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ANTIBACTERIAL ACTIVITY OF PAPUYU FISH SCALE CHITOSAN (*Anabas testudineus*) AGAINST *Staphylococcus aureus*

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ABSTRACT
Backgrounds*: Pulp disease due to caries can be treated through endodontic treatment, which aims to eliminate bacteria in the root canal so microorganisms cannot multiply. Staphylococcus aureus is a resistant microorganism causing root canal treatment failure. Chlorhexidine as a gold standard cannot remove biofilm and other organic debris. Utilization of nature, chitosan of papuyu fish scales (Anabas testudineus) has antibacterial properties that can be an alternative root canal irrigation material.* ***Purpose:*** *Analyze the antibacterial activity of chitosan of papuyu fish scales against Staphylococcus aureus based on the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).* ***Methods:*** *This research is a true experimental design with posttest only with control group design. The research groups were chitosan of Papuyu fish scales with concentrations of 25%, 50%, 75%, 100%, chlorhexidine gluconate 2% (positive control), and aquadest (negative control) with 6 repetitions.* ***Results:*** *The MIC value in the One-Way ANOVA test showed a significant difference (p<0.05). Data analysis followed by Post Hoc Games-Howell test showed all treatment groups had significant differences between each other (p<0.05). The MBC value in the Kruskal Wallis test shows that there is a significant difference (p<0.05). Data analysis followed by MannWhitney test showed significant differences between each other (p<0.05) except for the group of papuyu fish scale chitosan 25% concentration against papuyu fish scale chitosan 50% concentration, papuyu fish scale chitosan 100% concentration against chlorhexidine gluconate 2% (p>0.05).* ***Conclusion:*** *There is a difference in the antibacterial activity of chitosan of Papuyu fish scales concentrations of 25%, 50%, 75%, 100%, chlorhexidine gluconate 2% (positive control) and aquadest (negative control) against Staphylococcus aureus bacteria.*

Keywords: *Chitosan of Papuyu Fish Scales, MBC, MIC, Staphylococcus Aureus.*
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INTRODUCTION

Untreated caries can cause bacteria to penetrate the pulp, root canal and periodontal tissue, resulting in pulp disease.[1,2] Pulp disease can be treated through endodontic treatment which aims to eliminate bacteria in the root canal so that microorganisms cannot reproduce. The three main steps of root canal treatment or *endodontic triad* include biomechanical preparation, sterilization, and hermetic filling of the root canal.[1,2] Irrigation that is not carried out properly will leave behind microorganisms that cause infection in the root canal.[3] Often associated with failed root canal treatment is the bacterium *Staphylococcus aureus.*[4] *Staphylococcus aureus* is a facultative anaerobic gram-positive cocci, which is one of the bacteria that has the ability to penetrate the dentinal tubules, so that biomechanical preparation alone (cleaning and shaping) will not be effective in kill these bacteria.[4,5]

Irrigation agents such as chlorhexidine are the most widely used antibacterials in dentistry. The drawback of chlorhexidine is that it is unable to remove biofilm and other organic impurities. Another effort to inhibit the growth of bacteria in root canals is to use alternative irrigation materials obtained from natural ingredients.[2,6–8]

The use of chitosan as a natural ingredient in the medical field is growing rapidly.[9] Chitosan has the properties of biocompatibility, nontoxicity, biodegradability and antimicrobial activity. Chitosan is obtained from a biopolymer derived from chitin. Chitosan is obtained through three stages, namely deproteination, demineralization and deacetylation. Chitin is deacetylated to produce chitosan, which consists of *glucosamine* and *N-acetylglucosamine* units. The mechanism of action is through the interaction of chitosan polycations with the surface of negatively charged microorganisms. This interaction causes changes in cell permeability and results in leakage of cellular components.[10] Higher concentrations of chitosan can be more effective in inhibiting and killing bacteria.[11] Chitosan can be found in fish scales.[12]

