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ANTIBACTERIAL EFFECTIVENESS OF *Stenochlaena palustris* LEAVES EXTRACT AGAINST THE GROWTH OF *Streptococcus mutans*

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ABSTRACT
***Background*:** Dental caries is a common chronic infectious disease of dental hard tissue resulting from the interaction between tooth structure, the microbial biofilm formed on the tooth surface, dietary as well as salivary influences. *Streptococcus mutans* are considered to be the main bacteria that play role in the formation of cariogenic biofilms. *Chlorhexidine gluconate* 0,2% is a commonly used mouthwash and considered as the gold standard. The long-term use of chlorhexidine gluconate 0,2% may cause side effects so an alternative mouthwash with natural ingredients is needed. Kelakai leaves extract contains bioactive compounds which has antimicrobial effects such as flavonoids, alkaloids, tannins and saponins which can be used as an alternative mouthwash for *chlorhexidine gluconate* 0.2%. **Objective**: The purpose of this study was to observe the antibacterial effectiveness of kelakai leaves extract with concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, 75% and 100% with *chlorhexidine gluconate* 0.2% on growth of *Streptococcus mutans* bacteria. **Methods:** This study conducted through true experimental laboratories with a post-test only research design with control group design. This study used 9 treatments with 5 repetitions so that there were a total of 45 samples. The treatment group was kelakai leaves extract with concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, 75% and 100% with two control groups namely chlorhexidine gluconate 0.2% and distilled water against Streptococcus mutans. **Results:** The MIC of kelakai leaves extract was set at a concentration of 12.5% and the MBC at a concentration of 50%. **Conclusion**: Kelakai leaves extract concentrations 50%, 75% and 100% has antibacterial effectiveness which equivalent to *chlorhexidine gluconate* 0,2% against *Streptococcus mutans* bacteria.

Keywords: Antibacterial, *Stenochlaena palustris* leaves extract, *Streptococcus mutans*

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INTRODUCTION

Dental and oral health has an important role in supporting general health. Dental and oral health includes the teeth and their supporting tissues are free from disease and pain and can work optimally.[1] Dental caries is the most common dental and oral disease suffered by the people of Indonesia. Based on the 2018 Basic Health Research (RISKESDAS) data, the incidence of dental caries in Indonesia reached 45.3% and in South Kalimantan it reached 46.9%. Caries is a chronic infectious disease in dental hard tissues that caused by the interaction between tooth structure, biofilm on the tooth surface, microbes, and the influence of saliva. Caries is characterized by the occurrence of inorganic demineralization and damage to the organic substance of the teeth.[1,2,3] Bacteria that play a major role in the formation of cariogenic biofilms are *Streptococcus mutans*.[4] *Streptococcus mutans* is a gram-positive facultative anaerobic bacterium that has the ability to adhere to tooth enamel and metabolize carbohydrates.[5,6] Acidogenic bacteria from biofilms will produce organic acid byproducts due to fermentation of foods containing sugar. Continuous acidic conditions cause a decrease in pH. The low pH environment causes an increase in demineralization or erosion of the tooth surface

which is the initial stage of the emergence of dental caries.[7,8]

The use of antibacterial mouthwash such as *chlorhexidine gluconate* 0.2% can prevent caries by inactivating bacteria by inhibiting their growth and attachment to the tooth surface.[9] *Chlorhexidine gluconate* 0.2% is an effective gold standard mouthwash to reduce bacterial growth in the oral cavity.[10] However, long-term use of 0.2% *chlorhexidine gluconate* mouthwash can result in loss of taste sensation in the sense of taste, discoloration of teeth, restorative materials and tongue.[8,11] Therefore, it is necessary to have an alternative mouthwash with natural basic ingredients, namely the leaves of Kelakai plant.

Kelakai plant (*Stenochlaena palustris* (Burm) Bedd.) is a plant that is generally used by the people of South Kalimantan as a vegetable and traditional medicine that is able to treat anemia, fever and is used to increase postnatal energy and is believed to be able to increase and facilitate breast milk production.[12] Kelakai leaves contain various secondary metabolites, namely flavonoids, tannins, alkaloids and saponins that can act as antibacterial agents.[13,14] Based on research by Chear (2016), the flavonoid content of Kelakai leaves is 503.56 mg QE/g. Another study by Pertiwi (2019) found that the extract of the leaves of the Kelakai was proven to inhibit the growth of *Streptococcus sanguinis* bacteria.

