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**ANTIBACTERIAL EFFECTIVITY OF FLAVONOID FRACTION OF
RAMANIA LEAF EXTRACT (*Bouea macrophylla* Griffith) AGAINST
*Porphyromonas gingivalis***

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ABSTRACT

Background: Chronic periodontitis is a periodontal disease with 80% of all cases of periodontitis. The major causes are the accumulation of plaque and bacteria. The dominant bacteria in chronic periodontitis is *Porphyromonas gingivalis*. Treatment of chronic periodontitis can be done by scaling and root planing and supporting therapy by using mouthwash such as Chlorhexidine gluconate 0.2% which is the gold standard in the treatment of periodontal disease. Chlorhexidine gluconate 0.2% has disadvantages so that nowadays research on herbal plants is being done to find alternative medicines that are more effective. Ramania (*Bouea macrophylla* Griffith) leaf contains flavonoids that have antibacterial properties. **Objective:** To analyze the antibacterial effectivity of the flavonoid fraction of Ramania leaf extract against *Porphyromonas gingivalis* that causes chronic periodontitis. **Method:** True experimental study and post-test with control group design consisting of 5 treatment groups, namely flavonoid fraction of ramania leaf extract with concentrations of 0.1%, 0.3%, and 0.5%, chlorhexidine gluconate 0.2% as a control positive and aquadest as a negative control. Each group was repeated 6 times. Antibacterial tests using the dilution method with inhibitory rates calculated using a UV-Vis spectrophotometer and killing rates were calculated using a Colony Counter. **Results:** The average difference in absorbance values obtained inhibitory rates at concentrations of 0.1%, 0.3%, and 0.5%. One Way Anova Test showed a significance value of 0,000 ($p < 0.05$). The average number of colonies after 24 hours incubation showed the results of a kill rates in the concentration group of 0.3%, 0.5%, and positive control. The Kruskal Wallis test showed a significance value of 0,000 ($p < 0.05$). **Conclusion:** The minimum inhibitory concentration (MIC) was obtained at a concentration of 0.1% and the minimum bactericidal concentration (MBC) was obtained at 0.3% concentration.

Keywords: Flavonoid Fraction, MBC, MIC, *Porphyromonas Gingivalis*, Ramania Leaf Extract.

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INTRODUCTION

Periodontitis is a disease of periodontal tissue and a major factor causing tooth loss that affects about 10.5% to 12% of the world's population.^{1,2} The condition of oral health in Indonesia has its own spotlight where around 90% of Indonesia's population suffers from oral diseases.³ Riskesdas 2018 showed that the prevalence data for periodontitis cases in Indonesia was 74.1%.⁴ The most common periodontal tissue disease is chronic periodontitis, about 80% of all cases of periodontitis, generally occur in adults and begin at age 35 years.^{2,5} The main causes of chronic periodontitis are plaque and bacteria, where the

most dominant bacteria causing chronic periodontitis is *Porphyromonas gingivalis* with a prevalence of 80.5%.⁶

Treatment of chronic periodontitis can be done with mechanical and supportive therapy. Mechanical therapy consists of scaling and root planing, and maintenance of oral hygiene. Supporting therapy uses antibiotics or mouthwash to suppress the growth of periodontal bacteria and increase the benefits obtained from mechanical therapy.⁷ A mouthwash that is often used in the treatment of chronic periodontitis is Chlorhexidine gluconate 0.2% which is the gold standard in the treatment of periodontal disease.⁸ Excessive use of

Chlorhexidine can cause disturbances in taste perception, dryness of the oral mucosa, discoloration of the teeth, dorsal tongue and restorative material. Alternative research for the treatment of periodontitis is very relevant to be done in order to develop the use of natural materials and obtain more effective drugs.^{7,9} Research by Roni *et al* (2019) found that the extract of Ramania leaves using methanol and n-hexane solvents has bactericidal properties which is able to kill the growth of gram-negative bacteria, *Escherichia coli*. However, research by Fitri *et al* (2018) regarding the toxicity test of the extract of Ramania leaves using ethanol solvent concluded that the extract was toxic to vero cells. Researchers intend to do the fractionation or separation to obtain pure flavonoid compounds.^{10,11}

Based on the description above, the prevalence data for periodontitis cases in Indonesia was 74.1% with a percentage of chronic periodontitis was around 80%, so the researchers are interested in analyzing the antibacterial effectiveness of the flavonoid fraction of Ramania (*Bouea macrophylla* Griffith) leaf extract against *Porphyromonas gingivalis*, which is one of the bacteria that causes chronic periodontitis.

