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ANTIBACTERIAL ACTIVITY OF PAPUYU FISH SCALES CHITOSAN (*Anabas testudineus*) AGAINST *Enterococcus faecalis*

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ABSTRACT

Background: *Papuyu fish is widely consumed by Kalimantan people. Papuyu fish scales chitosan has antibacterial potential because it contains amino polysaccharides and enzyme lysozyme which inhibits the growth of root canal bacteria, specifically Enterococcus faecalis.* ***Purpose:*** *The purpose of this experimental is to analyze the antibacterial activity of papuyu fish (Anabas testudineus) scales chitosan against Enterococcus faecalis.* ***Methods:*** *This is a true experimental study with post-test only with a control group design using 15 samples of papuyu fish (Anabas testudineus) scales chitosan grouped into 5 treatment groups and 3 samples repetitions. The treatment groups consisted of papuyu fish (Anabas testudineus) scales chitosan concentrations of 25%, 50%, 75%, 100%, and distilled water (negative control). Absorbance calculation using a UV-Vis Spectrophotometer 722 for minimum inhibitory concentration (MIC) and Enterococcus faecalis colonies are visualized with a colony counter for minimum bactericidal concentration (MBC).* ***Results:*** *The MIC value of papuyu fish (Anabas testudineus) scales chitosan were obtained at 25% concentration and the MBC value were obtained at 100% concentration. The results of One-Way ANOVA test for MIC and Kruskal Wallis for MBC of papuyu fish scales chitosan concentrations of 25%, 50%, 75%, and 100% obtained significant results of $p<0,05$ and showed a significant difference between treatments.* ***Conclusion:*** *These are differences in the effectiveness of MIC and MBC papuyu fish (Anabas testudineus) scales chitosan concentrations of 25%, 50%, 75%, 100% and distilled water. Papuyu fish scales chitosan 100% concentration is the most effective concentration as an antibacterial against the growth of Enterococcus faecalis.*

Keywords: *Chitosan, Enterococcus faecalis, MBC, MIC, Papuyu fish scales*

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INTRODUCTION

Caries is a dental disease that many people often experience.[1] Based on RISKESDAS in 2018 from the Ministry of Health of the Republic of Indonesia reported that around 88.8% of the Indonesian population experienced caries, and in the province of South Kalimantan experienced problems with cavities, damage, or pain with a prevalence of 46.9%.[2] Caries can develop into pulp disease caused by infection from bacteria.[1,3] Bacteria infect the pulp, causing inflammation, and can undergo necrosis if treatment is not immediately done.[1]

Root canal treatment is required for necrotizing teeth so the pulp chamber is free of infected pulp tissue.[4] Root canal treatment consists of three main stages: root canal preparation, sterilization, and obturation.[5] One of the successes of root canal treatment is determined by irrigation.[6] The root canal irrigation solution kills resistant and dominant bacteria in the root canal. *Enterococcus faecalis* is one of the bacteria that cause root canal treatment failure.[7,8]

E. faecalis is a facultative anaerobic gram-positive bacterium that can survive in the root canal after root canal treatment.[9,10] This bacterium forms a *biofilm* on the root canal wall and can live alone without the help of other bacteria. In addition, these bacteria can produce toxins or play a role in the inflammatory process.

Further research is needed to find biomaterials that can be used as alternative materials for irrigation materials, one of which is chitosan from papuyu fish scales.[6,11] Papuyu fish or betok fish (*Anabas testudineus*) is a fish that the people of Kalimantan favor as a daily side dish that lives in brackish waters in the Kalimantan region.[11] The scales of papuyu fish result from the fishing industry, which is thrown away and becomes waste. If the waste scales are processed into chitosan, then the papuyu fish scales waste can provide benefits according to its usefulness.[12]

Chitosan has potential as an antibacterial, which is one of the ideal requirements for irrigation materials because it contains *amino-polysaccharides* and lysozyme enzymes that can inhibit bacterial growth. Chitosan is a derivative of chitin formed through the stages of demineralization, deproteination, and deacetylation.[12] It is *biodegradable*, *biocompatible*, and non-toxic to human cells.[12]

