Antibacterial evaluation of 2,4-dihydroxy benzoic acid on Escherichia coli and Vibrio alginolyticus

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Abstract. In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Therefore, research to discover the new anti-bacterial compounds is important to do. This study was carried out with an objective to synthesis and invaluated the antibacterial potentials of 2,4-dihydroxy benzoic acid. The aim of the study is to synthesis and assess the antimicrobial activity and to determine the zone of inhibition of on Escherichia coli and Vibrio alginolyticus. The synthesis of 2,4-dihydroxy benzoic acid was conducted by reacting 2,4-dihydroxy benzene and carbon dioxide. The characterization of the target compounds was performed by IR and MS spectrometers. The growth of the tested bacteria was observed using a colony counter to see the diameter of the resistance which was caused by the test solution. The antibacterial activity test indicated that 2,4-dihydroxy benzoic acid had the potential as an antibacterial against Escherichia coli and Vibrio alginolyticus. The synthesis product was obtained as a white solid, melting point 168-169 °C, 67.5 % yield and 97.5 % purity. The activity was known from its inhibition zone. At the concentration of 100 ppm, 2,4-dihydroxy benzoic acid solution showed the diameter of the inhibitory zone was 17.8 mm and 17.0 mm respectively, while amoxicillin antibiotic showed of 16.6 mm. These results indicate that 2,4-dihydroxy benzoic acid have a greater strength than amoxicillin in inhibiting bacterial growth Escherichia coli and Vibrio alginolyticus.

Keywords : antibacterial, synthesis, characterization, inhibition zone
1. Introduction

Infectious diseases are becoming a major cause of human and animal mortality and morbidity. This is further aggravated by the rapid development of multi-drug resistance, limited antibacterial spectrum and adverse effects of available antimicrobial agents [1]. Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses, not only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance [2, 3]. The emergence of this resistance caused by bacteria can adapt to the presence of antibiotics in clinical concentration and also can be caused by the wrong usage of antibiotic by patient. Various studies discovered that 40-62 % caused by usage of antibiotic for the diseases that not require antibiotics [4].

The resistance of microorganisms to antibiotics is a critical and dangerous medical problem [5]. Acid resistance (AR) is the ability of bacteria to protect themselves from extremely low pH (<pH 3.0). The low pH in the stomach (pH 1.5 to 3.0) is one of the first host defenses against foodborne enteric pathogens [6]. Some antibiotics that have been resistant as reported [7] are chloramphenicol (P.aeruginosa, K. pneumoniae, E. coli, S. typhimurium, V. cholerae), macrolides (Streptococcus pneumoniae, Enterococcus sps, Bacteroidessps, Pseudomonas sps and Enterobacteriaceae), tetracyclines (S. aureus, E. coli, A. baumannii, S. typhimurium), aminoglycosides (E. coli, P. aeruginosa, A. baumannii) and also beta-lactams (H. influenzae, P. aeruginosa, A. baumannii). In addition, there was increasing of 440,000 new cases due to multidrug-resistant tuberculosis (MDR-TB) each year which causes at least 150,000 cases of death each year. Indonesia ranked eighth out of 27 high MDR load countries [8].

There is two ways to develop new antibiotics such as; 1) Isolation of the active compounds in medical plants that traditionally used to treat diseases caused by bacterial [9,10], 2) synthesized the groups of compounds that have been known to have antibiotic activity. Economically, the first way is advantageous if the antibacterial content in plans or microorganism is present in large quantities. However, if the antibacterial content is presented in small quantities the second way will be more profitable. The discovery of new synthetic antibacterial agent effective against resistant microorganisms is important for medicinal chemists. Despite the discovery of many natural and synthetic antibiotics, the innovation of new antibacterial will help in solving the emergence of the microorganisms’ resistance problem [11].

The synthetic fenol compound derivates such us dihidroxy xantone possess interesting and have antibacterial activities [4, 12]. The weakness of these research is the route of dihidroxyxantone synthesis still long with the less of rendemend. In this study, in order to afford structural requirements for organic antibacterial with one step reaction, and evaluated their activity against Escherichia coli and Vibrio alginolyticus. Escherichia coli is a group of bacteria that can cause a number of diseases as a result of infection of the tissues of your body. Human pathogenic bacteria include among others Vibrio alginolyticus; a major cause of bacteremia, associated with higher morbidity and mortality compared to other bacteremia-causing pathogens [13].
2. Materials And Methods

2.1 Materials

The materials needed in this research are resorcinol, potassium hydrogen carbonate, sterile water, carbon dioxide, concentrated hydrochloric acid, decolorising carbon, Staphylococcus aureus, Mueller Hinton Agar medium, amoxicillin, beef broth, Agar powder, and anhydrous Na$_2$SO$_4$.

2.2 Instrumentation

Structure elucidation of the synthesized compounds was performed using $^1$H-NMR (Agilent 400), as well as FT-IR (Shimadzu, Prestige 21) and Mass spectrophotometers.

