

## The inhibitory effect of gourami fish scale (*Osphronemus gouramy*) extract-curcumin combination on *Porphyromonas gingivalis*

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### Abstract

**Background:** Gourami fish scales, often considered as a waste, turned out to be an alternative source for antibacterial components. Its extract contains alkaloid, an antibacterial agent, while also contains hydrogen bonds which have the effect of increasing the interaction with other substances. Curcumin is one of the active ingredients of turmeric, a spice commonly used as an herbal medicine in Indonesia. Curcumin could suppress bacterial proliferation by inhibiting virulence agents. The combination of these substances was expected to help inhibit *Porphyromonas gingivalis* growth, one of the key pathogens in periodontitis patients.

**Objective:** This study aims to determine the inhibitory effect of the gourami fish scale extract-curcumin combination on the growth of *P. gingivalis* bacteria.

**Method:** This research is an *in vitro* experimental analytical laboratory research. The concentrations used are 100%; 50%; 25%; 12.5%; 6.25%; 3.125%; 1.56%; and 0.78%. The inhibition testing on *P. gingivalis* bacteria cultures was carried out by spectrophotometric methods and colony counts.

**Results:** Gourami fish scale extract-curcumin combination with a concentration of 12.5%; 25%; 50%; and 100% killed colonies of *P. gingivalis* bacteria resulting in 0 colonies. A concentration of 6.25% inhibits bacterial growth by 92.9%. The statistical test result showed a significant difference ( $p < 0.05$ ).

**Conclusion:** Gourami fish scale extract-curcumin combination has an inhibitory effect on the growth of *P. gingivalis* bacteria with MIC value at the concentration of 6.25% and MBC of 12.5%.

**Keywords:** *Osphronemus gouramy*; Fish scale extract; Curcumin; Antibacterial; Periodontitis; *Porphyromonas gingivalis*

### 1. Introduction

Periodontal disease is a complex multifactorial disease caused by a polymicrobial infection on the periodontal tissue which consists of the periodontal ligament, gingiva, alveolar bone, and cementum [1, 2]. It usually starts with gingivitis, an inflammation centered on the gingival epithelium and connective tissue. It mainly occurs due to the invasion and accumulation of plaque bacteria on the periodontal tissues or tooth surfaces [3, 4]. If not being properly treated, gingivitis will continue to become periodontitis, an inflammation of the whole periodontal tissue. This could lead to tooth loss which furthermore will affect the patient's masticatory and phonetic function, diet intake, and self-confidence [5].

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Polymicrobial infection, along with the host's immune response and oral hygiene, are considered the etiology of periodontal disease [6]. One of the main bacteria causing periodontal disease is *Porphyromonas gingivalis*. It is a gram-negative, anaerobic, rod-shaped, and black-pigmented bacteria [7, 8]. Some virulence factors like lipopolysaccharide (LPS), fimbriae, gingipain, and other enzymes that could disturb the host's immune system, are produced by *Porphyromonas gingivalis* in plentiful amounts. These virulence factors play a major role in the progressivity of periodontal disease [9, 10].

Periodontal disease could be treated with a plaque removal procedure (scaling), root planing, resective and reconstructive surgical techniques, and the use of mouthwash & other antibacterial agents [4]. Antibiotics like metronidazole, amoxicillin, clindamycin, azithromycin, and clavulanic acid have been tested clinically and could be given systemically or orally in patients with periodontitis. But several studies show that *Porphyromonas gingivalis* is resistant to some of those antibiotics [11]. Therefore, lately antibacterial agents made from natural ingredients are being developed [12].

Gourami fish (*Osphronemus gouramy*) is a common fish found in Indonesia. The scales, often considered as waste, turns out to be a potential antibacterial agent due to its alkaloid substance [13, 14]. Alkaloid could disrupt the virulence factors of *Porphyromonas gingivalis* through binding with FtsZ protein, therefore inhibiting the bacteria's replication, and also inhibiting its DNA synthesis process [15, 16]. Curcumin, an active flavonoid substance from *Curcuma longa*, could also interfere with the proliferation of *Porphyromonas gingivalis* by inhibiting the *arginine-specific protease* (Arg-gingipain (RGP)) and *lysine-specific protease* (Lys-gingipain (KGP)) activity [17, 18]. But, according to several reviews, curcumin has a low bioavailability in the human body [19]. Gourami fish scales, on the other hand, had a tendency to bind with other substances so together they have a better bioavailability [20].