According to research by Hutami et al. (2020), chitosan from haruan fish scales (*Channa striata*) can effectively inhibit the growth of *Streptococcus sanguinis*.[13] According to research by Baharuddin et al. (2020), chitosan from feather clam shells has antibacterial properties against *Staphylococcus epidermidis*.[14] Based on these two studies, the potential of fish scale chitosan as an antibacterial was found. Irawan et al. (2018) stated that the deacetylated content of papuyu fish was 97.40%.[15] Based on this description, research is needed on the antibacterial activity of papuyu fish scale chitosan (*Anabas testudineus*) against *E. faecalis* bacterial growth based on the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

METHODS

The research method used was a *true experimental design* with *posttest only and a control group design* using five treatments and three repetitions of each treatment group, so 15 samples were obtained. This research has been declared ethically sound by the Ethics Commission of the Faculty of Dentistry, Lambung Mangkurat University with the number, No. 101/KEPKG-FKGULM/EC/X/2023. The treatment groups in this study were papuyu fish scale chitosan (*Anabas testudineus*) concentrations of 25%, 50%, 75%, and 100% and a negative control in the form of distilled water.

Preparing papuyu fish scale chitosan starts with collecting fish scales and putting them in an *ice box* to keep them fresh. The fish scales were cleaned from the remnants of meat and dirt using clean running water. Next, the fish scales are dried in an oven at 50°C for 24 hours. After that, the fish scales were pulverized into powder using a *blender* and stored in an airtight container.[16]

Chitin isolation goes through the stages of deproteination and demineralization. Furthermore, the process of converting chitin into chitosan is through the last process, namely the deacetylation process. Chitin powder of papuyu fish scales (*Anabas testudineus*) was mixed with 50% NaOH and then boiled on a *hot plate* at 80°C for 2 hours. After that, it was adjusted to room temperature for 30 minutes and washed using distilled water repeatedly until the pH became neutral, then continued with filtering using filter paper. The following process was drying for 24 hours in an oven at 50°C. The chitosan obtained is a creamy white powder.[17]

Chitosan of papuyu fish scales (*Anabas testudineus*) was diluted according to the required concentration using the p/v formula. Next, cover the test tube containing the *E. faecalis* bacterial culture with aluminum foil after the chitosan solution was diluted according to the concentration made 1 ml each, then add 1 ml of aquadest, then homogenize using a *vortex mixer*. Before incubation, the absorbance value was measured using a 722 UV-Vis spectrophotometer with a wavelength of 450 nm to obtain the initial absorbance value. Then, incubation was carried out under anaerobic conditions for 24 hours at 37°C. After that, the absorbance value was measured after incubation to determine the difference between the absorbance value before and after incubation to determine the minimum inhibitory concentration (MIC).[13]

Samples from each treatment group were each taken from 5 µl inoculation and distributed on *Nutrient Agar* (NA) media using micropipettes sterilized using 96% alcohol, then incubated for 24 hours under anaerobic conditions at 37°C. After that, the growth of *Enterococcus faecalis* bacteria was seen directly using a *colony counter* to obtain the minimum bactericidal concentration (MBC) value.[13]

Data on MIC values were subjected to *Shapiro Wilk* normality test ($p>0.05$) and *Levene's test* homogeneity test ($p<0.05$) so that the *One Way ANOVA* parametric test and *Games Howell Post Hoc test* were continued to determine which treatment groups had significant differences.20 Data on MBC values were subjected to *Shapiro Wilk* normality test ($p<0.05$) and *Levene's test* homogeneity test ($p<0.05$) so that

the *Kruskal Wallis* nonparametric test and *Mann Whitney Post Hoc test* were continued to determine which treatment groups had significant differences.[18]

RESULTS

The results of measuring the difference in absorbance values of each treatment group can be seen in Table 1.

