ISSN 0975-2366
DOI: https://doi.org/10.31838/ijpr/2020.SP3.104

Research Article

White shrimp of (*litopenaeus vannamei*) scalp waste edible chitosan film as inhibitor effect of *porphyromonas gingivalis* bacteria growth (*in vitro*)

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Received: 02.10.20, Revised: 02.11.20, Accepted: 02.12.20

ABTRACT

Background: Chitosan is a biopolymer that usually extracted from the shells of crustaceans such as crabs, shrimp, and lobster. The advantages of chitosan as an antimicrobial are abundant availability, low production costs, excellent biodegradability, biocompatibility, and bioresorbability, and sufficient chemical modification. The concept that underlies chitosan as an antimicrobial is the functional group of amines in chitosan that can form bonds with bacterial cell walls and cause intracellular leakage of constituents that leads to the bacteria extermination. Chitosan can show antibacterial activity against bacteria that cause periodontitis, one of which is *Porphyromonas gingivalis*. **Objective:** This research aims to observe and identify the effectiveness of chitosan edible films in inhibiting the growth of *Porphyromonas gingivalis* bacteria. **Method:** This study uses a laboratory experimental research type using the *Post-test only with control group* research design. In this study, six repetitions were carried out in 8 treatments with six repetitions each, namely chitosan extract with concentrations of 1%, 2%, and 3%, edible chitosan film 1%, 2%, and 3%, positive control (edible film metronidazole), as well as the negative control. The measuring instrument used in this study is the calipers in millimeters (mm). **Results:** Least Significance Different (LSD) result shows some significant differences in inhibiting the growth of Porphyromonas gingivalis bacteria ($p<0.005$) between each treatment. **Conclusion:** Edible chitosan film from white shrimp (*Litopenaeus vannamei*) scalp waste can inhibiting the growth of *Porphyromonas gingivalis* bacteria.

Keywords: *Edible Chitosan Film, Porphyromonas gingivalis, Antimicrobial.*

INTRODUCTION

Periodontal tissue is the supporting network of teeth consisting of the gingiva, cementum, periodontal ligament, and alveolar bone. Periodontal disease is a pathological condition affecting the periodontal tissue.[1] Periodontal disease ranges from gingivitis (mild, reversible gingival inflammation) to periodontitis (an irreversible pathological condition of the periodontal). Periodontitis will eventually cause erosion of the periodontal ligament, loss of cementum adhesions, resorption of alveolar bone, and exfoliation of the teeth.[2]

The World Health Organization (WHO) in 1978 concluded about the prevalence of periodontal disease based on various studies from communities around the world that periodontal disease is one of the most widespread diseases in humans. Based on the results of the Household Health Survey of the Ministry of Health of the Republic of Indonesia in 1995, the prevalence of periodontal disease patients in Indonesia (by measuring tartar and calculus) reached 42.8%.[3] Periodontitis is characterized by the presence of a periodontal pocket that forms in the gingival groove area when the groove is more than 4 mm deep. Based on the results of the research of tribes in South Sulawesi between the Bugis and Mandar tribes, it shows that the prevalence of periodontal pockets in the Bugis and the Mandar ethnic group is 58% and 44%, respectively. The prevalence was higher in the sample of men and old age compared to the sample of women and young people both in the Bugis and Mandar Tribe. Periodontal disease is multifactorial with risk factors related to genetics, age, sex, smoking, nutrition, stress, and economic factors.[4]

However, in general the etiology of periodontal disease is grouped into two, the first is local factors consisting of plaque and non-plaque, the second is systemic factors. Plaque is associated with initial factors such as bacterial plaque as the main cause

of periodontitis and bacterial plaque when it collects subgingival, can cause inflammation of the gingiva while systemic factors such as cardiovascular disease, stroke, pneumonia and premature birth with low body weight. Periodontal disease can be involved in systemic disease if pathogenic bacteria are present in the bloodstream, leading to extraoral infection.[5]

Periodontal disease is often associated with microbial infections caused by biofilms, plaques and calculus. Common bacteria that cause this disease are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. A number of publications state that microorganisms from the subgingival microbiota, in this case gram-negative anaerobes, are a major contributing factor for chronic and aggressive periodontitis. A study has identified several periodontal pathogens, one of which is *Porphyromonas gingivalis*.[6]

