A STUDY OF THE EFFECT OF OLIVE (*OLEA EUROPAEA*) STEM METHANOL EXTRACT AS AN ANTIBACTERIAL AGAINST *STAPHYLOCOCCUS AUREUS* BACTERIA

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ABSTRACT

Introduction: Herbal medicine is starting to become an alternative to treating various diseases. Based on proven research, olive plants *(Olea europaea)* have the potential to inhibit the growth of bacteria that causes infections. Drugs to treat bacterial infections are antibiotics, and high exposure to antibiotics could increase the possibility of resistance. At the end of the 1940s, it was shown that 28% of the *Staphylococcus aureus (S. aureus)* bacteria were already resistant to penicillin. Several classes of antibiotics have been created to deal with S. aureus infections. Still, these bacteria show their unique ability to respond quickly to some of the new antibiotics in producing resistance effect. That is why we need another alternative to overcome infections caused by these bacteria.
Aim: The purpose of this study is to know the effect of olive *(Olea europaea)* stem methanol extract as an antibacterial against Staphylococcus aureus bacteria.
Methods: This study uses an experimental study with the broth microdilution method, using extract concentrations of 200-51.200 µg/mL. Minimum Inhibitory Concentration (MIC) values were obtained from the results of spectrophotometer reading, and Minimum Bactericidal Concentration (MBC) was obtained from the results of streaking on agar media.
Result: In this study the MIC value at a concentration of 200-51.200 µg/mL could not be evaluated because it showed no linear lines, and the MBC value was not found. But there was a reduction in the number of colonies at a concentration of 51.200 µg/mL by approximately 50%.
Conclusion: Methanol extract of olive stems proved to have no antibacterial effect against Staphylococcus aureus bacteria at concentrations of 200-51.200 µg/mL.

Keywords : Antibacterial effect, methanol extract of olive stem, bacterial infection of *Staphylococcus aureus*.

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INTRODUCTION

Herbal treatments are starting to become alternatives in treating various diseases[1]. Indonesia is a tropical country that has much variety of plants, including olive plant *(Olea europaea)*. Based on proven research, olive plants have the potential to inhibit the growth of bacteria that cause infections[2].

Infectious diseases are still a significant public health problem.[3] One of the examples is a bacterial infection, and drugs that are used to treat bacterial infections are antibiotics. Over time, changes have occurred in health care practices. With more patients being hospitalized, the number of antibiotic resistance cases becomes higher, caused by frequent antibiotic exposure[4]. According to WHO, antibiotic resistance is an event where bacteria change their structure, which causes drugs that should be able to cure the infection of the bacteria becomes ineffective[5]. Antibiotic resistance is primarily due to the overuse of antibiotics as a treatment[6].

Staphylococcus aureus is a gram-positive bacteria of the genus Staphylococcus. Staphylococcus aureus is a major pathogen for humans; almost everyone has had an infection from these bacteria throughout their lives ranging from mild skin infections to life-threatening infections.[7]

Due to the many cases of antibiotic resistance, another alternative that can be used is needed as a substitute for antibiotics in dealing with *Staphylococcus aureus* bacterial infections. Therefore, we conducted a study of methanol extracts from olive stems to find out whether the stems of olive plants have Minimum Inhibitory Concentration (MIC) and Minimum Kill Concentration (MKC) against the Staphylococcus aureus bacteria. MIC is the smallest concentration of extract that can inhibit bacterial growth by ≥ 90% measured by microplate test results after being incubated for 24 hours by reading on the Microplate Reader. MKC is the smallest extract concentration that can kill 99.9% of bacteria carried out by growing bacterial test results on agar media with the streaking method. The growth of the bacteria was then observed.

METHODS

Materials and Method

1. Plant

   The plant material used is olive stem *(Olea europaea)*

2. Bacteria

   Staphylococcus aureus obtained from the Clinical Microbiology Laboratory of the Surabaya Central Laboratory of Health.

3. Media

   The media used are Mueller Hinton Agar media, Mueller Hinton Broth media, and Blood Agar.

4. Others

   Sterile distilled water, 96% alcohol, violet crystals, lugol, safranin / fuchsin, Penicillin G, NaCl 0.9%, lisol, spiritus, H2O2 3%, DMSO 2%

The Process of Making Olive Plant *(Olea europaea)* Stem Extract

Olive stem extracts are made with the maceration method. Olive stems are cut into small pieces and then dried. Pieces of dried stems are then mashed into powder by using a blender. Simplicia powder is macerated by immersing it with 95% methanol solvent until it completely submerged with a ratio of Simplicia and solvent of 1 to 4 (500 grams: 2 liters) for 24 hours. Then filtering is done using filter paper. The residue is then macerated again in the same way until the solvent is colorless or transparent. The extract is collected and then evaporated to separate the solvent. Evaporation is carried out using a rotary evaporator until the solvent has completely evaporated, and a concentrated, paste-shaped extract is obtained.

