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EVALUATING THE USE OF ANDALIMAN EXTRACT (*ZANTHOXYLUM ACANTHOPODIUM* DC) AS ANTIMICROBIAL AGENT AGAINST *STAPHYLOCOCCUS AUREUS* BACTERIA CAUSING FISH DISEASE

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ABSTRACT

Antimicrobial agent is any substances with ability to inhibit or decline the growth of microorganisms such as bacteria. Antimicrobial agents commonly found in the market are chemicals in the form of antibiotics, hence alternative of natural substances that is environmentally-friendly is necessary to reduce the long-term impact of antibiotics. One of natural substances with antimicrobial potency is Andaliman, a type of spice plant widely grows in North Sumatra. Study on *in vitro* test of Andaliman as antimicrobial agent is therefore required. Furthermore, *Staphylococcus aureus* was selected as the object of this study. *S. aureus* is pathogenic bacteria frequently cause disease in common carp (*Cyprinus carpio*), particularly the eye disease which affects fish brain and optic nerves. *In vitro*test by applying 4 (four) dosages of treatment, namely Extract of Andaliman 0% (*chloramphenicol as control*), 2%, 4%, and 6% was done in this study. Parameter observed included biochemical test for bacteria and diameter of inhibition zone. Result of study showed that *Andaliman*extract at dosage 2%, 4% and 6% had similar ability (P>0,05) to *Chloramphenicol*in inhibiting antimicrobial growth. Hence, Andaliman extract is possibly applied as substance to prevent or treat fish diseases caused by *S. aureus*.

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INTRODUCTION

Andaliman (*Zanthoxylum acanthopodium* DC**)** is one of spice plants widely distributed in the Regency of Toba Samosir and North Tapanuli, North Sumatera. The fruits of Andalimanare used as spices. Furthermore, its bark, roots, and leaves are traditionally utilized as medicine for stomachache, toothache, cough, rheumatic, and low back pain. Andaliman also has several biological activities including larvicidal, anti-inflammatory, analgesic, antimicrobial, antioxidant, and antifungal activities (Negi *et al*, 2011). This provides opportunity for Andaliman as raw materials of antioxidant and antimicrobial compounds in food and pharmaceutical industries. Thus, Andaliman is highly possible to be used as one of phytopharmaceuticals in aquaculture activity. The fruit of Andaliman (*Zanthoxylum acanthopodium DC)* is rich of essential oils and antioxidant. Several compounds have been successfully identified from Andaliman fruits, those are alkaloid, terpenoid, and flavonoid. For this reason, Andaliman has the potential to be used as one of compounds to inhibit the growth of pathogenic bacteria in fish, such as *Staphylococcus aureus*. *Staphylococcus aureus* is pathogenic bacterium found in fish with several characteristics such as invasive, causing hemolysis, forming coagulation, liquefying gelatin, and fermenting mannitol (Warsa, 1994 in Rahmaningsih *et al.*, 2012). Shah and Tyagi (1986) found this bacterium caused disease in common carp (*Cyprinus carpio*), particularly the eye disease which could affect fish brain and optic nerves. Fish suffer from this disease seems lazy and experiencing melanosis. Infection caused by *Staphylococcus aureus* is prevented and treated through the application of antibiotics which could inhibit the growth or even kill *Staphylococcus aureus.* However, frequent use of antibiotics will cause resistance, thus results in long-term adverse effect. The use of natural substance that is environmentally-friendly and has minimal side-effect is the most appropriate solution to apply. Therefore, it is necessary to conduct study about *invitro* use of Andaliman (*Zanthoxylum acanthopodium* DC) as antimicrobial agent against the viability of *Staphylococcus aureus* which causesfish disease. The aim of this study was to determine the best dosage and inhibitory potential of Andaliman extract ton the growth of *S. aureus*. Moreover, chemical antibiotics *chloramphenicol*was used as control. It is expected that Andaliman has similar ability to antibiotics in inhibiting the growth of *S. aureus*. In this study, the fruits of Andaliman were extracted into dry powder, allowing it to be stored and used for a long time.

MATERIALS AND METHODS

Tools used in this research included grinder, digital scale, beaker glass, vortex, aluminum tray,spectrophotometer, incubator, petri dish, erlenmeyer flask, paper disk, and other supporting tools. Materials used in this study were Andalimanbought from Pasar Senen (traditional market), East Jakarta which was previously extracted in the laboratory, bacterial isolate of *Staphylococcus aureus*obtained from the Agency of Fish Quarantine, Quality Control, and Safety of Fishery Product (BUSKIPM), growth media for bacteria, and *chloramphenicol*. Extraction was done by firstly cleaning and drying the fruits of Andaliman in a room at temperature of 23^0C. The dried Andaliman was further grinded into powder using grinder. Later, the extract was used for *in vitro* resistance test through the method of Kirby-Bauer disk diffusion susceptibility test.

