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Full Length Research Paper

Effect of ethanol extract of *Hibiscus sabdariffa* L. calyx on *Streptococcus sanguinis* viability *in vitro* biofilm based on crystal violet

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Most dental and gingival pathology were triggered by oral biofilm which consisted of *Streptococcus sanguinis* as an early colonizer. *S. sanguinis* could elicit some pathological events such as dental caries, periodontal diseases. One of the strategies to achieve such result is with antimicrobial therapy. The widely known gold standard is the utilization of chlorhexidine which has also been known to discolor the tongue and dentition, also change in sensation and mucosal irritation. Those disadvantages have resulted in some research toward the therapy from natural resources, such as the calyx of *Hibiscus sabdariffa*. *S. sanguinis* between 20 and 24 h in biofilm was exposed to the ethanol extracts of *H. sabdariffa* calyx at the concentration of 0.2% to 50% for 60 min. Subsequently, the cellular viability was tested with crystal violet assay. As the positive control group, chlorhexidine 0.1% and 0.2% were used. The viability was expressed in percents toward the negative controls. The results were analyzed with one way ANOVA (*P*<0.05). The viability of *S. sanguinis* was tending to decrease as the concentration increases. The ethanol extract of *H. sabdariffa* calyx is capable of reducing the *S.sanguinis* viability in the biofilm, where the effectiveness of the ethanol extract of *H. sabdariffa* calyx as antibacterial agent was affected by biofilm formation phase. The effectiveness of the ethanol extract of *H. sabdariffa* calyx in reducing *S. sanguinis* viability is equal to chlorhexidine, at the adherence phase (20 h) and maturated phase (24 h) of biofilm.

Key words: Ethanol extract, *Hibiscus sabdariffa* calyx, *Streptococcus sanguinis,* chlorhexidine, Biofilm, Viability.

INTRODUCTION

The biofilm community was a complex and dynamic structure, and being accumulated from a sequential bacterial colonization within the oral cavity (Hojo et al., 2009). One of the early colonizers in the biofilm was *S. sanguinis* (Kolenbrander et al., 2002; Hojo et al., 2009). The pioneering bacteria which consist of 85% of the biofilm were *Streptococcus oralis, Streptococcus mitis, Streptococcus gordonii* and *S. sanguinis.* Those early colonizers serve as the bridging microorganisms for the late colonizers. Those early colonizers have the ability to absorb the nutrition from proline-rich protein and also posses' adhesion receptors for the late colonizers. Bacterial co-aggregation occurs between those early colonizer and late colonizer. The co-aggregation is a

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specific cellular interaction between different bacterial cells and has been known as an important point in biofilm colonization (Kolenbrander et al., 2002; Hojo et al., 2009).

After colonization, biofilm starts to mature and grow at a slower rate. This slowing affects the biofilm resistance toward the antimicrobial substance. The extracellular matrix has also elicit a tendency for the biofilm to be resistant toward the antimicrobial substances in comparison to the planktonic bacterial (Levinson, 2010).

The biofilm formation phase can be divided into three different phases: Adherence phase (0 to 4 h), active accumulation phase (4 to 20 h), and plateau accumulated phase or maturated (after 20 h) (Kolenbrander et al., 2002). At the adherence phase, it was found that the dominant bacteria attached to the pellicle was *Streptococci* (61-78%) such as *S. sanguinis*, *S. mitis* and *S.oralis,* and *Actinomyces* (4 to 30%) (Kuramitsu et al., 2007; Ge et al., 2008). At the accumulation phase, the quantity of the biofilm cells was increasing exponentially due to the rapid growth and accumulation of the bacteria (Ge et al., 2008).

