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ANTIBACTERIAL ACTIVITIES OF CHITOSAN IN HARUAN FISH SCALES (*Channa striata)* TO THE GROWTH OF *Staphylococcus aureus*

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INTRODUCTION

Haruan fish *(Channa striata)* is a freshwater fish that can be found in all Indonesian waters. This fish has been utilized as a medicine which is associated with the benefits of albumin content for health sector, especially in assisting wound healing process[1]. High public consumption of haruan fish results in the increase of fish scales waste as well. However, public has yet known about the contents and the benefits of scented fish scales thus the disposal of this scented fish waste is inevitable. Even if the community can manage the waste of fish scales, it will become more valuable in improving the economy of South Kalimantan people as well as the welfare in health sector. According to Ifa (2018) study upon red snapper scales and Aziz (2017) on milkfish scales, it was revealed that freshwater and marine fish scales generally contain chitin. The chitin element can be used to make antibacterial agent after being processed into chitosan[2].

Chitosan is a natural biopolymer with the second-largest abundance after cellulose, a chitin deacetylation product, both produced from a chemical reaction and enzymatic reactions[3]. Chitosan is not toxic. It contains an amino polysaccharide group which can inhibit microbial growth[4,5]. It is one of the natural polymers used

frequently in the medical field which possess biocompatible and biodegradable properties[6].

These days, periodontal disease is one of dental and oral health problem in Indonesia that has a fairly high prevalence of around 96.58% for all ages[7]. It is considered as the number two disease in the world after caries[8]. Periodontal disease is generally induced by plaque, which then in contact with the gingival margin, causing an infection that triggers inflammation[6]. Plaque begins with the formation of pellicles on tooth surface derived from salivary glycoprotein which is followed by initial colonization formation characterized by the attachment of facultative gram-positive bacteria such as *Actinomyces viscosus (A.viscosus)* and *Streptococcus sanguis (S.sanguis)* on pellicle surface. Secondary colonization was formed by gram-negative bacteria *Fusobacterium nucleatum* and *Porphiromonas gingivalis* which were attached to previous bacterial cells, then the plaque maturation occurred. Any shifting in ecological balance will generate the pathogenicity from various strains of oral opportunistic microflora[9]. *Staphylococcus aureus (S.aureus)* is one of the gram-positive bacteria in the normal opportunistic flora which will become pathogenic if there is an imbalance or an increase in the number of colonies in the oral cavity[10]. Its adhesion to glycoproteins will be produced from previous plaque colonizing bacteria to form pathogenic colonies[11].

Due to its pathogenic ability, it is necessary to overcome the infection of *S.aureus*. Nonetheless, infection by these bacteria is difficult to manage because it is resistant to conventional antibacterial drugs, so new natural antibacterial agents are required in the hope of overcoming the infections and antibacterial resistance[12]. One of the natural antibacterial ingredients consumed by many people is haruan fish *(Channa striata)*, in which the scales of haruan fish can be processed into chitosan which is a natural antibacterial ingredient.

According to Goy et al. (2016), commercial chitosan from shrimp skin can inhibit the growth of *S.aureus* and *E.coli* bacteria in a varied concentration of 0%; 0.5%; 1%; 1.5%; and 2% chitosan where the Minimum Inhibitory Concentration (MIC) was observed at a concentration of 1.5%. Whereas Damayanti et al.(2016) reported that chitosan from shrimp skin can reduce the attachment of *E. coli bacteria*, *Bacillus subtilis* and *S.aureus* at various concentration of 1%; 2%; and 3% chitosan where the minimum inhibitory activity was found at a concentration of 1% and obtained a inhibitory zone of 14.6 mm; 5 mm; and 13.17 mm.

Based on the description above, it is known that chitosan from shrimp skin has antibacterial activity against gram-positive bacteria yet no research on chitosan from scented fish scales was issued. Thus, the authors wanted to examine the antibacterial activity of chitosan scales of fish *(Channa striata)* against *S.aureus* growth so that later it can be used to manage infectious diseases and the resistance of *S.aureus* bacteria.

MATERIALS AND METHODS

This study used a randomized pretest-posttest with control group design method. The population in this study was *Staphylococcus aureus* ATCC 6538 isolates.

The Manufacture of Chitosan from Fish Scales

Collection of haruan fish scales. The procedure of this study was initiated with the manufacture of chitosan. The scales was obtained from the spot cleaning industry for crackers manufacture which were brought using ice boxes filled with ice cubes. The scales was then cleaned and dried that soon the scales were mashed up using a blender until it becomes powder.