2.3 Procedures

2.3.1 Synthesis of 2,4-dihidroxy benzoic acid

A solution containing 40 g (0.364 mole) of resorcinol, 200 g potassium hydrogen carbonate and 400 mL of water were placed in a little flask fitted with a reflux condenser and gas inlet tube. Heat on a steam bath for 4 hours, and reflux vigorously over a flame for 30 minutes while passing a rapid stream of carbon dioxide through the solution. Acidity the solution while still hot by adding 180 mL of concentrated hydrochloric acid from a sparatory funnel with a long delivering acid to the bottom of the flask. Allow to cool to room temperature, cill in an ice bath and collect the crude 2,4-dihidroxy benzoic acid by filtration with suction. Recrystallise by boiling the crude acid with 180-200 mL of water in the presence of a little decolorising carbon, filter through a hot water funnel and cool in an ice-salt mixture with stirring. The pure 2,4-dihidroxy benzoic acid was collected and drying. The target compounds then was characterized by IR and mass spectrometers.

2.3.2 Antibacterial Activity Test

A. Sample Preparation

10 mg of the synthesized compound emulsified in 50 µL dimethyl sulfoxide and than diluted in 100 mL water to obtain the concentration of 100 ppm. Another tested solutions were prepared to be 20 ppm, 40 ppm, 60 ppm, and 80 ppm from the stock solution.

B. Medium Preparation

Nutrient Agar Medium was prepared by mixing 350 mL of sterile water, 350 mL of brief broth and 20 gram of agar powder. The mixture were heated until thickened in a beaker glass while stirring. Mueller Hinton Agar Medium was prepared diluting 20 grams of instant Mueller Hinton Agar in to 500 mL of sterile water and then the mixture was heated to be thickened and yellow in a beaker glass while stirring.

C. Rejuvenation of Tested Bacteria

The bacteria that used as the tested bacteria were inoculated in a 5 mL Nutrient Agar medium in the test tube using the ose needle, at 37ºC for 24 hours, using a scratch method.
D. Preparation of Comparative Solution

10.0 mg of amoxicillin antibiotic was dissolved adequately in sterile water up to 100 mL to reached a concentration of 100,0 ppm. This solution was used as a positive control.

E. Antibacterial Activity Testing Using Agar Diffusion Method (Sumuran)

Bacterial resistance was seen from the diameter of inhibitory zone. 10 mL of Mueller Hinton Medium poured on to sterile petri dishes and allowed to be freeze as the basic layer. After that, 5 mL of the rather cold Mueller Hinton Agar Medium with temperature 45-48 °C mixed well with bacteria up to 6 mL and homogenized. Then poured over the base layer of the medium and distributed evenly using a sterile spreader (pour plate method). Furthermore, the incisions were placed on the surface of the medium and filled with 0.2 mL of comparative solution and test solution. A medium consist of 7 tube that divided into a positive control tube which contain amoxicillin, 5 tube which contain difference concentration of 2,4-dihidroxy benzoic acid and a negative control tube which only contain of sterile water. The incisions were incubated at temperature of 37 °C for 24 hours. Then the diameter of inhibitory zone or the space that not overgrown by bacterial calculated by using colony counter.

3. Result

3.1 Characterisation of Target Compound

The IR spectrum showed strong absorptions at 3012 and 1642 cm\(^{-1}\) indicated the presence of the hydroxyl and carbonyl group of 2,4-dihidroxy benzoic acid, respectively. The analysis using mass spectrophotometer showed that the target compound was a single component and gave a molecular weight of 154 with the base peak of 136m/z which. The base peak resulted by H\(_2\)O released, whereas the peak of 108 resulted by the released of C–O group. All of fragmentations given were identical to 2,4-dihidroxy benzoic acid structure. The result of mass spectrophotometer analysis shown in Fig.1.

![Fig. 1 Mass Spectrum of 2,4-dihidroxy benzoic acid](image)

The 2,4-dihidroxy benzoic acid was obtained as a white solid, melting point 168-169 °C and 67,5 % yield. The synthesized 2,4-dihidroxy benzoic acid was characterized by infra red (IR) and mass spectrometers (MS). The route of synthesis of 2,4-dihidroxy benzoic acid is reported in Fig. 2.
3.2 Antibacterial Activity Test of 2,4-Dihydroxy benzoic Acid

The method used in antibacterial activity test of 2,4-dihydroxy benzoic acid was agar diffusion. In this method, the tested bacteria was bred in medium growth of bacteria then into each medium included various concentration tubes 2,4-dihydroxy benzoic acid which were 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. Result of antibacterial activity test of 2,4-dihydroxy benzoic against the growth of *Escherichia coli* listed in Table 1.

