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COMPARISON OF INHIBITORY ACTIVITY OF KELAKAI LEAF EXTRACT AND 0.2% *Chlorhexidine Gluconate* AGAINST *Streptococcus sanguinis* ATCC® 10556™

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ABSTRACT

Background: *Gingivitis is an early periodontal disease that occurs due to host response and dental plaque. Streptococcus sanguinis is pioneer of dental plaque colonization in oral cavity. Gingivitis treatment use 0,2% Chlorhexidine gluconate mouthwash, but long-term use causes side effects, so an alternative herbal mouthwash which has minimal side effects is needed. Kelakai leaf ethanol extract contains antibacterial compound, such as flavonoid, alkaloid, tannin and steroid, which can be herbal-based mouthwash as an alternative of 0,2% Chlorhexidine gluconate.* ***Purpose****: It was to compare the inhibitory zone of kelakai leaf ethanol extract and 0,2% Chlorhexidine gluconate against S. sanguinis.* ***Materials and methods****: This study was true experimental research and post-test only with control group design, that used 5 treatment groups with 4 replications of kelakai leaf ethanol extract, that were 25%, 50%, 75%, 100% concentrations and 0,2% Chlorhexidine gluconate against S.sanguinis with 20 total samples. All groups incubated for 24 hours at 37^{o}C, then it measured the diameter of inhibition zone using calliper.* ***Result:*** *One Way ANOVA and Post Hoc LSD tests showed significant difference between all groups after given with kelakai leaf ethanol extract and 0,2% Chlorhexidine gluconate. Kelakai leaf ethanol extract 25%, 50%, 75%, 100% concentrations and 0,2% Chlorhexidine gluconate has an average inhibition zone, which were 9,00 mm, 11,20 mm, 13,67 mm, 16,32 mm, and 18,12 mm against S.sanguinis.* ***Conclusion:*** *Kelakai leaf ethanol extract 25%, 50%, 75% and 100% concentrations has antibacterial activity against S.sanguinis, but not as strong as 0,2% Chlorhexidine gluconate.*

Keywords: *0,2% Chlorhexidine gluconate, Inhibitory activity, Kelakai leaf ethanol extracts, Streptococcus sanguinis.*

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INTRODUCTION

Gingivitis is an early stage periodontal disease due to host response toward plaque bacteria which causes gingival inflammation.[1] *Streptococcus sanguinis* is a pioneer bacteria in the formation of plaque colonization in the oral cavity because it acts as an anchor for the attachment of other oral cavity microorganisms that colonizes in the oral cavity, thus contributing on the development of caries, gingivitis, and periodontal disease.[2,3] Gingivitis prevention can be achieved by the use of chemotherapeutic agent, which is a mouthwash.[1] Today, 0.2% *Chlorhexidine gluconate* is thought to be the gold standard of periodontal disease treatment. However, it has several side effects if used for prolonged period.[4]

Herbal mouthwashes have been developed with the aim to search a more effective alternative material in controlling plaque-causing bacteria with no side effect and can promote oral health.[4] Kelakai

(*Stenochlaena palustris*) is a typical plant that grows on peatland. According to empirical evidence, kelakai leaf is used by the society as herbal medicine to cure anemia and skin disease. Antibacterial substances in kelakai leaf ethanol extract include flavonoid, alkaloid, tannin, and steroid.[5] Flavonoid is a secondary metabolite substance dominant to kelakai leaf.[6] *Quercetin* flavonoid that contained in kelakai leaf ethanol extract is 503.56 mg/g.[7]

According to Erwin (2016), kelakai leaf ethanol extract with 10%, 15%, 20%, 25%, and 30% concentrations could inhibit *Staphylococcus aureus*, which is a normal floral bacteria in the human skin, respiratory tract, and gastrointestinal tract with the average inhibition zone diameter of 7 mm, 7 mm, 8 mm, 9 mm, and 10 mm, respectively.[8] Currently, there is no study that investigates inhibitory activity of kelakai leaf ethanol extract that is comparable to 0.2% *Chlorhexidine gluconate* on normal flora bacteria of the oral cavity. Therefore, an in vitro comparison study of inhibitory activity between kelakai leaf ethanol extract and 0.2% *Chlorhexidine gluconate* on *Streptococcus sanguinis* ATCC® 10556™ was conducted.

