Antibacterial activity of nano-hydroxyapatite paste of snakehead fish bone against S.mutans

by Nurdiana Dewi

Submission date: 19-Jun-2024 05:27AM (UTC+0700)
Submission ID: 2404997542
File name: 51018-208872-1-PB.pdf (249.71K)
Word count: 3899
Character count: 21803

ORIGINAL ARTICLE

Antibacterial activity of nano-hydroxyapatite paste of snakehead fish bone against *S.mutans*: an in vitro study

Nurdiana Dewi[1*]
Afifah Rahmadella[2]
Isnur Hatta[3]
Maharani Laillyza Apriasari[4]
Deby Kania Tri Putri[5]

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin, Indonesia
[2]Undergraduate Preclinical Dentistry Programme, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin, Indonesia
[3]Department of Public Dental Health, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin, Indonesia
[4]Department of Oral Medicine, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin, Indonesia
[5]Department of Biomedicine, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin, Indonesia

* Correspondence:
nurdiana.dewi@ulm.ac.id

Received: 11 November 2023
Revised: 13 February 2024
Accepted: 23 March 2024
Published: 30 March 2024
DOI: 10.24198/pjd.vol36no1.51018

p-ISSN 1979-0201
e-ISSN 2549-6212

Citation: Dewi, N. Rahmadella, A. Hatta, I. Apriasari, ML. Putri, DKT. Antibacterial activity of nano-hydroxyapatite paste of Snakehead fish bone against S.mutans: an in vitro study. Padj J Dent, March. 2024; 36(1):1-8.

ABSTRACT

Introduction: Caries is the most common oral disease found in society. The prevalence of caries in South Kalimantan is as high as 46.9% in 2018. *S.mutans* is the causative microorganism in the initial occurrence of caries. Strategy that can be used to prevent caries is by adding nano-hydroxyapatite to the tooth paste. Nano-hydroxyapatite can be obtained from Snakehead (*Channa striata*) fish bone. Snakehead is a kind of fish that is abundant in Banjarmasin. This study aimed to analyze antibacterial activity of the nano-hydroxyapatite paste from snakehead (*Channa striata*) fish bone against *S.mutans* bacteria. **Methods:** The study consisted of 5 treatment groups: negative control (basic formula), positive control (casein phosphopeptide-amorphous calcium phosphate or CPP-ACP) and three treatment groups (nano-hydroxyapatite paste concentration of 10, 20 and 30%. The paste was made in the formulation of F1, F2, and F3. The antibacterial activity test by measuring MIC and MBC were performed using dilution method. **Results:** MIC of nano-hydroxyapatite paste was at a concentration of 10% with an average value of the difference absorbance of -0.468. MIC values in the concentration of 10%, 15%, 20% positive and negative control groups had a significant difference. MBC of nano-hydroxyapatite paste was at concentration of 15%. Concentration of 10% and the negative control group showed a significant difference, while concentration of 15% and 20% groups did not show a significant difference. **Conclusion:** Nano-hydroxyapatite paste from Snakehead fish bone has antibacterial activity in inhibiting and eliminating mutated *S.mutans* bacteria. The most effective concentration of antibacterial nano-hydroxyapatite paste to prevent caries was 10%. At this concentration, nano-hydroxyapatite can inhibit the growth of *S.mutans* without killing the bacteria.

KEYWORDS

fish bone, nano-hydroxyapatite, snakehead, *S.mutans*

INTRODUCTION

The most common oral cavity disease that can be found among the society is dental caries. Caries can occur in adults, adolescents, and even children.[1] According to the basic health research data (RISKESDAS) in 2013, the prevalence of dental caries in Indonesia was 25.9%, especially in South Kalimantan the prevalence of caries was 36.1%.[2] Based on the RISKESDAS of 2018, there was an

increase of prevalence in 2018, which was 57,6% for Indonesia caries prevalence, and 46.9% for South Kalimantan.[3] There are four main factors of caries, namely host, microorganism, substrate, and time. Microorganisms have a very important role in the caries process and are supported by other factors.[4] The initial process of caries is characterized by the increasing activity of microorganisms in the oral cavity. *S.mutans* is a causative microorganism that plays a role in the early occurrence of caries and can quickly adapt to sudden and substantial environmental changes in plaque, so that it becomes the main agent of caries.[5]