METHODS AND MATERIALS

The study began with the preparation of a research permit and ethical clearance issued by the Faculty of Dentistry, Lambung Mangkurat University No.002/KEPKG-FKGULM/EC/I/ 2020. The design of this study was true experimental study and post-test with control group design consisting of five groups, namely flavonoid fraction of Ramania leaf extract with concentrations of 0.1%, 0.3% and 0.5%, positive control of Chlorhexidine gluconate 0.2% and negative control of aquadest. Each group was repeated six times.

The research materials used consisted of 1.5 kg of ramania leaves, 95% ethanol, aquadest, n-hexane, ethyl acetate, *Porphyromonas gingivalis* isolate, Nutrient Agar (NA) media, Brain Heart Infusion Broth (BHIB) media, Chlorhexidine gluconate 0.2% and Mc Farland's 0.5 standard solution. The equipment used consisted of oven, blender, mesh sieve, analytical balance, glass vessel, magnetic stirrer, rotary evaporator, water bath, separatory funnel, petri dish, inoculation loop, sterile catton, sterile lamp, spiritus lamp, spectrophotometer, micropipette, colony counter, sterile tips, beaker glass, erlenmeyer flasks, test tubes and anaerobic incubators..

Ramania Leaf Extraction

Ramania leaf extraction process was carried out at the Laboratorium Pabrik Jamu Pucuk Sirih Banjarmasin. 1.5 kg of Ramania leaves were

cleaned and washed using tap water then dried using an oven at 60 °C for 48 hours. The leaves were made into dried simplicia powder using a blender and then sieved to obtain 785 grams of ramania simplicia leaves. The maceration method used 95% ethanol solvent with a ratio 1:10, so that 0.785 kg used 7.85 liters of 95% ethanol for 3 x 24 hours at a room temperature protected from sunlight. The blend was stirred every 6 hours and the solvent was changed every 24 hours, then the solvent was evaporated using a rotary evaporator at a temperature of 50 °C until it was ethanol free and then concentrated in a water bath. The final result was obtained as much as 78 grams of ethanol extract of Ramania leaves.

Flavonoid Fractionation of Ramania Leaf Extract

The extract of Ramania leaf was dissolved with aquadest until homogeneous with a ratio between extract and aquadest is 1: 2, then put into a separatory funnel, add n-hexane to the tune of 200 mL and homogenized, let stand until it forms 2 layers namely the distilled layer in the lower layer and the n-hexane layer in the upper layer. Fractionation with n-hexane was carried out until the solution was clear. The n-hexane fraction was separated from the aquadest fraction, then the aquadest fraction was added 200 mL of ethyl acetate solvent, homogeneous and fractionated using a separatory funnel, then allowed to stand until 2 layers were formed, the aquadest in the lower layer and the ethyl acetate layer above. The fractionation process used ethyl acetate until the solution was clear. The ethyl acetate layer was taken, then concentrated using a water bath until viscous. The viscous fraction was a flavonoid fraction of 8 grams.

Antibacterial Test of Flavonoid Fraction of Ramania Leaf Extract against *Porphyromonas gingivalis*

Porphyromonas gingivalis ATCC 33277 pure isolates from the Microbiology Laboratory of Airlangga University were cultured in NA media, then incubated into anaerobic incubators at 37 °C for 24 hours. Then the bacteria were inoculated into the BHIB media using inoculation loop. The suspension was added with sterile aquadest until the turbidity equivalent to Mc Farland's 0.5 standard. Six tubes were prepared for one treatment group then added with 1ml solution of treatment group. There were five treatment groups consisted of aquades, chlorhexidine gluconate 0.2% and a flavonoid fraction group with three different concentrations, namely 0.1%, 0.3% and 0.5%, so the total were 30 tubes. Standardized bacterial

suspension with Mc Farland turbidity 0.5 was put into each test tube 1 ml.

Each test tube absorbance value was measured using a spectrophotometer ($\lambda = 600 \text{ nm}$). The inhibitory level was determined by the value of the absorbance difference, which obtained from the absorbance value after incubation minus the absorbance value before incubation. Concentrations that can inhibit bacterial growth were shown with negative results. The kill rate was determined by taking 0.2 ml of BHIB media solution subculture into a petri dish which was filled with sterile NA media, then incubated for 24 hours at 37 °C in an anaerobic incubator. Calculation of the number of bacteria using a colony counter, the kill rate was obtained if the result of the calculation was 0.¹²

RESULTS

Research on the effectiveness of this antibacterial has been carried out using the delusion method. The minimum inhibitory concentration (MIC) was determined by calculating the difference in absorbance value (optical density) using a UV-Vis 722AP spectrophotometer ($\lambda = 600 \text{ nm}$). The results of inhibitory data can be seen in table 1.