Based on the anti-bacterial resistance ability, the bacteria in biofilm can be 100 to 1000 times more resistant compared to planktonic bacterial (Levinson, 2010). *S. sanguinis* in biofilm could elicit a dental caries and periodontal diseases (Kreth et al 2008; Hojo et al 2009). *S. sanguinis* has also been known for causing infective endocarditis which is a serious endovascular disease (Paik et al., 2005; Ge et al., 2008). The strategy for this purpose is by administering antibacterial agents. One of the synthetic antibacterial substances to inhibit biofilm formation is chlorhexidine (Lewis, 2001). Chlorhexidine is effective toward gram positive and gram negative bacteria. This substance has a bactericidal and bacteriostatic property, and disrupts the bacterial membrane (Decker et al., 2003; Quirynen et al 2005).

Some research has shown that chlorhexidine has an antibacterial effect toward *S.sanguinis*. Chlorhexidine has proven to have minimum inhibitory level of 50 µg/ml on the *S. sanguinis* biofilm at 24 h (Larsen and Fiehn, 1996). However, some studies reveals the disadvantages of chlorhexidine, which turned out to be discoloring the dentition and the tongue, altered sensation, oral mucosa irritation, and increasing the calculus formation (Lorenz et al., 2006; Berchier et al., 2010). Those disadvantages have resulted in some research toward the therapy from natural resources, and in the present study, we would like to investigate the antibacterial effect of ethanol extract from the calyx of *H. sabdariffa* toward *S. sanguinis* in the biofilm and its' effectiveness in comparison to the chlorhexidine.

H. sabdariffa has been widely known and used by Indonesian as a traditional medication, food coloring agent, and for food itself. The calyx of *H. sabdariffa* has an active biologic property owing to their chemical compounds, particularly their secondary metabolites which could interfere in an organism cellular system. The calyx of *H. sabdariffa* was reported to have an antibacterial effect (Borisutpeth, 2005; Hwang et al., 2000), antiseptic, anti-inflammation (Ali et al., 2003), analgesic, antipyretic (Amos et al., 2003; Ali et al., 2003; Reanmongkol and Itharat, 2007), anti-hypertensive (Herrera-Arellano et al., 2004) and has been known to decrease blood cholesterol, uric acid, and triglyceride in the urine (Olantuji et al., 2005; Zarrabal et al., 2005; Lin et al., 2007).

The antibacterial property belongs to active substances such as flavonoids and polyphenols (anthocyanin-delphinidin-3-glucosyl-xyloside or hibiscin) and cyanidin-3-glucosyl-xyloside (Herrera-Arellano et al., 2004; Lin et al., 2007). Flavonoids play an important role to inhibit the growth of microorganisms due to their ability to form a complex compound with protein through hydrogen bonding. Polyphenols play an important role due to their ability to elicit protein denaturation and disrupt the bacterial plasma membrane (Herrera-Arellano et al., 2004). Previous study has been conducted on the infusion of *H. sabdariffa* calyx to investigate the minimum inhibitory level, and the author suggested that the infusion of *H. sabdariffa* have shown an excellent antibacterial effect on *S. sanguinis*. The present research was aimed at investigating the effectiveness of the ethanol extract of *H. sabdariffa* calyx as antibacterial agent was affected by biofilm formation phase. The antibacterial potency of ethanol extract form *H. sabdariffa* was compared to the chlorhexidine. *H. sabdariffa* and *S. sanguinis* as reported in this work.

MATERIALS AND METHOD

This research was an *in vitro* laboratory experiment. The research was done to evaluate the exposure effects of the ethanol extraction of *H. sabdariffa* for an hour to *S. sanguinis* between 20 and 24 h in biofilm. The research was conducted in three fold and was repeated 3 times. It includes 3 stages orientation: Determination of the *S. sanguinis* bacterial sample concentration, determination of the concentration of ethanol extract of *H. sabdariffa*, testing the viability of the *S. sanguinis* between 20 and 24 h in biofilm.