Porphyromonas gingivalis is a pro-dominant periodontal pathogen that is able to attack the oral epithelium in vitro. This bacterium is a group of Black-pigmented Gram-negative anaerobic bacteria colonies and includes melanogenic, non-carolytic bacteria. These bacteria are found in dental plaque and will cause pathological changes in the periodontal tissue through the activation of immune and inflammatory responses from host cells, and directly affect the periodontium cells.[7]

In maintaining healthy teeth and mouth, Indonesian people stick to the method of brushing their teeth twice a day. However, this is often ineffective in ensuring the cleanliness of the teeth from plaque that causes periodontitis. Therefore, the importance of other efforts to prevent periodontitis include the use of antimicrobial agents. Antibiotics are antimicrobial compounds that have been used so far. But in use, antibiotics have disadvantages such as causing allergies, toxicity, and resistance to long-term use. An antimicrobial alternative that is safer and in a form that is simple, inexpensive, and easy for the community to use is needed.[8]

One that can be developed as an alternative to antimicrobials is chitosan. Chitosan is a biopolymer that can be extracted from the shells of marine crustaceans such as crab, shrimp, and lobster. The advantages of chitosan as an antimicrobial are that it is abundantly available in nature, low production costs, good biodegradability, biocompatibility, and bioresobsibility, as well as sufficient chemical modification. The main concept underlying chitosan's antimicrobial properties is due to the presence of an amine functional group in chitosan which can form bonds with bacterial cell walls and result in leakage of intracellular constituents so that the bacteria will die.[9]

Seeing these problems, one of the breakthroughs made in this study was to use chitosan edible film from shrimp head waste as an antimicrobial to inhibit the growth of *Porphyromonas gingivalis*. Edible film preparations have several advantages, namely being biodegradable, edible (biocompatibility), and having antimicrobial activity.[10]

MATERIALS AND METHODS

This study uses a laboratory experimental research type. This study also used a post test only research design with control group design. The research was conducted at the Phytochemical Laboratory of the Faculty of Pharmacy, Hasanuddin University, Laboratory of Biology and Microbiology, College of Pharmacy. The study was conducted in July - Completed in 2019. The samples from this study were Porphyromonas gingivalis bacteria provided from the Microbiology Laboratory of the Hasanuddin University Faculty of Medicine and white shrimp scalp waste (Litopenaeus vannamei) obtained from the white shrimp cultivation development center (*Litopenaeus vannamei*) .

The inclusion criteria of this study were the cultured *Porphyromonas gingivalis* bacteria. Then, white shrimp scalp waste (*Litopenaeus vannamei*) chitosan edible film preparation. The exclusion criteria of this study were contaminated *Porphyromonas gingivalis* bacteria. Then, the waste of white shrimp scalp (*Litopenaeus vannamei*) from the edible film of chitosan has been contaminated.

Raw Material

White shrimp head waste (*Litopenaeus vannamei*) which has been obtained from the white shrimp cultivation center, is then cleaned by separating the shrimp scalp and the contents of the shrimp head. Then, the shrimp scalp is cleaned again using clear water or running water in order to remove the slippery part of the surface of the shrimp scalp, so that a completely clean sample will be obtained. Weigh the shrimp scalp (wet weigh). After that, dry the shrimp scalp, can use the oven with a temperature of 110-120ºC for ±1 hour or take advantage of 1-2 days of sunshine (if using the sun, shrimp scalp must be covered with black cloth) The dry raw material is then mashed using a blender, then weighing the shrimp's scalp is carried out (dry weighing). Store in a jar (not clear).

Chitosan Extract

Demineralization

The finely ground shrimp head waste powder is put into an erlenmeyer flask that has been given a stirrer stone, then 1.5 M HCl is added (measured

using a measuring cup and a beaker with a ratio of 1:15 (w/v), then stirring using a stirrer. After that, the shrimp shell waste powder and 1.5 M HCl solution were heated at a temperature of 60-70°C for 2 hours while stirring at a speed of 100 rpm using a stirrer engine.