One of the materials that is widely used as a caries prevention agent is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP). It has been proposed that CPP-ACP is a substance that can help restore the minerals in dental enamel. ACP supersaturation in relation to tooth enamel is made possible by CPP's capacity to buffer unbound calcium and phosphate ions, which lowers demineralization and increases remineralization. CPP and ACP together exhibit potent antibiofilm action against *S.mutans*, which covers an additional anticariogenic impact.[6] Hydroxyapatite has also been used as a caries-preventing ingredient in toothpaste, because nano-hydroxyapatite has an ability to help remineralization of teeth. Brushing the teeth using toothpaste is the first step to controlling the plaque.[7]

Nano-hydroxyapatites are hydroxyapatite particles with sizes ranging from 1 to 100 nm.[8] It can be integrated into the enamel and infiltrate the demineralized dentin collagen structure.[9] This material has many advantages such as antibacterial ability, increasing remineralization, as well as reducing sensitivity.[10] Furthermore, El-Gar et al. showed that nano-hydroxyapatite aqueous suspensions has potent antibacterial and antiadhesive actions that can prevent formation of *S. mutans*.[11] This result was in line with Hariani et al. which explained that Snakehead (*Channa striata*) fish bone nano-hydroxyapatite solution has antibacterial properties against *S. aureus* and *E. coli*.[12] There has been no research examining the antibacterial ability of nano-hydroxyapatite paste of Snakehead (*Channa striata*) fish bone against *S.mutans.*

A great number of Snakehead fish can be found in South Kalimantan, especially Banjarmasin, and this makes it easy to obtain snakehead fish bones. Fish bones are usually disposed of or used as a mixture of animal feed. Fish bone waste actually can be used for human health. Devitasari, et al (2019) stated that hydroxyapatite paste from fish bones was proven to trigger remineralization and increase enamel hardness with concentrations of 10% and 15%.[13] A study by Scribante, et al in 2020 stated that nano-hydroxyapatite solution was proven to increase enamel hardness after demineralization.[14]

Currently, many products containing nano-hydroxyapatite have been developed, such as paste for teeth and sports drinks, which have a certain effect on inhibiting tooth demineralization.[9] There has been no research examining the antibacterial ability of nano-hydroxyapatite from snakehead fish (*Channa striata*) bones. Antibacterial ability can be determined by carrying out MIC and MBC analysis.[15,16] The aim of this study was to analyze antibacterial activity of the nano-hydroxyapatite paste from snakehead (*Channa striata*) fish bone against *S.mutans* bacteria.

METHODS

This research was true experimental with post-test using control group design. The sample used was pure isolate of *S.mutans* ATCC 25175 as much as 15 samples and divided into 5 treatment groups.

CaO preparation was conducted by cutting the Snakehead fish bones into pieces in 2-5 mm of length. As much as 1 kg of fish bones were boiled for 1 hour, washed, and dried for ± 1 day. A total of 100 g of fish bones were soaked for 2 hours in 1 N HCl solution, then washed to pH ± 7 and dried for 2 hours in an oven at 105°C. Dried fish bones were crushed and filtered through 60 mesh. The fish

bone powder was calcined for 4 hours in a furnace at 900°C. The calcined CaO powder was ground using ball milling for 1 hour with a weight ratio of CaO:ball being 1:5.

