Determination of minimum inhibitory and minimum bactericidal concentration of ketapang (Terminatia catappa) leaves extract against Vibrio harveyi

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Abstract. Vibrio harveyi is a bacteria that causes Vibriosis in shrimp and can cause mass death. The use of antibiotics and chemicals continuously can harm the environment and humans as consumers. Ketapang leaves (Terminalia catappa) are natural ingredients that can be an alternative to antibiotics and chemicals used to suppress V. harveyi bacteria. The purpose of this study was to determine the antibacterial activity of ketapang leaf extract against V. harveyi in vitro using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods. The experimental study used a completely randomized design (CRD) with 14 treatments and three replications. The results showed that ketapang leaf extract was able to inhibit and kill V. harveyi bacteria in vitro. The minimum concentration of ketapang leaf extract which can inhibit V. harveyi bacteria was 1.56% and the one who was able to kill was 3.12%. This proves that ketapang leaves can be used as an antibacterial against V. harveyi.

1. Introduction

Vibrio harveyi is known as a major pathogen in the larval stage which often causes 100% death [1]. Not only the larval stage, but even Vibriosis also causes death in the post-larval stage, juveniles, sub-adults and also adult shrimp. An outbreak of vibriosis can cause almost 100% death [2]. Shrimp with vibriosis show clinical symptoms: carapace reddening, melanosis of the skin, tail experiencing necrosis, swimming legs, and foot pathway reddened and reddened hepatopancreas tend to be dark [3].

The widespread and effective use of antibiotics in aquaculture has resulted in the development of serious health problems in aquaculture, other animals, and humans. The use of various kinds of antibiotics in large numbers, including non-biodegradable antibiotics, causes antibiotic-resistant bacteria to appear in the aquatic environment [4]. Sensitivity test results indicate that isolates of Vibrio vulnificus, Vibrio mimicus, Vibrio parahaemolyticus are resistant to erythromycin, enrofloxacin, and oxytetracycline [3].

Medicinal herbs play an alternative role to tackle this problem [5]. Several studies related to the antibacterial activity of herbs in Indonesia have been carried out such as: adas (Foeniculum vulgare) fruit extract against Micrococcus luteus [6], patikan kebo (Euphorbia hirta) leaf extract against Aeromonas hydrophyla [7], and kersen (Muntingia calabura) leaf extract against A. hydrophyla [8]. Other herbs that have antibacterial activity and are safe for recommending this plant for use as an antibacterial agent are T. catappa leaves [9, 10, 11]. As a natural product, T. catappa leaf extract can overcome the problem of chemical residues and antibiotic resistance in fish farming [9].
The ability of *T. catappa* as an antibacterial agent, due to the crude phytochemical component of *T. catappa* extract consisting of saponin glycosides, saponins, steroids, digitalis glycosides (cardiac), tannins, and phenols [12]. Aqueous leaf extracts *T. catappa* contain 3.20% alkaloids, 11.66% saponins, cyanogenic glycosides 7.84%, flavonoids 10.49%, tannins 1.24%, and phenols 0.80% [13].

The crude extract of *T. catappa* has been shown to have antimicrobial activity *Bacillus subtilis*, *Staphylococcus aureus* (ATCC 103207), and *Staphylococcus aureus* (clinical) with 2000 ug/ml concentration, but cannot inhibit the growth of *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* [12]. Research on the utilization of ketapang leaf extract (*T. catappa*) for prevention and treatment has been carried out on catfish (*Pangasionodon hypophthalmus*) infected with *Aeromonas hydrophila* in vitro. The lowest dose of ketapang leaf extract which is effective in inhibiting the growth of *A. hydrophila* bacteria is 60 g/l [10]. Low concentration of *T. catappa* water extract can eliminate *Vibrio parahemolyticus* [9]. The methanol extract of young *T. catappa* leaves has an antibacterial activity of *V. parahemolyticus* compared to adult leaves by the method of disk diffusion technique [14]. Testing the antibacterial power of Ketapang leaf extract (*T. catappa*) against *V. harveyi* bacteria in vitro still needs to be done to determine whether it also can inhibit or even as a bactericide against *V. harveyi* that causes Vibriosis in shrimp. Information obtained from this study can help the development of antimicrobial agents from natural ingredients *T. catappa* which is certainly safer to use in shrimp farming, to reduce mortality rates.