Deproteination

Furthermore, the waste powder to the filtered shrimp is then added with NaOH 4% ratio of 1:10 (w/v) (measured using a measuring cup and beaker). After that, the heating is done at a temperature of 60-70°C for 2 hours at a speed of 100 rpm using a stirrer engine. Perform re-filtering using filter paper, funnel and Erlenmeyer flask. The precipitate obtained is rinsed several times then the pH is calculated until a normal pH is obtained (pH 7).

Deacetylation

The final stage in the manufacture of chitosan extract is through the deacetylation process. The chitin obtained at the deproteination stage was then added with NaOH 60% ratio of 1:20 (w/v) then heated to 65ºC for 2 hours. The results obtained are then filtered using a funnel and filter paper. The precipitate is then rinsed with distilled water to a neutral pH (pH 7).

Chitosan Edible Film

The chitosan extract that has been obtained previously will be made in edible film preparations with various concentrations (1%, 2% and 3%). Making chitosan edible film begins with dissolving the modified starch in distilled water. The solution was heated at 60°C to 70°C and stirred on a hot plate stirrer to form a thickened solution. Glycerol and HPMC were added to distilled water and then added with the chitosan extract 1%, 2%, and 3% respectively, then stirred until gelatinized and homogeneous.

The starch solution and HPMC solution were mixed into a beaker then stirred and heated on a hot plate stirrer for 1 hour, at 65°C, pour the solution on a large surface so that it will form a thin film, then dry it in a dryer at temperature 37°C for 1 day, and removed from the mold then cut into pieces 3x4 mm. The final result will be obtained white shrimp scalp chitosan in dosage forms respectively 1%, 2% and 3%.

Mueller Hinton Agar (MHA) Medium

Making Mueller Hinton Agar (MHA) medium weighed as much as 17 grams then put into 500 ml Erlenmeyer. After that, add the distilled water while shaking and heat it until it boils. Then cover with cotton and put the media into the autoclave. Close the autoclave and pipe valve tightly, sterilize at 121°C for 15 minutes. After enough time, the pipe valve is opened, then the temperature will drop little by little. Remove the MHA (Mueller Hinton Agar) medium from the autoclave, then pour into each of the petri dishes.

Inhibition Test and Observation of Zone of Inhibition

1. Prepare the tools and materials (paper disk, negative and positive controls, chitosan extract, chitosan edible film with a concentration of 1%, 2% and 3%, and prepare pure isolates of *Porphyromonas gingivalis* bacteria that have previously been inoculated by swabbing on MHA medium).
2. Next, the inhibitory power test will be assessed by inserting a paper disk in each sample that has been given different treatments (6 treatments). Place aseptically (do not tear).
3. Incubate the petri dishes at 37°C for 24-48 hours. Incubation was carried out without turning the petri dishes.
4. Measure the inhibition zone around the paper disk for each treatment. Observe the zone of inhibition every 6 hours to see the activity of these bacteria qualitatively and take measurements using a caliper as quantitative data. The clear zone formed is measured using a caliper, the measurement is carried out by measuring three sides of the clear zone, namely horizontally, vertically, and obliquely. The sizes obtained are then averaged. The diameter of the clear zone is in millimeters (mm).

RESULTS

Inhibition test in this study was carried out with 6 repetitions of the 8 treatments obtained by different inhibition zones. The results showed that the results of the normality test using Shapiro-Wilk obtained a value of $p>0.05$, namely $p=0.062$, which means that the data was normally distributed so that it was continued with the parametric statistical test, namely One Way Anova. Anova test is used to see the difference between one treatment and another. The results of research on the inhibition of chitosan edible film from white shrimp (*Litopenaeus vannamei*) waste have found that chitosan edible film is in the strong category in inhibiting *Phorphyromonas gingivalis* bacteria.

The results of the measurement of the inhibition zone diameter of each petri dish can be seen in table 1 below.

Table 1: Results of Inhibition Zone Diameter Measurement.