Test of Bacterial Viability: The test procedure of Andaliman resistance to the viability of *S. aureus*bacterium was done using the method of Kirby-Bauer disk diffusion susceptibility test (Koneman, 1983). The dried isolate of *Staphylococcus aureus* was added with 1 mL of sterile aquadestat bacterial density of 10^9 CFU/mLfor 30 minutes before being transferred to 10 mLof*Tryptic Soy Broth*(TSB) to be further incubated for 24 hours at temperature of 35^0C. After ±24-hours incubation, about 1 mL of suspension was spread on Mueller Hinton Agar(MHA) surface. Later, paper dish contained antimicrobials to be tested was put on the surface of culture media. Paper dish contained *chloramphenicol*as control (Andaliman 0%), Andaliman 2%, 4%, and 6%, of each. Culture of media and bacteria were kept in the incubator at temperature of 35^0C for 24 hours to be further observed for the growth. Testing of isolate was performed with 3 replicates of treatment. Result of test was determined by measuring the diameter of inhibition zone formed around the paper disk using ruler. Result of the resistance of isolate to 0%, 2%, 4%, 6% was referred to CLSI (2017). Moreover, biochemical test for bacteria was carried out through conventional method of biochemistry and molecular biology (sequencing) of BLAST sequencing. It was found that 99,27% of bacteria used in this study were identical with *Staphylococcus aureus*strain B3A22 16S ribosomal RNA gene, partial sequence, ACC Number KX023358.1.

Data Analysis: Experimental design applied in this study was the Completely Randomized Design (CRD) with a single factor consisted of 4 treatments and 4 replicates of each. Moreover, the dosage of treatments are as follows:

A : Control, Andaliman dosage of 0%
B : Andaliman dosage of 2%
C : Andaliman dosage of 4%
D : Andaliman dosage of 6%

Data of the diameter of inhibition zone obtained in this study were previously tested for the normality and homogeneity. Data were confirmed to be normally distributed and homogenous. Furthermore, analysis of variance (ANOVA) at confidence level of 95% was applied using SPSS. If analysis of variance (ANOVA) reported a statistically significant result, Duncan post-hoc test was done to estimate differences between treatments.

RESULTS AND DISCUSSION

Test for investigating the use of Andaliman extract at different dosages on the diameter of inhibition zone of *S. aureus*bacteria resulted in a insignificantly different effect (P>0,05). Detail of test result is presented in Table 1. Based upon the statistical test result on inhibitory zone, control treatment was observed to have similar value as treatment of Andaliman dosage of 2%, 4%, and 6%. This indicates that Andaliman ability equals the ability of *chloramphenicol*to inhibit *in vitro*bacterial growth. Therefore, Andaliman is potentially used to prevent and treat fish disease caused by *S. aureus*. *Chloramphenicol* is one of antibiotics frequently used in aquaculture. In livestock and fishery industry, *chloramphenicol* has been used for many years as additiveinfeed functioned as medicine to treat fish diseases (Adam, 2002).

Table 1. Test Result of The Zone of Inhibition

No	Treatment (%)	The Diameter of Inhibition Zone (mm)
1	*Chloramphenicol* (Control)	5,07±0,32[a]
2	2%	3,50±1,55[a]
3	4%	4,91±1,06[a]
4	6%	4,05±1,24[a]

Note: Values with the same superscript letter within a column are insignificantly different (P> 0,05).

Table 2. Result of Biochemical Test for *Staphylococcus aureus*

No	Test	*Staphylococcus aureus* *Reaction* confirmed result
1	Coagulase	+
2	Catalase	+
3	Thermostable nuclease production	+
4	Anaerobic fermentation of glucose	+
5	Anaerobic fermentation of mannitol	+