Table 1. Measurement of Difference in Absorbance Value for Antibacterial Activity Test based on MIC

Treatment	N	Absorbance		Diff.	Dev Std
		Before	After		
KSP 25%	3	2	1,265	-0,735	0,008
KSP 50%	3	2	1,024	-0,975	0,003
KSP 75%	3	2	0,807	-1,192	0,002
KSP 100%	3	2	0,605	-1,394	0,006
Aquadest	3	0,659	1,535	+0,876	0,039

The (-) sign in Table 1 indicates a decrease in *E. faecalis* bacteria, while the (+) sign indicates an increase in bacteria. Papuyu fish scale chitosan 25%, 50%, 75%, and 100% decreased the average absorbance value, which means the number of *E. faecalis* bacteria decreased, or it inhibited the growth of these bacteria. At the same time, the distilled water data increased the average value, which means the number of bacteria increased. Chitosan papuyu fish scales (*Anabas testudineus*) concentration of 25% is seen to be the beginning of a decrease in the average absorbance value, so this concentration can be said to be the minimum inhibitory concentration (MIC).

Table 2. Calculation of the Number of *E. faecalis* Colonies for Antibacterial Activity Test based on MBC

Treatment	N	Average (CFU/µL)
KSP 25%	3	1024
KSP 50%	3	712
KSP 75%	3	129
KSP 100%	3	0
Aquadest	3	948

Table 2 shows the number of colonies of *E. faecalis* bacteria in papuyu fish scale chitosan solution (*Anabas testudineus*) based on the average number of colonies that grew on NA media for 24 hours. Papuyu fish scale chitosan (*Anabas testudineus*) 25%, 50%, 75%, and distilled water are known to have bacterial colony growth still or have not been able to kill the growth of *E. faecalis* bacteria. In comparison, papuyu fish scale chitosan (*Anabas testudineus*) 100% concentration shows no growth of *E. faecalis* bacteria. It can concluded that papuyu fish scale chitosan (*Anabas testudineus*) 100% concentration is the minimum bactericidal concentration (MBC) value.

The research data for the difference in absorbance values were tested for normality ($p>0.05$) and homogeneity test ($p<0.05$). Based on the results of the normality test and homogeneity test, the *One-Way ANOVA* parametric test can be used, and the p-value is obtained <0.05 ($p=0.000$), which means that H0 is rejected so that there is a significant difference in at least one treatment from the difference in absorbance data for each group. Significant differences can be seen through the *Post Hoc Games-Howell test* because the data results were not homogeneous in the previous homogeneity test, and the results are obtained in Table 3. Based on the results of the *Post Hoc Games-Howell test* in Table 3 show that all treatment groups have significant differences from each other because they have a significance of $p\leq0.0$.

Table 3. Results of *Post Hoc Games-Howell Test* Difference in Absorbance Value for Antibacterial Activity Test of Papuyu Fish Scale Chitosan (*Anabas testudineus*) against *E. faecalis* Bacterial Growth based on the Minimum Inhibitory Concentration (MIC) value

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

Table 4. Results of *Post Hoc Mann-Whitney Test* of Colony Counts of *E. faecalis* Bacteria for Antibacterial Activity Test of Papuyu Fish Scale Chitosan (*Anabas testudineus*) based on Minimum Bactericidal Concentration (MBC) value

Treatment	KSP 25%	KSP 50%	KSP 75%	KSP 100%	Aquadest
KSP 25%		0,050*	0,050*	0,037*	0,827
KSP 50%			0,050*	0,037*	0,127
KSP 75%				0,037*	0,050*
KSP 100%					0,037*
Aquadest					

The research data on the number of bacterial colonies were tested for normality ($p<0.05$) and homogeneity test ($p<0.05$). Based on the results of the normality test and homogeneity test, the *Kruskal Wallis* nonparametric test can be used and obtain a significance value of $p\leq0.05$ ($p=0.014$), which means H_0 is rejected so that there is a significant difference in at least one treatment from the data on the number of bacterial colonies in each group. Significant differences can be seen through the *Post Hoc Mann-Whitney test* and the results obtained in Table 4 above.

The 25% papuyu fish scale chitosan with distilled water showed no significant difference ($p=0.827$). The 50% papuyu fish scale chitosan with distilled water showed no significant difference ($p=0.127$). All other treatment groups showed significant differences as they had $p\leq0.05$.