Based on this description, it is necessary to conduct research on the extract of the kelakai leaves (*Stenochlaena palustris* (Burm) Bedd.) which has the potential as an antibacterial against *Streptococcus mutans* bacteria. The aim of this study was to examine the antibacterial effectiveness of the extracts of 3.125%, 6.25%, 12.5%, 25%, 50%, 75% and 100% concentrations of the bacteria *Streptococcus mutans* by measuring MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) through the dilution method.

MATERIALS AND METHODS

This study was conducted through ethical licensing and has been declared ethically feasible by the Health Research Ethics Commission of Lambung Mangkurat University No. 021/KEPKG-FKGULM/EC/II/2021. This study is a true experimental laboratory study with a post test only with control group design. This study consisted of treatment groups, namely the concentration of the kelakai leaves extract of 3.125%, 6.25%, 12.5%, 25%, 50%, 75%, 100% and 0.2% *chlorhexidine gluconate* as a positive control and distilled water as a control negative. The number of replications for each treatment group was 5 times based on calculations using the Lemeshow formula, so that a total sample of 45 samples was obtained.

The determination test of the Kelakai plant and the manufacture of extracts were carried out at the Basic Laboratory of FMIPA and the Biochemistry Laboratory of the Faculty of Medicine, University of Lambung Mangkurat Banjarbaru. The antibacterial effectiveness test was conducted at the Biomedical Laboratory, Faculty of Dentistry, Banjarmasin.

The MIC was measured using the liquid dilution method to calculate the absorbance value using a UV-Vis spectrophotometer (λ = 420-740nm) and the MBC with solid dilution to calculate the number of bacterial colonies using a colony counter.

Kelakai Leaves Extraction

The kelakai leaves were picked and obtained from the swamps of the Anjir Region, Barito Kuala Regency, South Kalimantan as much as 5 kg with the criteria, namely adult kelakai leaves. Mature leaves are leaves with a greenish color and have a thicker texture. The leaves were washed and cut into smaller shapes and dried at room temperature and then placed in the oven for 4 hours at 40°C. The dried leaves are then sifted to become simplicia powder. The simplicia powder was macerated with 2 liters of 96% ethanol solvent for 3x24 hours while stirring with the help of a shaker. The maceration results were filtered using a cloth and evaporated using a vacuum rotatory evaporator with low pressure at a temperature of 50-60°C for 4-6 hours. Then it was heated over a water bath until all the solvent had evaporated and 86 grams of brownish liquid residue was obtained and made into primary liquor 100% concentrated extract was added with 10% DMSO solution in a ratio (1:1). The extract was diluted with distilled water so that the concentration according to the treatment group was obtained using the dilution formula, as follows:

$V1.C1 = V2.C2$

V1= Volume of solution to be diluted (ml)
C1 = Concentration of the preparation of the extract of the leaves (%)
V2= Desired volume of solution (ml)
C2= Concentration of the preparation of the extract of the leaves to be made (%)

Culture of *Streptococcus mutans*

Several colonies of *Streptococcus mutans* derived from the growth of MSA media were cultured as pure isolates on NA media (Nutrient Agar), then the NA medium was put into an incubator and incubated under anaerobic conditions for 1x24 hours at 37°C.

Production of *Streptococcus mutans* Suspension

Taking *Streptococcus mutans* bacteria from the culture medium using an ossicle then put it into a test tube containing 1 ml of sterile BHIB then put it into an incubator and incubated under anaerobic conditions for 2x24 hours at 37ºC after that, do the dilution by adding sterile distilled water and homogenized until the turbidity is proportional to standard Mc Farland 0.5 (1.5x108 CFU/ml).