On that account, the combination of gourami fish scale extract and curcumin is expected to be an alternative therapy option and giving a maximal therapeutic effect on patients with periodontal disease. In this study, the authors wanted to observe the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of gourami fish scale extract-curcumin combination on the growth of *Porphyromonas gingivalis* bacteria through a spectrophotometric method and colony count test.

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## 2. Material and methods

This study was conducted from August 2022 until September 2022 at the Airlangga University Dental Medicine Research Center. Gourami (*Osphronemus gouramy*) fish scales were extracted in the Chemistry Laboratory of Science and Technology Faculty at Airlangga University according to the protocol of the laboratory.

This study is an experimental analytical laboratory research with post-test only control group design consisting of 10 treatment groups: gourami fish scale extract-curcumin combination with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, positive control, and negative control. After doing the calculation using the Federer formula, it is found that the repetition should be held 3 times at a minimum, therefore the total sample would be 30.

### 2.1. Materials and Equipment Preparation

The materials used in this experiment are gourami fish scale extract-curcumin combination, 96% ethanol, sulfuric acid, acetic acid, pure *Porphyromonas gingivalis* isolate, Brain Heart Infusion Broth (BHIB) media, Muller-Hinton Agar (MHA), McFarland standard 0.5, and sterile aquades. The equipment used are a maceration vessel, stirring rod, plastic container, strainer, aluminum foil, filter paper, funnel, volume pipette, analytical balance, measuring glass, blender, vacuum rotary evaporator, water bath, micropipette, test tube, petri dish, incubator, round ossicle, autoclave, bunsen, Uv-Vis BKD-560 spectrophotometer, vortex mixer, colony counter, eppendorf, erlenmeyer flask, sterile cotton, sterile yellow and blue tips, tube rack, and magnetic stirrer.

### 2.2. Preparation of Gourami Fish Scale Extract

#### 2.2.1. Deproteinization

A total of 100 grams of gourami fish scales were soaked in 1N NaOH solution in a ratio of 1:7. The mixture was then heated at 100 °C for 1 hour with continuous stirring. After that, the mixture is rested at room temperature until it has slightly cooled. The mixture is then separated between the filtrate and the precipitate by filtering it using a Buchner funnel. The precipitate obtained was then washed repeatedly with aquades until the pH was neutral.

### 2.2.2. Demineralization

The precipitate obtained from the deproteinization stage was then dissolved in 1N HCl solution in a ratio of 1:5 at room temperature for 30 minutes. The precipitate was then separated using a Buchner funnel and washed repeatedly until the pH was neutral. The precipitate is then baked at a temperature of 50-60 °C until it's dried.

### 2.2.3. Deacetylation

The precipitate resulting from the demineralization process was then soaked with 50% NaOH solution in a ratio of 1:5 and heated at a temperature of 100 °C for an hour. The precipitate was then separated using a Buchner funnel and washed repeatedly until the pH was neutral. The precipitate is then once again baked at a temperature of 50-60 °C until it's dried.

## 2.3. Combination Process of Gourami Fish Scale Extract and Curcumin

The gourami fish scale extract was then combined with the curcumin using a nanoencapsulation procedure. A total of 10 mg of curcumin was dissolved in a mixture of 0.1N NaOH solution and 50% alcohol in a ratio of 1:1 (Solution A). Then, 10 mg of gourami fish scale extract and 10 mg of polyvinyl alcohol (PVA) were dissolved in a 2% acetic acid solution (Solution B). Solution A and Solution B were then mixed and ultrasonicated at 60% power for 5 minutes. The results of encapsulation of curcumin with gourami scale extract and PVA were characterized by dynamic light scattering (DLS), fourier transform infrared (FTIR), thermogravimetric analysis (TGA), and scanning electron microscopy (SEM) [21].