Plant collection

The calyx of the *H. sabdariffa* was taken from Balai Tanaman Obat dan Aromatik (BALLITRO), Bogor, Indonesia, from February to March, 2010 and the calyx weight was measured, plant was identified by Dr. Wahyu Widiono from Indonesian Institute of Science, Research Center for Biology, with voucher specimen number 291/IPH.1.02/If.8/III/2010. The calyx was then dried in the open air at room temperature for 14 to 21 days, the calyx weight was then measured and recorded as the sample dry weight. The extraction process was done by maceration technique using 70% ethanol at room temperature, in 1: 3 ratios. The maceration process was done in 3 days, and the extraction was then filtered. The maceration results was then distilled and evaporated in low pressures using rotary evaporator (Rotavapor® R-200 Buchi, Switzerland) in temperature not higher than 50°C, until the extract increased in consistency.

Preparation of crude extracts

The ethanol extract of *H. sabdariffa* calyx was diluted in 1% Dimethyl Sulfoxide (DMSO). Then 50% extraction was made by diluting 50g of *H. sabdariffa* calyx ethanol extract in 100 ml 1% DMSO (Balakin et al., 2006).

Preliminary phytochemical analysis

From the analysis, secondary metabolite components such as flavonoid, triterpenoid, saponin, tannin, phenolic, and glycoside was found.

The determination of *S. sanguinis* concentration

A suspension of *S. sanguinis* ATCC 10556 to a concentration of 10^{-8} was sub-cultured on a fresh thioglycollate agar plate for 48 h. Inocula were prepared by transferring several *S. sanguinis* to a centrifuge tube with 1 ml BHI broth. The microbial cell suspension was mixed to homogeneity and dilution of the bacteria from the concentration of 10^{-1} to 10^{-8} suspension was performed by preparing eight 15 ml centrifuges and BHI agar with a subsequent labeling for 10^{-1} to 10^{-8} bacterial suspensions. Each 15 ml centrifuge tube was filled with 4500 µl of thioglycollate broth. The main solution (500 µl) was placed on the first tube and being homogenized with mini vortexer to obtain 10^{-1} bacterial suspension. From the suspension, 500 µl was placed in the second tube and was homogenized using mini vortexer to obtain 10^{-2} bacterial suspension. From each 10^{-1} to 10^{-8} bacterial concentration, 10 µl was placed inside BHI agar and being dispersed evenly with wire loop. The Petri dish was subsequently placed inside an anaerobic jar and being filled with CO_2. Anaerobic jar was placed in the incubator at 37°C for 48 h. All of the procedures were performed thrice.

The counting of *S. sanguinis* colony

The colony of *S. sanguinis* from 10^{-1} to 10^{-8} concentration which was performed thrice was counted with colony counter and the mean was determined. Based on the colony observation, the bacteria suspension colony which was countable was in the concentration of 10^{-6} CFU/ml and these were confirmed by viable counts. This will be the standard concentration throughout this study. Other observations were made with microplate reader. The result was read in 490 nm wavelengh. The microbial cell suspension was mixed to homogeneity to give a final density. The colony counting or Colony Forming Unit (CFU) was conducted by converting those results to CFU/ml. Therefore, each centrifuge tube would require 230 µl of bacteria and 79.770 µl thioglycollate broth (Engelkirk and Engelkirk, 2008).

The concentration range determination of ethanol extract from the calyx of *H. Sabdariffa.*

In this orientation study, the concentrations used were 2 µg/ml which were equal to 0.2%, followed by 0.8%, 1.6%, 3.2%, 5.0%, 10%, 20%, 30%, 40% and 50%. The stock solution was determined to be 50%. We prepared 10 centrifuge tube of 15 ml which were labelled as the dilution vessels for the ethanol extract of *H. sabdariffa* micropipette and DMSO solution.

After extracting the ethanol extract of *H. sabdariffa* in 0.2% to 50% concentration, the centrifuge tube was added with ethanol extract of *H. sabdariffa.* Chlorhexidine *CHX* 0.1% and chlorhexidine 0.2% were wrapped with aluminum foil and stored in the refrigerator till usage. The negative control was the bacteria that were given no treatment while the positive control was the bacteria which has been exposed to chlorhexidine 0.1% and 0.2%.