No	Treatment	P						Total	Average
		Repetition to-							
		I	II	III	IV	V	VI		
1	Edible 3%	10,53	10,46	12,2	12,85	10,46	11,86	68,36	11,39
2	Edible 2%	11,91	12,56	11,41	11,88	11,13	11,15	70,04	11,67
3	Edible 1%	11,8	10,95	12,34	10,39	9,11	10	64,59	10,76
4	Extract 1%	7,72	7,33	7,58	7,52	6,86	7,24	44,25	7,37
5	Extract 2%	8,97	9,53	8,21	7,54	9,38	7,76	51,39	8,56
6	Extract 3%	8,36	8,97	7,88	9,13	9,49	10,63	50,93	8,48
7	Control +	10,9	11,05	12,65	7,83	10,33	11,18	63,94	10,65
8	Control -	9,93	9,95	8,26	8,35	9,9	10,35	56,74	9,45

The test results in Table 1 for the extract test material concluded that the addition of 2% chitosan extract after averaging out of 6 repetitions the result was 8.56 mm and became the treatment with the greatest inhibition of the chitosan extract category while the addition of 1% chitosan extract and 3% after averaging out of 6 repetitions, the results were 7.37 mm and 8.48 mm, respectively. These three results fall into the moderate inhibition zone category because they only range from 5-10 mm.

The chitosan extract was then made in the form of edible film and showed an increase in the inhibition zone from the addition of 1%, 2% and 3% chitosan respectively. It can be observed that the 2% concentration of chitosan edible film has the largest inhibition zone size, namely 11.67 mm. For chitosan edible the concentration of 1% was 10.76 mm and for chitosan edible film with a concentration of 3% the inhibition zone was 11.39 mm. For negative control, it shows a size of 9.45 mm, indicating that the inhibition zone of negative control is the smallest zone of inhibition, while the positive control using metronidazole has an inhibition of 10.65 mm.

It can be concluded that the 1% concentration of chitosan edible film has a minimum inhibition zone while the 2% concentration of chitosan edible film has a maximum inhibition zone compared to other treatments. The five chitosan edible films were in the strong category because they ranged from 10-20 mm. Furthermore, the normality test data is processed using the Shapiro - Wilk test method.

Table 2: Results of Normality Test Data Processing

Data Normality	Statistic	Df	P Value
Inhibition	955	48	0,062

* Shapiro Test – Wilk

From table 2 above, it is known that the results of the normality test in the Saphiro-Wilk column the probability value of the data obtained is p=0.062. The probability value can be said to be normally distributed if p>0.005, so the data in table 2 is said to be normally distributed, so that it can be continued for the One Way Anova parametric test.

Table 3: One Way Anova Parametric Test Results

Group	Data	
	Mean	SD
Edible 2%	11,68	0,56
Edible 1 %	11,39	1,05
Edible 3%	10,77	1,19
Negative Control	9,46	0,91
Positive Control	10,66	1,58
Extract 1%	7,38	0,31
Extract 2%	8,57	0,85
Extract 3%	8,49	0,89
P Value	0.000*	

The One Way Anova test is carried out to see the differences in each treatment and show the results obtained are significant or insignificant. From the table above, the value of p=0.000<0.05, then Ho

is rejected, which shows a significant difference in each type of treatment so that it can be concluded that the inhibition test carried out can inhibit the growth of the Phorphyromonas gingivalis bacteria. Furthermore, the further difference test (Post Hoc Test) uses the Small Significant Difference (LSD) test or better known as the Least Significant Differences (LSD) test. This method uses the LSD value as a reference in determining whether the average of two or more treatments is statistically different or not. The LSD test results can be seen as follows:

Table 4: LSD Test Results

Group		Mean Difference	P value
Edible 2%	Edible 1 %	0,28	0,621
	Edible 3%	0,91	0,117
	Negative control	2,22	0,000
	Positive control	1,02	0,080
	Extract 1%	4,30	0,000
	Extract 2%	3,11	0,000
	Extract 3%	3,19	0,000
Edible 1 %	Edible 3%	0,63	0,276
	Negative control	1,94	0,002
	Positive control	0,74	0,202
	Extract 1%	4,02	0,000
	Extract 2%	2,83	0,000
	Extract 3%	2,91	0,000
Edible 3%	Negative control	1,31	0,027
	Positive control	0,11	0,850
	Extract 1%	3,39	0,000
	Extract 2%	2,20	0,000
	Extract 3%	2,28	0,000
Negative control	Positif control	-1,20	0,041
	Extract 1%	2,08	0,001
	Extract 2%	0,89	0,125
	Extract 3%	0,97	0,096
Positive control	Extract 1%	3,28	0,000
	Extract 2%	2,09	0,001
	Extract 3%	2,17	0,000
Extract 1%	Extract 2%	-1,19	0,043
	Extract 3%	-1,11	0,057
Extract 2%	Extract 3%	0,08	0,893