Chitin Isolation

Deproteination. Deproteination was done to eliminate the protein content in the scale. The fish scales that have been pulverized were put into a glass beaker which then boiled with 4% NaOH temperature of 80^0C for 1 hour. Further, the pH was neutralized and the isolate was dried using an oven.

Demineralization. The procedure was preceded to demineralization process to eliminate mineral content within fish scales. The dried scales powder was put into a 1% HCl solution in a 1:4 for powder-HCl ratio inside the beaker glass and soaked for 24 hours. The mixture was then neutralized using distilled water and dried in an oven.

Chitin Test. The fish scale powder containing chitin was tested first to detect the presence of chitin within the scales using Van Wesselink color reaction. Haruan fish scales powder was reacted with I2-KI 1% solution to stimulate changes on powder color to brown. Any changes in powder color to violet after the addition of pure H_2SO_4 powder demonstrating a positive result of chitin.

The Making of Chitosan

Deacetylation. The next procedure was deacetylation for chitosan production. Chitin powder of haruan scales was mixed with 50% NaOH then boiled at 80°C temperature for 2 hours on a hot plate. After the chitin scales of haruan fish obtained neutralized pH, the sample was then dried using an oven.

Antibacterial Test

Manufacturing Chitosan Concentration. After the chitosan powder was obtained,

antibacterial testing was subsequently conducted. The first stage was preparing chitosan concentrations which followed by the moving of bacterial cultures into a vacuum tube. Chitosan which had been diluted was added according to the concentration required. Positive control was comprised of 0.2% Chlorhexidine while negative control was 1% acetic acid. The mixtures were covered with sterile cotton and homogenized with a vortex mixer.

Measurement of Minimum Inhibitory Concentration (MIC). Minimum Inhibition Concentration (MIC) was measured in two stages, before and after incubation, to see the difference in absorbance. Chitosan which was with already homogeneous bacterial culture was then measured for the absorbance before 24 hours incubation using the spectrophotometer Biobase B-KD 560 UV-Vis with a wavelength of 450 nm. Furthermore, the sample was incubated into an incubator in anaerobic condition for 24 hours at 37°C. And then proceed for absorbance evaluation using spectrophotometer Biobasve BKD-560 UV-Vis with a wavelength of 450 nm.

Minimum Bactericidal Concentration (MBC). After the MIC from concentration of chitosan was obtained, a test to evaluate Minimum Bactericidal Concentration (MBC) was performed. As much as 5μL research samples with a concentration of 1.5%; 2%; 2.5%; 3%; 3.5%; 4%, 0.2% chlorhexidine and 1% acetic acid was transferred to NA using a micropipette which had been sterilized with alcohol, then flattened. The transferred samples were incubated into incubator in an aerobic state for 24 hours at 37°C. The results were then read and Minimum Bactericidal Concentration (MBC) was determined using colony counter .

RESULTS

Minimum Inhibitory Concentration (MIC) test result of chitosan scales *(Channa striata)* on the growth of *S.aureus* were obtained from measuring the absorbance using spectrophotometry Biobase B-KD 560 UV-Vis as presented in Table 1.

Table 1. Minimum Inhibitory Concentration (MIC) test result of chitosan scales *(Channa striata)* on the growth of *Staphylococcus aureus*

Concentration	N	incubation (0 hours)	incubation 24 hours	Difference	Information
KSIH 0.5%	3	0.207	0.221	0.014	Up
KSIH 1%	3	0.220	0.226	0.006	Up
KSIH 1.5%	3	0.235	0.225	-0.01	Down
KSIH 2%	3	0.283	0.237	-0.046	Down
KSIH 2.5%	3	1.051	0.336	-0.715	Down
KSIH 3%	3	1.088	0.425	-0.663	Down
KSIH 3.5%	3	1.164	0.434	-0.73	Down
KSIH 4%	3	1.190	0.485	-0.705	Down
KLR 0,2%	3	1.798	1.387	-0.411	Down
AA1%	3	0.235	0.329	0.094	Up

Information :
KSIH 0.5% : Chitosan Haruan Fish Scales *(Channa striata)* 0.5%
KSIH 1% : Chitosan Fish Scales Haruan *(Channa striata)* 1%
KSIH 1.5% : Chitosan Haruan Fish Scales *(Channa striata)* 1.5%
KSIH 2% : Chitosan Fish Scales Haruan *(Channa striata)* 2%
KLR 0.2% : 0.2% chlorhexidine gluconate
AA 1% : 1% acetic acid
N : Number of repetitions

After the result of the Minimum Inhibitory Concentration (MIC) was obtained, the measurement of Minimum Bactericidal Concentration (MBC) was performed. The following results of MIC was obtained.