**Figure 1** Gourami fish scale extract-curcumin combination

## 2.4. MIC & MBC Test on *Porphyromonas gingivalis*

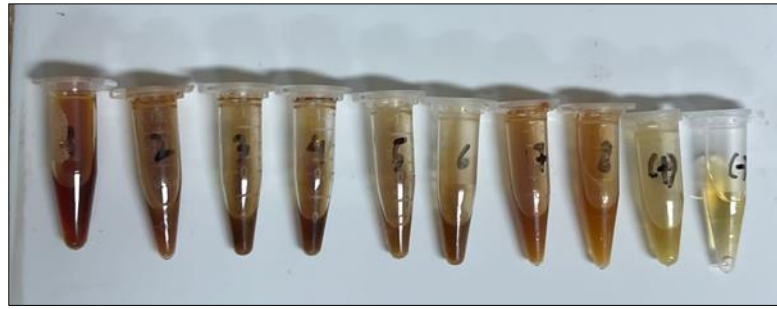
The next step is the preparation of the pure isolates of *Porphyromonas gingivalis* bacteria obtained from the Research Center of the Faculty of Dental Medicine, Airlangga University. The pure isolates were then grown on Muller-Hinton Agar (MHA) media and incubated anaerobically into an incubator for 1x24 hours at 37 °C to create bacterial cultures. After the incubation, the suspension was made by taking *Porphyromonas gingivalis* from the culture medium using an ossicle, then putting it in a test tube containing 1 ml of sterile Brain Heart Infusion Broth (BHIB) and then putting it in an incubator, and incubated anaerobically for 1x24 hours at 37 °C. Next, the dilution was carried out by adding sterile aquades and homogenized until the turbidity was comparable to the standard Mc Farland 0.5 ( $1.5 \times 10^8$ ).

The bacterial suspension that had been standardized with Mc Farland turbidity 0.5 ( $1.5 \times 10^8$ ) was put into each test tube containing 1 ml of gourami scale extract-curcumin combination with 8 different concentrations, namely 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, test tubes containing bacteria culture and media as positive controls, and test tubes containing only the media as negative controls. After that, each test tube was incubated for 1x24 hours at 37 °C. The test tubes were then measured to determine the inhibitory effect of the gourami scale extract-curcumin combination on the growth of *Porphyromonas gingivalis* by observing MIC and MBC using a UV-Vis spectrophotometer BKD-560 ( $\lambda = 560 \text{ nm}$ ) and a colony counter.

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## 3. Results

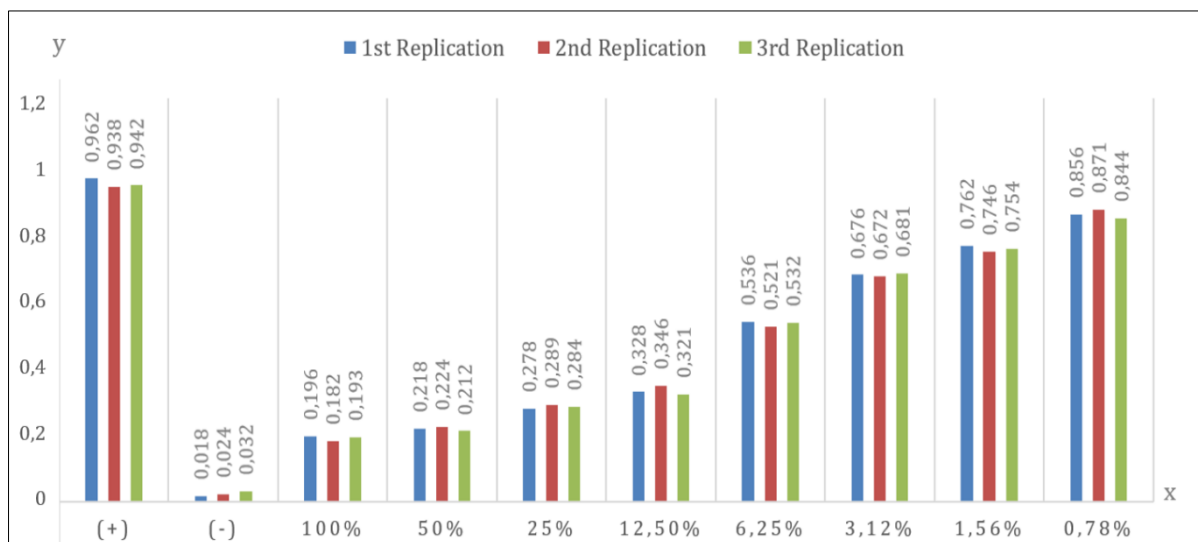
The antibacterial inhibitory effect of the gourami scale extract-curcumin combination was tested using spectrophotometric and colony count test methods to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) on the growth of *Porphyromonas gingivalis* bacteria.



