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Research article

Lime peel extract *(Citrus Aurantifolia)* inhibit the growth of bacteria *Lactobacillus Acidophilus* in childhood dental caries

Putu Yetty Nugraha[1*], Eko Sri Yuni Astuti[2], Ida Ayu Putu Meilya Citra Saraswati[3]

[1,2,3]Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Mahasaraswati-Denpasar
*Correspondence author email: putuyetty@gmail.com

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Abstract: Caries occur due to the presence of pathogenic microorganisms such as *Lactobacillus Acidophilus* bacteria. One way to prevent caries is to inhibit and kill *Lactobacillus Acidophilus* bacteria. This study used the dilution method to determine the inhibition and killing power of lime peel extract (*Citrus Aurantifolia*) against *Lactobacillus Acidophilus* bacteria, lime peel extract obtained by maceration method and made with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% and 0.78%. Based on the results of the study, it was found that lime peel extract with a concentration of 1.56% proved to be able to inhibit bacterial growth, at concentrations of 100%, 50%, 25%, 12.5%, 6.25% and 3.125% showed no growth of bacteria on the media. In the normality test used the *Shapiro-Wilk* method, the results obtained by the value of ρ (Sig.) > 0.05 so that the data is said to be normally distributed. Followed by the homogeneity test using the *Levene Test* and the value of ρ (Sig.) = 0.001 <0.05 so that the data is declared to be inhomogeneous. The *Kruskall-Wallis* test to determine the differences between the treatment groups and the value of ρ (Sig.) < 0.05, which means there was a significant difference and continued with the *Mann-Whitney* test to determine which groups were significantly different. The content of lime peel extract that affects the inhibition and killing power of bacteria are flavonoids, tannins, essential oils, saponins and triterpenoids which have antibacterial properties and are able to inhibit and kill Lactobacillus Acidophilus bacteria.

Keywords: *Caries, Lactobacillus Acidophilus, Lime peel extract (Citrus Aurantifolia).*

INTRODUCTION

The caries process can occur due to the activity of microorganismsin sweet carbohydrates, especially sucrose, which can be fermented, as well as microbial activity on a carbohydrate undergoing fermentation characterized by demineralization of the hard tooth tissue followed by decay the organic

material. Dental caries is also caused by several factors (*multiple factors*) which influence each other are divided into internal factors and external factors.[1]

Internal factors namely *hosts*, microorganisms, substrate (food) and time while external factors are race, gender, age, economic status and attitude and behavior.[2] The caries mechanism starts from sucrose or sugar from food residue which the bacteria then process and adhere to at a certain time begins to damage the hard tooth tissue.[3]

The dominant bacteria that play a role in caries are bacteria *Streptococcusmutants* and *Lactobacillus Acidophilus*. *Streptococcus* role at the moment the beginning (initial) of caries, meanwhile *Lactobacillus* play a role in development and subsequent process of caries, *Lactobacillus* the dominant one. Dental caries in the oral cavity is caused by *Lactobacillus Acidophilus*.[4]

Lactobacillus Acidophilus are which are gram positive bacteria, facultative anaerobes, no spores. Generally *Lactobacillus Acidophilus* cells that are in the form of a chain with smooth, round surface with flat edges can ferment carbohydrates and produce acid so that the pH in the oral cavity decreases and demineralization process occurs in the teeth. These bacteria have the ability to grow in an acidic environment and metabolizes sugars from food withaquick process so that it becomes an organic acid, namely lactic acid.

Lactobacillus Acidophilus believed to be a precursor bacteria in advanced caries because these bacteria are more isolated in deeper caries compared to the development of caries and the onset of tooth decay.[5]

Various methods have been used to reduce the incidence of caries carried out such as plaque control, diet, flour use and the use of mouthwash to prevent caries chemically. Existing mouthwash products on the market currently has a high alcohol composition and tends to has a high level of acidity so it can cause various negative effects for users. Various negative effects such as dehydration on mucosal tissue, burning sensation and dry mouth.[6]

Nowadays there are more and more alternative treatments using ingredients naturally as an antimicrobial, because this natural ingredient has side effects low, less toxic and has higher biodegradability if compared to conventional drugs.[7] One of the natural ingredients is a herbal plant that is

widely used in Indonesia which is the Citrus fruit that can cure or prevent diseases (*Citrus Aurantifolia*). Lime is a fruit that is rich in benefit. Lime leaves, fruit and peel have benefits as antibacterial because it contains essential oils, namely flavonoid compounds which can inhibit bacterial growth. Lime peel contains active ingredients such as tannins, saponins, flavonoids and triterpenoids are believed to be able to provide an antibacterial effect.[8]

