Effectiveness of The Antibacterial Activity of n-Hexane Andaliman \((Zanthoxylum Acanthopodium DC)\) Extract Against \textit{Bacillus subtilis}, \textit{Salmonella typhi}, and \textit{Staphylococcus aureus}

N Susanti*\textsuperscript{,}, E Situmorang, W Fitri

Department of Chemistry, Faculty of Mathematic and Natural Sciences, Universitas Negeri Medan, Jl. Willem Iskandar, Pasar V, Medan, 20221, North Sumatera, Indonesia

* nora.susanti.s2@gmail.com

Abstract. Infection caused by bacteria is a problem found in various countries, including Indonesia. Different pathogenic bacteria become resistant to some antibiotics. One way to inhibit the activity of these bacteria is to study natural materials that have antibacterial activity. One of the natural ingredients that have these activities is andaliman. It evidenced by the results of the analysis that the diffusion test of Andaliman extract discs uses two types of solvents: n-hexane and ethyl acetate. In the inhibition zone diameter of three types of bacteria, namely: \textit{Salmonella typhi}, \textit{Bacillus subtilis} and \textit{Staphylococcus aureus} and for the antibacterial test several variations of concentration of andaliman extract were used, namely: 12.5, 25, 50, and 75%, the best results for n extract -hexane at a level of 50%, namely: 26.7 mm for the best ethyl acetate extract at a concentration of 75%, 21.5 mm, both of the \textit{Staphylococcus aureus} bacteria. These results indicate that andaliman extract is useful as an antibacterial.

1. Introduction

Nowadays, more and more infections found that Indonesian attack people\cite{1}. The disease is a common health problem suffered by the community due to the growth of microorganisms, one of which is pathogenic bacteria. The bacteria that cause the most cases of infection in the city are pathogenic bacteria of the species \textit{Staphylococcus aureus} (\textit{S. aureus}) and \textit{Escherichia coli} (\textit{E. coli})\cite{2}.

Indonesia has an extraordinary biodiversity, which is 40,000 plant species of that number, 1,300 of which used as traditional medicine. Based on this potential, conventional Indonesian medicinal products can be widely developed, for example, like herbal medicine, standardized herbal medicine or phytopharmaca. One of the typical plants in Indonesia that have medicinal properties is andaliman which grows in the Tapanuli region\cite{3}.

Traditionally, andaliman fruit widely used as an aromatic ingredient, tonic, appetite stimulant, stomach-ache medicine, and diarrhoea\cite{4}. Indian society uses andaliman fruit to treat paralysis and various skin diseases, such as boils and leprosy. Andaliman fruit also used as a spice in North Sumatra, especially North Tapanuli. Plants of the genus \textit{Zanthoxylum} contain many phenol hydroquinone, flavonoids, steroids / triterpenoids, tannins, glycosides, essential oils, alkaloids, coumarin, lignans, amides and terpenes\textsuperscript{[5]}.  

Based on the research of A Muzafri, et al. in 2018 about "The extraction of antimicrobials component of andaliman (\textit{Zanthoxylum aca}nthopodium \textit{DC})" Moreover, its application on catfish...
(Pangasius sutchi) fillets” explains that the fruit of andaliman can use as an antibacterial. In the study of A Muzafri, et al. used the bacteria Escherichia coli, Staphylococcus aureus and Salmonella typhimurium and then tested on catfish which showed that the quality of catfish cuttings is promising for the food industry [6].

The Antibiotic Activity of Andaliman Fruit Extracts on the Growth of Staphylococcus aureus Bacteria In Vitro conducted by Nurliana, et al in 2010. They found that he Andaliman Fruit Extract (Zanthoxylum acanthopodium DC) has an antibiotic effect on the growth of Staphylococcus aureus bacteria in vitro [7]. Based on research conducted by Adolf JN The 2006 Parhusip on "Effect of Polarity and Concentration of Andaliman Extract on the Growth of Salmonella typhimurium” using the well diffusion method stated that the Andaliman fruit could inhibit the growth of Salmonella typhimurium bacteria [8]. It is hoped that andaliman extract can be used as an alternative to the antibacterials of Bacillus subtilis, Salmonella typhi, and Staphylococcus aureus” [9].