MATERIALS AND METHODS

This study was conducted in Microbiology Laboratory of Natural Sciences Faculty, Lambung Mangkurat University, Industrial Consultation Research Center, and Microbiology Laboratory of Faculty of Dentistry Research Center, Airlangga University. The study was begun by obtaining permission and ethical clearance from the Ethical Committee of Faculty of Dentistry, Lambung Mangkurat University No. 096/KEPKG-FKGULM/EC/XII/2018. The method which used in this study was true laboratory experiment with post-test only with control group design using 5 treatment groups. The minimal number of repetition for each treatment was 4 times using Lemeshow formula. The sample size used was 20.

Kelakai leaf ethanol extract was made using maceration method. Two kilograms of kelakai leaves was cleaned and dried in oven at 40^{o}C for 4 hours, then blended and sifted to obtain simplisia powder. The powder was then immersed in 96% ethanol solvent for 1 x 24 hours and stirred with the help of a shaker. The solvent was evaporated using rotary evaporator at 50-60^{o}C and heated on a water bath until the solvent has completely evaporated to obtain 200 g kelakai leaf thick extract with 100% concentration. Test for ethanol content was conducted by adding several drops of potassium dichromate ($K_2Cr_2O_7$).

Afterwards, kelakai leaf ethanol extract was diluted to several concentrations, i.e. 25%, 50%, 75%, and 100%. The concentration obtained was in accordance with the formula as follows:

$$V_1 \times N_1 = V_2 \times N_2$$

V_1 = baseline volume
N_1 = baseline concentration
V_2 = final volume
N_2 = final concentration

Kelakai leaf ethanol extract with 25%, 50%, 75%, and 100% concentrations and 0.2% *Chlorhexidine gluconate* were subjected to inhibitory activity test on *Streptococcus sanguinis* obtained from pure isolate in Research Center Microbiology Laboratory, Airlangga University. Several colonies of *Streptococcus sanguinis* ATCC 10556 from pure isolate were placed on MHA media, then incubated for 1 x 24 hours at 37^{o}C. Afterwards, 0.5 ml bacteria were inoculated in 5 ml liquid BHI and incubated for 2 x 24 hours at 37^{o}C inside incubator. The suspension was diluted with liquid BHI media until the turbidity reached 0.5 *McFarland* standard.

Inihibitory activity test was conducted by agar diffusion method by smearing *Streptococcus sanguinis* with 0.5 *McFarland* standard with sterile cotton stick on MHA media. Then, 0.01 ml Kelakai leaf ethanol extract with 25%, 50%, 75%, and 100% concentrations, and 0.2% *Chlorhexidine gluconate* were dripped onto empty paper disks using micropipette. The paper disks containing kelakai leaf ethanol extract with 25%, 50%, 75%, 100% concentrations and 0.2% *Chlorhexidine gluconate* were placed on MHA media containing bacteria using tweezers, then were incubated at 37^{o}C for 24 hours. The next step was measuring inhibition zone which was formed using caliper.

RESULTS

The results of average inhibition zone diameter of kelakai leaf ethanol extract with 25%, 50%, 75%, 100% concentrations and 0.2% *Chlorhexidine gluconate* on *Streptococcus sanguinis* can be seen as follows.

Table 1. The average inhibition zone diameter of kelakai leaf ethanol extract and 0.2% *Chlorhexidine gluconate* on *Streptococcus sanguinis*.

