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Research Article

The Antibacterial Activity of Chitosan from Haruan (*Channa striata*) Fish Scales on the Growth of *Streptococcus sanguinis*

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KEYWORDS

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ABSTRACT

Introduction: Recurrent Aphthous Stomatitis (RAS) is the most common oral lesion in Banjarmasin, with a prevalence of 45.42%. *Streptococcus sanguinis* (*S. sanguinis*) is thought to be one of the factors causing RAS. One natural ingredient that is often used by the people of South Kalimantan is the Haruan fish. The high Haruan fish consumption will eventually leave fish scales that have the potential to pollute the environment if not addressed immediately. Fish scales contain chitin, which when deacetylated produces chitosan, which has antibacterial properties. **Objectives**: The purpose of this study was to determine the effectiveness of the chitosan from the scales of the Haruan fish (*Channa striata*) at inhibiting *S. sanguinis* growth. **Methods**: This study used a randomized pretest-posttest with control group design using five treatments. The five treatments were subjected to liquid dilution using the UV-Vis Spectrophotometer method to obtain minimum inhibitory concentration (MIC) and a solid dilution test using the Total Plate Count method to obtain minimum bactericidal concentration (MBC). **Results**: The results showed that Haruan scale chitosan proved to be effective as an antibacterial against *S. sanguinis*, with a MIC of 1.25% and a minimum lethal concentration of 2.5%. One-Way Anova test results showed significance for the MIC test (p = 0,000) and MBC test (p = 0.000; p < 0.05). **Conclusion**: Chitosan from Haruan fish scales is both inhibitory and lethal to *S. sanguinis.*

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INTRODUCTION

The people of South Kalimantan like to use natural ingredients as traditional medicines. One ingredient that is often used by the people of South Kalimantan is the Haruan fish.[1] The Haruan fish is known to accelerate wound healing and possesses anti-inflammatory properties. In addition to medical treatment, Haruan fish (*Channa striata)* is also a staple food for the people of South Kalimantan.[2]

Budirahardjo (2010) found that fish scales contain several important compounds, one of which is chitin.[3] Faridah et al. (2012) also found that chitin is present in cells under the dermis layer of fish scales.[4] Chitin that is deacetylated either chemically or enzymatically will produce chitosan. Chitosan has special properties in terms of biocompatibility, biodegradation, and biological activity; it is non-toxic and does not cause allergies.[5]

Chitosan has the potential to be used as an antibacterial material because it contains an amino polysaccharide group that can inhibit bacterial growth.[6] Liu (2004) argued that chitosan compounds can kill bacteria by damaging cell membranes.[7]

According to Putri et al. (2020), chitosan from Haruan fish scales has a deacetylation degree (DD) of 85.25%.[8] Mursida et al. (2018) stated that that the purity of chitosan is determined by the degree of deacetylation (DD), the more acetyl groups that can be removed and replaced by amino groups, the higher the value of the degree of deacetylation.[9]

Kim et al. (2018) said that the nature of chitosan polycation in acidic conditions made it able to react with negative molecules such as the carbohydrates, fats, and proteins that make up bacterial cell walls. Thus, chitosan with a higher deacetylation degree is more effective at bacterial inhibition.[10]

According to Aliasghari et al. (2016), blue crab chitosan at concentrations of 1.25%, 2.5%, and 5% can inhibit the growth of *Streptococcus sanguinis (S. sanguinis)* in vitro, with its minimum inhibitory power at a concentration of 1.25%.[11]

Based on the description above, it can be seen that chitosan from the blue crab displays antibacterial activity against *S. sanguinis*. However, no one has examined the antibacterial activity of chitosan from Haruan fish scales (*Channa striata*) against the growth of *S. sanguinis*. Therefore, this research needed to be done to determine the antibacterial activity of chitosan from Haruan fish scales against *S. sanguinis* growth.

MATERIALS AND METHODS

Making Chitosan from Haruan Fish Scales (*Channa striata*)

Fresh scales from the waste industry of Banjarmasin City cracker fish were collected and put into iceboxes, then cleaned with clean tap water. As much as 3 kilograms of Haruan fish scales that have been cleaned were dried in an oven for 24 hours at 50°C. The scales were crushed by blending to powder and stored in an airtight container.[12, 13]

Deproteination: Haruan fish scales powder was placed in a measuring cup and soaked in a boiling sodium hydroxide (NaOH) (4% m/v) for 1 hour to dissolve protein and sugar. After the sample was boiled in sodium hydroxide, the beaker contains a sample of Haruan scales powder cooled at room temperature. Washed samples using distilled water to neutralize pH. Next, the sample was filtered and put into the oven to dry for 24 hours at 50°C. [13, 14]