2. Materials and Methods

2.1. Chemicals and equipment

The research sample was the andaliman ripe fruit (Zanthoxylum acanthopodium DC) of the Rutaceae family, which took at Medan Traditional Market. The materials used in this study are n-hexane (merck), aquades, filter paper, concentrated HCl (merck), anhydrous acetate (merck), concentrated H2SO4 (merck), FeCl3 1% (merck), FeCl3 5% (merck), Dimethyl sulfoxida (DMSO), chloramphenicol (oxoid), disk paper, Aluminum foil, MHA media (merck), Dragenndroff reagent (merck), physiological NaCl 0.9%, Salmonella typhi bacteria, Staphylococcus aureus and Bacillus subtilis [10,11,12,13]

The tools that used in this research are vacuum rotary evaporator (Heidolph), petridish dish, beaker glass (approx), Erlenmeyer (pyrex), test tube (pyrex), incubator (memmert), autoclave (Tomy), oven (memmert) , Laminar flow (18-one V 915 S buchner (100 mm), measuring cup (pyrex), ose, cotton, cling wrap, and hotplate [14]

2.2. Research procedure

2.2.1. Sample preparation

A total of 3 kg fresh andaliman then washed. Drained and then dried by leaving it indoors while turning it over and over. The drying process is carried out in a place protected from sunlight for six days. Drying did in a shady place intended to avoid damaging the metabolite content of the sample due to direct contact with sunlight [15,16,17,18]

2.2.2. Andaliman extraction

Andaliman macerated as much as 1000 grams with n-hexane and ethyl acetate as much as 2 L each and for 3 x 24 hours. Then the extract is filtered using a buncher. After that, the filtrate obtained is concentrated with a vacuum rotary evaporator so that the extraction results obtained. The extract is stored in a dark bottle and covered with aluminium foil.

2.2.3. Phytochemical Test

a. Flavonoid Compound Test. Test for the presence of flavonoid compounds is carried out by adding two drops of 5% FeCl3 solution to five drops of sample on the drip plate. The change of colour to greenish or blackish blue indicates the presence of flavonoids.

b. Alkaloid Compound Test. Test for the existence of alkaloid compounds was carried out by adding 0.5 gram of sample with 1 mL of HCl 2N and supplemented with 9 mL of distilled water and then combined with five drops of Dragendorff reagent, the presence of red brick precipitate indicated the presence of alkaloids.

c. Steroid and Triterpenoid Compound Test. Tests for the existence of steroid and terpenoid compounds are carried out by adding 0.5 gram of extract with ten drops of acetate anhydrous added
d. Saponin Group Compound Test. Test for the presence of saponin compounds is done by adding 1 mL of the sample with 10 mL of distilled water until 10 minutes and leaving the foam and then waiting for up to 10 minutes if the foam does not disappear, add 2N HCl. If the foam does not go, indicating the presence of compound saponins.

e. Tanin Group Compound Test. Test for the existence of tannin compounds was carried out by adding 0.5-gram extract with 10 mL aqua dest and combined with three drops of FeCl3 1%, the appearance of a blackish green colour showed decisive containing tannins [19,20]

2.2.4. Making andaliman concentration
In this antibacterial test, research concentration was carried out. Making andaliman concentration, made into four concentrations with units of weight / volume 25% concentration, 50 % concentration and 75 % concentration.

2.2.5. Equipment and media sterilization
Glass tools are sterilized using an oven at 100° C for 2 hours. Metal tools are cleaned using an incandescent lamp. In contrast, for machines that cannot withstand heat and the heating medium at high temperatures, they are cleaned by autoclaving at 121 ° C pressure of 2 atm for 15 minutes.

2.2.6. Making Media Agar
A total of 2.3 g NA was put into the beaker glass, then added with 100 mL of distilled water. The mixture is stirred using isopods until it is homogeneous. After being comparable, the medium is put 20 mL into a 100 mL Erlenmeyer then placed in an autoclave sterilized. Then prepare the medium and pour into a petri dish.

2.2.7. Rejuvenation of Pure Culture and manufacture of bacterial suspensions
Test bacteria in the form of Bacillus subtilis, Salmonella typhi, and staphylococcus aureus rejuvenated before being used for bacterial testing. Bacteria were bred on a sterilized slant agar, then incubated for 24 hours at 37 ° C. The results of bacterial culture were taken from sense and inoculated into a petri dish containing 20 mL of sterile MHA media. Then incubated for 24 hours. After that, the bacteria were suspended into a 0.9% NaCl solution to the McFarland 0.5 standard

2.2.8. Antibacterial Activity Testing with Paper Disc Diffusion Method
The bacterial culture of the test took as much as 100 μl inoculated on MHA medium by the spread plate method. Paperdisk placed on the medium and then 20 μl ethyl acetate extract is dropped. Test solutions used were andaliman fruit ethyl acetate extract with various concentrations (12.5, 25, 50, 75%) chloramphenicol as positive control and DMSO as a negative control. Then the petri dish was incubated in an incubator at 37 ° C for 24 hours. A clear area is a sign of bacterial sensitivity to antibiotics or other antibacterial agents used as test material expressed in inhibition zone diameters. Inhibition zone diameters are measured in millimetres (mm) using a scale callipers

3. Result and Discussion

3.1. Sample preparation
The initial color of the Andaliman sample was blackish green, after the color of the Andaliman sample was dried to black. The mass of samples obtained after the drying and refining process of 3 kg of fruit andaliman (Zanthoxylum aacanthopodium DC) obtained andaliman fruit powder as much as 1,000 grams (33.3%) with a reduction in water content of 66.7%.