Antibacterial Effectiveness Test

The antibacterial effectiveness testing of the extracts of the leaves of kelakai *(Stenochlaena palustris* (Burm) Bedd.) using the liquid and solid dilution method. The suspension of *Streptococcus mutans* which had been equalized with 0.5 Mc Farland solution was transferred into 1 ml test tubes each. The extract of kelakai leaves were added into test tube and diluted according to the concentration which is made of 1 ml each, for positive control 0.2% *Chlorhexidine gluconate* was added. The vacuum tube was covered with sterile cotton and then measured the absorbance value with a Uv-Vis Biobase BKD-500 spectrophotometer before incubation. Furthermore, the test tubes were incubated for 24 hours at 37ºC to determine the effect of the extract of the leaves of kelakai (*Stenochlaena palustris* (Burm) Bedd.) on the growth of *Streptococcus mutans* bacteria. After that, the tubes that have been incubated for 24 hours will be measured for absorbance with UV-Vis Spectrophotometer Biobase BKD-500. The results of MIC can be seen from the difference in absorbance results before and after 24 hours incubation.

Next, a test was carried out to determine the MBC by taking 10 L from each treatment group and then adding it to a petri dish containing sterile NA media and then incubating for 24 hours at 37ºC, then counting the number of bacteria with a colony counter, if the result of counting the number of bacterial colonies is zero (no bacteria) then the MBC is obtained.

Table 1. Mean and Standard Deviation of Difference In Absorbance Value and Number of Colonies

Sample	N	Difference In Absorbance Value			Number of Colonies		
		Mean	±	SD	Mean	±	SD
3.125%	6	0,395	±	0,067	216,4	±	34,341
6.25%	6	0,304	±	0,056	30	±	6,892
12.5%	6	-1,384	±	0,086	12,6	±	3,362
25%	6	-1,411	±	0,040	3,8	±	1,304
50%	6	-1,256	±	0,043	0	±	0,000
75%	6	-0,991	±	0,049	0	±	0,000
100%	6	-0,755	±	0,026	0	±	0,000
K(+)	6	-0,043	±	0,032	0	±	0,000
K(-)	6	0,548	±	0,042	386,8	±	171,881

RESULTS

The results of the research on the antibacterial effectiveness of the kelakai leaves extract were obtained through the MIC test by measuring the absorbance value before and after incubation for 24 hours using a UV-Vis spectrophotometer with a wavelength of 420 nm and 740 nm so that the difference in absorbance values and the MBC test was obtained by counting the number of colonies on the media with a colony counter. The research data can be seen in table 1.

Based on the table above, it shows that at concentrations of 3.125%, 6.25% and aquadest negative control, there was an increase in the difference in absorbance values which indicated the growth of *Streptococcus mutans* bacteria indicating that bacterial growth had not been inhibited. The MIC was obtained at a concentration of 12.5% because it showed a decrease in the difference in absorbance values which indicated that the growth of *Streptococcus mutans* was inhibited with a lower mean value than the negative control.

Table 1 also shows the average value of the number of colonies in the treatment group. Concentrations of 3.125%, 6.25%, 12.5% and 25% showed that there was still bacterial growth indicating that these concentrations had not been able to kill *Streptococcus mutans* bacteria. Concentrations of 50%, 75% and 100% showed no bacterial colonies which indicated that these concentrations had the ability to kill *Streptococcus mutans* bacteria. The MBC is obtained at the smallest concentration that can kill bacteria, namely a concentration of 50%.

The data that has been obtained was tested for normality by Shapiro Wilk. The data from the MIC test results showed that the data were normally distributed ($p>0.05$) and continued with the Levene's Test homogeneity test with a sig value 0.127 ($p>0.05$) which indicates a homogeneous data variance so that the *One Way ANOVA* parametric data analysis was carried out and the results showed that there were differences

between groups so the LSD Post Hoc test was continued (Table 2.)

The data from the MBC test results obtained a concentration of 25% sig value. 0.021 ($p<0.05$) which indicates that the data is not normally distributed, so *Kruskal Wallis* non-parametric data analysis was carried out and the results showed that there were differences between groups so that the *Mann Whitney* test was continued (Table 3.)