**Figure 2** The result from serial dilution test of gourami fish scale extract-curcumin combination in Brain-Heart Infusion Broth

Serial dilution was done to make the various concentrations of the 10 treatment groups. As can be seen in (Figure 2), tube 1 contained a mixture of gourami fish scale extract-curcumin combination with a concentration of 100% in 1 mL of Brain-Heart Infusion Broth with 1 mL of the bacterial suspension. Tube 2, 3, 4, 5, 6, 7, and 8 contained a mixture of 1 mL bacterial suspension with 1 mL gourami fish scale extract-curcumin in 1 mL of Brain-Heart Infusion Broth with each concentration of about 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%. Tube (+) was a positive control, made from 1 mL bacterial suspension in 1 mL Brain-Heart Infusion Broth, and Tube (-) was a negative control, made from 1 mL gourami fish scale extract-curcumin combination with concentration 100% in 1 mL Brain-Heart Infusion Broth.

Then, a spectrophotometry test was conducted to determine whether the gourami fish scale extract-curcumin combination actually has antibacterial activity against *Porphyromonas gingivalis*, by observing the differences in general between all the absorbances.

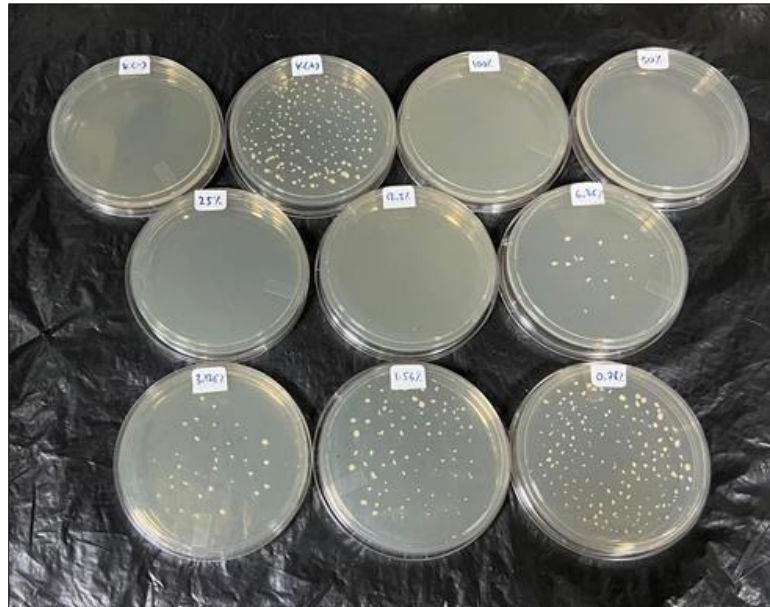


**Figure 3** Absorbance Results Using a Spectrophotometry (OD)

Based on (Figure 3), it can be seen that the average of the highest spectrophotometric test results in *Porphyromonas gingivalis* bacteria was  $0.947 \pm 0.013$  OD, namely when given positive control treatment. The average of the lowest spectrophotometric test results was when the negative control treatment was given, namely  $0.025 \pm 0.007$  OD.

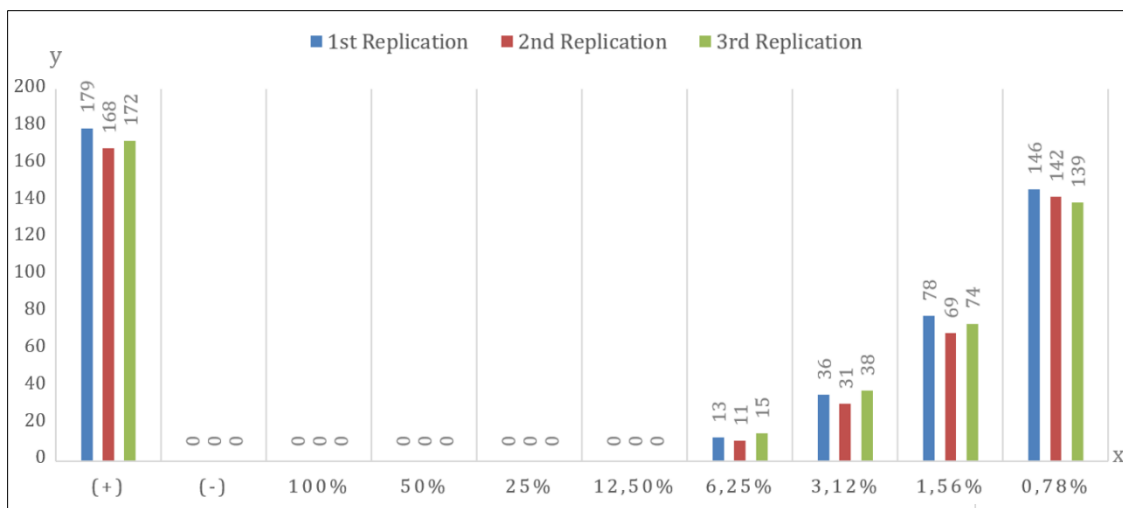
The spectrophotometry results concluded that the concentration level of the gourami fish scale extract-curcumin combination is directly related to the decrease in the number of the *Porphyromonas gingivalis* bacterial colonies. The decrease of the bacterial colonies can be seen from the reduction in the value of optical density along with the increasing levels of the concentration of the gourami fish scale extract-curcumin combination that was given.