Testing the viability of *Streptococcus sanguinis* in biofilm

Biofilm model production

Biofilm models were made in three stages: saliva preparation, pellicle production and bacterial exposure. Saliva from healthy volunteers (50 ml) was placed on 15 ml centrifuge tubes and centrifuged for 20 min at 4500 rpm, 4°C. From this centrifugation process, the bacteria would precipitate as a pellet and salivary protein would be contained within the supernatant. The supernatant would then be removed to the new 50 ml tube.

The salivary supernatant was filtered with 0.22 µm sartorius stedim filters. The result of this filtration process was added to (Phenylmethane Sulfonyl Fluoride) PMSF in 1 µmol concentration as protease inhibitor. PMSF was added to the pure saliva in ratio 1: 9. The saliva was then cultured in (Brain Heart Infusion) BHI (10 µl) to ensure the saliva was free from bacteria and fungi. The culture was stored in the incubator at 37°C for 24 h. However, the remaining saliva was being stored in the freezer at -80°C. After the culture revealed that the saliva was free from the bacteria and fungi, the saliva was diluted 50 times with PBS. Subsequently, the biofilm assay preparation was done by coating the saliva. We placed 100 µl saliva on each well (96 microwell plate tissue culture), and they were placed on the orbital shaker at 80 rpm for 90 min in an incubator at 37°C. Those wells were then rinsed with 100 µl (Phosphate Buffered Saline) PBS once to obtain the sticking pellicle.

Eventually, 100 µl bacterial suspensions were added on each well (6 microwell-plate for tissue culture). The mixture was then placed in an anaerobic jar which has been filled with gas and incubated at 37°C. For the adherence phase of the biofilm, 3 microwell plate were incubated for 20 h, while the remaining 3 wells were used for maturation phase and were incubated for 24 h. Afterward, each well was rinsed once with PBS 100 µl to remove the planktonic bacteria.

The exposure of ethanol extracts from *H. sabdariffa* calyx toward the bacteria

The effectiveness test on ethanol extracts from the calyx of *H. sabdariffa*, each biofilm containing well was washed and exposed to 100 µl of ethanol extracts from the calyx of *H. sabdariffa* and incubated for an hour. On the positive control group, 100 µl chlorhexidine of 0.1 and 0.2% were also being exposed to each well. On the negative control group, antibacterial agent was not given and the specimens were only being incubated in the medium. These treatments were done in threefold and repeated thrice. Three well plates were incubated for an hour at 37°C and placed inside the anaerobic jar filled with gas. Subsequently, the wells were washed once with PBS 100 µl.

Crystal violet test

The violet crystal 4% solution was made by diluting 4ml violet crystal in 96ml distilled water, while the ethanol 96% solution was obtained from diluting 96 ml pure ethanol with 4 ml sterile distilled water. On each washed well, 200 µl violet crystal of 4% were placed and incubated at a room temperature for 45 min. The violet crystal was then disposed and the well were rinsed with 100 µl distilled water twice. Subsequently, 200 µl ethanol was poured in each well and incubated at a room temperature for 45 min, the end

Table 1. The mean of *S. sanguinis* colony (CFU/10 µl) from serial dilution 10^{-1} - 10^{-8}.

***S. sanguinis* dilution**	**Colony count (CFU/10 µl)**
10^{-1} (100µl main bacterial solution + 900 µl BHI)	> 7000
10^{-2} (100µl bacterial solution 10^{-1} + 900 µl BHI)	> 5000
10^{-3} (100µl bacterial solution 10^{-2} + 900 µl BHI)	> 5000
10^{-4} (100µl bacterial solution 10^{-3} + 900 µl BHI)	> 3000
10^{-5} (100µl bacterial solution 10^{-4} + 900 µl BHI)	> 2000
10^{-6} (100µl bacterial solution 10^{-5} + 900 µl BHI)	636
10^{-7} (100µl bacterial solution 10^{-6} + 900 µl BHI)	61
10^{-8} (100µl bacterial solution 10^{-7} + 900 µl BHI)	8

results were then read on a microplate reader (Biorad) in 490 nm wavelength (Maclean et al., 2008).