3.2. Phytochemical test
Phytochemical test results of alkaloids, flavonoids, saponins, tannins, steroids and triterpenoids in ethyl acetate extract are presented in Table 2.
Table 1. Secondary Phytochemical Tests on n-hexane Extracts

| No. | Phytochemical Tests | n-hexane extract | ethyl acetate extract |
|-----|---------------------|------------------|---------------------|
| 1.  | Flavonoid           | +                | +                   |
| 2.  | Saponin             | +                | +                   |
| 3.  | Tannin              | -                | -                   |
| 4.  | Steroid             | -                | -                   |
| 5.  | Alkaloid            | +                | +                   |
| 6.  | Triterpenoid        | -                | -                   |

Note: (+) = there; (-) = nothing

3.3. *Antibacterial Activity Test*

Diffusion well test is a test to show the inhibition of andaliman fruit extracts against the bacteria Bacillus subtilis and Salmonella typhi. Antibacterial activity measured from the diameter of the clear zone on the MHA media compared to the standard antibacterial chloramphenicol compound as a positive control and DMSO as a negative control. In testing this diffusion well, andaliman extract made variations of concentration that is 25, 50, and 75%. The following are the results of the antibacterial activity test of the n-hexane and ethyl acetate extract against the bacterium Bacillus subtilis, Salmonella typhi and Staphylococcus aureus.

Table 2. Anti-bacterial Activity Test Results for n-hexane and ethyl acetate extract

| No. | Bacteria           | Concentration | Inhibitory Zone Diameter |
|-----|--------------------|---------------|--------------------------|
|     |                    |               | n-hexane | ethyl acetate |
| 1.  | Bacillus subtilis  | 12.5%         | 10.2 mm | 7.2 mm       |
|     |                    | 25%           | 6.6 mm  | 8.5 mm       |
|     |                    | 50%           | 6.8 mm  | 11.7 mm      |
|     |                    | 75%           | 8.4 mm  | 12.4 mm      |
|     | Chloramphenicol    |               | 21.1 mm | 17.0 mm      |
|     | DMSO               |               | 5.3 mm  | 5.6 mm       |
| 2.  | Salmonella typhi   | 12.5%         | 7.9 mm  | 12.3 mm      |
|     |                    | 25%           | 11.2 mm | 12.2 mm      |
|     |                    | 50%           | 15.4 mm | 13.9 mm      |
|     |                    | 75%           | 8.0 mm  | 15.1 mm      |
|     | Chloramphenicol    |               | 17.5 mm | 18.1 mm      |
|     | DMSO               |               | 5.3 mm  | 5.8 mm       |
| 3.  | Staphylococcus aureus | 12.5%     | 9.3 mm  | 7.2 mm       |
|     |                    | 25%           | 12.9 mm | 12.4 mm      |
|     |                    | 50%           | 12.3 mm | 17.2 mm      |
|     |                    | 75%           | 16.6 mm | 16.9 mm      |
|     | Chloramphenicol    |               | 22.3 mm | 20.3 mm      |
|     | DMSO               |               | 5.2 mm  | 5.3 mm       |

3.4. Discussion

The method used is the disk diffusion method. The principle of diffusion method is a potential test based on observations of the area of bacterial growth inhibition due to the diffusion of antibacterial from the initial point of administration to the diffusion area. In the Petri dish paper disc is inserted, then drops of extract. The extract used consisted of four different concentrations. This process is carried out with two repetitions with three types of bacteria.

Referring to the general standard issued by David Stout, it stated that microbes are declared sensitive to antibacterial if the plant has a size of inhibition of 10-20 mm. Based on the strength of antimicrobial power with inhibition zone diameter can be grouped into 4 parts, namely: a) weak, inhibition zone 5 mm or less; b) medium, 5-10 mm inhibition zone; c) active inhibition zone of 10-20 mm; and d) very strong, inhibition zones of 20 mm or more.
Pelczar and Chan (1986) state that Gram-negative bacterial cells have a multi-layered structure and relatively higher fat content (11-12%), so they are more resistant to environmental changes caused by chemicals. In general, the type of Gram-positive bacteria has a more straightforward cell wall structure that is 90% in the cell wall, which consists of a peptidoglycan layer. The other layer is teichoic acid (Fardiaz, 1989). This is thought to cause Gram-positive bacterial cell walls to be easily damaged by antibacterial compounds from the Andaliman extract rather than Gram-negative bacteria. According to Dewi (2010), Teichoic acid as a constituent of cell walls of Gram-positive bacteria is a water-soluble polymer that functions as transport of positive ions to get in and out. This water-soluble nature shows that Gram-positive bacterial cell walls are more polar. Thus, polar bioactive compounds easily enter the cell wall and damage the polar layer of peptidoglycan rather than the non-polar lipid layer. This also supported by the opinion of Ningtyas (2010), which states that polar compounds are challenging to pass through Gram-negative cell walls. The content of Gram-negative bacterial cell walls consists of more lipid content than Gram-positive bacterial cells whose cell wall content is peptidoglycan.