Table 1. The Average and Standard Deviation of the *Porphyromonas gingivalis* Absorption Difference in the Flavonoid Fraction of Ramania Leaf Extract, Chlorhexidine gluconate 0,2% and Aquadest.

<i>Descriptives</i>				
Treatment Groups		N	Statistic	SD
0,1%	Mean	6	-,459	,0446
0,3%	Mean	6	-,316	,0297
0,5%	Mean	6	-,324	,0208
C (+)	Mean	6	,535	,0737
C (-)	Mean	6	,328	,0511

Table 1 shows the analysis results of the average and standard deviation of the absorbance difference in each treatment group that measured using a spectrophotometer. Concentrations of 0.1%, 0.3%, and 0.5% indicated negative values or absorbance decreases, while for positive control of chlorhexidine gluconate 0.2% and negative controls of aquadest showed positive values or an increase in absorbance.

The inhibitory concentration data of the research results were analyzed, starting with Shapiro Wilk's normality test which showed significance value > 0.05, meaning that the data was distributed normally. It could be continued using the One Way Anova parametric test and the Levene

homogeneity test to determine the Post Hoc test. The data showed the significance value of (0.265) ($p > 0.05$) which means that the data was homogeneous. The One Way Anova test showed a significance value as many as 0,000 ($p < 0.05$) which means that there were significant differences among the averages. The next test to find out which groups had the significant differences was conducted by the Post Hoc Bonferroni test.

Table 2. The Results of Post Hoc Bonferroni Test of the Absorption Difference of *Porphyromonas gingivalis* in the Flavonoid Fraction of Ramania Leaf Extract, Chlorhexidine gluconate 0,2% and Aquadest.

Multiple Comparisons					
Dependent Variable: Minimum Inhibitory Concentration					
Bonferroni					
Treatment Groups	0,1%	0,3%	0,5%	Control (+)	Control (-)
0,1%	-	,000	,000	,000	,000
0,3%	,000	-	1,000	,000	,000
0,5%	,002	1,000	-	,000	,000
Control (+)	,000	,000	,000	-	,000
Control (-)	,000	,000	,000	,000	-

*. The mean difference is significant at the 0.05 level.

The Post Hoc Bonferroni test results showed that there were significant differences between the average of all treatment groups, except between treatment group concentrations of 0.3% and 0.5% with a significance value (1,000) > 0.05 which means that there was no significant difference between the two concentrations.

The inhibitory results were obtained then the BHIB media solution was subcultured into a petri dish which was filled with sterile NA media, then incubated for 24 hours at 37 °C in anaerobic incubators. The minimum bactericidal concentration (MBC) was obtained by counting the bacterial colonies using a colony counter. The results of MBC data can be seen in table 3.

Table 3. The Results of Average Values and Standard Deviation of *Porphyromonas gingivalis* Colonies in the Flavonoid Fraction of Ramania Leaf Extract, Chlorhexidine gluconate 0,2% and Aquadest

<i>Descriptives</i>				
Treatment Groups		N	Statistic	SD
0,1%	Mean	6	734,67	398,635
0,3%	Mean	6	,00	,000
0,5%	Mean	6	,00	,000
C (+)	Mean	6	,00	,000
C (-)	Mean	6	1842,67	890,521

Table 3 shows the analysis results of the average and standard deviation of the bacterial colonies from each treatment group which was calculated using a colony counter. A concentration of 0.1% indicated an average of 734.67 CFU/ml, a concentration of 0.3%, 0.5% and positive control of chlorhexidine gluconate 0.2% showed a result of 0 CFU/ml, while a negative control of aquadest showed an average of 1842.67 CFU/ml of *Porphyromonas gingivalis* colony.

Data on the bactericidal concentration carried out by the normality test using the Shapiro Wilk test that showed a significance value of < 0.05 . It was meant that the data was not distributed normally, then continued to analyze using the Kruskal Wallis non-parametric test and the Post Hoc Mann Whitney test. The non-parametric Kruskal Wallis test showed a significance value of 0,000 ($p < 0.05$), meaning that there were significant differences among the means, then to find out which groups showed a significant difference in the Post Hoc Mann Whitney test was performed.