2. Materials and methods

2.1. Materials

Materials used in this study include: ketapang leaf extract, pure culture of *V. harveyi* bacteria from the Juanda Fish Quarantine Center, Sidoarjo, Nutrient Agar (NA), Thiosulfate Citrate Bile Salt Sucrose Agar (TCBSA), Nutrient Broth (NB), distilled water sterile, ethanol 96%, Dimethylsulfoxide (DMSO) 10%, Methyl Red (MR), Voger Procaeur (VP), Gelatin, Triple Sugar Iron Agar (TSIA), Motility Indol Ornithine (MIO), Oxidative / Fermentative Media (O / F), sugar test media (maltose, lactose, arabinose, inositol, mannitol, sucrose, sorbitol), violet crystals, safranin, lugol, alcohol, and acetone.

2.2. Methods

Research with experimental methods to determine the minimum concentration of MIC and MBC test of ketapang leaf extract against *V. harveyi* bacteria. The experimental design used was a Completely Randomized Design (CRD), consisting of 14 treatments with three replications.

2.2.1. Ketapang (*Terminalia catappa*) leaf extract

Ketapang leaves used are leaves that have fallen from the tree, yellowish-green, and dry conditions because the leaves have better antibacterial properties than fresh ketapang leaves [15]. A total of 1 kg of ketapang leaves is washed thoroughly with water and dried, then crushed with a blender and sieved to get 400 grams of a dark green fine powder, and ready to be macerated. Maceration is done by soaking the ketapang leaf powder in 96% ethanol, for 3 x 24 hours at 28-30°C [16]. Then filtered with filter paper. The residue was macerated again, in the same way, three times. Macerated extracts are collected and evaporated with a rotary evaporator at a temperature of 70°C - 80°C, until the solvent has evaporated, resulting in 20 ml thick extract of dark green ketapang leaves.

Ketapang leaf extract was diluted using 10% dimethyl sulfoxide (DMSO) solvent to obtain the desired concentration for MIC and MBC determination. Raj et al. [17] using 10% DMSO as a solvent for brown alga extract, *Stoechospermum marginatum*. According to [7], DMSO is a material used as a solvent for organic and inorganic materials and is commonly used in the drug industry. The concentration of ketapang leaf extract that will be used for this research is 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, 0.19%, 0.09%, 0.04%, 0.02% and 0.01%.
2.2.2. Suspension of Vibrio harveyi

V. harveyi isolate was obtained from the Fish Quarantine Center (BKI) Juanda, Sidoarjo. The V. harveyi bacterial colony was rejuvenated by planting it on Nutrient Agar (NA) media plus 2% NaCl, then Gram staining and biochemical tests were carried out on V. harveyi bacterial isolates.

Pure culture of V. harveyi on NA media was planted in Nutrient Broth (NB) which had been given NaCl 2% as much as 4-5 Ose streaks and incubated at 30°C for 24-48 hours [18]. The following day, NB was turbid with an equivalent bacterial density of 3.0 x 10^8 CFU / ml according to Mc Farland 1 standard [19].

2.2.3. Minimum inhibitory concentration (MIC) test

Every concentration from 25% to 0.01% is added with 1 ml of V. harveyi bacteria containing 3.0 x 10^8 CFU / ml. The positive control tube contained 1 ml of ketapang leaf extract + 1 ml of a 10% DMSO solution and the negative control contained 1 ml of V. harveyi bacteria + 1 ml of a 10% DMSO solution. Incubate at 30°C for 24 hours [18].

Observation of MIC test results by looking at turbidity visually on all tubes. If the tube looks clear as in the positive control tube, it means that the ketapang leaf extract can inhibit the growth of V. harveyi bacteria. If the tube looks cloudy as in a negative control tube, it means that the ketapang leaf extract is unable to inhibit the growth of V. harveyi bacteria. To distinguish the turbidity level, continued by using a spectrophotometer at a wavelength of 492 nm to determine the value of optical density (OD).

2.2.4. Minimum bactericidal concentration (MBC) test

The MIC results from all concentrations including positive and negative controls were planted on Nutrient Agar (NA) media with 2% NaCl added. Incubation temperature of 30°C for 24 hours [18]. If the NA media contained V. harveyi bacterial growth, a negative result means that the ketapang leaf extract was not bactericidal. If there is no growth of V. harveyi bacteria, the results are said to be positive, meaning that the ketapang leaf extract is bactericidal.

2.2.5. Analysis of data

Data of optical density (OD) results of MIC observations were analyzed by Analysis of Variants (Anava). If the treatment has a significant effect, then it is followed by Duncan's Multiple Range Test [20].

