



## The Effect of Antibacterial Agent of *Pangasius sutchi* Bone Extract Against *Porphyromonas gingivalis*

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### Abstract

**Introduction:** Periodontal disease is the most common dental problem mainly due to bacterial infection. The highest virulence bacteria found in periodontal disease is *Porphyromonas gingivalis*. *Pangasius sutchi* bone extract contains bioactive peptides which have the potential effect to inhibit the growth of Gram-negative bacteria such as *P. gingivalis*. **Purpose:** This study aimed to determine the effect of an antibacterial agent of *Pangasius sutchi* bone extract against *Porphyromonas gingivalis*. **Methods:** This research was *in vitro* laboratory experimental study with a post-test-only control group design. The treatment groups consisted of *Pangasius sutchi* bone extract using concentrations of 10%, 20%, 40%, 60%, and 100%, positive control (cefixime) and negative control (placebo). **Results:** The results showed that each concentration of *Pangasius sutchi* bone extract had antibacterial properties. The largest diameter of the inhibitory zone was at 100% concentration. The minimum Bactericidal Concentration (MBC) was at a concentration of 40%, and the Minimum Inhibitory Concentration (MIC) was 20%. **Conclusion:** *Pangasius sutchi* bone extract had an antibacterial effect against *Porphyromonas gingivalis*.

**Keywords:** antibacterial; *Pangasius sutchi*; *Porphyromonas gingivalis*

### Introduction

Periodontal disease is an infectious disease that damages the soft tissue around teeth. It can be acute or chronic inflammation. This inflammation involving the periodontium occurs progressively, which includes the gingival tissue, alveolar bone, cementum, and periodontal ligament.<sup>1</sup> The main factor causing periodontal disease is the presence of bacteria in plaque. Bacteria that cause periodontal disease include *Tanerella forsythia*, and *Treponema denticola*, a cluster of red-complex Gram-negative anaerobic bacteria. One of the bacteria that cause periodontal disease with high virulence is *Porphyromonas gingivalis*.<sup>2</sup>

*Porphyromonas gingivalis* is a type of pathogenic obligate anaerobic bacteria found in the oral cavity, especially in the gingival sulcus and pocket.<sup>3</sup> The virulence factors of *P. gingivalis* are lipopolysaccharide (LPS), fimbriae, capsules, lipoteichoic acids, and outer membrane proteins.<sup>4</sup> LPS and fimbriae act as endotoxins that trigger inflammatory responses



by inducing the production of TNF- $\alpha$  and interleukin- $\beta$  by macrophage cells leading to cell damage in the periodontium.<sup>5</sup>

The treatment of choice in periodontal disease is maintaining oral hygiene, supragingival scaling, and antibiotic therapy.<sup>6</sup> Administering antibiotic drugs, such as cefixime, can inhibit the growth of *P. gingivalis*, but according to several studies, this antibiotic can make bacteria resistant.<sup>7</sup> It is necessary to carry out research that utilizes natural ingredients to treat *P. gingivalis*.

Previous studies have tested the antibacterial power of natural ingredients derived from animals, one of which is fish.<sup>8</sup> Natsir et al. explained that the bones of yellowfin tuna (*Thunnus albacares*) can be used as a Gram-negative antibacterial agent.<sup>9</sup> According to a survey by the Central Bureau of Statistics (BPS) in 2014, fish production in South Sumatra was 48,186.5 tons of seawater fish and 48,481.4 tons of freshwater fish.<sup>10</sup> Survey of the Ministry of Fisheries and Maritime Affairs in 2018 stated that one of the types of freshwater fish whose production is high in South Sumatra is catfish (*Pangasius sp*), reaching 137,662.05 tons.<sup>11</sup> This is expected to result in massive consumption. Thus, the resulting fish bone waste is also high, so it needs to be utilized.

One type of *Pangasius* that is cultivated in South Sumatra is *Pangasius sutchi*. Mahmoodani stated that *Pangasius sutchi* bones have important ingredients such as collagen and bioactive peptides.<sup>12</sup> Bioactive peptides are obtained through the hydrolysis process of collagen content from the fish bone extract by an acid such as acetic acid or hydrochloric acid.<sup>13</sup> Collagen and bioactive peptides contained in the fishbone extract have many benefits for some medications such as a wound dressing, triggering cell regeneration, and antibacterial agent.<sup>14</sup> Collagen from the bone extract of catfish sutchi contains bioactive peptides such as arginine, leucine, isoleucine, and hydroxyproline.<sup>13,14</sup> These active ingredients are also found in yellowfin tuna (*Thunnus albacares*), which has been proven to inhibit the growth of *Escherichia coli* at concentrations of 10%, 15%, and 20%.<sup>8</sup> Another study by Li (2020) showed that D-arginine peptide isolate has the ability to eliminate the biofilm of *Porphyromonas gingivalis*.<sup>15</sup> The presence of bioactive peptides in *Pangasius sutchi* bone extract has the potential to inhibit the growth of Gram-negative bacteria, such as *Porphyromonas gingivalis*. The research was conducted to determine the effect of an antibacterial agent of *Pangasius sutchi* bone extract against *Porphyromonas gingivalis*.