Group	*Mean*±SD
EDK 25%	9,00±0,14
EDK 50%	11,20±0,21
EDK 75%	13,67±0,18
EDK 100%	16,32±0,22
CHX 0,2%	18,12±0,09

Kelakai leaf ethanol extract with 25% concentration had an average inhibition zone diameter of 9 mm and 50% was 11.20 mm. The 75% concentration had the diameter as much as 13.67 mm. Least, 100% concentration had 16.32 mm of diameter. 0.2% *Chlorhexidine gluconate* had average inhibition zone diameter of 18.12 mm. The results of this study showed that kelakai leaf ethanol extract with 100% concentration had the highest average of inhibition zone diameter compared to 75%, 50%, and 25% concentrations. However, kelakai leaf ethanol extract with 100% concentration had lower average of inhibition zone diameter than the 0.2% *Chlorhexidine gluconate*.

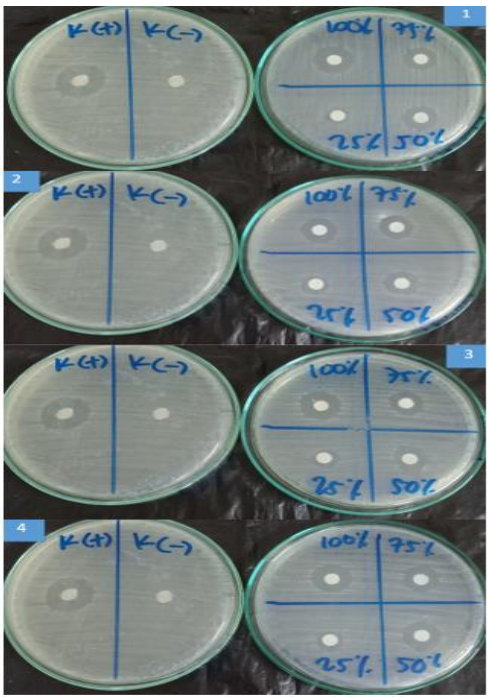

Figure 1. Inhibition zone of kelakai leaf ethanol extract and 0.2% *Chlorhexidine gluconate* on *S.sanguinis* with 4 replications.

Data obtained from each treatment was tested for normality using *Shapiro-wilk* test and *Levene's* homogeneity test with required p value of > 0.05. The result of normality test on kelakai leaf ethanol extract with 25% concentration was p=0.161, 50% concentration with p value= 0.577, 75% concentration with p=0.086, 100% concentration with p=0.789, and 0.2% *Chlorhexidine gluconate* showed p value= 0.272. Normality test of all treatment group showed p value > 0.05, thus the data were normally distributed. Homogeneity test showed p value=0.588 (p>0.05) which means that the data were homogenous.

Normally distributed and homogenous data were then analyzed using *One-Way* ANOVA with confidence interval of 95%. The result of *One Way* ANOVA was p=0.000 (p<0.05) which meant that there were significant differences between treatment groups. The administration of kelakai leaf ethanol extract and 0.2% *Chlorhexidine gluconate* had different effect on the growth of *Streptococcus sanguinis*, thus *Post Hoc Least Significant Difference* (LSD) was used to determine which group showed significant differences.

Table 2. The result of *Post Hoc* LSD inhibition zone diameter of kelakai leaf ethanol extract and 0,2% *Chlorhexidine gluconate* on *Streptococcus sanguinis*.

Treatment	CHX 0,2%	EDK 100%	EDK 75%	EDK 50%	EDK 25%
CHX 0,2%		0,000*	0,000*	0,000*	0,000*
EDK 100%			0,000*	0,000*	0,000*
EDK 75%				0,000*	0,000*
EDK 50%					0,000*
EDK 25%					

* : significant differences (p<0,05).