One of the fish scales that can be used in making chitosan is the scales of betok fish or papuyu fish. Betok fish or Papuyu (Banjar) (*Anabas testudineus*) is a fish that lives in the swampy waters of Kalimantan.[13] Papuyu fish scale chitosan (*Anabas testudineus*) has a DD of 97.48%, where the DD is higher than the SNI (Indonesian National Standard) for chitosan, namely ≥75%, this shows that Papuyu fish scale chitosan has great antibacterial activity and low toxicity. In Titien's (2022) research, papuyu fish scale chitosan (*Anabas testudineus*) with concentrations of 25%, 50%, 75% and 100% experienced a decrease in absorbance until it was proven to inhibit the growth of the activity of *Streptococcus mutans* bacteria. In line with the statement in Rohmah's research (2021) that Papuyu fish scale chitosan is said to be non-toxic because cell viability is ≥ 60%.[14]

Based on the studies above, the researchers were interested in analyzing the antibacterial activity of papuyu fish scale chitosan (*Anabas testudineus*) against *Staphylococcus aureus* bacteria which had not previously been studied.

METHODS

This research has been declared ethically feasible through the ethical clearance certificate No. 100/KEPKG-FKGULM/EC/X/2023. The research method used was a true experimental design with a posttest only with control group design with six treatments. The population used in this study was the bacterial isolate *Staphylococcus aureus* ATCC 6538 obtained from the MBRIO Biotekindo Bogor Laboratory with 36 samples of papuyu fish scale chitosan (*Anabas testudineus*) divided into 6 treatment groups and 6 sample repetitions.

Making Chitosan from Papuyu Fish Scales

The collected fresh papuyu fish scales (*Anabas testudineus*) are put into an ice box and washed until clean. A 3000 g sample of fish scales was dried in the oven for 24 hours at 50ºC. Fish scales are ground using a blender until they become powder and stored in an airtight place.[11,15]

Chitin Isolation

Deproteination

Deproteination aims to dissolve proteins and sugars. Papuyu fish scale powder was put into a 1000 ml measuring cup and boiled in sodium hydroxide (NaOH) (4% w/v) for one hour. Samples that had been boiled in sodium hydroxide were cooled for 30 minutes and rinsed with aquadest until the pH was neutral. Next, the sample was dried in an oven for 24 hours at a temperature of 50°C.[11,15]

Demineralization

Demineralization aims to remove the mineral content in fish scales using HCI 1% with a sample ratio of 1:4 HCl and the time required for soaking is 24 hours using a beaker to release minerals (especially calcium carbonate). The resulting sample was filtered and washed with aquadest to neutralize the pH. Next, the sample was dried in an oven for 24 hours at a temperature of 50°C.[11,15]

Chitin Test

The chitin test was carried out to determine the presence of chitin in the sample using the *Van Wesslink method*. Chitin is reacted with I2-KI 1% solution which produces a brownish yellow color, then 1 M H_2SO_4 is added to become purplish red or violet. A positive reaction to chitin is characterized by a color change from brownish yellow to violet.[11,15]

Deacetylation

The deacetylation stage was carried out by adding NaOH 50% to the chitin sample and boiling on a hot plate for 2 hours at 100°C. The sample is then left to cool at room temperature for 30 minutes and filtered and then washed with the remaining cold NaOH 5%. Then the sample was washed with aquadest until the pH was neutral and filtered to separate the solid, namely chitosan. The samples were then dried in an oven for 24 hours at 50°C. Chitosan from deacetylation is a creamy white powder.[11,15]

Antibacterial Test

Prepare a chitosan solution that has been diluted according to concentration, positive control (+) chlorhexidine gluconate 2% and negative control (-) aquadest.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration was obtained by measuring the initial absorbance value using a Spectrophotometer 722 UV-Vis with a wavelength of 450 nm. Samples were incubated for 24 hours in anaerobic conditions at 37ºC. The solution of chitosan from papuyu fish scales and *Staphylococcus aureus* bacteria that was incubated was then measured again using a Spectrophotometer 722 UV-Vis with a wavelength of 450 nm to obtain the final absorbance value and the difference between the absorbance value after incubation and before incubation, which is the MIC value.[11,15]

Minimum Bactericidal Concentrations (MBC)

Solution chitosan fishpapuyu and bacteria *Staphylococcus aureus* 5 µl was taken from the inoculation and spread on Nutrient Agar (NA) media with a micropipette, then incubated for 24 hours in anaerobic conditions at a temperature of 37ºC, then the growth of *Staphylococcus aureus* bacteria was seen directly through a *colony counter* to obtain MBC.[11]

RESULTS

The results of MIC measurements with concentrations of 25%, 50%, 75%, 100%, positive control (+) chlorhexidine gluconate 2% and negative control (-) aquadest can be seen in table 1.