Demineralization: Samples were demineralized with 1% HCl, a ratio of sample and HCl is 1:4 and immersion time is 24 hours to remove minerals (especially calcium carbonate). Samples were filtered and washed using distilled water to neutralize pH. The sample after washing was filtered back to get chitin. Next, the sample was dried in the oven for 24 hours with a temperature of 50°C. [13, 15]

Chitin Test: To detect the presence of chitin in the sample, the Van Wesslink color reaction were used. Chitin was reacted with a 1% iodine-potassium iodide (I_2-KI) solution which will give a brownish yellow color, then 1 M sulfuric acid (H_2SO_4) was added to turn into purplish-red or red-violet. The color changed from brownish yellow to purplish-red showed a positive reaction to chitin.[16]

Deacetylation: The deacetylation process was carried out by added 50% NaOH to the chitin sample and boiled it on the hot plate for 2 hours at 100°C. The sample was then cooled to room temperature and filtered and then washed with the remaining 50% NaOH. Then, the samples were washed with distilled water to a neutral pH and filtered to maintain solids, namely chitosan. The sample was then dried in the oven for 24 hours at 50°C. Chitosan obtained from the results of deacetylation in the form of creamy white powder. [13, 17]

This study used a true experimental design with a randomized pretest-posttest with control group design using three treatments and two controls.

Treatment 1: 1.25% chitosan from Haruan (*Channa striata*) fish scales (CFH) on *S. sanguinis* growth.
Treatment 2: 2.5% chitosan from Haruan (*Channa striata*) fish scales (CFH) on *S. sanguinis* growth
Treatment 3: 5% chitosan from Haruan (*Channa striata*) fish scales (CFH) on *S. sanguinis* growth.
Positive control: 0.2% chlorhexidine gluconate on *S. sanguinis* growth.
Negative control: 1% acetic acid on *S. sanguinis* growth.

Bacterial Culture

A culture of *S. sanguinis* (ATTC 10556) was inoculated into 30 mL Brain Heart Infusion Broth (BHI-B) (NutriSelect™ Plus, Darmstadt, Germany), incubated at 2×24 hours at 37°C under anaerobic conditions with an anaerobic jar. The suspension was added with sterile distilled water until the turbidity was comparable to the McFarland 0.5 standard.

Determine MIC and MBC of Chitosan Haruan Scales (*Channa striata*) Against *Streptococcus sanguinis*

Five hundred milligrams of chitosan powder of Haruan fish scales are dissolved in 5 mL of 1% acetic acid. Chitosan solution which has been diluted according to the concentration made each 1 mL, positive control of 1 mL chlorhexidine 0.2%, and negative control of 1 mL 1% acetic acid, added to the culture of *S. sanguinis* bacteria, covered with sterile cotton, then homogenized with vortex mixer, then the absorbance value was measured before incubation by using the Biobase BK-D560 UV-Vis Spectrophotometer at 450 nm wavelength (Biobase Biodustry Co., Ltd, Qingdao, China) to obtain the initial absorbance value. After that, all samples were incubated at 37°C for 24 hours under anaerobic conditions. The chitosan solution of Haruan fish scales and the incubated *Streptococcus sanguinis* bacteria were measured again using the Biobase BK-D560 UV-Vis Spectrophotometer at 450 nm wavelength (Biobase Biodustry Co., Ltd, Qingdao, China) to determine the MIC. After determining the MIC, 5 µL of the corresponding bacterial suspension was spread on Nutrient Agar (NA) (Merck KGaA, Darmstadt, Germany) with micro pipets. It was incubated at 37°C for 24 h under anaerobic condition. The numbers of colonies growing from each of the test tubes were counted and the absence of colony growth in NA was recorded as the confirmation for MBC. All experiments were conducted in quintuplicate for each concentration.

Statistical Analysis

The data obtained were then analyzed using the *Saphiro-Wilk* normality test, the homogeneity test of *Levene's Test*, *One Way Anova Test*, and the *Dunnet T3 Post Hoc test*.

RESULT

The results of the MIC and MBC normality tests using the Shapiro-Wilk process show that all treatment groups have a value of $p > 0.05$, which means that the data is normally distributed. The results of Levene's test for homogeneity show a value of $p < 0.05$, which means that the data is not homogeneous. Data obtained was normally distributed followed by a parametric analysis using One-Way Anova testing with a confidence level of 95%. The results of the One-Way Anova parametric analysis showed $p = 0{,}000$ ($p < 0.05$), which showed a significant difference between each treatment group. The administration of Haruan chitosan scales at concentrations of 1.25%, 2.5%, 5%; the 0.2% chlorhexidine gluconate positive control; and the 1% acetic acid negative control had different effects on the inhibition and growth of *S. sanguinis* colonies. Next, the Dunnett T3 Post Hoc test was conducted to find any significant differences between groups. The results of the Post Hoc Dunnett T3 test showed that each treatment group had significant differences ($p < 0.05$) when examining for MIC against *S. sanguinis* bacteria in Haruan scale chitosan at concentrations of 1.25%, 2.5%, and 5%; 0.2% chlorhexidine gluconate; and 1% acetic acid. The results of the Post Hoc Dunnett T3 test also showed that not every treatment group had a significant difference when testing compared to that of 0.2% chlorhexidine gluconate and 1% acetic acid.