The result of *Post Hoc* LSD showed p value = 0.000 for each group, thus concluded that all treatment groups showed significant difference of *Streptococcus sanguinis* average inhibition zone diameter due to the administration of kelakai leaf ethanol extract and 0.2% *Chlorhexidine gluconate.*

DISCUSSION

The results of inhibitory activity comparison between kelakai leaf ethanol extract (*Stenochalena palustris*) and 0.2% *Chlorhexidine gluconate* on *Streptococcus sanguinis* showed that kelakai leaf

ethanol extract with 25%, 50%, 75%, and 100% concentrations could inhibit the growth of *Streptococcus sanguinis*.

Agar diffusion method was used in this study to analyze antibacterial activity comparison by measuring inhibition zone diameter of kelakai leaf ethanol extract with 25%, 50%, 75%, and 100% concentrations and 0.2% *Chlorhexidine gluconate* on the growth of *Streptococcus sanguinis*. Inhibition zone measurement was used to determine antibacterial activity that the material had has and showed the most effective concentration of kelakai leaf ethanol extract to inhibit the growth of *Streptococcus sanguinis*.

According to Ngajow (2013) and Apriasari (2015), bacterial antibiotic activity measurement using David-Stout method stated that to be seen from inhibition zone, antibacterial power can be classified into very strong antibacterial if the inhibition zone formed was 20 mm or more, strong was 10-20 mm, moderate was 5-10 mm, and weak was 5 mm.[9,10] According to this standard, the inhibitory activity of kelakai leaf ethanol extract with 50%, 75%, 100% concentrations and 0.2% *Chlorhexidine gluconate* had strong antibacterial effect on *Streptococcus sanguinis*, because the average inhibition zone diameter was between 10-20 mm.

Kelakai leaf ethanol extract with 100% concentration had the highest average inhibition zone in inhibiting the growth of *Streptococcus sanguinis* compared to 25%, 50%, and 75%. This was in accordance with Zuraini (2010) that the inhibitory power of kelakai leaf ethanol extract on *Staphylococcus aureus* at 100% concentration had the highest inhibition zone with 14 mm.[11] The correlation between concentration of kelakai leaf ethanol extract and inhibitory power of *Staphylococcus aureus* was directly proportional, which means that the higher the concentration used, the higher the inhibitory action achieved.[12] Higher concentration causes increasing in osmotic pressure, thus liquid tends to pass through bacterial cell toward lower concentration, which lead to cell shrinkage and cell death because of inability to function.[13] Increased concentration will escalate the active substance of antibacterial, thus increasing its ability in eradicating bacteria.[14]

Secondary metabolite substance contained in kelakai leaf, include flavonoid, alkaloid, tannin, and steroid.[5] Maharani (2015) stated that the dominant antibacterial content in kelakai leaf was flavonoid.[6] *Quercetin* flavonoid which contained in kelakai leaf was 503.56 mg/g.[7] Flavonoid, in its function in inhibiting the growth of *Streptococcus sanguinis*, had an action mechanism of inhibiting the synthesis of nucleic acid by bonding with sub unit GyrB from DNA gyrase and inhibiting the activity of ATPase enzyme, thus bacteria failed to replicate. Bacterial cytoplasm membrane function was inhibited by increasing membrane inner permeability and loss of membrane potential, thus disturbing the process of proton electrochemical gradient in passing through membrane. It is important to maintain ATP synthesis capacity, membrane transportation, and bacterial movement.[15]

Flavonoid, alkaloid, and steroid disturb the component which forming cell wall peptidoglycan layer of *Streptococcus sanguinis*, thus the cell wall unable to completely form. Alkaloid has base group which will disrupt bacterial peptidoglycan. Flavonoid and steroid had hydrogen bond which will react with cell wall, thus damaging bacterial cell wall. Afterwards, cytoplasm leakage follows, and bacteria undergo lysis.[16,17]

