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Lethal concentration of Golden Sea Cucumber killed *Vibrio cholerae*

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ABSTRACT

Introduction: Diarrhea is still a considerable health problem today, especially in developing countries, including Indonesia. One common etiology of diarrhea is *Vibrio cholerae*, which still causes many problems, such as inappropriate use of antibiotics causes antibiotic resistance. The golden sea cucumber (*Stichopus hermanii*) has antibacterial potential against *Vibrio cholera*. This study aims to determine the effect and lethal concentration of golden sea cucumber extract on the growth of *Vibrio cholerae* bacteria.

Methods: This research is an experimental laboratory using the post-test-only control group design method. Dilution is conducted, where six groups are consisting of 4 treatment groups and two control groups. The concentration of the treatment group is 100%, 50%, 25%, and 12.5%, while for positive control using tetracycline and distilled water as a negative control.

Results: The results of this study indicate that the extract of golden sea cucumbers can inhibit the growth of *Vibrio cholerae* bacteria, as evidenced by a dilution test, where the higher the concentration of the golden sea cucumber extract, the more *Vibrio cholerae* bacteria are inhibited its growth. With a value of $p = 0.001$. The lethal concentration of golden sea cucumber extract obtained from this study through a dilution test starting from a concentration of 25%.

Conclusion: Golden sea cucumber extract (*Stichopus hermanii*) can inhibit the growth of *Vibrio cholerae* bacteria through dilution test, regardless of concentration.

Keywords: *Vibrio cholerae*, golden sea cucumber, *Stichopus hermanii*, antibacterial.
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INTRODUCTION

Diarrhea is still a significant health problem today, especially in developing countries, including Indonesia. In 2016, 38 countries reported a total of 132,121 cases. Cases were reported from all regions, including 17 countries in Africa, 12 countries in Asia, four countries in Europe, four countries in America, and one country in Oceania. Of the cases reported globally, 54% came from Africa, 13% from Asia, and 32% from Hispaniola. Diarrhea due to *Vibrio cholera* represents an estimated burden of 1.4 to 4.0 million cases and 21,000 to 143,000 deaths per year worldwide. Many cholera patients died mainly in Sub-Saharan Africa and Hispaniola, which clearly shows that cholera remains a significant public health problem.[1] In East Java, Indonesia, the prevalence of diarrhea was 4.7%. This prevalence shows the incidence of diarrhea in East Java is still relatively high.[2]

Diarrhea is a condition where someone defecates with a soft or liquid consistency, it can even be in the form of water only, and the frequency is more frequent (usually three times or more) in one day.[3] One of the causes of diarrhea is *Vibrio cholerae*. Vibrio cholerae is a bacterium that causes cholera with severe diarrhea and vomiting manifestations due to enterotoxins produced by *Vibrio cholerae* bacteria.

The treatment for acute diarrhea is fluid rehydration, diet, diarrhea medication, and antibiotics. The administration of fluid rehydration combined with medicines offers advantages because antibiotics can reduce the severity of symptoms by reducing the volume of diarrhea so that the use of antibiotics can reduce the amount of fluid needed for rehydration.[3] The first-line antibiotics used to treat Vibrio cholerae are doxycycline and use tetracycline, ciprofloxacin, azithromycin, cotrimoxazole, and furazolidone as second-line antibiotics (CDC, 2015).[4] Several studies have shown the presence of multidrug-resistance *Vibrio cholerae* against several second-line antibiotics, such as sulfamethoxazole, trimethoprim, cotrimoxazole, tetracycline, streptomycin, ampicillin, nalidixic acid, and gentamicin.[5,6] Resistance occurs because of the many inappropriate uses of antibiotics by the community.[7]

Based on the explanation above, alternative treatments are needed to overcome antibiotic resistance, which is expected to provide effective results in helping to reduce the use of oral rehydration and the severity of diarrhea. These alternative treatments can use natural ingredients. Natural resources in Indonesia are very abundant, but many natural resources have not been explored, primarily aquatic biota. Tens of thousands of marine biota species, both in freshwater and in the sea, have enormous potential.

One of them is the golden sea cucumber (*Stichopus Hermanii*).

Stichopus hermanii has various beneficial properties, including calcium, phosphorus, essential amino acids, non-essential amino acids, glycoprotein, collagen, glycosaminoglycans, hyaluronic acid, chondroitin sulfate, heparin, heparin sulfate, proteoglycans, EPA-DHA, flavonoids, saponins, triterpenoids, and cell growth factor.[8] Flavonoid compounds and saponins work as antimicrobials. The mechanism of action of flavonoids as an antibacterial by forming complex compounds against extracellular proteins that interfere with the integrity of bacterial cell membranes, whereas saponins cause leakage of proteins and enzymes from within the cell.[9] Therefore, this study aims to determine the effect and lethal concentration of golden sea cucumber extract (*Stichopus Hermanii*) on the growth of *Vibrio cholerae* bacteria.