## Methods

The study was *in vitro* laboratory experiment with a post-test-only control group design. The samples of this study were *Porphyromonas gingivalis* strain ATCC 33277. Samples were divided into 7 groups; Groups A to E were treated with catfish bone extract concentrations of 10%, 20%, 40%, 60%, and 100%, respectively. The positive and negative control group was treated with cefixime 500 mg and a placebo, respectively.

*Pangasius sutchi* was obtained from Banjar Pinang Fish Seed Center, Musi Banyuasin, South Sumatra. The fish's mass weight was + 1.2-1.5 kg with ages over 6 months. Separation of the head bones, ribs, fin bones, and tail bones of *Pangasius sutchi* from the meat and skin was done using a knife.

The bones were soaked in a citric acid solution for 48 hours to convert the collagen into gelatin. Softened fish bones (ossein) were separated from the acid solution using a centrifuge at 4000 rpm for 15 minutes. Extraction was carried out by heating the ossein solution to 75° C for 5 hours in a water bath. The extract solution was filtered using Whatman grade 4 paper to separate the pure extract solution from the residue. The pure gelatin solution of catfish bone extract was stored in a test tube and stored in the refrigerator at 4°C until used.

Pure gelatin bone extract was incubated at 50°C in an incubator for 10 minutes. Then, 6% flovorenzim enzyme was added. The enzymatic process was carried out for 8 hours, then stopped by immersing the test tube in boiling water at 100°C for 20 minutes. Separation of liquid and gelatin was done using a centrifuge at 1000 rpm for 15 minutes. The finished collagen catfish bone extract containing bioactive peptides was stored again in the refrigerator at 18°C until used. Concentrations of 10%, 20%, 40%, 60%, and 100% were prepared by adding distilled water to bone extract.

*Porphyromonas gingivalis* ATCC 33277 strain was cultured on Brain Heart Infusion (BHI) media which had been given menadione (0.5 µg/mL) and hemin (5 mg/mL). Cultures were carried out under anaerobic conditions (5% CO<sub>2</sub>, 10% H<sub>2</sub>, and 85% N<sub>2</sub>) at 37°C for up to five to seven days. *Porphyromonas gingivalis* strain was harvested from BHI media and dissolved in units of 10<sup>8</sup> CFU/mL. The BHI agar medium was poured into a petri dish with a thickness of 5 mm. After the BHI had solidified, *P. gingivalis* colonies were inoculated using a cotton bud on the media.

The wells were made with a drill with 5 mm diameter with a minimum distance of 5 cm between the wells. Various concentrations of collagen extract, placebo, and cefixime 500



mg which had been dissolved were dripped over the wells of the *Porphyromonas gingivalis* colony as much as 20  $\mu$ L using a micropipette. *P. gingivalis* colonies that had been treated were incubated at 37°C for 24 hours. The inhibition zone of *P. gingivalis* was observed and measured using calipers.

100  $\mu$ L of *Porphyromonas gingivalis* suspension and BHI were put in a microplate. Various concentrations of catfish bone extract, placebo, and cefixime as positive controls as much as 100  $\mu$ L were included in the microplate. The microplate containing *Porphyromonas gingivalis* and the test solution were incubated for 24 hours at 37°C. The pour plate method was used by taking 50  $\mu$ L of *Porphyromonas gingivalis* suspension which had been previously incubated, transferring it to a petri dish containing BHI, and incubating again for 24 hours. Minimum Inhibitory Concentration (MIC) calculation is done by using a colony counter. The above steps are repeated four times.

Various concentrations of bone extract were included in a 96 microwell microplate. 100.00 ppm of BHI broth was added to the microplate. 5 ml of *Porphyromonas gingivalis* ATCC 33277 bacterial suspension was put into the microplate. Determination of Minimum Bactericidal Concentration (MBC) by looking at the presence or absence of *Porphyromonas gingivalis* colonies on microplates that had been incubated at 37°C for 48 hours.

Data were subjected to Oneway ANOVA and the mean comparisons were performed by post-hoc LSD using SPSS version 20.0. Differences between means were considered significant at  $p$ -value < 0.05.

## Results

The antibacterial effect can be seen from the diameter of the inhibition zone formed around the disc paper. The average diameter of the inhibition zone measurement results is illustrated in Table 1. It showed that *Pangasius sutchi* bone extract had an inhibition zone starting from a concentration of 20%. The largest inhibition zone was in the positive control group (cefixime 500 mg).