After conducting the spectrophotometry test, the dilution test results were then cultured into Muller-Hinton Agar to do a colony count test. Each plate represents each tube with the concentration. There was no growth of *Porphyromonas gingivalis* colony on plate 100%, 50%, 25%, and 12.5%, while plate 6.25%, 3.125%, 1.56%, and 0.78% shows growth of *Porphyromonas gingivalis*.



**Figure 4** Results of colony counts test

The MIC and the MBC value were determined based on the average number of bacterial colonies growing on MHA media. The results of the colony count test can be seen in Figure 5.



**Figure 5** Colony Count Results with Colony Counter (CFU/ml)

While on (Figure 5), it can be seen that the highest average colony count test result in *Porphyromonas gingivalis* bacteria was 173.00 CFU/ml when positive control was given. The lowest average colony count test results were when the concentration of gourami fish scale extract and curcumin were 100%, 50%, 25%, and 12.5%, and negative control where the colony count test results reached the lowest point or the best, namely 0 CFU/ml.

Gourami fish scales extract and curcumin combination with a concentration of 6.25% had a colony count test result of 13 CFU/ml. The less gourami scale extract and curcumin combination is given, the higher the colony count test results. This can be seen from the concentration of 3.125% having a colony count test result of 35 CFU/ml, a concentration of 1.56% having a colony count test result of 73.67 CFU/ml, and a concentration of 0.78% having a colony count test result of 142.33 CFU/ml.

In order to acknowledge the MIC and the MBC value, the calculation of the bacterial inhibition percentage and average colony growth was conducted. The table following contains the calculation result:

**Table 1** Average Colony Growth and Bacterial Inhibition Percentage

Group		Average Colony Growth (CFU/ml)	Bacterial Inhibition Percentage (%)
Concentrations	0.78%	142.33	17.7
	1.56%	73.67	57.4
	3.125%	35	79.8
	6.25%	13	92.5
	12.5%	0	100
	25%	0	100
	50%	0	100
	100%	0	100
Positive Control		173	0
Negative Control		0	-

Gourami fish scale extract-curcumin combination with a concentration of 6.25% can be expressed as MIC (Minimum Inhibitory Concentration) because with this concentration the growth of bacterial colonies can be suppressed by 92.5% (>90%). Meanwhile, MBC (Minimum Bactericidal Concentration) is determined as the smallest concentration that can kill bacteria so that no colonies can grow, which is found at a concentration of 12.5%.

In this study, statistical data analysis and determination of MIC and MBC were done using the data from bacterial colony growth on Muller-Hinton (MH) agar media. The data obtained was then tested for statistical analysis with the normality test using the *Shapiro-Wilk* test. The test results show that all treatment groups have a value  $p > 0.05$ , thus the data is normally distributed. The test is continued with the homogeneity test, using the *Levene* test. The test obtained a  $p$ -value of 0.007, so because it is  $< 0.05$ , it means that the data is not homogeneous. With a non-parametric test, Kruskal Wallis, the  $p$  result is 0.001, which means the data has a significant difference. The result of the post hoc Mann Whitney test (Table 2) showed that concentrations of 6.25%, 3.125%, 1.56%, and 0.78% have significant differences with concentrations of 100%, 50%, 25%, and 12.5%.