Table 1. Calculation results for the growth of *Vibrio cholerae* bacteria on Mueller Hinton agar.

Replication	Control		*Sticopus hermanii* concentration			
	(+)	(-)	100%	50%	25%	12.5%
I	No growth	2582	123	261	865	1564
II	No growth	2550	94	231	738	1774
III	No growth	2575	85	192	852	1710
IV	No growth	2448	84	172	839	1462

Table 2. Descriptive Statistics.

		Mean	Standard Deviation	Minimum	Maximum
Control	(-)	2538.75	62.03964	2448	2582
	(+)	0	0	0	0
Concentration	12.5%	1627.5	141.05673	1462	1774
	25%	823.5	57.97988	738	865
	50%	214	39.77436	172	261
	100%	96.5	18.23001	84	123

NB: Each calculation result is multiplied by 10^3 in units of CFU / ml

MATERIAL AND METHODS

Experimental design

This experimental study uses the post-test-only control group design method, which is an empirical study that is only done once after getting treatment. This design allows researchers to measure the effect of treatment (intervention) in the experimental group by comparing the intervention group with the control group.

Conducting Bacterial Sensitivity

Conducting bacterial sensitivity test by diffusion with the following steps, the step were following: take bacteria from Mueller Hinton order, then planted in liquid media Mueller Hinton then incubated at 34-37^{0}C for 18-24 hours, then, equalize bacterial turbidity so that it is equivalent to McFarland 0.5. Scrape with a sterile cotton swab squeezed on the inner wall of the 0.5 McFarland bacterial suspension tube on the media for Mueller Hinton. Scratches are done three times by rotating the plate so that 60° per scrape. Wait for the breed to dry for 5-10 minutes at room temperature. Dip sterile disc paper into each test solution with varying concentrations. Place each disc paper on the surface of the media in a petri dish planted with *Vibrio cholerae* bacteria. The positive control is tetracycline disc, and negative control is aquadest. Incubate at 36-37^{0}C for 18-24 hours.

Bacterial growth observations are made in each petri dish. Measure the bland zone using a digital calliper in millimeters. The measurement of the bland zone obtained is the average measurement of the 3-way measure of the diameter of the bland zone. Conduct bacterial sensitivity test by dilution with the following steps: Make standard bacteria up to 0.5 Mc Farland. Each tube is filled with 1 ml of gold sea cucumber extract with various concentrations, then adds 1 ml of bacterial suspension to each of them, which is positive control with tetracycline and negative control with aquadest, further incubated at 36-37°C for 18-24 hours. Observe turbidity and compare with controls. Plant bacteria to have Mueller Hinton then incubated at 36-37°°C for 18-24 hours. Then, count the number of bacteria that grow on each petri dish. The number of bacteria can be multiplied by 1000 because it is done 3x dilution.

The group of this research was divided into: Negative control uses aquades (K1); Positive control using tetracycline (K2); Golden sea cucumber extract concentration of 100% (E1); Golden sea cucumber extract concentration of 50% (E2); Golden sea cucumber extract concentration of 25% (E3); Golden sea cucumber extract concentration of 12.5% (E4); Observation (O).

Statistical analysis

The control group and the independent variable data (golden sea cucumber extract) were nominal. In contrast, the dependent variable data (the number of growths of *Vibrio cholerae* bacterial colonies) were numerical scales. Data analysis was conducted with Kruskal Wallis test and Mann-Whitney U post hoc test to determine whether there were differences between the two groups. All the statistical analysis was done using the SPSS program.

RESULTS

The dilution test appeared evident on the positive control tube, but for negative control and various concentrations of golden sea cucumber extract looked turbid. Then the suspension solution from the boxes is planted on Mueller Hinton agar according to the procedure previously described; the results are as follows:

Each calculation result is multiplied by 10^3 with units of Colony Forming Units (CFU)/ml. The results in Table 1 show in the first replication, *Vibrio cholera* bacteria grew on Mueller Hinton agar as much as 2582 x 10^3 CFU/ml for the negative control, 123 x 10^3 CFU/ml for those given golden sea cucumber extract at a concentration of 100%, 261 x 10^3 CFU/

ml for those given golden sea cucumber extract with a concentration of 50%, 865 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 25%, and 1564 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 12.5%.