Nano-hydroxyapatite synthesis was carried out by heating 6 g of CaO powder and 5 mL of demineralized water at 30°C for 30 minutes, then 50 mL of 1.66 M (NH4)2HPO4 was added to the mixture. 1M NH_4OH solution was added gradually to the mixture until it reached pH ± 10, while stirring using a magnetic stirrer and heated for ± 2 hours, then the solution was left for 24 hours. The white precipitate formed (nano-hydroxyapatite) was separated by filtration and dried in an oven at 105° C for 2 hours. Nano-particles <100 nm in size were determined according to research by Hariani, et al.[12]

Snakehead *(Channa* striata) fish bone nano-hydroxyapatite paste was made with Na-CMC as a gelling agent. To make the paste, distilled water was heated then nipagin and Na-CMC were added and stirred until homogeneous. The refined nano-hydroxyapatite powder was moistened with glycerin. Menthol was dissolved using alcohol, then mixed with moistened nano-hydroxyapatite powder, then menthol was added to the prepared nano-hydroxyapatite powder which was mixed and stirred until homogeneous to produce a paste. The paste formula contains nano-hydroxyapatite named as F1 (10%), F2 (15%), F3 (20%) and control negatives can be seen in Table 1. The positive control used in this research was CPP-ACP.

Table 1. Nano-hydroxyapatite paste of snakehead (*channa* striata) fish bone formulas

	Paste Formulas			
	F1	**F2**	**F3**	**Control (-)**
Nano-hydroxyapatite	10 g	15 g	20 g	0 g
Aquadest ad	52.65 g	47.65 g	42.65 g	62.65 g
Paste content	Basic formula*	Basic formula*	Basic formula*	Basic formula*

*Basic formula contain of Na-CMC (2g), Glycerin (35 g), Nipagin (0.25 g), Alcohol (0.05 g), Menthol (0.05 g)

Laboratory equipment was sterilized using autoclave at a temperature of 121°C for 30 minutes before antibacterial activity test. Nutrient agar medium was used as culture medium for bacterial isolates. Agar medium was made by dissolving 2 grams of nutrient agar in 100 ml distilled water in an Erlenmeyer flask. The Erlenmeyer flask was heated until all ingredients were completely dissolved. The Erlenmeyer flask then was covered with cotton and aluminum foil and sterilized using an autoclave. The sterile medium then was poured aseptically into a petri dish until the surface was covered with agar medium, then the agar medium was allowed to stand until it hardened and was closed to prevent contamination.

S.mutans suspension was made by taking cultured *S.mutans* bacteria from agar media using a sterile tube needle, then placing it in a test tube containing 1 ml of Brain Heart Infusion Broth (BHIB). The reaction tube was incubated in an incubator for 1x24 hours at 37°C under anaerobic conditions, then diluted by adding Brain Heart Infusion Broth (BHIB) and homogenized until the turbidity was in accordance with the McFarland standard of 0.5 (1.5×10^8 CFU/ml).

Antibacterial activity of nano-hydroxyapatite paste of snakehead (*Channa striata*) fish bone against the growth of *S.mutans* bacteria was conducted using solid and liquid dilution methods. 1 ml of *S.mutans* bacterial suspension that was appropriate with 0.5 McFarland's solution was added to each test tube containing nano-hydroxyapatite of snakehead fish bones (*Channa striata*) in concentrations of 10, 15, and 20%, the positive control (CPP-ACP), and the negative control (paste without nano-hydroxyapatite). Each test tube was covered with sterile cotton and homogenized using a vortex mixer.

MIC is the lowest concentration for inhibiting bacterial growth.[15] The absorbance value, before incubation, was measured using a UV-Vis

Spectrophotometer with a wavelength of 450 nm, then all samples were incubated for 24 hours at 37°C under anaerobic conditions. The absorbance results after 24 hours of incubation were measured again using a UV-Vis Spectrophotometer with a wavelength of 450 nm. The MIC value was obtained from the difference in the absorbance results between before and after the incubation of each sample.

MBC is the lowest concentration for killing 99.9% of bacteria.[16] MBC was determined by transferring 5 µL of each sample into a petri dish containing sterile nutrient agar media, then incubating it for 24 hours at 37°C under anaerobic conditions. MBC was obtained by counting the number of bacterial colonies after incubation using colony counters. If the calculation results obtained were 0 CFU/µL (not found), then it was recorded as MBC.

All experiments were done in triplicate and the results were expressed as mean ± SD. Statistical analysis was done by using one-way ANOVA followed by Games Howell test using SPSS software version 25.0 and p-value at a level of 95% confidence limit.