In the determination of the antibacterial activity of the adaliman fruit, extracts used a standard atypical antibiotic commonly used in treatment as a positive control, namely chloramphenicol. The response given by the bacteria Bacillus subtilis and Salmonella typhi to the extract and chloramphenicol was different. Where the bacteria Bacillus subtilis and Salmonella typhi used are more effectively inhibited by chloramphenicol with inhibition zone diameter produced with ethyl acetate extracts on the bacteria bacillus subtilis, salmonella typhi and Staphylococcus aureus, respectively 17.0 mm, 18.1 mm and 20.3 mm. Chloramphenicol has a more significant inhibition zone compared to andaliman fruit extracts. That means that chloramphenicol has a broad spectrum with high inhibitory strength in inhibiting and killing bacteria. The inhibitory potential of andaliman fruit extract compared to chloramphenicol can be seen in the following table:

| Bacteria                        | Effectiveness inhibitory (%) |
|---------------------------------|------------------------------|
|                                 | 12.5% 25% 50% 75%            |
| Bacillus subtilis               | 48.3% 42.3% 31.2% 50.0% 32.2% 68.8% 39.8% 72.9% |
| Salmonella typhi                | 45.1% 67.9% 64.0% 67.4% 88.0% 76.7% 45.7% 83.4% |
| Staphylococcus aureus           | 41.7% 35.4% 57.8% 61.1% 55.1% 84.7% 74.4% 83.5% |

In theory, the potential of Andaliman fruit extract as an antibacterial is higher with increasing concentration. It means that the higher the concentration, the more antibacterial that plays a role in bacterial inhibition. However, in Bacillus subtilis the second repetition of the concentration is 50% greater in diameter than the diameter at a concentration of 75%, this is because the drops of Andaliman extract did not seem wholly diffuse. It also happened to the Staphylococcus aureus bacteria test on the first test. Their interaction with the microbes tested, the number of microbes tested, the speed of growth of the test microbes and the level of sensitivity of microbes to the antimicrobial material. Antimicrobials of test material are called potent inhibitors if they have inhibition zones more significant than 11 mm, moderate inhibitions with 6-11 mm inhibition zones, whereas if inhibition zones smaller than 6 mm are weak / low inhibits (Nurliana et al., 2009).

Andaliman ethyl acetate extract activity in each growth phase had a very significant effect (p <0.01). The higher the concentration of ethyl acetate extract, the higher the diameter of the inhibitor. The mechanism of phenolic antibacterial components will generally interact with proteins present in cell walls or cytoplasm through hydrogenic bonds and hydrophobic interactions (Naidu and Davidson 2000). In this study, it suspected that the sensitivity of the bacteria Bacillus subtilis and Salmonella typhi, and staphylococcus aureus, due to the presence of secondary metabolites in andaliman fruit extracts in the form of alkaloids, tannins, saponins.

Dzulkarnain and Sundari (1996) also suggested that tannins have anti-spasmyloitic properties, which shrink the intestine so that intestinal peristalsis reduced. However, this spasmyloitic effect might shrink the cell wall or cell membrane so that it interferes with the cell's permeability. As a result of the
disruption of permeability, cells cannot carry out living activities so that their growth is stunted or even dies. According to Winarno and Sundari (1996), the alkaloids in some traditional medicines such as Guadiva Psidium leaves are also antibacterial. Brotoali alkaloids can interfere with the formation of bridges across the cross components of the peptidoglycan in bacterial cells so that the cell wall layers are not formed intact and cause cell death. Thus, it is suspected that inhibition of Bacillus subtilis, Salmonella typhi, and staphylococcus aureus, might also be caused by the presence of alkaloids in andaliman fruit extracts.

4. Conclusion
Based on research that has done, it concluded that phytochemical test carried out by positive secondary metabolite compounds in Andaliman fruit ethyl acetate extract are flavonoids, alkaloids, and saponins, these compounds which act as antibacterial. Mased on the measurement of the effectivenes of andaliman extract, it found that the best effectiveness of andaliman extract which inhibits bacterial activity is that of n-hexane extract andaliman n-hexane extract andaliman has the highest effectiveness, namely at a 50% concentration of Salmonella typhi bacteria where the effectiveness reaches 88.0% While the effectiveness of andaliman ethyl acetate extract, the highest effectiveness reached 84.7%, namely at a concentration of 50%.

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