Table 1. Minimum Inhibitory Concentration in the Antibacterial Activity Test of Papuyu Fish Scale Chitosan (*Anabas testudineus*) as a Root Canal Irrigation Material against *Staphylococcus aureus* Bacteria

Concentration	N	Absorbance		Difference	Std. Dev
		Before	After		
KSP 25%	6	2	1,072	-0.927	0.007
KSP 50%	6	2	0.885	-1,114	0.012
KSP 75%	6	2	0.777	-1,223	0.015
KSP 100%	6	2	0.570	-1,430	0.019
Control (+)	6	0.966	1,428	0.463	0.037
Control (-)	6	0.680	1,833	1,154	0.032

Type: KSP (Papuyu Fish Scale Chitosan)

Based on Table 1, it shows the results of observations of turbidity levels after and before incubation to obtain the MIC value. Minimum Inhibitory Concentration was obtained from the difference in absorbance after incubation minus before incubation in each treatment group. Concentrations that can inhibit bacterial growth are indicated by a negative value and positive values if the concentration is unable to inhibit bacterial growth. The largest difference in absorbance value was found in the negative control with an average value of 1.154, while the absorbance value in all papuyu fish scale chitosan concentration groups showed a decrease in absorbance values, indicating that papuyu fish scale chitosan can inhibit the growth of *Staphylococcus aureus* bacteria. Papuyu fish scale chitosan with a concentration of 25% was designated as the MIC, because this concentration is the smallest concentration capable of inhibiting the growth of *Staphylococcus aureus* bacteria after incubation for 24 hours at 37°C.

Table 2. Average Amount Colony Bacteria *Staphylococcus aureus* on the Antibacterial Activity Test of Papuyu Fish Scale Chitosan (*Anabas testudineus*) as a Root Canal Irrigation Material

Concentration	N	Average (CFU/µL)
KSP 25%	6	662
KSP 50%	6	552
KSP 75%	6	37
KSP 100%	6	0
Control (+)	6	0
Control (-)	6	1,021

Type: KSP (Papuyu Fish Scale Chitosan)

Table 2 shows the average MBC values for the number of colonies growing on NA for 24 hours, which was calculated using a *colony counter*, showing that the Papuyu fish scale chitosan group with concentrations of 25%, 50%, 75% and the negative control still had bacterial colony growth or has not been able to kill the growth of *Staphylococcus aureus* bacteria. The papuyu fish scale chitosan group with a concentration of 100% and the positive control had an average number of bacterial colonies with a value of "0", which means there was no growth of *Staphylococcus aureus* bacteria at that concentration. Papuyu fish scale chitosan at a concentration of 100% shows that there is no longer any bacterial growth found at that concentration, so papuyu fish scale chitosan at a concentration of 100% is declared as MBC to kill the growth of *Staphylococcus aureus* bacteria.

The MIC research data was tested for normality using the *Shapiro-Wilk* test and the data was said to be normally distributed. Next, homogeneity testing was carried out using *Levene's test* and the data was declared not homogeneous. The next test carried out was the *One-Way ANOVA* test with a confidence level of 95% and the *Post-Hoc Games Howell* test, showing significant differences in each group. The results of the analysis are as follows.

Table 3. *Post-Hoc Games Howell* Test Results on MIC Data

Treatment	KSP 25%	KSP 50%	KSP 75%	KSP 100%	CHX 2%	Aquadest
KSP 25%		0,000*	0,000*	0,000*	0,000*	0,000*
KSP 50%			0,000*	0,000*	0,000*	0,000*
KSP 75%				0,000*	0,000*	0,000*
KSP 100%					0,000*	0,000*
CHX 2%						0,000*
Aquadest						

Table 3 states that the data above have significant differences between each other because the results are p<0.05 (p=0.00).

The normality test on the Minimum Bactericidal Concentrations (MBC) data obtained from each treatment was stated to be not normally distributed. Next, a homogeneity test was carried out on the data using *Levene's test* (p<0.05) and it was stated that the data was not homogeneous. The next tests carried out were the non-parametric *Kruskal Wallis* test with a confidence level of 95% and the *Mann-Whitney* test. In the *Kruskal Wallis* test, it was found that there were significant differences between treatments. The results of further analysis are shown below.