DISCUSSION

The results of the average difference in absorbance values of papuyu fish scale chitosan (*Anabas testudineus*) 25%, 50%, 75%, and 100% in this study decreased in absorbance value along with the higher concentration, the difference in absorbance values before and after incubation obtained was also more significant.[19,20] The inhibition of bacterial growth is improving along with the higher concentration because the higher the chitosan concentration, the more amine groups (-NH2) are contained.[21] The positively charged amine groups (-NH2) help attract negatively charged amino acid molecules that form proteins in bacteria so that the more amine groups (-NH2) contained, the greater the inhibition of bacterial growth.[19,20]

The results of the calculation of the number of bacterial colonies in the chitosan of papuyu fish scales (*Anabas testudineus*) at a concentration of 100% stated that there were no *E. faecalis* bacterial colonies or the number of colonies was zero. These results indicate that 100% papuyu fish scale chitosan has a killing power against *E. faecalis* bacteria and can be determined as the Minimum Bactericidal Concentration (MBC). Based on these results, it can concluded that a 100% concentration of papuyu fish scale chitosan has optimal killing power against *E. faecalis* bacteria.

The mechanism of action of 100% papuyu fish scale chitosan in inhibiting the growth of *E. faecalis* bacteria is that chitosan contains positively charged amine (-NH2) functional groups and lysozyme enzymes.[14,22] The positively charged amine (-NH2) group will bind to negatively charged amino acid molecules, forming glutamate proteins, which are one of the components of the bacterial cell membrane.[14,23,24] The bond that occurs is an electrostatic interaction and causes an electrostatic force that disturbs the cell.[13] The cell disturbance that occurs affects changes in intracellular membrane permeability to increase.[13,23,24]

Two factors cause intracellular membrane permeability to increase. First, there is a bond between chitosan and choline, a cell membrane phospholipid. Second, there is help from the enzyme lysozyme, which blocks the permeability of the bacterial cell membrane by breaking the β-(1, 4) bond between *N-acetylmuramate* (NAM) and *N-acetylglucosamine* (NAG). As a result, the peptidoglycan layer, an important part of the bacterial cell wall, is damaged.[23,24] The increased cell membrane permeability causes leakage of bacterial intracellular constituents in the cytoplasm and lysis.[13,23,24]

Another mechanism that occurs is that the polycationic nature of chitosan can form an impermeable layer around bacterial cells, which blocks the entry of nutrients through the bacterial cell membrane.[25,26] This inhibits the process of transporting substances needed by bacteria for survival and metabolism.[26] Chitosan will also penetrate the cell membrane and interact with bacterial DNA.[27] The interaction caused by chitosan binding to bacterial DNA.[28] Antibacterial compounds such as chitosan can also inhibit DNA replication, mRNA formation, and protein synthesis.[28,29] An essential mechanism for bacterial growth is that bacterial DNA will undergo a transcription process by forming an RNA molecule called *messenger* RNA (mRNA).[30] Then ribosomes bind and read mRNA and incorporate amino acids sent by tRNA into new proteins.[30] After that, the ribosome synthesizes proteins from the RNA's information, which is called the translation process.[30] However, the mechanism is inhibited by protein synthesis, namely by inhibiting

translation and transcription due to antibacterial compounds.[31] The whole mechanism results in cell damage, which continues into cell death.[23,28]

The results of this study state that papuyu fish scale chitosan (*Anabas testudineus*) with concentrations of 25%, 50%, 75%, and 100% has the potential as an alternative biomaterial solution for root canal irrigation because its ability has been proven in this study in inhibiting the growth of *E. faecalis* bacteria which is the dominant bacteria in cases of dental root canal infection. This theory states that a good and ideal irrigation material has antibacterial properties.[32,33] Papuyu fish scale chitosan with a concentration of 100% can be noted as the most effective concentration as an antibacterial because it has inhibition and killing power against the growth of *E. faecalis* bacteria.

This study concludes that there is an antibacterial activity of papuyu fish scale chitosan (*Anabas testudineus*) with 25% concentration determined as the minimum inhibitory concentration (MIC) and 100% concentration determined as the minimum bactericidal concentration (MBC). Papuyu fish scale chitosan (*Anabas testudineus*) with distilled water as a negative control has a different effect based on MIC and MBC.

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