Data analysis

The test used to analyze the viability percentage results for each treatment group on *S. sanguinis* was ANOVA test and to determine any significant difference within the compared group, the post hoc analysis was done by using Post-Hoc (Hill and Lewicki, 2007; Ruxton and Beauchamp, 2008).

RESULTS

A bacterial suspension dilution of *S. sanguinis* from the culture counts in 10^{-1} to 10^{-8}, it was found that the culture count in 10^{-1} to 10^{-5} concentration was difficult to be performed due to the high colony's density. At concentration of 10^{-6} the colony's density decreased and could be easily counted. However, at 10^{-7} to 10^{-8} concentration, the colony grew at a minimum count. Therefore in this study, dilution was being determined at 10^{-6} concentration due to the facts that it would harvest a less dense culture and will be countable. The mean bacteria colony at 10^{-6} concentration was 636 CFU. The result is presented in Table 1.

On the antibacterial study, we performed antibacterial test from various extracts of *H. sabdariffa* calyx (0.2 to 50% concentration) toward the viability of *S. sanguinis* adherence phase (20 h) and maturation phase (24 h) using violet crystal test. This test was done in threefold and repeated thrice. The optical density value (OD) obtained (read at 490 nm wavelength) were averaged. The result on the 20 h and the 24 h biofilm with various ethanol extract from *H. sabdariffa* calyx exposed for an hour using violet crystal test were shown at the Tables 2 and 3. This result shows that bacteria which has been exposed to the extract of *H.sabdariffa* from concentration of 0.2 to 50% has the tendency to decrease.

The results of Kolmogorov Smirrnov normality test shows that all the obtained data had normal distribution ($p>0.05$). From the homogeneity test on statistical test ANOVA, it was found that all the data were homogeny ($p>0.05$).

The mean results of OD, viability percentage of *S. sanguinis*, and the one way ANOVA test results on negative control, positive control, and ethanol extract of *H. sabdariffa* for 60 min using violet crystal group can be viewed in Table 2 and 3. Table 2 shows that the *S. sanguinis* viability in 20 h old biofilm towards the ethanol extract of *H. sabdariffa* was lower than the negative control group. The OD mean value for the chlorhexidine 0.1% positive control group (20.47%) was nearly equal to the ethanol extract of *H. sabdariffa* 0.8% group (20.75%). The OD mean value for the chlorhexidine 0.2% (10.66%) was comparable to the ethanol extract of *H. sabdariffa* 10% (10.73%) and 20% (10.80%).

Table 3 shows that the *S. sanguinis* viability in the 24 h biofilm at the ethanol extract of *H. sabdariffa* group was lower than the negative control group. The mean OD value in chlorhexidine 0.1% positive control group (18.47%) was nearly the same with the ethanol extract of *H. sabdariffa* 1.6% group (18.12%). The OD mean value of chlorhexidine 0.2% group (9.26%) was nearly equal to the ethanol extract of *H. sabdariffa* 10% (9.22%).

From these data, the hypothetical statement that ethanol extract of *H. sabdariffa* petals can be used to decrease the *S. sanguinis* viability in biofilm is accepted. And the hypothesis about the effectiveness of ethanol extract of *H. sabdariffa* in decreasing the *S. sanguinis* viability is equal to chlorhexidine 0.1% and 0.2% in biofilm test.

DISCUSSION

S. sanguinis is a specie mostly found at the early biofilm formation (Kolenbrander et al., 2002; Marsh and Martin, 2009). In this particular study, the effect of ethanol extract of *H. sabdariffa* calyx to the viability of *S. sanguinis* was tested. The test was conducted between 20 and 24 h in biofilm. This was done due to the slow growth of the bacteria colony (20 h) which eventually stopped (24 h).