In table 1, it can be observed that there are differences in the absorbance results of each chitosan concentration on the growth of *S.aureus*. The statement "Up" shows the increase of *S.aureus* bacteria growth which means that there is no antibacterial activity of chitosan existed in inhibiting the growth of *S.aureus*. The "Down" description shows a decrease in the growth of *S.aureus* bacteria which means that chitosan possesses antibacterial activity to inhibit the growth of *S.aureus*. In the absorbance difference column, an increase in the growth of *S.aureus* bacteria is written with a positive number, whereas a decrease in the growth of *S.aureus* bacteria is written with a negative number. Table 1 shows that the concentrations of 0.5% chitosan and 1% still cannot inhibit *S.aureus* growth while 1.5%; 2%; 2.5%; 3%; 3.5%; 4% chitosan concentration and 0.2% chlorhexidine gluconate already inhibited the growth of *S.aureus*. Based on this result, the concentration of 1.5% was determined as the Minimum Inhibitory Concentration (MIC) of

chitosan from haruan fish scales *(Channa striata)* on the growth of *S.aureus*.

Data which has been obtained from each concentration was tabulated and normality test was performed using Shapiro Wilk under the condition where the data was less than 50 ($p<50$). The results of the normality test for Minimum Inhibitory Concentration (MIC) of 0.5%; 1%; 1.5%; 2%; 2.5%; 3%; 3.5%; 4% chitosan, 0.2% Chlorhexidine gluconate and 1% acetic acid on the growth of *S.aureus* obtained $p> 0.05$ so that it was acquired that data was normally distributed. The data from chitosan scales *(Channa striata)* performed homogeneity tests using Levene's test. The obtained p value is equal to 0.001 (sig <0.05) which indicates that the data variance is not homogeneous. Notwithstanding the non-homogenous data variance, some studies mentioned that data homogeneity is not an absolute requirement for conducting a parametric analysis of One Way ANOVA with a confidence level of 95%. One Way ANOVA test obtained p value equal to 0,000 ($p <0.05$) which represents significant differences among treatment groups. The data analysis was then continued with Post Hoc Dunnet test to find out which group presented a significant difference. According to the Post-Hoc Dunnet analysis, chitosan at the concentration of 0.5%; 1%; 1.5%; 2%; 2.5%; 3%; 3.5%; 4%; 0,2% chlorhexidine gluconate ; and 1% acetic acid showed a difference in inhibiting activity on the growth of *Staphylococcus aureus*.

After the Minimum Inhibitory Concentration (MIC) was obtained, the measurement of Minimum Bactericidal Concentration (MBC) was performed and following results was obtained as presented in the table 2.

Table 2. The mean value of Minimum Bactericidal Concentration (MBC) test result on the growth of *Staphylococcus aureus*

Concentration	N	Mean (CFU/μL)	Std. Deviation
KSIH 1.5%	3	688.67	89.226
KSIH 2%	3	316.00	7.211
KSIH 2.5%	3	120.00	18.083
KSIH 3%	3	48.00	6.083
KSIH 3.5%	3	0	-
KSIH 4%	3	0	-
KLR 0,2%	3	0	-
AA 1%	3	1685.00	64.583

Based on table 5.2, it can be seen that chitosan at the concentration of 1.5%; 2%; 2.5%; and 3% have been able to inhibit *S.aureus* growth yet unable to kill the colony due to its growth. Moreover, chitosan at the concentration of 3.5% and 4% and 0.2% Chlorhexidine gluconate demonstrated bactericidal activity towards *S.aureus* thus 3.5% concentration of chitosan was determined as the Minimum Bactericidal Concentration (MBC) on *S.aureus* growth. The normality test results from the Minimum Bactericidal Concentration (MBC) 1.5%; 2%; 2.5%; 3%; 3.5%; 4%; 0,2% Chlorhexidine gluconate; and 1% acetic acid obtained $p>0.05$ which represent normal data distribution. Data from chitosan scales (*Channa striata*) was evaluated for variant's homogeneity using Levene's test. The data was resulted in non-homogenous category yet ANOVA tests could still be performed where several study mentioned its immunity to non-homogeneous data. The test continued with One-way ANOVA parametric analysis with a confidence level of 95%. The results of One-way ANOVA test obtained $p = 0,000$ ($p<0.05$) which showed a significant difference among the treatments. Data analysis was continued with Post Hoc LSD test to determine which groups presented with significant differences. According to Post-Hoc Dunnet analysis, chitosan at the concentration of 0.5%; 1%; 1.5%; 2%; 2.5%; 3%; 3.5%; 4%; 0,2% Chlorhexidine gluconate; and 1% acetic acid showed differences in bactericidal activity on the growth of *Staphylococcus aureus*.

