyze using one way Anova and continued by Post Hoc Test of Gomes Howell. The damage of *Pseudomonas aeruginosa* bacteria could be observed through the morphological change on cell structure due to the application of single garlic essential oil containing antibacterial compounds. The observation was conducted using SEM with magnification of 25,000x.

3. Results and Discussion

3.1. Bacterial Identification and Biochemical Test

Figure 1 indicates that gram stain obtained rod-shaped bacterial cells, gram-negative was red in color and biochemical test was conducted using Microbact System Test. Based on the result of the identification test, it can be proved that bacteria used were indeed *P. aeruginosa* bacteria.

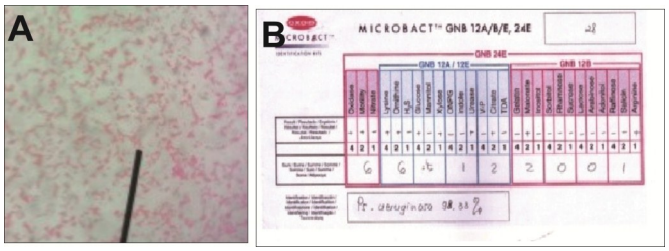

Figure 1. Bacterial identification. A: the Result of Gram Stain, B: Microbact System Test

Figure 2 indicates that disc of Ceftazidime antibiotic of 30µg/ml (positive control) could inhibit the growth of *P. aeruginosa* with diameter of inhibition zone of 14 mm compare to those 1% DMSO (negative control) that resulted no inhibition zone. The result of inhibition zone in concentration of 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml was, respectively, 7.9mm, 8.4mm, 9.3mm, and 11.1mm. The result of inhibition test of the extract of essential oil on *P. aeruginosa* can be seen in Figure 2.

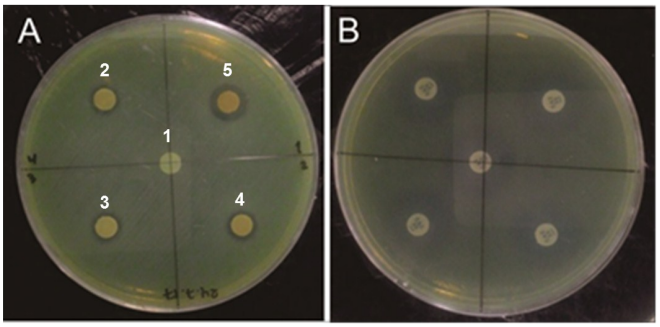

Figure 2. The diffusion method for measuring the inhibition zone of P. *areuginosa* bacteria. A: Single garlic oil inhibition zone of *P. aeruginosa* in various concentrations (1 = DMSO as a negative control; 2 = 25mg/ml, 3 = 50mg/ml, 4 = 75mg/ml, 5 = 100mg/ml). B: Positive controls using Ceftazidime 30µg/ml.

Table 1 shows that the diameter of inhibition zone in each concentration of the extract of single garlic essential oil of 100 mg/ml, 75 mg/ml, 50 mg/ml, 25 mg/ml, and 0 mg/ml on the growth of *P. aeruginosa* bacteria had different values; however, the criteria of antibacterial strength in concentration of 100 mg/ml and positive control was the same, which was in the strong category since the diameter was in the range of 10-20 mm (Greenwood, 1995). It indicates that the extract of single garlic essential oil in concentration of 100 mg/ml contained strong antibacterial substances in inhibiting the growth of *P. aeruginosa* bacteria.

Table 1. The Result of the Measurement of Inhibition Zone Diameter of the Extract of Single Garlic Essential Oil on the Growth of *Pseudomonas aeruginosa* Bacteria

Concentration of Single Garlic Essential Oil	Diameter of Inhibition Zone (mm)	Criteria of Anti-bacterial Strength
K4 (100 mg/ml)	11.1	Strong
K3 (75 mg/ml)	9.3	Weak
K2 (50 mg/ml)	8.4	Weak
K1 (25 mg/ml)	7.9	Weak
Ceftazidime 30µg/ml (K+)	14	Strong
DMSO 1% (K-)	6	Weak

The damage in the bacteria due to the application of single garlic essential oil can be observed morphologically through scanning electron microscope (Figure 3).

Figure 3 indicates the cells of *P. aeruginosa* bacteria with the application of single garlic essential oil in a concentration of 100 mg/ml based on the result of

observation using SEM with a magnification of 25000x. Red arrows indicate the surface of the cell membrane that experienced shrinkage along with a thin form and there were some parts of the cells that looked empty. Green arrows indicate the surface of the cell membrane that experienced shrinkage and lysis. Whereas, in *P. aeruginosa* with the application of Ceftazidime antibiotic in concentration of 30mg/ml based on the result of observation using SEM with magnification of 25000x, red arrows indicate the surface of cell membrane that experienced shrinkage along with a thin form and some parts of cells that looked empty and green arrows indicate the surface of cell membrane that experienced shrinkage and lysis. Regarding *P. aeruginosa* in the medium of Nutrient Broth, based on the result of observation using electron microscope (SEM) with a magnification of 25,000x, it can be seen that blue arrows indicate intact bacterial cell membrane.