The ethanol extract of *H. sabdariffa* calyx in this study was diluted with DMSO 1% as in Hwang's study; this demonstrates that DMSO of 1% has no effect on *S. sanguinis* (Hwang et al., 2000).

Table 2. The viability (%) of *S. sanguinis* in adherence phase biofilm (20 h) exposed at control and treatment group.

Group	Rerata OD (x) ±SD	Viability (%)	Significant compare	Significant compare	Significant compare
Negative control			(-) control	chlorhexidine0.1	chlorhexidine 0.2
No treatment	0.155 ± 0.001	100%	-	0.000*	0.000*
Positive control					
CHX 0.1%	0.031 ± 0.002	20.47%	0.000*	-	0.000*
CHX 0.2%	0.016 ± 0.001	10.66%	0.000*	0.000*	0.000*
Ethanol extract					
0.2%	0.053 ± 0.002	34.14%	0.000*	0.000*	0.000*
0.8%	0.032 ± 0.002	20.75%	0.000*	0.824	0.000*
1.6%	0.030 ± 0.000	19.75%	0.000*	0.226	0.000*
3.2%	0.028 ± 0.003	18.39%	0.000*	0.040*	0.000*
5.0%	0.021 ± 0.001	13.81%	0.000*	0.000*	0.000*
10%	0.016 ± 0.000	10.73%	0.000*	0.000*	0.482
20%	0.016 ± 0.000	10.80%	0.000*	0.000*	0.494
30%	0.024 ± 0.002	15.60%	0.000*	0.001*	0.000*
40%	0.035 ± 0.003	22.69%	0.000*	0.099*	0.000*
50%	0.037 ± 0.004	24.26%	0.000*	0.021*	0.000*

*p<0.05

Table 3. The viability (%) of *S. sanguinis* in maturation phase biofilm (24 h) exposed at control and treatment group.

Group	Rerata OD (x) ±SD	Viability (%)	Significant compare	Significant compare	Significant compare
Negative control			(-) control	chlorhexidine0.1	chlorhexidine 0.2
No treatment	0.077 ± 0.001	100%	-	0.000*	0.000*
Positive control					
CHX 0.1%	0.014 ± 0.003	18.47%	0.000*	-	0.000*
CHX 0.2%	0.007 ± 0.000	9.26%	0.000*	0.000*	0.000*
Ethanol extract					
0.2%	0.025 ± 0.001	32.66%	0.000*	0.000*	0.000*
0.8%	0.017 ± 0.001	22.44%	0.000*	0.024*	0.000*
1.6%	0.014 ± 0.000	18.12%	0.000*	0.262	0.000*
3.2%	0.011 ± 0.009	15.25%	0.000*	0.226	0.000*
5.0%	0.008 ± 0.001	11.62%	0.000*	0.002*	0.002*
10%	0.007 ± 0.000	9.22%	0.000*	0.000*	0.755
20%	0.007 ± 0.000	10.23%	0.000*	0.000*	0.000*
30%	0.008 ± 0.000	11.12%	0.000*	0.000*	0.000*
40%	0.013 ± 0.002	17.55%	0.000*	0.246	0.000*
50%	0.016 ± 0.001	21.58%	0.000*	0.226	0.000*

*p<0.05

The viability test using violet crystal is a colorimetric assay measuring cells quantity based on the colouring intensity of the violet crystal to the cell's membrane. The elevation of the colouring intensity will alleviate higher OD value and will be interpreted as a high bacterial concentration. Should the read violet crystal colour become bluish purple, the quantity of viable *S. sanguinis* is high and is able to attach to the biofilm surface.