The average results of MIC that can be seen as absorbance value of Haruan fish scale chitosan on *S. sanguinis* growth can be seen in Table 1. The average results of the MBC value of the chitosan on *S. sanguinis* growth can be seen in Diagram 1. As seen in Table 1, the average results of the MIC test using a UV-Vis spectrophotometer showed that Haruan scale chitosan concentrated at 1.25%, 2.5%, and 5% was able to inhibit the growth of *S. sanguinis* bacteria. At a concentration of 1.25%, it was seen that there had been a decrease in absorbance, which showed that there had been inhibition of bacterial growth. A concentration of 5% chitosan was the most effective at inhibiting the growth of *S. sanguinis*. Table 1 also shows that in the negative control, 1% acetic acid was not able to inhibit the growth of *S. sanguinis* bacteria.

In Figure 1, showed that the average MBC test using colony counting, results found that a chitosan concentration of 2.5% is the smallest concentration that can kill *S. sanguinis* bacteria (MBC), as characterized by

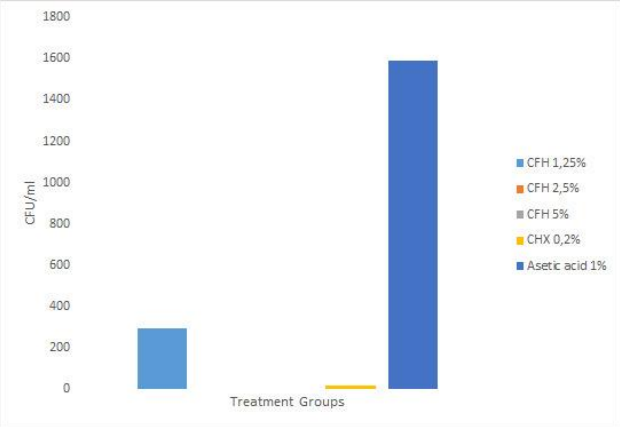

Figure 1. Results of the Colony Counting for chitosan from Haruan fish scales against the growth of *S. sanguinis*

Table 1. Results of Spectrophotometry Absorbance for chitosan from Haruan fish scales against the growth of *S. sanguinis.*

Sample	0 hour	24 hours	Difference
CFH 1.25%	0.303	0.219	-0.084*
CFH 2.5%	0.405	0.236	-0.169*
CFH 5%	0.618	0.255	-0.363*
CHX 0.2%	1.179	0.370	-0.809*
Acetic acid 1%	0.265	0.292	0.027

* : Able to inhibit *S. sanguinis*

the absence of bacterial on petri dishes. The 2.5% chitosan concentration was still more effective at killing *S. sanguinis* bacteria compared to the positive control, i.e., 0.2% chlorhexidine gluconate. One percent of acetic acid as negative control did not kill *S. sanguinis* bacteria.

DISCUSSION

Chitosan from the scales of Haruan fish (*Channa striata*) showed antibacterial effect on *S. sanguinis* growth with concentrations of 1.25%, 2.5%, and 5%, characterized by a decrease in absorbance after incubation, whereas concentrations of 2.5% and 5% were capable of killing *S. sanguinis* bacteria, as indicated by the absence of *S. sanguinis* colonies on nutrient agar media after incubation.

The results showed that a 1.25% chitosan concentration decreased the absorbance value, which means it could inhibit the growth of *S. sanguinis* bacteria. Warokka et al. (2016) stated in their study that higher concentration of extracts used will result with lower bacterial growth activity, the greater inhibition could be seen because the smaller clusters of bacteria absorbed less light at certain wavelengths.[18] This is what was seen in the chitosan from Haruan fish scales. The chitosan concentration of 2.5% and 5% has the same effectiveness and both are superior to chitosan 1.25% at inhibiting the growth of *S. sanguinis*. Thus, the effectiveness of Haruan chitosan in inhibiting and killing *S. sanguinis* bacteria is influenced by its concentration. This echoes the research conducted by Aliasghari et al. (2016), which also found that greater concentration of chitosan showed, higher ability to inhibit and kill bacteria. This occurs because of the difference in the number of amine groups ($-NH_2$), which are positively charged on chitosan and will bind to the surface of the negatively charged bacterial cell and making it more effective at inhibiting growth and killing bacteria.[11]