Table 4. *Mann-Whitney* Test Results on MBC Data

Treatment	KSP 25%	KSP 50%	KSP 75%	KSP 100%	CHX 2%	Aquadest
KSP 25%		0.016	0.004	0.002*	0.002*	0.004*
KSP 50%			0.004*	0.002*	0.002*	0.004*
KSP 75%				0.002*	0.002*	0.004*
KSP 100%					1,000	0.002*
CHX 2%						0.002*
Aquadest						

Based on the results of the *Mann-Whitney* test, it showed that in the treatment group, Papuyu fish scale chitosan had a concentration of 25% against Papuyu fish scale chitosan, a concentration of 50% (p= 0.016), Papuyu fish scale chitosan had a concentration of 100% against chlorhexidine 2% (p= 1.000), no there was a significant difference (p>0.05). All other treatment groups had significant differences between each other because they had a p value <0.05

DISCUSSION

If caries is left untreated and not treated from the start, it can cause bacteria to enter the pulp and root canals, causing infection and pulp disease. Chlorhexidine as the *gold standard* in root canal irrigation has disadvantages, namely that it can cause taste disturbances, the formation of lesions, changes in the color of teeth, restorations and the tongue, so chitosan can be used as an alternative root canal irrigation material.[16] The properties of chitosan are proven to be non-toxic, biocompatible and Biodegradable makes it a very useful biomaterial in the medical field compared to other polymers. Chitosan can be found in papuyu fish scales. Chitosan is effective in inhibiting growth bacteria.[9]

The results of calculating the difference in absorbance using a Spectrophotometer 722 UV-Vis showed that the Papuyu fish scale (*Anabas testudineus*) chitosan group with concentrations of 25%, 50%, 75%, 100% experienced a decrease in absorbance values. In this study, Papuyu fish scale chitosan with a concentration of 25% was the MIC value because this concentration began to inhibit the growth of *Staphylococcus aureus* bacteria. This research proves that Papuyu fish scale chitosan has antibacterial properties against *Staphylococcus aureus*.

The research continued with observations of killing power. Observation of the killing power in *nutrient agar* (NA) media after incubation for 24 hours under anaerobic conditions showed that 100% concentration of papuyu fish scale chitosan did not have the growth of *Staphylococcus aureus* bacterial colonies. Papuyu fish scales with a 100% concentration have the power to kill *Staphylococcus aureus* bacteria so that this concentration is expressed as the MBC value.

The treatment concentration showed differences in absorbance values and differences in the average number of bacterial colonies, followed by an increase in the concentration of papuyu fish scale chitosan. One of the antibacterial mechanisms of chitosan is by binding to molecular weight. Low molecular weight chitosan can penetrate bacterial cell walls, bind DNA, and inhibit the process of DNA transcription and mRNA synthesis from bacteria, while high molecular weight chitosan is able to bind to bacterial cell wall components that have a negative charge which causes changes in cell permeability. and inhibits the transport of molecules such as nutrients into bacterial cells.[16] Research conducted has proven that 100% concentration of Papuyu fish scale chitosan is more effective in inhibiting the growth of *Staphylococcus aureus* bacteria than other concentrations. At concentrations of 25%, 50%, 75, the chitosan content is less, making the antibacterial mechanism at these concentrations not work optimally. A higher concentration of chitosan has higher antibacterial activity as well, this indicates that an increase in this concentration is able to degrade the reactive group bonds of chitosan, namely the amine group (-NH2) so that it is more efficient in damaging the structure of bacterial cell walls.[17] Based on Rohmah's research (2021) stated that Papuyu fish scale chitosan treatment up to a concentration of 100% did not have a toxic effect because it showed a cell viability percentage of >60%. In line with Widayaningrum's (2019) research on the antibacterial activity of haruan fish scale chitosan in the growth of *Staphylococcus aureus*, it shows that this bacterium contains protein and antigenic polysaccharides, both of which are important components in the formation of cell walls consisting of a thick peptidoglycan layer, so they cannot inhibit the growth of *Staphylococcus aureus*.[11]