The elevation of the ethanol extract of *H. sabdariffa* calyx concentration exposed has a tendency to reduce the attachment of *S. sanguinis* to the biofilm not only at the adherence phase (20 h) but also at the maturation phase (24 h). But at the adherence phase (20 h), the peak extract's concentration of 30%, 40% and 50% has no further improvement compared to the lesser concentration. While at the maturation phase (24 h), the peak extract's concentration of 20%, 30%, 40% and 50% showed a higher rate of growth compared to the lesser concentration. This is probably due to the mirror effect of the ethanol extract of *H. sabdariffa* calyx, that has an antibacterial effect at the lower concentration, while at the peak concentration this extract will alleviate the viability of *S. sanguinis*.

The reduction of the *S. sanguinis* viability as the extract's concentration increases is due to antibacterial activity of polyphenol. Polyphenol is capable of causing a mass destruction to the cell membrane. Delphinidin-3 glucoxyloxide is a member of polyphenol family and this compound owns hydrocarbon – OH branch. This hydrocarbon branch is strongly correlated to the antibacterial effect of the Delphinidin-3 glucoxyloxide (Herrera-Arellano et al., 2004).

With the destruction of the bacterial membrane, the attachment capability of those bacterial to the biofilm will be diminished. This was due to the fact that *S. sanguinis*' ability to attach strongly depends on its phillia or adhesins. *S. sanguinis* as a member of the early colonizer has an important role in building a relationship between pellicle and the bacterial adhered next (Kuramitsu et al., 2007; Hojo et al., 2009). When *S. sanguinis* lysis, the late colonizer bacterial will be off balance. This will interfere with the biofilm formation.

The viable *S. sanguinis* is mostly attached to the maturation phase of the biofilm (24 h). After exposure of the ethanol extract of *H. sabdariffa* calyx the viability of *S. sanguinis* is higher in the 20 h in biofilm. This demonstrates that the ethanol extract of *H. Sabdariffa* is effective against *S. sanguinis* in biofilm at adherence phase or maturation phase.

There are a few theories about the building mechanism of biofilm resistance to antimicrobial agent. One of the triggering action of this resistance building was the slowing rate of their growth. When the nutrient of the bacterial colony starts to depleted, their growth rate will decrease. This will affect their resistance to antibacterial agent. The decrease of their growth rate can be observed in the maturation phase of the biofilm (Mah et al., 2001). Therefore, the matured biofilm will be more resistant to antibacterial agent compared to the immature one.

Chlorhexidine is used as positive control in this study due to its gold standard as an antibacterial agent and has been proven clinically. Statistically, there was significant difference in bacterial viability exposed to chlorhexidine 0.1% and 0.2% compared to the exposure of ethanol extract of *H. sabdariffa* calyx. On the viability graph for biofilm 20 h old, the bacterial viability percentage at the chlorhexidine 0.1% exposure was close to the ethanol extract of *H. sabdariffa* calyx 0.8% exposure. While the viability at the exposure of chlorhexidine 0.2% was close to the ethanol extract of *H. sabdariffa* calyx 10% and 20%. This demonstrates that there were equation between the antibacterial effect of ethanol extract of *H. sabdariffa* calyx and chlorhexidine 0.1% and 0.2%.

On the viability graph for biofilm 24 h old, the bacterial viability percentage at the chlorhexidine 0.1% exposure was close to the ethanol extract of *H. sabdariffa* calyx 1.6% exposure. While the viability at the exposure of chlorhexidine 0.2% was close to the ethanol extract of *H. sabdariffa* calyx 10%. This demonstrated that there were equation between the antibacterial effect of ethanol extract of *H. sabdariffa* calyx and chlorhexidine 0.1% and 0.2%.

CONCLUSIONS

The ethanol extract of *H. sabdariffa* calyx is capable of reducing the *S. sanguinis* viability in the biofilm, where the effectiveness of the ethanol extract of *H. sabdariffa* calyx as antibacterial agent was affected by biofilm formation phase. The effectiveness of the ethanol extract of *H. sabdariffa* calyx in reducing *S. sanguinis* viability is equal to chlorhexidine 0.1% and 0.2% in both adherence phase (20 h) and matured phase (24 h).

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