Table 2. *Post Hoc Least Significant Difference* (LSD) Test Results of Minimum Inhibitory Concentration (MIC) Kelakai Leaves Extract Against the Growth of *Streptococcus mutans* Bacteria

Sample	3.125%	6.25%	12.5%	25%	50%	75%	100%	K(+)	K(-)
3.125%	-	.009*	.000*	.000*	.000*	.000*	.000*	.000*	.000*
6.25%		-	.000*	.000*	.000*	.000*	.000*	.000*	.000*
12.5%			-	.407	.000*	.000*	.000*	.000*	.000*
25%				-	.000*	.000*	.000*	.000*	.000*
50%					-	.000*	.000*	.000*	.000*
75%						-	.000*	.000*	.000*
100%							-	.000*	.000*
K(+)								-	.000*
K(-)									-

(* = there is a significant difference $p<0.05$)

Table 3. *Mann Whitney* Test Results of Minimum Bactericidal Concentration (MBC) Kelakai Leaves Extract Against the Growth of *Streptococcus mutans* Bacteria

Sample	3.125%	6.25%	12.5%	25%	50%	75%	100%	K(+)	K(-)
3.125%	-	.009*	.009*	.008*	.005*	.005*	.005*	.005*	.117
6.25%		-	.009*	.008*	.005*	.005*	.005*	.005*	.009*
12.5%			-	.008*	.005*	.005*	.005*	.005*	.009*
25%				-	.005*	.005*	.005*	.005*	.008*
50%					-	1.000	1.000	1.000	.005*
75%						-	1.000	1.000	.005*
100%							-	1.000	.005*
K(+)								-	.005*
K(-)									-

(* = there is a significant difference $p<0.05$)

DISCUSSION

The results of the study on the antibacterial effectiveness of the extracts of the leaves of Kelakai (*Stenochlaena palustris* (Burm) Bedd.) at concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, 75% and 100% against the growth of *Streptococcus mutans* bacteria were obtained at concentrations of 12.5%, 25%, 50%, 75% and 100% were proven to be able to inhibit the growth of *Streptococcus mutans* which was indicated by a decrease in the average absorbance value after incubation, the 12.5% concentration of kelakai leaves extract was designated as MIC. Concentrations of 50%, 75%, 100% and *chlorhexidine gluconate* 0.2% were proven to be able to kill *Streptococcus mutans* bacteria which were marked as no growth was found in the media after incubation and the 50% concentration of the kelakai leaves extract was designated as MBC.

Based on the results of the analysis of the Post Hoc LSD MIC test, it was found at a concentration of 12.5% which can be seen from the difference in the average absorbance value which decreased by 1.384 compared to the extract of the kelakai leaves with concentrations of 3.125% and 6.25% with a difference in the average absorbance value experienced an increase of 0.395 and 0.304, respectively, which indicated that there was still bacterial growth so that concentrations of 3.125% and 6.25% could not inhibit the growth of *Streptococcus mutans*. The results of the analysis of the concentration of 12.5% did not have a significant difference with a concentration of 25% in inhibiting the growth of *Streptococcus mutans* bacteria with a value ($p>0.05$). At concentrations of 12.5%, 25%, 50%, 75% and 100% of kelakai leaves extract, it is known that there is antibacterial activity seen from the decrease in the average value of absorbance after the bacteria was given the extract

of the leaves of the plant which showed a decrease in the population of *Streptococcus mutans* bacteria. The inhibition of bacterial growth was caused by the presence of secondary metabolites such as flavonoids, alkaloids, tannins and saponins contained in the leaves of the kelakai which are antibacterial. The content of flavonoids contained in the leaves of the kelakai as much as 503.56 mg QE/g.[15]

The mechanism of inhibition of bacterial growth from secondary metabolites is through cell membrane penetration. The first active substances that will work against *Streptococcus mutans* bacteria are alkaloids, tannins and saponins which will disrupt the outermost layer of bacteria, namely the peptidoglycan wall.[16] Peptidoglycan is a component of the cell wall of *Streptococcus mutans* bacteria so that the presence of this disorder will result in the cell wall layer being not fully formed and causing bacterial cell death.[17]

The mechanism of action of tannins as antibacterial against *Streptococcus mutans* bacteria is by interfering with the synthesis of peptidoglycan compounds in bacteria. In *Streptococcus mutans* bacteria the molecule in the cytoplasm of the peptidoglycan cell wall serves to protect bacteria from high internal osmotic pressure, in the presence of tannin compounds, the formation of bacterial cell walls is inhibited or incomplete and in the presence of osmotic or physical pressure, bacterial cells will lyse.[16,18,19] Tannins also inhibit the enzyme reverse transcriptase and DNA topoisomerase so that bacterial cells cannot be formed.[20]