Gram-positive bacteria, such as *S. sanguinis* have a negative charge due to the presence of teichoic acid and contain repeat-chain glycerol phosphate and ribitol phosphate. This negative charge attracts a protonated amino group from the chitosan layer, which can interact with the surface of the bacterial cell wall.[19]

The ability of chitosan to inhibit bacterial growth is due to the electrostatic interactions that occur between chitosan and Gram-positive bacteria such as *S. sanguinis*. The electrostatic interaction is due to the chitosan chain, which is positively charged, being attracted to the negatively charged teichoic acid on the surface of the bacterial cell wall. This chain will bind to more than one bacterial cell and form a bridge between cells, which will eventually create a mass of clots (flocs) that cannot attach to a surface.[20] According to Tan et al. (2013), the inhibition of bacterial growth by chitosan occurs because chitosan has a free amino group that is protonated to become polycationic in an acidic atmosphere, so that chitosan polysaccharide is positively charged. This natural polycation will compete with Mg^{2+} ions to interact with the anionic group in the cell wall. This interaction forms an impermeable layer around bacterial cells. The impermeable layer will block the transport of molecules needed by bacterial cells so that they cannot metabolize, and eventually, bacterial growth is disrupted.[21]

The mechanism used by chitosan in shutting down the growth of *S. sanguinis* bacteria is caused by the interaction of the positively charged NH_2+ amino group with the negatively charged OH- carboxylic group of bacterial cell membranes, forming an electrostatic bond which makes the permeability of the bacterial cell membrane unstable and results in the leakage of bacterial intracellular constituents such as K+ ions, proteins, nucleic acids, and glucose, which will eventually cause

bacterial cell death.[22] Chitosan can also penetrate into the bacterial nucleus and bind to DNA to inhibit the DNA transcription process, RNA synthesis, and protein synthesis.[19,23]

The NH_2, hydroxyl, and H_2O groups in the chitosan chain are also known to have the ability to bind to essential metals such as Ca^{2+} and Mg^{2+} to form a complex. This results in teichoic acid in the *S. sanguinis* cell wall being unable to bind Ca^{2+} and Mg^{2+}, disrupting the molecular transfer process into the cell.[19, 24] Adhesin production, toxin production, and bacterial growth are inhibited because the bacteria are unable to uptake the necessary metal ions from the environment.[24] Adhesin molecules play a role in binding to receptors and producing strong, irreversible adhesion between bacteria and epithelial cells in the oral cavity. If adhesin production is inhibited, the attachment of bacteria to epithelial cells will also be inhibited.[25] *S. sanguinis* can attach to epithelial cells (initial adhesion) through lectins on the cell surface, which will bind specifically to carbohydrates in epithelial cells.[26] NH_2 molecules in chitosan cause this initial adhesion to fail so that RAS infections can be quickly cured.

This study used chlorhexidine gluconate 0.2% as a positive control, which showed different activities at different concentrations. At high concentrations (>2%) chlorhexidine is bactericidal, while at low concentrations (0.2%) it is bacteriostatic.[27,28] Chlorhexidine can cause changes in the permeability of bacterial cell membranes such that the cell cytoplasm and cell components are expelled, resulting in cell death.[29]

In this study, chitosan from Haruan scales was superior at concentrations of 2.5% and 5% compared to the positive control of 0.2% chlorhexidine gluconate due to the antibacterial content found in chitosan from the scales of Haruan fish, such as amine groups which have various synergistic mechanisms. The mechanisms described above make chitosan bactericidal; it is effective at inhibiting and killing *S. sanguinis* bacteria at relatively low concentrations, while chlorhexidine gluconate is bactericidal at higher concentrations (> 2%).

Haruan fish scales have the potential to be used as an alternative medicine made from natural ingredients because they contain antibacterial compounds that can inhibit bacterial growth, kill the *S. sanguinis* bacteria that cause RAS, and are biocompatible with mucosal tissue so that they can improve the quality of the patient's oral health.

The limitation of this study is that UV-Vis spectrophotometry is less selective for distinguishing research samples from other particles or contaminants that absorb light of the same wavelength.

CONCLUSION

According to the results of this study, chitosan from Haruan (*Channa striata*) fish scales is an effective inhibitor of the growth of *S. sanguinis*. The minimum inhibitory dosage was found to be at a concentration of 1.25% and the minimum lethal dosage at a concentration of 2.5%. The author hopes that there will be further research on derivative compounds from Haruan (*Channa striata*) chitosan fish scales that can be more specific to the growth of *S. sanguinis.*

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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