The single garlic oil had an influence on the inhibition of the growth of *P. aeruginosa* bacteria since it contained more than 100 compounds of secondary metabolites that biologically useful (Jakobsen et al, 2012). The compounds were mostly contained sulfur (Zhang, 1999). There were three organosulfur compounds in single garlic with high concentration, which were alliin (41.1 g/mL), allicin (26.8 g/mL), and ajoene that divided into E-ajoene (10.1g/mL) and Z-ajoene (25.1g/mL).

The working mechanism of allicin as an antibacterial is by inhibiting the synthesis of RNA and damaging the wall of bacterial cells (Durairaj, 2010). The inhibition of RNA synthesis was conducted by forming a strong bond in the bacterial enzyme, which was DNA Dependent RNA Polymerase thus it could inhibit the synthesis of bacterial RNA (Jawets et al., 2005). The inhibition of the cell wall was conducted by inhibiting the biosynthesis of peptidoglycan that would give strength and rigidity on the cell wall (Brooks et al, 2013). The compounds in *Allium cepa* and garlic effective used as antimicrobials in *Staphylococcus aureus* and *Salmonella enteritidis* bacteria (Benkeblia, 2004), 30 strains of mycobacteria, consisting of 17 species, were inhibited by various concentrations of garlic (Delaha & Garagusi, 1985).

The damage in cell wall could be observed microscopically using SEM. In the application of the extract of single garlic essential oil of 100mg/ml and Ceftazidime of 30mg/ml damage was visible in cell wall marked with the shrinking of the surface of cell membrane. The shrinking cell surface was due to the active compounds containing in the oil that had hydrophilic property thus it could penetrate the outer membrane of cell covered with hydrophilic lipopolysaccharide (LPS). In addition, there were an empty part of cell surface and the thinning cell shape due to the increase in membrane permeability that caused the loss of cell components. Mangoni et al. (2004) stated that cell damage that caused membrane permeability could cause the formation of ghost cell. Ghost cell is a cell structure that looks thin and empty.

The increase in permeability in the cell membrane of *P. aeruginosa* bacteria caused shrinkage and it was thinning if contacted with phospholipid. A phospholipid is part of the special component in the cell wall of gram-negative bacteria, which is the outer membrane in double layered structure and the inside layer has a similar composition to cytoplasm membrane, whereas the outer phospholipid is replaced by lipopolysaccharide (LPS) molecules (Brooks, 2013). Antibacterial compounds attacking phospholipid will make the phospholipids break down into such compounds as glycerol and phosphoric acid. Therefore, phospholipids are unable to maintain the form of the cell membrane and the consequence, leakage occurs in the cell membrane of the bacteria. The leakage is seen morphologically in ghost cell-shaped (Rustanty, et al, 2013).

The shrinking cell membrane of *P. aeruginosa* bacteria will cause the cell into lysis. Morphologically, some of the

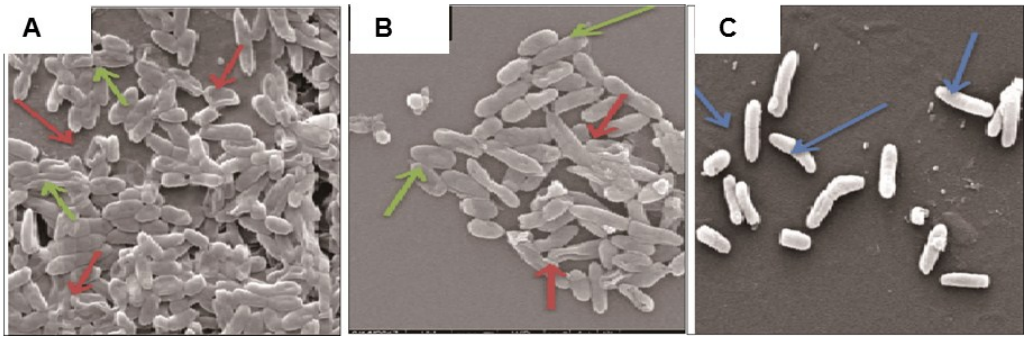

Figure 3. The Bacterial Damage Test using Scanning Electron Microscope (SEM). A: Bacterial damage to SGO 100mg/ml; B: Bacterial damage to Ceftazidime 30µg/ml; C: Bacterial in the medium of liquid nutrient. Red arrow indicates the surface of the cell membrane that experienced shrinkage, green arrow indicates the surface of the cell membrane that experienced shrinkage and lysis and blue arrow show normal cell. Magnifications of 25000X.

lysed cells are partially visible whilst others are visible in full. Davidson and Branen (1980) stated that antibacterial compounds could react with phospholipid components from gram-negative bacterial cells and it causes lysis on the cell wall. The lysed cell wall could cause the cell wall to be completely or partially removed and it is called as spheroplast.

In the surface of cell membrane of *P. aeruginosa* bacteria with the application of growing media of Nutrient Broth, the cell surface was intact and flat. It indicates that there was no contact between cell membrane and the antibacterial material thus the surface was visible morphologically.

4. Conclusions

Single garlic oil (SGO) is potential as an antimicrobial of *Pseudomonas aeruginosa* bacteria by inhibiting the growth and damaging cell membrane. SGO has the same potential as well as the antibiotic of Ceftazidime.

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