Result of the study also showed that Andaliman dosage of 2% was already able to inhibit the growth of *S. aureus*. It is possible since Andaliman contains flavonoid which is expected to effectively inhibit bacterial growth. It is easier for polar flavonoid to penetrate peptidoglycan layer of microbes and nonpolar lipid layer. The cell walls of *Staphylococus aureus* consist of polysaccharides, that is water-soluble polymers which function to transfer positive ions in and out the cell walls. Cell wall disruption will cause cell lysis, other antimicrobial mechanism of flavonoid is inhibiting the function of cell membrane through the disruption of cell membrane and inhibition of enzyme binding, such as between ATPase and phospholipase (Rijayanti, 2014). Factors affecting the appearance of the zone of inhibition include the diffusion of antimicrobial agent into the media and its interaction with the tested microbes, amount of microbes tested,growth rate of the tested microbes, and sensitivity level of microbes to antimicrobial agents. The zone of inhibition produced by Andaliman dosage of 4% and 6% revealed similar statistical value to the dosage of 2%. Hence, the use of Andaliman at lower dosage was already able to provide excellent result which will further lead to lower cost of application in fish disease prevention and treatment. The parameter tested in this study besides inhibition zone was biochemical test for *S. aureus*. This test was aimed to prove that *S. aureus* cultured in this study might cause fish disease according to its pathogenic characteristics. Detail result of the test is listed in Table 2.

Result of the characteristic of coagulase test identified the bacteria as Staphylococcus aureus for providing positive coagulase response through the presence of clumps in the test tube. Coagulase positive is commonly produced by Staphylococcus aureus, yet coagulase-negative Staphylococcus aureus is also found. Coagulase negative acts as opportunistic pathogen (Yurdakul *et al*., 2013). Coagulase is an enzyme-like protein with ability to coagulate oxalate or citrate plasma by use of a factor in serum. Coagulase-reacting factor in serum reacts with coagulase to produce esterase and blood clotting activity through the same mechanism, that is activating prothrombin to thrombin (Jawetz *et al*. 2001). Phagocytosis process of coagulase-positive Staphylococcus aureus could be reduced through blood clotting reactionwhich is an inhibitory mechanism that might originated from fibrin on the surface of organism. Coagulase enzyme reacts to such complex to convert fibrinogen into fibrin, results in blood clots. Fibrin is also found on the surface of Staphylococcus aureus, which could protect bacteria against cell damage due to cell phagocytosis action. The production of coagulase is related to the invasive pathogenic potential (Prescott and Langsing 1999). Catalase test performed on *Staphylococcus aureus* bacteria showed a positive result. Catalase or peroxidase enzymes play crucial role in microbial survivability. Positive reaction produced by Staphylococcus aureus in this study as observed from the formation of gas bubble in the test tube. Toelle *et al*. (2014) reported that result of catalase positive is indicated by gas bubble (O_2) produced by the genus of Staphylococcus. Furthermore, Staphylococcus spp. uses catalase to protect itself from hydrogen peroxide (H_2O_2) by transforming it to

water and oxygen (Locke *et al*. 2013). Catalase test is useful in the identification of certain groups of bacteria. Result of thermostable nuclease test on S. aureus bacteria provided a positive response with characteristic of pink ring formation. Nuclease is phosphodiesterase enzyme with endonucleolytic and exonucleolytic cleavage activities which could break DNA or RNA. This enzyme is composed of single polypeptide chain with compact globular structure and found inside, on, or around the cell surface of Staphylococcus aureus. The structure of this enzyme will change due to heating at65ºC, yet it is a reversible action, means that enzyme will return to its original structure when temperature is rapidly decreasing (Joklik *et al*., 1992). Nuclease enzyme could break down nucleic acid (Prescott and Langsing, 1999). The presence of this enzyme allows S. aureusto attack intracellular part of fish and even cause nucleic acid lysis. If fish immune system could not defend and resist this attack, clinical sign like wound and other diseases might occur. Observation result of carbohydrate fermentation showed that Staphylococcus aureus positively fermented Mannitol and Glucose. Lay (1994) confirmed that changes in the media occurred since bacteria were able to ferment carbohydrate to produce acid, thus reduced pH which eventually changed the color of indicator. The ability of microbes to ferment carbohydrate varies along with the various products of bio-oxidation in carbohydrate fermentation. Yanti and Dali (2013) mentioned that acid production from carbohydrate could occur in both aerobic and anaerobic conditions. The characteristic of carbohydrate fermentation is often used to differentiate bacterial species within a certain genus for the purpose of identification.

CONCLUSION

The results showed that Andaliman extract at doses of 2%, 4% and 6% had the same ability (P>0.05) as Chloramphenicol in inhibiting antimicrobial growth. Based on that, Andaliman extract can be used as an ingredient to prevent or treat diseases in fish caused by *S. aureus.*

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