DISCUSSION

Based on the above results, it was found that chitosan at 0.5% and 1% concentration possess no inhibitory activity against the growth of *S.aureus*. This is because *S.aureus* contains polysaccharides and antigenic proteins as the important substances in cell walls structure with thick peptidoglycan layers. Peptidoglycan is a polysaccharide polymer that contains incorporated subunits and is an exoskeleton that that maintains the rigidity of cell walls[10,13]. Chitosan at the concentrations of 0.5% and 1% still showed low antibacterial activity that it cannot penetrate bacterial cell walls. These concentrations resulted in no inhibitory ability towards the growth of *S.aureus*.

Chitosan at 2%; 2.5%; and 3% concentration demonstrated inhibitory activity on the growth of *S.aureus* which is depicted by the decrease in absorbance value. Hence, chitosan at the concentration of 1.5% was determined as the Minimum Inhibitory Concentration (MIC) of chitosan from Haruan scales *(Channa striata)* on the growth of *S.aureus*. This is because chitosan contains a free amino group (NH_2^+) which is positively charged so that it can bind to other compounds that possess a negative charge. The

surface of gram-positive bacteria generally has a negatively-charged teichoic acid[13]. Positive charges on chitosan will interact with the negative charge of bacteria which then absorbed to form a kind of layer that inhibits the channel transformation of bacterial cells so that bacterial cells lack of the substance to develop. This interaction is expected to disrupt the formation of peptidoglycan. The disruption will lead to the absence of cell wall layer presenting cell in sturdy condition that is easy to experience lysis as the results of bacterial metabolic activity inhibition[14,15].

Chitosan at the concentration of 3,5% and 4% demonstrated bactericidal activity against *S.aureus* bacteria. This can be seen from the absence of *S.aureus* colonies growth on the surface of NA therefore 3.5% concentration of chitosan was determined as the Minimum Bactericidal Concentration (MBC) for the growth of *S.aureus*. It is explained by Broek et al (2015) who mentioned that chitosan has an amine group which shows antimicrobial properties against bacteria. The antibacterial mechanism is through several paths, namely by interfering bacterial mechanism and preventing the occurrence of RNA transcription. The polycationic nature of chitosan interferes with bacterial metabolism using negative charges (electrostatic) on the surface of bacterial cells. In addition, low molecular weight in chitosan can generate the entry of this compound to cell nucleus (adsorption on DNA molecules), thus preventing the occurrence of RNA transcription from DNA[16]. According to Killay (2013), chitosan has a very strong affinity in binding to bacterial DNA, which can disrupt mRNA activity and protein synthesis. This process will result in bacterial growth inhibition and microbial death.

Chitosan at the concentration of 3,5% and 4% possessed bactericidal activity that it is able to kill bacteria[17]. The concentration of chitosan 1% and 0.1%, according to previous research by Damayanti et al (2016) and Yeul and Rayalu (2013), it was found that chitosan generally showed a strong bactericidal effect for gram-positive bacteria *(Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus Plantarum, L. Brevis, and L. bulgaris)* than gram-negative bacteria *(E. coli, Pseudomonas fluorescens, Salmonella Typhimurium, and Vibrio parahaemolyticus)*[16].

In the MIC test of this study, it is obtained that the results were not linear as it can be seen in Table 1. The 2.5% concentration of chitosan decreased the rate of bacterial growth by 0.715; 3% concentration of chitosan decreased the growth rate of bacteria by 0.663, 3.5% concentration of chitosan decreased the growth rate of bacteria by 0.73, and 4% concentration of chitosan decreased the growth rate of bacteria by 0.705. According to a research by Wuon et al (2018) and Warokka et al (2016), this result is caused by the contamination persisted in the serial dilution that dead bacteria can also be observed from the evaluation using Spectrophotometry UV-Vis.

A large standard deviation was obtained in this study which might occur due to several factors. Several procedures such as inoculating bacteria on the surface of media or carrying out serial dilution later than it ought to be may allow bacterial growth on media surface. This will result in exceeded result of bacterial colony calculation using colony counter which should be around 30-300 colonies per plate[18]. According to Breed and Dotterrer (1916), data that exceeds the calculation requirements will affect the results of statistical calculations. It is based on the description above that the standard deviation value in this study was increase. Lastly, it can be concluded that there are differences in the antibacterial activity of haruan scales *(Channa striata)* chitosan at the concentration of 0.5%; 1%; 1.5%; 2%; 2.5%; 3%; 3.5%; 4% on the growth of *Staphylococcus aureus*.

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