The Process of Making Bacterial Suspension

In making an excellent bacterial suspension, it must be adjusted to the 0.5 McFarland turbidity standard. First, mix 1% sulfuric acid and 1.175% barium chloride to create a 0.5 McFarland turbidity standard. Bacteria are then suspended into a tube containing 5 ml of NaCl 0.9% solution until its turbidity is following the standard turbidity of 0.5 McFarland, so it obtains a bacterial suspension containing 1.5 x 108 CFU/mL.8

Antibacterial Test of Olive Plant (Olea *europaea*) Stem Extract

1. Microdilution Method

   The antibacterial test was carried out by the microdilution method. 50µL of Mueller Hinton Broth (MHB) was dripped into Well microplate, then 50µL of olive plant (*Olea europaea*) stem extract was added. The first wells were made with the highest concentration of 3200 µg/mL, the second wells with concentrations of 1600 µg/mL, the third wells with concentrations of 800 µg/mL, the fourth wells were 400 µg/mL and the fifth wells with concentrations of 200 µg/mL. After that, the bacteria were added to the MHB mixture and extracts of the olive plant *(Olea europaea)* by 50µL. Repetition is done to make five sets of treatments so that we can get valid data. Then the microplate was incubated at 37°C for 24 hours.

2. Determination of MIC and MBC.

   The microplate was evaluated for OD values using a spectrophotometer. The MIC value is obtained if olive stem extract can inhibit bacterial growth by ≥ 90%. After that, the results of microdilution were grown by streaking on solid media and incubated for 24 hours at 37 ° C to be observed and determine the MBC value. MBC value determination can be obtained if there is no bacterial growth, or 99.9% of bacteria do not grow on the media.

METHOD

Minimum Inhibitory Concentration

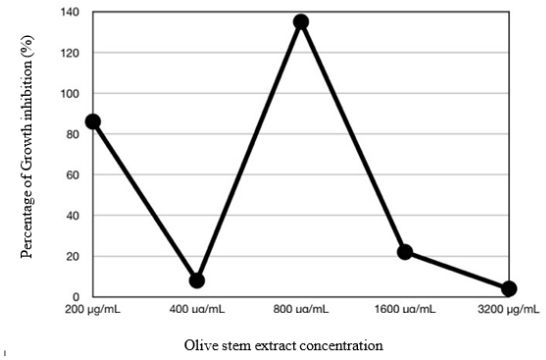

Fig 1. Percentage graph of inhibition of olive stem extract against S. aureus bacteria in the first phase of the study.

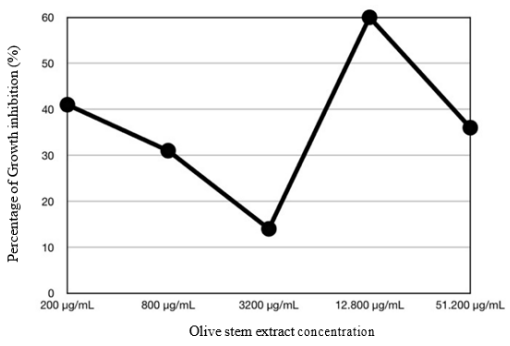

Fig 2. Percentage graph of inhibition of olive stem extract against S. aureus bacteria in the second phase of the study.

Table 1 First phase control.

Control	Growth
K1	-
K2	+
K3e (200 µg/mL)	-
K3d (400 µg/mL)	-
K3c (800 µg/mL)	-
K3b (1600 µg/mL)	-
K3a (3200 µg/mL)	-
K4	-
K5	+

Minimum Bactericidal Concentration (MBC)

Table 2 First phase testing.

Tested	Growth
P5 (200 µg/mL)	+
P4 (400 µg/mL)	+
P3 (800 µg/mL)	+
P2 (1600 µg/mL)	+
P1 (3200 µg/mL)	+

Table 3 Second phase control.

Control	Growth
K1	-
K2	+
K3e (200 µg/mL)	-
K3d (800 µg/mL)	-
K3c (3200 µg/mL)	-
K3b (12.800 µg/mL)	-
K3a (51.200 µg/mL)	-
K4	-
K5	+

Table 4 Second phase testing.