Table 4. Test Results of Post Hoc Mann Whitney test of the Average Number of *Porphyromonas gingivalis* Colonies in the Flavonoid Fraction of Ramania Leaf Extract, Chlorhexidine gluconate 0,2% and Aquadest

Multiple Comparisons					
Dependent Variable:					
Minimum Bactericidal Concentration					
Mann-Whitney					
Treatment Groups	0,1%	0,3%	0,5%	Control (+)	Control (-)
0,1%	-	,002	,002	,002	,016
0,3%	,002	-	1,000	1,000	,002
0,5%	,002	1,000	-	1,000	,002
Control (+)	,002	1,000	1,000	-	,002
Control (-)	,016	,002	,002	,002	-

*. The mean difference is significant at the 0.05 level.

Post Hoc Mann Whitney Test showed results that there were significant differences among all treatment groups, except between treatment groups concentrations of 0.3%, 0.5% and positive control. Based on these data, it can be concluded that the concentrations of 0.3% and 0.5% have no significant difference with the positive control of chlorhexidine gluconate 0.2%. This is because the concentrations of 0.3%, 0.5% and positive controls have the same result that there is not a single bacterial colony on NA media, meaning that all three groups have a killing rates against *Porphyromonas gingivalis*.

DISCUSSION

The flavonoid fraction of Ramania leaf extract concentration of 0.1% found a significant difference because the value of the absorbance difference was more significant at -0.459, compared to the concentration of 0.3% decreased the absorbance with a difference -0.316 and a concentration of 0.5% obtained a difference -0.332. At concentrations of 0.3% and 0.5%, concentrations higher than 0.1% should have decreased absorbance values more significantly, but in this research, the opposite results were obtained. Based on the research by Wuon *et al* (2018), the less significant decrease was not due to bacterial growth, but was thought to be influenced by a more concentrated color density at higher concentrations, so the light absorption by dead bacterial cells in liquid media could be affected.^{13,14}

The positive control showed a positive absorbance difference of 0.535 as well as a negative increase in absorbance. This was probably due to the structure of residual bacteria or dead bacteria left after exposure to chlorhexidine gluconate 0.2% accelerated the formation of calculus and functioned as an ideal substrate for attachment of new bacteria. Research by Yamaguchi *et al* (2013) stated that treatment using chlorhexidine for five minutes on *Porphyromonas gingivalis* biofilms does not reduce the external structure or volume of protein and carbohydrates.^{15,16} Another possibility that causes an increase in absorbance is thought to be not due to the positive control of chlorhexidine gluconate 0.2% unable to inhibit the growth of *Porphyromonas gingivalis* bacteria but is caused by the UV-Vis spectrophotometer in the positive control group being unable to distinguish between dead and living bacterial cells. Research by Wuon *et al* (2018) that gave the same result, the positive control which should decrease instead of an absorbance increase which is influenced by the lack of UV-Vis spectrophotometer. The result of negative control shows that the absorbance difference is positive, that is 0.328. It means that there is an increase in absorbance value which indicates an increase in bacterial growth in the media.^{13,14,17}

The antibacterial study of Ramania leaf extract flavonoids obtained inhibitory levels, in line with the study of Valentina *et al* (2019) who examined the antibacterial flavonoids of propolis against gram-negative bacteria *Porphyromonas gingivalis* there were inhibitory levels at 0.1% concentration. Inhibitory levels are obtained because the antibacterial effect possessed by flavonoids can inhibit bacteria by the mechanism of action inhibits the formation of biofilms, inhibits the synthesis of bacterial cell wall and inhibits bacterial toxins such as peptidoglycan which is an important component of bacterial cell walls.

Research on the derivatives of flavonoid namely apigenin, sacuranetin and quercetin has been shown to inhibit the dehydration of 3-hydroxyacyl-ACP the enzyme from *Helicobacter pylori*.^{18,19}

The flavonoid fraction of Ramania leaf extract concentration of 0.1% has been able to inhibit the growth of *Porphyromonas gingivalis* bacteria, but it has not been able to kill the bacteria as evidenced by the growth of *Porphyromonas gingivalis* colony on NA media with an average of 734.67 CFU/ml, allegedly because of the active flavonoids at that concentration is not enough to kill the *Porphyromonas gingivalis*. The minimum kill rate was only obtained at a concentration of 0.3% which in NA media, it did not find any bacterial colonies. It is suspected that at this concentration, the active flavonoid contained was able to kill the *Porphyromonas gingivalis*.