RESULTS

The MIC test in this study was to determine the minimum concentration value of the active compound of Snakehead *(Channa striata*) fish bone nano-hydroxyapatite paste which can inhibit the growth of *S.mutans* ATCC 25175 bacteria. The results of the MIC test can be seen in Table 2.

Table 2. Minimum inhibitory concentration (mic) of snakehead fish bone nano-hydroxyapatite paste against *s.mutans.*

Treatment	Absorbance		Difference of absorbance (Mean ± SD)
	Before incubation (Mean)	After incubation (Mean)	
F1	2.000	1.532	-0.468 ± 15.821
F2	2.000	1.356	-0.644 ± 9.073
F3	2.000	1.279	-0.721 ± 5.859
Control (+)	2.000	1.166	-0.834 ± 4.041
Control (-)	1.026	1.783	0.757 ± 106.240

The absorbance before incubation in the treatment group of snakehead fish bone nano-hydroxyapatite paste (*Channa striata*) at F1, F2, F3 as well as the positive control group, was higher than after incubation. This value indicated that there was bacterial inhibition. The MIC of nano-hydroxyapatite was F1, which was the lowest concentration capable of inhibiting the growth of *S.mutans*. The highest value of bacterial inhibition was the positive control group. In the treatment group, the difference in absorbance increased with increasing concentration. An increase in absorbance was seen in the negative control group, indicating bacterial growth.

Table 3. Post-hoc games howell of mic of snakehead (*channa striata*) fish bone nano-hydroxyapatite paste

	F1	F2	F3	Control (+)	Control (-)
F1		0.001*	0.001*	0.001*	0.007*
F2			0.003*	0.000*	0.006*
F3				0.000*	0.005*
Control (+)					0.005*
Control (-)					

*Significantly different

One Way ANOVA result showed a significant value ($p < 0.05$), which means that there was a difference in the effect of snakehead fish (*Channa striata*) bone nano-hydroxyapatite paste at F1, F2, F3, positive and negative control on MIC values of *S.mutans* bacteria. The data were continued with Games Howell post-hoc test which aimed to see which groups showed differences. The results of the

post-hoc Games Howell test in Table 3 showed that the MIC values in the F1, F2, F3, positive and negative control groups had a significant difference.

MBC in this study was defined as the lowest concentration of Snakehead (*Channa striata*) fish bone nano-hydroxyapatite paste that yielded no colony growth by subculturing on agar plates. The results of the MBC test can be seen in Table 4.

Table 4. Minimum bactericidal concentration (mbc) of snakehead fish (*channa striata*) bone nano-hydroxyapatite paste against *s.mutans*

Treatment	Number of colony (Mean)
F1	235.00
F2	0.00
F3	0.00
Control (+)	0.00
Control (-)	574.33

The MBC of Snakehead fish (*Channa striata*) bone nano-hydroxyapatite paste against *S.mutans* bacteria was present at F2 which indicated that there was no growth of bacterial colonies, while at the negative control it still showed bacterial colony growth. The number of bacteria in the negative control was still relatively high, then it decreased at F1 and there was no bacterial growth at F2, F3 and positive control groups.

Table 5. Mann whitney test results of mbc of nano-hydroxyapatite paste from snakehead (*channa striata*) fish bone

	F1	F2	F3	Control (+)	Control (-)
F1		0.037*	0.037*	0.037*	0.050
F2			1.000	1.000	0.037*
F3				1.000	0.037*
Control (+)					0.037*
Control (-)					

*Significantly different

The results of the Mann-Whitney test in Table 5 show that at F2, there was no significant difference to F3 and the positive control. At F3, there was no significant difference to the positive control. In the F1 and negative control groups, there were significant differences in all treatment groups. This showed that at F1 and negative control, there was a significant difference in reducing the number of *S.mutans* bacteria compared to F2, F3 and positive control. In conclusion, based on the Mann-Whitney test, the F1, F2, and F3 showed a significant difference with the negative control, F1 showed a significant difference with F2 and F3, while the F2 and F3 groups did not show any significant difference.