In Table 4, there is a significant difference between the 2% concentration of chitosan edible film with negative control, 1%, 2%, and 3% extracts. Then in the 1% concentration of chitosan edible film, there was a significant difference in negative control, 1%, 2%, and 3% extract, 3% concentration of chitosan edible film showed a significant difference in negative control, 1%, 2% and 3% extract. The negative control saw a significant difference to the 1% extract, the positive control saw a significant difference towards the 1%, 2%, and 3% extracts, the 1% extract saw a significant difference against the 2% extract, then for the 2% extract it looked insignificant against the 3% extract.

DISCUSSION

Chitosan is a polysaccharide that is mostly distributed in nature, being the main component of the exoskeleton of crustaceans such as shrimp and insects.[11] The export value of Indonesian shrimp has now reached 142,000 tons with a total waste of both skin and head which is not utilized reaching 60,000 tons. In the shrimp shell, it is rich in chitin and chitosan compounds, which can be used in various fields and purposes. Chitosan is more often used than chitin so that it is dubbed the Magic of Nature.[12]

Both lactate chitosan and carboxymethyl chitosan show significant antimicrobial activity against *Aggregatibacter actinomycetemcomitans* and *Porphyromonas ginigvalis* and can be used as potential antimicrobials in periodontal therapy. The results of the research conducted by both of them showed that there was no statistically significant difference due to differences in the concentration of chitosan given to certain microbes. The inhibition zone obtained by both

variants of chitosan was statistically significant ($p<0.01$) against microorganisms, thus proving the antimicrobial ability of chitosan.[13]

In its activity, amino groups of chitosan when in contact with protonated physiological fluids and bind to anionic groups of microorganisms will cause agglutination of microbial cells and inhibit the growth of these microbes. When interacting with bacterial cells, chitosan will increase the transfer of Ca^{++} from the membrane anionic site resulting in cell damage.[14]

Chitosan has been able to show low toxicity and the development of resistance has not occurred, and chitosan is able to show anti-inflammatory activity by modulating PGE2 levels through the JNK pathway which can be useful in the prevention or treatment of periodontal inflammatio.[15]

Edible films can be made from chitosan which can be used as an antimicrobial agent. It has been studied and proven in recent years the mechanism of antimicrobial action of chitosan that in its activity, chitosan involves the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes.[15]

Strong effect of chitosan on periodontal pathogens through inhibition of biofilm formation with registered activity even after 168 hours and against multiple species biofilms, thus indicating that chitosan can disrupt bacterial coaggregation. In this study, chitosan extract was made which will later be made into edible film. Extract of white shrimp scalp waste chitosan (*Litopanneaus vannamei*) was prepared by dissolving the extract with each concentration of 1%, 2%, 3% using a 1% acetic acid solution and showed the ability to attract the active substances contained in it. According to Dunn, the characteristics of chitosan which can only be dissolved in dilute acid solutions, such as acetic acid, are possible because the carboxyl groups in acetic acid will facilitate the dissolution of chitosan due to the hydrogen interaction between the carboxyl groups and the amine groups of chitosan.[15]

Metronidazole-based chitosan gel against *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and concluded that metronidazole-based chitosan gel 0.125%, 0.25%, 0.5% , 1%, 2% were very effective in inhibiting the growth of *Porphyromonas gingivalis*. Chitosan gel without metronidazole did not form a clear zone in the Mueller Hinton Agar medium, so it can be concluded that the chitosan gel was not effective in inhibiting the growth of the bacteria *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*.[16,17]

CONCLUSION

Based on the results and observations made in this study, it can be concluded that the edible film of white shrimp scalp waste chitosan (Litopenaeus vannamei) has antimicrobial properties in inhibiting the growth of Porphyromonas gingivalis bacteria. The data show that the greatest inhibition of the edible film is at a concentration of 2%. For 1% and 3% less have good inhibitory properties, this depends on the consistency of chitosan.

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