3. Results and discussion

3.1. Results

The results of observations of the MIC test showed that the ketapang leaf extract (T. catappa) had the activity of inhibiting the growth of V. harveyi bacteria with a minimum concentration of 1.56%. Results of observations of the MIC test visually (Table 1), in tubes 25% to 1.56% (appear clear), while those that appear cloudy indicate bacterial growth is found in negative controls and tubes with concentrations of 0.78% to 0, 01%.

The observations showed control (+) there was no growth of V. harveyi bacterial colonies and no growth of other bacterial colonies, meaning that there was no contamination during the dilution of ketapang leaf extract. The observations showed that control (-) there was a growth of bacterial colonies, meaning that the suspension of V. harveyi bacteria 3.0 x 10^6 CFU/ml that was used for the living conditions was not contaminated by other bacteria, as evidenced by the formation of bacterial colonies by bacterial colonies V. harveyi.
Table 1. Results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

| Ketapang leaf extract concentration (%) | MIC test visual results | MIC test OD average | MBC test results |
|----------------------------------------|-------------------------|---------------------|-----------------|
| 25                                     | Clear, reddish brown    | 0.683 ± 0.031bc     | -               |
| 12.5                                   | Clear, orange           | 0.851 ± 0.022b      | -               |
| 6.25                                   | Clear, orange           | 0.806 ± 0.225a      | -               |
| 3.12                                   | Clear, light yellow     | 0.469 ± 0.073ad     | -               |
| 1.56                                   | Clear, light yellow     | 0.406 ± 0.017ad     | +               |
| 0.78                                   | Murky, colorless        | 0.333 ± 0.021a      | +               |
| 0.39                                   | Murky, colorless        | 0.293 ± 0.044a      | +               |
| 0.19                                   | Murky, colorless        | 0.310 ± 0.019a      | +               |
| 0.09                                   | Murky, colorless        | 0.318 ± 0.046a      | +               |
| 0.04                                   | Murky, colorless        | 0.310 ± 0.027a      | +               |
| 0.02                                   | Murky, colorless        | 0.334 ± 0.044a      | +               |
| 0.01                                   | Murky, colorless        | 0.300 ± 0.012a      | +               |
| Control (+)                            | No Color                | 3.354 ± 0.407a      | -               |
| Control (-)                            | Murky, colorless        | 0.265 ± 0.015a      | +               |

Note: Different superscripts in the same column show a significant difference (p<0.05). Control (+) = 1 ml ketapang leaf extract + 1 ml DMSO 10%, Control (-) = 1 ml of 10% DMSO + 1 ml of V. harveyi bacterial suspension, (+) = There is a growth of V. harveyi colony because ketapang leaf extract is unable to kill bacteria, and (-) = There is no growth of V. harveyi colony because ketapang leaf extract can kill bacteria.

3.2. Discussion
The research proves that the ketapang leaves extracted with ethanol can kill V. harveyi bacteria at a minimum concentration of 3.12% and inhibit the growth of V. harveyi bacteria at a minimum concentration of 1.56% (Table 1). According to [21], [22], ethanol is a good solvent for extracting antimicrobial substances from plants. The plant extraction with ethanol showed better bactericidal activity [23].

The antibacterial agent in T. catappa extracts acts by inhibiting nucleic acids, proteins, cell walls and membrane phospholipid biosynthesis [11]. According to [24], there are four main mechanisms by which antibacterial compounds work in inhibiting or killing bacteria including disorders in the cell wall synthesis process, inhibiting protein synthesis, disruption in the process of nucleic acid synthesis, and inhibiting metabolic pathways. Nadirah et al. [14] argue the mechanism of action of T. catappa leaf extract against Vibrio sp through interference with cell membranes.

This study did not detect the components contained in ketapang leaf extract, but based on [13], flavonoids contained in aqueous leaf extracts of T. catappa were 10.49%. According to [25], the possibility of flavonoids which are responsible for antibacterial activity. Proven by [26], the flavonoids contained in the T.catappa extract can inhibit bacterial growth.

The existence of tannin components in the ethanol extract of T. catappa leaves has been proven by [27]. The antimicrobial activity of tannins is by inhibiting extracellular microbial enzymes [28], [29]. Tannin takes the substrate needed for microbial growth by complexing metal ions by tannins [28], [30] or through oxidative phosphorylation inhibition [28].

4. Conclusion
Ketapang leaves can be used as an antibacterial against V. harveyi with a minimum concentration that is still able to inhibit the growth of V. harveyi bacteria in vitro is 1.56% and the minimum concentration that is still able to kill the V. harveyi bacteria in vitro is 3.12%.
5. References

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