Table 1. The Inhibitory Zone Diameter of against 2,4-dihydroxy benzoic acid against the growth of *Escherichia coli*

| No | Treatment | Diameter of Inhibitory Zone (mm) | Total | Average |
|----|-----------|----------------------------------|-------|---------|
|    |           | I | II | III |      |         |       |
| 1  | 20 ppm    | 13.4 | 13.3 | 13.45 | 40.15 | 13.38 |
| 2  | 40 ppm    | 14.54 | 14.65 | 14.32 | 43.51 | 14.50 |
| 3  | 60 ppm    | 15.75 | 15.65 | 15.60 | 47.00 | 15.67 |
| 4  | 80 ppm    | 16.31 | 16.19 | 16.20 | 48.70 | 16.23 |
| 5  | 100 ppm   | 17.70 | 17.90 | 17.80 | 53.40 | 17.80 |
| 6  | K⁺        | 16.50 | 16.60 | 16.70 | 51.00 | 16.60 |
| 7  | K⁻        | - | - | - | - | - |

Caption: K⁺ is amoxicillin as the positive control and K⁻ is water as the negative control.

The data in Table 1 showed that the 2,4-dihydroxy benzoic acid solution has an inhibitory effect on the growth of *Escherichia coli* indicated by the mean diameter of different inhibitory zones in each treatment. The increased concentration was in line with the increased of inhibitory zone diameter. According to [14], if the diameter of the inhibitory area is 5 mm or less, the inhibiting activity is categorized as weak, 6-10 is categorized as moderate, 11-19 mm is categorized as strong, and 20 mm or more is categorized as very strong. This research revealed that 2,4-dihydroxy benzoic acid acid provided a strong inhibitory activity because it had inhibitory zone diameter between 11 and 19 mm at concentration of 100 ppm. Amoxicillin was used as a comparative solution because amoxicillin proved 80% resistance to *Escherichia coli*. Based to the result in the table 1, inhibitory zone diameter of amoxicillin was 16.6 mm. in other word it was less than inhibitory zone diameter of 2,4-dihydroxy benzoic acid. The higher concentration caused the greater released of antimicrobials, thus facilitating the penetration of the compound into the cell. But the concentration of 100 ppm of 2,4-dihydroxy benzoic acid solution can be quite good in inhibiting bacterial growth compared to the exciting studies. [10] declared on his research
that at concentration of 100 ppm cinnamaldehyde of *Cinnamomun burmanii* inhibited the growth of *Escherichia coli* with the inhibitory zone diameter of 15.4 mm. Then the result of this research was better than the exciting studies because 2,4-dihidroxy benzoic acid solution of this research provided a considerable inhibitory than the tested solution of the exciting studies. The results of this study also showed that the diameter of inhibition zone of the test compound was greater than that of *Curcuma aeruginosa Roxb* which has an inhibition zone diameter of 10.97 mm at a concentration of 100,000 ppm.

[16] also stated that a tested solution provided a potential as an antibacterial if it had the standard inhibition of 14 mm. According to the statement, can be conclude that concentration of 100 ppm 2,4-dihidroxy benzoic acid had the potential as an antibacterial with inhibitory of 15.4 against *Escherichia coli*. Result of antibacterial activity test of 2,4-dihidroxy benzoic acid against the growth of *Vibrio alginolyticus* listed in Table 2.

| No | Treatment | Diameter of Inhibitory Zone (mm) | Total | Average |
|----|-----------|----------------------------------|-------|---------|
|    |           | I      | II     | III     |        |         |
| 1  | 20 ppm    | 12.40  | 12.60  | 12.45   | 37.45  | 12.48   |
| 2  | 40 ppm    | 13.34  | 13.65  | 13.35   | 40.34  | 13.48   |
| 3  | 60 ppm    | 14.75  | 14.65  | 14.80   | 44.20  | 14.73   |
| 4  | 80 ppm    | 16.50  | 16.40  | 16.20   | 49.10  | 16.37   |
| 5  | 100 ppm   | 17.00  | 16.90  | 17.10   | 51.00  | 17.00   |
| 6  | K⁺        | 16.50  | 16.60  | 16.70   | 49.80  | 16.60   |

Caption: K⁺ is amoxicillin as the positive control and K⁻ is water as the negative control.

The data in Table 2 showed that the 2,4-dihydroxy benzoic acid solution has an inhibitory effect on the growth of *Vibrio alginolyticus*. This research revealed that 2,4-dihydroxy benzoic acid acid provided a strong inhibitory activity because it had inhibitory zone diameter between 11 and 19 mm at concentration of 100 ppm. Based to the result in the table 2, inhibitory zone diameter of amoxicillin was 16.6 mm. in other word it was less than inhibitory zone diameter of 2,4-dihydroxy benzoic acid.

The ability of compound 2,4-dihydroxy benzoic acid as an anti-bacterial is caused by the presence of three hydroxyl groups and one carbonyl group. As is known these functional groups are able to release one free radical H which is able to inhibit the development of a pathogenic bacteria. While in the amoxicillin molecule there are no hydroxyl fuctional groups.

4. Conclusion

The 2,4-dihydroxy benzoic acid can be synthesized from resorcinol by using carboxylation method. The product was obtained as a white solid, melting point 169 °C and 67.5 % yield. Tested solution of with the concentration of 100 ppm showed that the compound provided the potential as an anti-bacterial against *Escherichia coli and Vibrio alginolyticus* with the strong categorized.
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