Tested	Growth
P5 (200 µg/mL)	+
P4 (800 µg/mL)	+
P3 (3200 µg/mL)	+
P2 (12.800 µg/mL)	+
P1 (51.200 µg/mL)	+ (Colonies reduction Fig. 3 section P1)

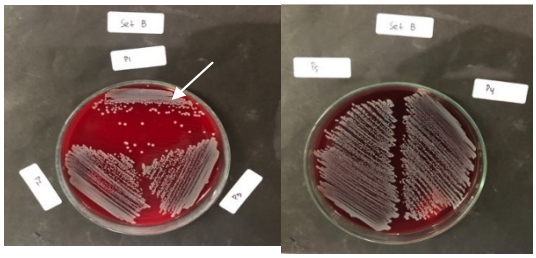

Fig 3. Result of streaking on agar media

DISCUSSION

This study was divided into two phases; the first phase was carried out using a concentration of 3200 µg/mL, 1600 µg/mL, 800 µg/mL, 400 µg/mL, and 200 µg/mL. In the first phase, the results of the microplate reader to determine the value of the Minimum Inhibitory Concentration (MIC) cannot be evaluated because of the value of Optical Density (OD) in waves of 595 nm and 620 nm are unstable. The results of streaking on the media showed no ability to kill S. aureus bacteria at concentrations of 200 -3200 µg / mL; this is proven by the growth of bacteria on the media with no reduction in the colonies.

This is thought to occur because at a concentration of 200 – 3200 µg/mL, the amount of the compound contained in the extract is insufficient to penetrate the cell membrane of the S. aureus bacteria. The second phase was done to confirm the research by increasing the concentration to 51.200 µg/mL, 12.800 µg/mL, 3200 µg/mL, 800 µg/mL, and 200 µg/mL. In the second phase of the study, the MIC value cannot be determined because the OD values at the wavelength of 595 nm and 620 nm tended to get unstable like what happened in the first phase. This is due to the precipitate at the bottom of the well because the olive stem extract is not completely dissolved in the solvent; this precipitate causes the solution to become unstable, creating a saturated solution.9 This unstable solution causes the absorption of light by molecules to be erratic, so the absorbance value tends to fluctuate due to variations in the concentration.[10]

The MBC value in this second phase of the study also could not be found, but at a concentration of 51,200 µg/mL, it was shown that the methanol extract of the olive stem has a killing power against S. aureus bacteria, this is proven by the reduction of colonies from bacteria on the results of streaking in agar media (Figure 3 section P1). This is thought to occur because at a concentration of 51.200 µg/mL compounds found in the olive stem extracts such as Oleuropein, Hydroxytyrosol, and Maslinic acid began to penetrate the cell membrane of S. aureus bacteria, causing interference with peptidoglycan synthesis and also damaging cell membranes from bacteria which causes lysis of bacteria.[11,12] But the reduction of the colony on the results of streaking cannot be said to be a Minimum Bactericidal Concentration (MBC) value because it only inhibits about 50% of growth alone, the amount of olive stem extract compound at a concentration of 51.200 µg / mL is not enough to kill 99% of bacteria. In a study conducted by

Kishikawa et al. (2015) using ethanol solvents to produce olive, the MIC and MBC of plant stem extracts against S. aureus bacteria could not also be found. This is because to produce the killing power of olive stem extract against S. aureus bacteria; the concentrations are required to be high; this was proven in this study as a 50% reduction in colony occurred when administering a concentration of 51.200 µg/mL.

The reduction of bacterial colonies happened when given a concentration of 51.200 µg/mL; this was thought to happen because S. aureus bacteria are Gram-positive bacteria that have thick peptidoglycan walls which make it difficult for extracted compounds to penetrate the peptidoglycan walls and thus require large numbers of dissolved particles to be able to give effect to these bacteria.[13]

In addition to concentration and bacterial factors, extraction method factor is also expected to be involved in this study, maceration extraction method used in this study are thought to be unable to extract specific active ingredients that have an antibacterial effect, the maceration method removes all components contained in both material whether the components that have an antibacterial effect or the components that do not have an antibacterial effect. So, at the time of the test, a large concentration is required to get the effect wanted from components that have antibacterial effects.

In a study conducted by Korukluoglu (2010) using the soxhlet method to extract olive leaves with ethanol solvent, the MIC and MBC for S. aureus bacteria were only 55 µg/mL and 110 µg/mL, as well as with acetone solvents against S. aureus bacteria the MIC and MBC is only 50 µg/mL and 110 µg/mL.[14] The study was able to find MIC and MBC with concentration values that did not reach 51.200 µg/mL because of the olive leaf extraction process using the soxhlet method; this method can specifically attract the active compounds needed and utilized in antibacterial testing. The statistical test in this study cannot be done because of the OD values in the Microplate reader calculation tend to fluctuate and gets unstable so that these values are not possible to be included in the statistical test.