The results of the *Shapiro-Wilk* normality test from all the treatment and control groups showed that the data were normally distributed ( $p > 0.05$ ). It was further analyzed with *Levene's* homogeneity test, showing that the data were homogenous ( $p > 0.05$ ). Furthermore, the parametric *One-Way ANOVA* was done to determine the significance of the difference in the



average diameter of the inhibition zone between study groups. The results showed a statistically significant difference in the average value of the inhibition zone between the treatment group (significance value of 0.000 ( $p < 0.05$ )). Statistical analysis continued with *the Tamhane Post-hoc* test to see which groups have significant differences. The results of the Tamhane Post-hoc test can be seen in Table 2.

**Table 1.** Means of inhibitory zone

No	Groups	n	Inhibition zones	p
1	The concentration of 10%	5	0.00 ± 0.00	
2	The concentration of 20%	5	10.71 ± 0.44	
3	The concentration of 40%	5	13.79 ± 0.30	
4	The concentration of 60%	5	16.79 ± 0.22	0.00*
5	The concentration of 100%	5	19.64 ± 0.29	
6	Positive control	5	22.81 ± 0.32	
7	Negative control	5	0.00 ± 0.00	

\* one-way ANOVA,  $p=0.05$

**Table 2.** Compatibility of the inhibitory zone between groups

Group	10%	20%	40%	60%	100%	cefixime	placebo
10%		0.00*	0.00*	0.00*	0.00*	0.00*	-
20%			0.00*	0.00*	0.00*	0.00*	0.00*
40%				0.00*	0.00*	0.00*	0.00*
60%					0.00*	0.00*	0.00*
100%						0.00*	0.00*
cefixime							0.00*
placebo							

\* *Post-hoc* Tamhane test,  $p=0.05$



Table 2 showed that all groups had a significance value of less than 0.05 ( $p < 0.05$ ). It could be concluded that there were significant differences between the *Pangasius sutchi* bone extract test groups, the positive control, and the negative control.

## Discussion

The results of this study proved that *Pangasius sutchi* bone extract with concentrations of 20%, 40%, 60%, and 100% had antibacterial properties. The inhibition zone was visible in those groups, and the largest diameter at 100% concentration. The higher the concentration of *Pangasius sutchi* bone extract, the greater the inhibition zone, so it was more effective in inhibiting bacterial growth. Li *et al.* (2020) found that D-arginine in *Pangasius sutchi* bone extract could inhibit the growth of Gram-negative bacteria.<sup>15</sup> Previous studies stated that the antibacterial effect of a material was directly proportional to its concentration.<sup>16,17</sup>

*Pangasius sutchi* bone extract has antibacterial properties because it contains bioactive peptides, such as histidine, serine, arginine, glycine, aspartate, glutamate, threonine, alanine, proline, cystine, lysine, tyrosine, methionine, valine, isoleucine, leucine, and phenylalanine.<sup>18</sup> The hydrophobic nature of bioactive peptides acted as a negative charge that would bind to the positive charge on the bacterial membrane lipids.<sup>19</sup> This positive-negative charge bond caused holes in the bacterial membrane so that the bacterial cell would lyse.<sup>16</sup> Arginine, an essential amino acid, has guanidine and amine groups in its chain structure.<sup>17</sup> Those were able to reduce attachment and destroyed *Porphyromonas gingivalis* colonies.<sup>19</sup> Lysine and histidine contained in tuna and squid gelatin showed the inhibition of bacterial growth. Proline could bind with tannins to form complexes that cause damage to bacterial cell walls.<sup>20</sup> Phenylalanine produced phenylalanine acid that has proven as a broad-spectrum antimicrobial compound against bacteria and fungi.<sup>21</sup>

In this study, the inhibition zones formed in the extract groups with concentrations of 20%, 40%, 60%, and 100% were categorized as strong.<sup>6</sup> However, the diameter of the inhibition zones formed was smaller than the positive control. The structure of the cell wall of pathogenic bacteria influences antibacterial activity. The cell wall structure of Gram-negative bacteria *in vitro* was more complex than Gram-positive bacteria because it had an outer membrane layer, peptidoglycan, and lipopolysaccharide, which blocked antibacterial compounds from entering the bacterial cell.<sup>22</sup>





The colony counter determined that the minimum bactericidal concentration was 40% because there was no bacterial growth, while at a concentration of 20%, there was still bacterial growth. The minimum inhibitory concentration was 20%. The results also showed that inhibition zones in media treated with cefixime positive control were clearer than the groups treated with *Pangasius sutchi* bone extract.

## Conclusion

It can be concluded that *Pangasius sutchi* bone extract had an antibacterial effect against *Porphyromonas gingivalis*

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