In the second replication, *Vibrio cholera* bacteria grew on Mueller Hinton agar as much as 2550 x 10^3 CFU/ml for the negative control, 94 x 10^3 CFU/ml for those given golden sea cucumber extract at a concentration of 100%, 231 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 50%, 738 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 25%, and 1774 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 12.5%.

On the third replication, *Vibrio cholera* bacteria grew on Mueller Hinton agar as much as 2575 x 10^3 CFU/ml for the negative control, 85 x 10^3 CFU/ml for those given golden sea cucumber extract at a concentration of 100%, 192 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 50%, 852 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 25%, and 1710 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 12.5%.

In the fourth replication, *Vibrio cholera* bacteria grew on Mueller Hinton agar as much as 2448 x 10^3 CFU/ml for the negative control, 84 x 10^3 CFU / ml for those given golden sea cucumber extract with a concentration of 100%, 172 x 10^3 CFU / ml for those given golden sea cucumber extract with a concentration of 50%, 839 x 10^3 CFU / ml for those given golden sea cucumber extract with a concentration of 25%, and 1462 x 10^3 CFU / ml for those given golden sea cucumber extract with a concentration of 12.5%.

From the dilution test, it can be analyzed with descriptive statistics in the table above.

Analysis of the results of the study on the dilution test listed in Table 2 shows the average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar was 2538.75 x 10^3 CFU/ml for the negative control, 1627.5 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 12.5%, 823.5 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 25%, 214 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 50%, and 96.5 x 10^3 CFU/ml for those given golden sea cucumber extract with a concentration of 100%.

Among the four concentration groups of golden sea cucumber extract, the highest number of *Vibrio cholerae* bacterial colonies was found at a concentration of 12.5% with an average of 1627.5 x 10^3 CFU/ml (141.06 x 10^3 CFU/ml), and the lowest was at a concentration of 100% with the mean 96.5 x 10^3 CFU/ml (18.23 x 10^3 CFU/ml).

The average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar in Table 2 above can be compared between the concentration of 12.5% golden sea cucumber extract and negative control results of 0.641. Thus, around 0.641 *Vibrio cholerae* bacteria grow on Mueller Hinton agar with a concentration of 12.5% golden sea cucumber compared to those without adverse treatment/control. This shows that at a concentration of 12.5%, the golden sea cucumber extract in this study could kill around 0.395 *Vibrio cholerae* bacteria.

The average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar with a 25% sea cucumber golden extract concentration compared with a negative control was 0.324. Thus, around 0.324 *Vibrio cholerae* bacteria grow on Mueller Hinton agar with a concentration of 25% sea cucumber golden extract compared to its negative control. This shows that at a concentration of 25%, the golden sea cucumber extract in this study killed around 0.676 *Vibrio cholera* bacteria.

The average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar with a concentration of 50% sea cucumber golden extract compared with negative control of 0.084. Thus, there are still around 0.084 *Vibrio cholerae* bacteria growing on Mueller Hinton agar with a 50% sea cucumber golden extract concentration compared with its negative control. This shows that at a concentration of 50%, golden sea cucumber extract in this study could kill about 0.916 *Vibrio cholera* bacteria.

The average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar with a 100% sea cucumber golden extract concentration compared with negative control of 0.038. Thus, around 0.038 *Vibrio cholera* bacteria grow on Mueller Hinton agar with a concentration of 100% sea cucumber golden extract compared to its negative control. This shows that at a concentration of 100%, the golden sea cucumber extract in this study can kill about 0.962 *Vibrio cholera* bacteria.

From the comparison of the average number of *Vibrio cholerae* bacteria growing on Mueller Hinton agar above, more than 50% of *Vibrio cholerae* bacteria were killed starting at a concentration of 25% in this study. So starting from a concentration of 25% can be defined as a lethal concentration of 50. that is, concentrations that cause death in 50% of *Vibrio cholerae* bacteria.

DISCUSSION

Based on the results of research on the dilution test on Table 2, it is known that at a concentration of 12.5%, the average number of *Vibrio cholerae* bacteria that grew less than the negative control showed that starting a concentration of 12.5% extract of golden sea cucumbers in this study was able to inhibit the growth of *Vibrio bacteria* cholera. The greater the attention of the golden sea cucumber extract tested, the less the *Vibrio cholera* bacteria that grow.