DISCUSSION

Based on the results in Table 2 and 3, Snakehead (*Channa striata*) fish bone nano-hydroxyapatite paste at concentrations of 10, 15, and 20% were proven to inhibit the growth of *S.mutans* bacteria, marked by a decrease in absorbance values before and after incubation. This result is in accordance with Hariani, et al which conducted research on *E. coli* and *S. aureus* bacteria.[12] The bacterial growth inhibition ability increased significantly as the concentration of nano-hydroxyapatite paste increased. The MIC and MBC values obtained from the results of this study were 10% and 15%. The optimal concentration for caries prevention paste in the treatment group was 10%. At a concentration of 10%, the paste was able to inhibit the growth of *S.mutans* bacteria without killing the bacteria. This result is in accordance with the research results of Nambiar, et al and Seyedmajidi, et al which stated that application of hydroxyapatite for 48 hours could inhibit the growth of *S.mutans* and did not cause the death of all *S. mutans*.[17,18] *S.mutans* are oral commensal bacteria, and the use of antibacterial agents that kill all *S.mutans* can cause an imbalance in the microbiome.

Imbalances of the oral microbiome and dysbiosis have traditionally been linked to the occurrence of teeth and oral diseases.[10,11] This is probably because of HAp/nHAp's antibacterial properties, which are contingent upon its dimensions, surface area, morphology, porosity, crystallinity, stoichiometry, and ion types and concentration.[19]

In this study, no analysis was carried out on the mechanism of inhibiting bacterial growth, but based on previous research it was stated that the inhibition mechanism of nano-hydroxyapatite paste can occur when the OH- group in nano-hydroxyapatite destroys the bacterial cell wall and protein structure. The OH- group combines N-acetyl muramic acid into a mucopeptide structure in damaging the bacterial cell wall, while the cause of the damage to the bacterial protein structure is the occurrence of protein denaturation due to chemicals.[12] Denaturation and coagulation of proteins will cause lack in bacteria's physiological activity, and as the result, its function cannot work properly. The protein structure that altered bacterial cell wall will increase cell permeability, resulting in inhibition of cell growth, then this mechanism will cause the damage to whole bacteria structure, and in the end, this results in death of *S. mutans*.[19] Growth inhibition is also related to the bacterial cell wall through the mechanism of peptidoglycan destruction. The hydrogen bonds between the H atoms on the hydroxyapatite and the O atoms on the peptidoglycan disrupt the tetrapeptide bonds so that the bonds in the peptidoglycan become unstable so that the peptidoglycan is easily destroyed.[12] Nano-hydroxyapatite also caused the increase of calcium and phosphate ions concentration in dental biofilm. The bacteria will absorb the phosphate as food and then the cell will be destroyed.[20]

Snakehead (*Channa striata*) fish bone nano-hydroxyapatite has the ability to kill all *S.mutans* at concentration of 15% as shown in Tables 4 and 5. This result is different from the results of research by Nambiar, et al which showed that hydroxyapatite did not cause the death of all *S.mutans* in 24 hour incubation even though the hydroxyapatite concentration was increased. In Nambiar's research, *S.mutans* experienced total death after application for 48 hours at a concentration of 20%.[18] Mousavi *et al.*,[21] conducted research on the antibacterial ability of nano-hydroxyapatite synthesized from eggshell and sheep bones waste against other gram-positive bacteria, namely *S. aureus*, and the results showed that nano-hydroxyapatite has the ability to kill all bacteria at concentrations 500µg/mL. This difference in concentration is likely due to the antibacterial ability of hydroxyapatite being influenced by size, external surface and type of bacteria.[19] Bactericidal performance of nano-hydroxyapatite can result from increasing the surface charge potential of the nanomaterials. The antibacterial mechanism is centered on the ability of the nanostructures to stretch the bacterial membrane in the process of bacterial adhesion.[22,23] By reducing the particle size it will increase the surface area, and increase contact with the surrounding environment, so the release of ions is faster than regular-sized particles.[17]