Papuyu fish scale chitosan has a positively charged amine group (-NH2) which reacts with the negatively charged microbial layer. The cell membrane is one of the elements that form bacteria as a boundary between cells and their surroundings, where in the extracellular and intracellular parts of the membrane there is a fluid containing ions and nutrients needed for cell life.[18] The positively charged cell membrane is found in the extracellular and the negatively charged is found in the cell membrane. intracellular part. The cell membrane is positively charged on the outside (*extracellular*) and negatively charged on the inside (*intracellular*). Cell membranes have ion channels that function as entrances and exits for fluid, thereby causing membrane traffic. The movement of the membrane will create a difference in ion concentration, causing a potential difference in the membrane, and exiting to the extracellular environment which then invades the intracellular.[19,20] with potential extraction of lipids which can bind the positively charged amine groups of chitosan resulting in distortion of the cell wall so that the contents in the cytoplasm come out due to osmotic differences caused by damage and rupture of the bacterial cell wall.[21,22] The positive charge on chitosan will interact with the negative charge of the bacteria. which is then absorbed to form a layer that blocks the bacterial cell's transportation path so that the bacterial cell lacks substances to grow. This interaction will interfere with the formation of peptidoglycan. This disturbance causes the cell wall layer to degrade in an unstable condition and easily undergo lysis due to inhibition of bacterial metabolic activity. [11] Chitosan has a very strong affinity for binding to bacterial DNA so that it can interfere with mRNA activity and protein synthesis. This process will result in inhibited bacterial growth and microbial death.[23]

The antibacterial activity of Papuyu fish scale chitosan comes from the important structure of positively charged polycations which is associated with DD or the degree of deacetylation of chitosan which affects the positive charge density. Papuyu fish scale chitosan with a DD of 97.48% causes a higher positive charge density, which provides stronger antibacterial activity against *Staphylococcus aureus*.[14,24]

The chlorhexidine gluconate 2% group as a positive control was bacteriostatic and bactericidal. Chlorhexidine gluconate 2% is a broad spectrum antibacterial agent that effectively kills gram-positive and gram-negative bacteria. Chlorhexidine gluconate 2% is able to bind electrostatically to the surface of negatively charged bacteria and can cause damage to the outer layer of the cell wall so that the bacteria can penetrate it.[25] This is in line with research by Marieke (2022) that exposure to bacteria attached to chlorhexidine causes membrane damage in *Staphylococcus aureus* bacteria. After exposure to chlorhexidine, bacteria experience increased cell wall deformation which indicates cell wall damage and intracellular leakage, thus the loss of intracellular pressure causes continued deformation.[26] In this study the results of examination with a spectrophotometer were inversely proportional and showed an increase in the absorbance of chlorhexidine. The negative control group used aquadest treatment as a comparison in this study. Aquadest has no effect on bacterial growth because the compounds in aquadest are neutral and do not have antibacterial properties.[27] This research proves that the absorbance value of aquadest has increased and cannot inhibit bacterial growth.

This research has theoretical differences, the MIC value of chlorhexidine gluconate 2% should be able to inhibit bacterial growth. This value is in line with research by Pratiwi, *et al* (2022) which states that the higher value after incubation occurs because the formation of the *Staphylococcus aureus* bacterial biofilm has gone through a maturity stage so that inhibitory antibacterial compounds penetrate less into the biofilm matrix. Bacterial self-defense in the biofilm in the long incubation phase will form *extracellular polymeric substances* (EPS). The two main components of the EPS matrix in *Staphylococcus aureus are poly-N-acetyl-dglucosamine* (PNAG) and extracellular DNA (eDNA).[28] This is assumed to be due to the main weakness of chlorhexidine which is unable to dissolve or remove biofilm and other organic tissue.[2]

The conclusion of this research is that there is antibacterial activity of Papuyu fish scale chitosan (*Anabas testudineus*) against the growth of *Staphylococcus aureus* bacteria based on the Minimum Inhibitory Concentration (MIC) at a concentration of 25% and the Minimum Bactericidal Concentrations (MBC) at a concentration of 100%. Based on this research, it is also known that 100% concentration of Papuyu fish scale chitosan has the same antibacterial power as chlorhexidine gluconate 2%, while aquadest cannot inhibit and kill *Staphylococcus aureus* bacteria.

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