The chemical content of golden sea cucumbers that can inhibit bacterial growth is flavonoids and saponins. The higher the concentration of the golden sea cucumber extract tested in this study, the fewer *Vibrio cholerae* bacteria grew on Mueller Hinton agar. This means that the chemical content in the golden sea cucumber extract can inhibit the growth of test bacteria. The mechanism of action of flavonoids is antibacterial because of complex compounds against extracellular proteins that interfere with the integrity of the bacterial cell membrane. The mechanism of action is by denaturing bacterial cell proteins and damaging cell membranes without being repaired.[10] Flavonoid compounds can form complexes with bacterial cell proteins through hydrogen bonds. The structure of cell walls and bacterial cytoplasmic

membrane containing proteins becomes unstable because the structure of bacterial cell proteins becomes damaged due to hydrogen bonding with flavonoids, consequently, the permeability function of bacterial cells is disrupted, and bacterial cells will lysis resulting in bacterial cell death.[11]

The mechanism of action of saponins as an antibacterial is to cause leakage of proteins and enzymes from within the cell.[9] Surface-active substances are like detergents. As a result, saponins reduce the surface tension of bacterial cell walls and damage membrane permeability. Damage to this cell membrane disrupts bacterial survival. Saponins diffuse through the outer layer and cell walls of the vulnerable and then bind to the cytoplasmic membrane so that it interferes with and reduces the stability of the cell membrane. This causes the cytoplasm to leak out of the cell resulting in cell death. Antimicrobial agents that inhibit cytoplasmic membranes are bactericidal.[12] Saponins act on the phosphate group of the phospholipid cell membrane and enter the cell. This causes leakage of nitrogen and phosphorus elements. Agents enter cells, denaturation of their proteins, and damage cell membranes.[13] Through this mechanism, the bacteria eventually die.

In a previous study, golden sea cucumber methanol extract was proven to inhibit the growth of *Vibrio cholerae* biotype elector bacteria by forming inhibition zones of 12 mm around the disc paper in the diffusion test**,** not a dilution test like this research.[14,15] The advantage of this study compared to Rasyid is that in this study, the dilution test and the concentration of the extract of the golden sea cucumber (*Stichopus hermanii*) were reduced by half to 12.5% to find out what concentrations could inhibit the growth of *Vibrio cholerae* bacteria effectively.

Other studies have shown the results of the antimicrobial activity of *Sticopus hermanii* extract in inhibiting the growth of *Escherichia coli*, *Pseudomonas* species, *Vibrio voinovich*, and *Staphylococcus aureus* by screening phytochemicals to see the main antimicrobial components by confirming the presence of saponin as a secondary metabolite along with tannin, flavonoid, terpenoids, and steroids. And it turns out that alkaloids are not found in *Sticopus hermanii*.[16]

Sea cucumbers use saponins in high concentrations as a defense mechanism against predators. Saponins can be used as antimicrobial compounds. Pure sea cucumber extracts containing holotoxin, whose effect was found to be similar to antimycin in a dose of 6.25-25 mg/ml. The process of making sea cucumber extract largely determines the results of research conducted as conducted by Rasyid (2012) which found the antibacterial activity of *Sticopus hermanii* extracted with methanol by maceration without a heating process where the maceration was carried out on fresh sea cucumber simplicia and continued its Maserati centrifugation, as well as Nimah, S et al., 2012 obtained research results on the presence of *Sticopus hermanii* inhibitory zones against *Pseudomonas aeruginosa* from methanol extracts from macerated simplicia of fresh sea cucumbers followed by evaporation at 38^0 C.[14,17]

Other factors that influence the secondary metabolite content of antibacterial compounds are the species and origin of the biological material. In contrast to primary metabolites (amino acids, nucleotides, sugars, and lipids) found in almost all plants, secondary metabolites are only found in one particular species.[18] biological materials originating from different regions cause unequal soil nutrient content. The difference in soil nutrient content causes different types of secondary metabolites produced.[19] The golden sea cucumber used in this study came from Sapeken island, Madura, East Java, Indonesia.

CONCLUSION

Golden sea cucumber extract can inhibit the growth of *Vibrio cholerae* bacteria as evidenced through dilution tests, where the higher the concentration of gold sea cucumber extract, the more vibrio cholerae bacteria are inhibited growth. Lethal dose (LD50) of golden sea cucumber extract obtained from this study through dilution tests starting from a concentration of 25%.

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DISCLOSURES

Conflict of Interest

All authors declare no conflict of interest.

Ethical Statement

The Ethical Committee of Faculty of Medicine Universitas Hang Tuah approved the study protocol with registration number: 023/HC/DU/KEPUHT/VI/2019.

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Author Contribution

All authors contributed equally.

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