Based on the results of the Games Howell post hoc test (Table 3), the ability to inhibit the growth of *S.mutans* bacteria in the treatment group was significantly lower compared to CPP-ACP. While the ability of nano-hydroxyapatite paste at a concentration of 15% (MBC value) is comparable to CPP-ACP in killing *S.mutans* bacteria (Table 5). No previous studies have compared the antibacterial effectiveness of nano-hydroxyapatite with CPP-ACP. Previous studies compared the effectiveness of nano-hydroxyapatite remineralization with CPP-ACP.[24,25] Positive control CPP-ACP exhibited a propensity for initiating the nucleation and crystallization processes of calcium phosphates within well-developed biofilms. CPP-ACP also induced the formation of deposits within the interstices among immobilized bacteria in the biofilm, resembling structures akin to calcium phosphate microcrystals. Interestingly, this deposit displayed minimal adhesion to the bacterial surface, implying the creation of unfavorable binding sites for bacteria. Consequently, the existence of this deposit on the surface could

potentially serve as a mechanism to impede the development of bacterial biofilms.[6]

In the negative control group, there was still bacterial growth after being given treatment. The MIC and MBC values in both the treatment group and the positive control group showed significant differences from the negative control group because the treatment given was only the basic formula without the addition of nano-hydroxyapatite. The results are in accordance with the results of Nambiar, et al and Wadu *et al.,*[26] which indicates that the basic formula does not have antibacterial ability.[18]

The limitation of this research was that it was carried out in vitro under controlled conditions. There are various components in the oral cavity that can influence research results.

CONCLUSION

Nano-hydroxyapatite paste from Snakehead (*Channa striata*) fish bone with a concentration of 10% is effective in inhibiting *S.mutans* growth without killing the bacteria, but its inhibitory ability is still lower than the positive control CPP-ACP. Therefore, the implication of research is that prospective use as a toothpaste material is validated. Further research with modifications is needed to increase the effectiveness of nano-hydroxyapatite paste as an antibacterial agent in preventing dental caries. In vivo and in situ studies are also needed to see the effectiveness of nano-hydroxyapatite paste in the oral cavity.

Acknowledgement
The authors wish to gratefully acknowledge Lambung Mangkurat University to conduct the research opportunity and obtaining the grant

Author Contributions: Conceptualization, ND; Methodology, ND and IH; software, AR; validation, ND, AR, MLA and DKTP.; formal analysis MLA; investigation, AR and NH; resources, AR and NH; data curation, AR; writing original draft preparation, ND; writing review and editing, DKTP ang MLA ; visualization, DKTP, supervision, IH and MLA; project administration, AR.; funding acquisition, ND and DKTP. All authors have read and agreed to the published version of the manuscript."

Funding: Ministry of Education, Culture, Research and Technology

Institutional Review Board Statement: Ethical review and approval were waived for this study due to "Not applicable" for studies not involving humans or animals.

Informed Consent Statement: "Not applicable" for studies not involving humans.

Data Availability Statement: Data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study.

Conflicts of Interest: The authors declare no conflict of interest.

Antibacterial activity of nano-hydroxyapatite paste of snakehead fish bone against S.mutans

ORIGINALITY REPORT

14%	7%	9%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Poedji Loekitowati Hariani, Muryati Muryati, Muhammad Said, Salni Salni. "Synthesis of Nano-Hydroxyapatite from Snakehead (*Channa striata*) Fish Bone and its Antibacterial Properties", Key Engineering Materials, 2020 Publication	6%
2	jurnal.unpad.ac.id Internet Source	5%
3	Deby Kania Tri Putri, Fadel Amirda, Hilman Muzadi, Amy Nindia Carabelly, Isyana Erlita, Rahmiati. " The Antibacterial Activity of Actinomycetes Against the Growth of and ", BIO Web of Conferences, 2020 Publication	1%
4	Maharani Laillyza Apriasari, Selviana Rizky Pramitha, Dewi Puspitasari, Diah Savitri Ernawati. "Anti-Inflammatory Effect of Musa acuminata Stem", European Journal of Dentistry, 2020 Publication	1%

5	www.researchgate.net Internet Source	1 %
6	jurnal.unissula.ac.id Internet Source	1 %

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		