The mechanism of action of saponins as antibacterial is by reducing the surface tension of the bacterial cell wall and increasing membrane permeability. Saponins diffuse through the outer membrane and vulnerable cell walls and then bind to the cytoplasmic membrane and cause cytoplasmic components to leak and result in cell lysis.[21,22] When the bacterial wall is not perfectly shaped and is damaged, the next secondary active substance, namely flavonoids, will easily enter into the cell, the cellular part of the bacteria and damage the bacterial nucleus.[16]

The mechanism of action of flavonoids as antibacterial agents against *Streptococcus mutans* bacteria works by inhibiting the function of bacterial cell membranes, namely binding to bacterial cell membranes and forming complex compound bonds with soluble extracellular proteins so that the integrity of bacterial cell membranes is disrupted followed by the release of intracellular compounds. Disturbances in cell membrane permeability will result in impaired ATP synthesis, membrane transport and bacterial movement. The mechanism of flavonoids in inhibiting nucleic acids is carried out through ring B on flavonoids which have an important role in the intercalation process or hydrogen bonding by accumulating nucleic acid bases that inhibit DNA and RNA synthesis which results in the destruction of lysosomes and bacterial microsomes.[16,17] This study is supported by a study by Pertiwi et al (2019) about the extract of the leaves of the kelakai can inhibit the growth of gram-positive bacteria, namely *Streptococcus sanguinis*. Gram-positive bacteria have a cell wall structure consisting of less lipids, more peptidoglycan and contain polysaccharides (teichotic acid).[18] The cell wall structure of Gram-positive bacteria is simpler, which is single layered with low lipid content (1-4%) and peptidoglycan with a thickness (20-80 nm) making it easier for bioactive ingredients to enter the cell.[18,23] Teichoic acid as a constituent of the cell walls of gram-positive bacteria is a water-soluble polymer that functions as a transport of positive ions to enter and leave so that gram-positive bacteria are more polar. Polar bioactive compounds will more easily enter the polar cell wall and damage the peptidoglycan layer compared to nonpolar cell walls such as the lipid layer which is found in many gram-negative bacteria.[22]

The MBC from this study was found in the extract of the leaves of Kelakai (*Stenochlaena palustris* Burm) Bedd.) with a concentration of 50%. At the smallest concentration of 3.125% the average number of colonies was 216.4 CFU/µL, 6.25% concentration was 30 CFU/µL, 12.5% concentration was 12.6 CFU/µL, 25% concentration was 3.8 CFU/µL and concentrations of 50%, 75%, 100%, positive control of 0 CFU/µL. MBC is set at the smallest concentration where there is no bacterial growth, namely at a concentration of 50%. Based on the results of statistical analysis, the concentration of 50% did not have a significant difference with concentrations of 75%, 100% and 0.2% *chlorhexidine gluconate* in killing *Streptococcus mutans* bacteria. The 50 % concentration of kelakai leaves extract can kill *Streptococcus mutans* bacteria as in the positive control of 0.2% *chlorhexidine gluconate*. This is in accordance with the theory that the higher the concentration used, the more active compounds contained so that the antibacterial activity will be greater.[16]

Chlorhexidine gluconate 0.2% was used as a positive control in this study. *Chlorhexidine gluconate* 0.2% is a broad-spectrum antibacterial agent that is strong against gram-positive and gram-negative bacteria. The mechanism of 0.2% *chlorhexidine gluconate* as an antibacterial compound against *Streptococcus mutans* is by binding to the positively charged 0.2%

chlorhexidine gluconate molecule (cation) with the negatively charged bacterial cell wall (anion). This causes the strong attachment of 0.2% *chlorhexidine gluconate* to the bacterial cell membrane causing penetration into the cytoplasm. *Chlorhexidine gluconate* 0.2% will cause changes in the permeability of bacterial cell membranes, resulting in leakage of components in the cytoplasm and components whose molecules are lower in the end causing the death of microorganisms.[24,25] The results of this study indicate that the extract of the leaves of kelakai has the potential as an alternative mouthwash of 0.2% *chlorhexidine gluconate* in inhibiting the growth of *Streptococcus mutans* bacteria that causes dental caries. Based on the results of the study, it can be concluded that the 50, 75%, and 100% concentration of the extract of the leaves of kelakai have antibacterial effectiveness equivalent to 0.2% *chlorhexidine gluconate* against *Streptococcus mutans* bacteria with MIC obtained at a concentration of 12.5% and MBC at a concentration of 50%.

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