A concentration of 0.5% is also able to kill the *Porphyromonas gingivalis*. According to the theory which stated that the higher concentration of antibacterial substances, the higher antibacterial effect. At this concentration, it has been able to kill as well as higher concentrations.²⁰ Positive control of Chlorhexidine gluconate 0,2% showed the same results with concentrations of 0.3% and 0.5%. There were 0 bacterial colonies, while negative controls showed that the bacteria continued to grow as evidenced by the colony of *Porphyromonas gingivalis* which amounted to an average of 1842.67 CFU/ml.

Flavonoids inhibit and kill the *Porphyromonas gingivalis* in various ways, including damaging membranes by attacking phospholipids on bacterial cytoplasmic membranes. Phospholipids are unable to maintain cytoplasmic membranes resulting in leakage of the cytoplasmic membrane and substances that function to metabolize bacterial cells wasted out and occur bacterial death.^{21,22,23} The mechanism of flavonoids damage the cell wall is to form an alcohols group, then it will react with lipids and amino acids which are bacterial cell wall structures so that cell wall damage occurs. When damage occurs in the cell walls, flavonoid compounds will continue to enter the bacterial cell nucleus, then it will contact with deoxyribonucleic acid (DNA) which ultimately causes damage to the DNA lipid structure so that it lysis the bacteria and then the cell will die.^{19,23}

The research of Amanda *et al* (2019) drew the conclusion that pure flavonoid Propolis extract of *Trigona Sp* (*Trigona thorasica*) has the ability to inhibit bacteria at a concentration of 0.1%; 0.3% and able to kill bacteria at a concentration of 0.5% against *Porphyromonas gingivalis*.²³ Based on these results, it can be compared that the pure flavonoid fraction of Ramania leaf extract is more effective than the pure flavonoid fraction of

Propolis extract because at a concentration of 0.3%, it has been able to kill the *Porphyromonas gingivalis*.

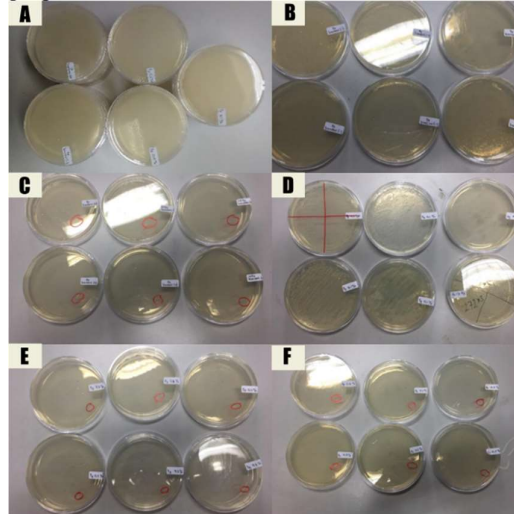


Figure 1. Results of the research before and after incubation. (A) NA media have been inoculated by bacteria before incubation; (B) Negative control results after 24 hours incubation; (C) Positive control results after 24 hours incubation; (D) The result of 0.1 concentration after 24 hours incubation; (E) The results of 0.3% concentration after 24 hours incubation; (F) The results of 0.5% concentration after 24 hours incubation.

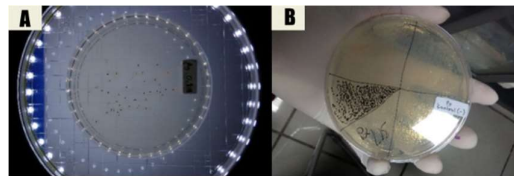


Figure 2. Calculation of the number of *Porphyromonas gingivalis* colonies. (A) Calculation of the bacterial colony using a colony counter; (B) Recording number of bacterial colonies.

Antibacterial testing with the dilution method has limitations. In this research, that is the lack of a UV-Vis spectrophotometer which is not able to distinguish between dead and life bacterial cells.²⁴ Spectrophotometers also have other weaknesses, such as the ability to distinguish samples from other particles or contaminants that can absorb light at the same wavelength, so that subsequent research can conduct antibacterial tests using other methods such as antibacterial testing with diffusion method.^{13,25}

Based on the results of the research that has been done, it is found that the inhibition levels of flavonoid extracts of Ramania leaf are obtained at concentrations of 0.1%, 0.3% and 0.5% with MIC at a concentration of 0.1%, while the kill rates is

obtained at a concentration of 0.3% and 0.5% with MBC at a concentration of 0.3%.

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