The explanation above is following the research hypothesis that the olive stem extract of methanol has an antibacterial effect against S. aureus bacteria, this was proven by a 50% reduction in the colony at a concentration of 51.200 µg/mL. If this research is continued by increasing the extract concentration above 51.200 µg/mL, then the MBC value of the S. aureus bacteria will be found.

However, this extract cannot be used as an antibiotic therapy because the MIC value from the administration of olive stem extracts to these bacteria is not found. On the microplate reader, the Optical Density (OD) value fluctuates, tends to get unstable, and forms a non-linear line

(figures 1 and 2). This is because, one of the standards of the Clinical and Laboratory Standards Institute (CLSI) criteria is that a compound can be used as antibiotic therapy only if the MIC value forms a linear line so that the compound can be evaluated and classified as susceptible, resistant, or intermediate. Susceptible means that the concentration of certain compounds can inhibit a bacterium if the recommended dose is given to the bacterial infection.[15]

CONCLUSION

Based on the results of research that has been done, it can be concluded that:

- The methanol extract of olive stems at a concentration of 200-51.200 µg/mL has been shown to have no Minimum Bactericidal Concentration (MBC) against S. aureus bacteria. Still, if the concentration is increased beyond 51.200 µg/mL, it is possible that the MBC value can be found.
- Olive stem methanol extract as an antibiotic therapy against bacterial infection of S. aureus cannot be used because on the calculation curve the value of Minimal Inhibitory Concentration (MIC) forms a non-linear line

REFERENCES

1. Departemen Kesehatan Republik Indonesia. Kebijakan Obat Tradisional Nasional. Departemen Kesehatan Republik Indonesia. 2007.
2. Bubonja-Sonje M, Giacometti J, Abram M. Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. Food Chemistry. 2011;127(4):1821-1827.
3. Kementerian Kesehatan RI. Pedoman penggunaan antibiotik. Jakarta: Departemen Kesehatan RI; 2011.
4. Mardiastuti HW. Emerging resistance pathogen: situasi terkini di Asia, Eropa, Amerika Serikat, Timur Tengah dan Indonesia. 2007;5:76
5. World Health Organization What is antimicrobial resistance? [Internet]. 2019 [accessed in 24 January 2019]. Accessed from: https://www.who.int/features/qa/75/en/
6. Laxminarayan Re. Antibiotic resistance: the need for global solutions. – PubMed - NCBI [Internet]. Ncbi.nlm.nih.gov. 2019 [accessed in 26 February 2019 Accessed from: https://www.ncbi.nlm.nih.gov/pubmed/24252483
7. Carroll K, Hobden J, Miller S, Morse S, Mietzner T, Detrick B et al. Jawetz, Melnick & Adelberg's medical microbiology. New York: Mcgraw-Hill Education; 2016.
8. Bailey W, Tille P. Bailey & Scott's diagnostic microbiology. St. Louis: Elsevier; 2017.
9. Reger D, Goode S, Ball D. Chemistry: principles and practice. Belmont, CA: Brooks/Cole, Cengage Learning; 2009. p475-476
10. Kesalahan Spektrofotometri [Internet]. ACADEMIA. 2019 [cited 28 September 2019]. Available from: https://www.academia.edu/6736800/KESALAHAN_SPEKTROFOTOMETRI
11. Haris Omar S. Oleuropein in Olive and its Pharmacological Effects. Scientia Pharmaceutica. 2010;78(2):133-154.

12. Pavel I, Danciu C, Oprean C, Dehelean C, Muntean D, Csuk R et al. In Vitro Evaluation of the Antimicrobial Ability and Cytotoxicity on Two Melanoma Cell Lines of a Benzylamide Derivative of Maslinic Acid. Analytical Cellular Pathology. 2016;2016:1-6.
13. Chouhan S, Sharma K, Guleria S. Antimicrobial Activity of Some Essential Oils- Present Status and Future Perspectives. MDPI. 2017;12-1.
14. Korukluoglu M, Sahan Y, Yigit A, Ozer E, GÜcer S. ANTIBACTERIAL ACTIVITY AND CHEMICAL CONSTITUTIONS OF OLEA EUROPAEA L. LEAF EXTRACTS. Journal of Food Processing and Preservation. 2010;34(3):383-396.
15. Leekha S, Terrell C, Edson R. General Principles of Antimicrobial Therapy. Mayo Clinic Proceedings. 2011;86(2):156-167.