**Table 2** The Result of Post Hoc Mann Whitney test

Groups	Control (+)	Control (-)	100%	50%	25%	12.5%	6.25%	3.125%	1.56%	0.78%
Control (+)										
Control (-)	0.037*									
100%	0.037*	1.000								
50%	0.037*	1.000	1.000							
25%	0.037*	1.000	1.000	1.000						
12.5%	0.037*	1.000	1.000	1.000	1.000					
6.25%	0.050	0.037*	0.037*	0.037*	0.037*	0.037*				
3.125%	0.050	0.037*	0.037*	0.037*	0.037*	0.037*	0.050			
1.56%	0.050	0.037*	0.037*	0.037*	0.037*	0.037*	0.050	0.050		
0.78%	0.050	0.037*	0.037*	0.037*	0.037*	0.037*	0.050	0.050	0.050	

\*Shows significant differences

#### 4. Discussion

Secondary infection incident in the middle of the process of alveolar bone resorption treatment with the bone graft addition method has a high prevalence. Some anaerobic bacteria which cannot be eliminated by liquid medication, such

as chlorhexidine, turns into pathogenic bacteria that contribute to the continuation of the disease. *Porphyromonas gingivalis* is one of the bacteria mentioned above. Hence, an antibacterial material is needed in the bone graft to fight the bacteria. On the other hand, the demand for alternative bone graft materials at a more affordable price than those available in the market also increases, causing researchers to look for other potential materials, and one of the materials being considered is gourami fish scales.

Treatment with scaffold material is also expected along with the provision of antibacterial agents, to overcome the invasive action of anaerobic bacteria that reside in the subgingival area, dental apices, and alveolar bone surfaces. Moreover, based on the study conducted before, curcumin had become a choice of antibacterial material to be paired with gourami fish scale scaffold material. Other than its function as a scaffold material, gourami fish scale is also known to contain an alkaloid material, which is useful in antibacterial activity. The antibacterial mechanism of alkaloids is through the process of inhibiting cell division, by obstructing dihydrofolate reductase, and topoisomerase I, enzyme activity. Thus, resulting in inhibition of the nucleic acid synthesis. Alkaloids can also interfere with GTPase activity, by binding into the protein that regulates cell division, the FtsZ protein. Moreover, alkaloids can induce elongation thereby interfering with DNA replication, membrane structure, nucleoid segregation, and inhibition of bacterial cell division [15].

As a polyphenolic compound, curcumin has two active sites for phenolic hydroxyl and diketone. During the reaction, the active sites are in the two phenolic hydroxyl groups, with a mechanism involving hydrogen atoms, and electron transfer. In addition, intermediates auto-oxidation and curcumin by-products are also known to exhibit biological activity. Furthermore, curcumin is a tautomeric compound in the form of diketone, and enol. Curcumin can inhibit bacterial growth by targeting bacterial cell membranes, cell walls, proteins, and DNA, as well as with the quorum sensing (QS) system [22, 23]. Curcumin is also known to inhibit arginine-specific protease (Rgp) and lysine-specific protease (Kgp), by binding to form hydrogen bonds, interfering with the proliferation process of *Porphyromonas gingivalis* [18, 22].

In this study, after serial dilution was done, two methods were used for determining the inhibitory effect of gourami fish scale extract-curcumin combination: spectrophotometry test and colony counts test. According to the absorbance result (optical density) using a spectrophotometer (Figure 3), the absorbance value increases as the concentration decreases. On the 100% concentration, *Porphyromonas gingivalis* growth was still found. Meanwhile, based on the colony counts test, at a concentration of 12.5%; 25%; 50%; and 100%, there was no sign of *Porphyromonas gingivalis* growth. These results are in accordance with the statement that the use of spectrophotometry has a weakness, which is that the residues from dead bacteria could affect the turbidity of the solution [24].

In addition, the measurement of Minimum Bactericidal Concentration (MBC) using spectrophotometry is difficult to do, since the media used is a liquid media, and on the 100% concentration, there are some components that were still found in BHIB media although there were inhibition and a decrease in the bacterial growth. However, colony counting using spectrophotometric tools has the advantage such as it can be used quickly, is relatively inexpensive, and is non-destructive [25, 26]. Therefore, in this study, statistical data analysis and determination of MIC and MBC were done using the data from bacterial colony growth on Muller-Hinton (MH) agar media.

Lastly, even though the result stated that the gourami scale extract-curcumin combination does have an antibacterial effect on the *Porphyromonas gingivalis*, it is still unknown which active components that participate most in inhibiting the growth of *Porphyromonas gingivalis* bacteria. Thus, further research was required to reveal it.

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## 5. Conclusion

The conclusion is the gourami fish scale extract-curcumin combination has an inhibitory effect on the growth of *Porphyromonas gingivalis* bacteria with MIC value at the concentration of 6.25% and MBC at the concentration of 12.5%.

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## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors have no conflict of interest to declare.

*Statement of ethical approval*

This research study was approved ethically by Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission, with an ethical clearance letter number: 799/HRECC.FODM/X/2022.

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