Incomplete cell membrane aids in alkaloid, tannin, flavonoid, and steroid substances to penetrate into cell. Base group containing nitrogen in alkaloid changes cell protein to denaturize and react to amino acid that forms cell membrane and bacterial DNA. This reaction causes changes in structure and composition of amino acid which will change the genetic balance in DNA chain, thus causing damage and promoting lysis. Base group will increase cell membrane permeability, then damaging membrane. Cell membrane damage will lead to bacteria losing their most important component from the cell or through direct path from cell membrane which causes polarity damage of the cell membrane, leading to cell death.[16,18]

Tannin forms a complex bond that is irreversible to proline-rich protein and inhibiting the synthesis of bacterial protein.[19] Tannin can form hydrogen bond with protein which contained in bacterial cell. If this bond formed between tannin and protein, then denaturation of the protein will form which causes metabolism disturbance in the bacteria.[20,21] The reaction of tannin with protein will form tannin-protein bond. Reactive protein had the ability to bond with tannin, which is a peptide bond, hydroxyl group and amide. The formation of hydrogen bond between tannin and protein causes shape change in protein molecule, thus the protein is denaturized, and bacterial biochemical activity decreased.[21]

Steroid forms a bond with lipid membrane and suppress its action, thus causing lipid membrane

leakage. Lipid membrane with sensitivity toward steroid component will cause leakage on bacterial liposome. Steroid interacts with cell phospholipid membrane that is permeable to lipophilic substance, so decreasing membrane integrity and change cell membrane morphology, leading to vulnerability and lysis.[22]

Antibacterial substances contained in kelakai leaf ethanol extract, such as flavonoid, alkaloid, tannin, and steroid, cause complex damage on *Streptococcus sanguinis* bacterial structure, that causing lysis. Lysis causes cell death which means that there is no growth of *Streptococcus sanguinis*. The death of *Streptococcus sanguinis* as the pioneer bacteria that forms plaque causes decrease the ability to form plaque accumulation in oral cavity, so that decrease the potential of gingivitis.[22]

This study used 0.2% *Chlorhexidine gluconate* as positive control which has bactericidal and bacteriostatic effect on gram-positive and gram-negative bacteria in plaque. *Chlorhexidine gluconate* had positive-charged molecule which bonds with negative-charged molecule in bacterial cell wall, then damaging cell wall and disrupting osmosis. In low concentration, it will affect the integrity of cell wall. After damaging the cell wall, 0.2% *Chlorhexidine gluconate* will enter the cell and attacks cytoplasm membrane (inner membrane). Smooth semipermeable cytoplasm membrane causes leakage of cell components, thus causing cell death.[23]

Kelakai leaf ethanol extract with 100% concentration had lower inhibition zone than 0.2% *Chlorhexidine gluconate* in inhibiting the growth of *Streptococcus sanguinis*. This occurred because 0.2% *Chlorhexidine gluconate* is a broad spectrum antibacterial, effective in fighting gram-positive, gram-negative bacteria and fungi. *Chlorhexidine gluconate* can inactivate microorganism with broader spectrum than other antimicrobial substances with faster average killing ability.[23] This was in accordance with Sinaredi (2014) that 0.2% *Chlorhexidine gluconate* had the strongest antibacterial effect compared to povidone iodine and fluoride.[24]

Kelakai leaf ethanol extract has the potential to be used as herbal-based mouthwash because it contains antibacterial substance which can inhibit *Streptococcus sanguinis*, which is the pioneer bacteria that forms plaque colonization in the oral cavity. In this study, kelakai leaf ethanol extract was investigated to become an alternative to synthetic mouthwash, like 0.2% *Chlorhexidine gluconate* as plaque control and gingivitis prevention. It is expected to decrease the side effect of prolonged use of 0.2% chlorhexidine gluconate. In conclusion, kelakai leaf ethanol extract has antibacterial activity, which was shown by inhibition zone. However, the inhibitory activity of kelakai leaf ethanol extract with 25%, 50%, 75%, and 100% concentrations is not as strong as inhibitory activity of 0.2% *Chlorhexidine gluconate* on the growth of *Streptococcus sanguinis*.

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