Research testing the antibacterial activity of lime peel extract (*Citrus Aurantifolia*) with concentrations of 12.5%, 25%, 50% and 100% reported that the results of the antibacterial activity test of lime peel extract have inhibitory power against bacteria *streptococcus mutans.* The most effective effect occurs in concentration 50%. Lime peel extract (*Citrus Aurantifolia*) which has the most effective effect is at the concentration of 100%.[9]

Research on the effect of lime peel extract with (*Citrus Aurantifolia*) 10% concentration on enzyme activity glucosyltransferase *streptococcus mutans* reported test results of the GTF enzyme in lime peel extract is able to inhibit enzyme activity GTF significantly. So the lime peel in the study proven to inhibit the activity of the GTF enzyme *streptococcus mutans* because it contains polyphenolic compounds, especially flavonoids.[10]

METHODS

The research method that is used in this research is laboratory experimental. The research design used is Posttest Only-control Design.[11] The sample subjects that is used in this study were lime peel extract (*Citrus Aurantifolia*) and *Lactobacillus Acidophilus* ATCC (American Type Culture Collection) 4356. In this experiment the concentration of the ingredients is divided into 10 groups, namely 8 extract treatment groups and 2 control groups.:

1. Group I : 100% of lime peel extract (*Citrus Aurantifolia)*
2. Group II : 50% of lime peel extract (*Citrus Aurantifolia)*
3. Group III : 25% of lime peel extract (*Citrus Aurantifolia)*
4. Group IV : 12,5% of lime peel extract (*Citrus Aurantifolia)*
5. Group V : 6,25% of lime peel extract (*Citrus Aurantifolia)*

6. Group VI : 3,125% of lime peel extract (*Citrus Aurantifolia)*
7. Group VII : 1,56% of lime peel extract (*Citrus Aurantifolia)*
8. Group VIII : 0,78% 0f lime peel extract (*Citrus Aurantifolia)*
9. Group IX : positive control (*Chlorhexidine 0,2%*)
10. Group X : negative control (sterile distilled water)

Based on the calculation above, it takes as many as 3 samples in each treatment, so the total number of samples required is 30 samples.

Making the lime peel extract (Citrus Aurantifolia)

Preparation of 5 kg of lime peel extract, cleaned, then the skin of the lime is peeled and separated from the dried flesh. The dried lime peel is mashed with a blender. Next, lime peel powder as much as 16 mg was added with 96% ethanol solvent and thus obtained lime peel extract (Citrus Aurantifolia). Fruit peel extractlime (Citrus Aurantifolia) used in this research are concentration 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%.

Concentration The purpose of the solution used is to determine the minimum inhibitory concentration and minimum kill concentration. Lime peel extract is obtained by using the maceration method, namely by soaking the skin simplicia powder lime in 96% ethanol.[12] The finished product will then be tested phytochemically to find out Ingredients in lime peel extract (*Citrus Aurantifolia)*.

Preparation of Bacterial Suspension Lactobacillus Acidophilus

Making Bacterial Suspensions *Lactobacillus Acidophilus* ATCC 4356 by taking cultured bacteria using a loop needle and inoculated into MRS-B as much as 2 ml then the media incubated for 24 hours at 37°C in an anaerobic atmosphere. Furthermore, suspension results were adjusted to Mc Farland turbidity 0.5 (content 1.5x108cells/ml). In this study, the antibacterial effectiveness test was determined using method *broth macrodilution.*

1. Sterilization of tools

Sterilize glassware and media in an autoclave at 121°C for 15 minutes and

sterilization of the tube wire and tweezers is carried out by burning directly over the fire.

2. Making the medium
   a. *Brain Heart Infusion Broth*

      Medium creation *Brain Heart Infusion Broth* performed with Dissolve the medium in distilled water then heat it until the medium boils and is completely mixed. Medium then sterilized in an autoclave at a temperature of 121°C and an air pressure of 1-2 atmfor ±15 minutes. Before being put into the test tube, the medium cooled at 40-45°C.

   b. *Trypticase Soy Agar*

      Media powder *Trypticase Soy Agar* put into the tube *Erlenmeyer*then dissolved with 1 L of distilled water and heated until boiling. The mouth of the tube was plugged with sterile cotton and covered with The paper is then sterilized using an autoclave at 121°Cfor ±15 minutes. After sterilizing the media *Trypticase Soy Agar* Cool for 45 minutes then pour into a Petri dish 15 ml each and left until it solidifies.

Research Protocol

Test the inhibitory power of lime peel extract (*Citrus Aurantifolia)*to Bacteria *Lactobacillus Acidophilus* carried out using the dilution method.

a. Bacterial suspension *Lactobacillus Acidophilus* which has been adjusted to the levelthe turbidity is removed with a sterile cotton swab, remove excess solution or The suspension is taken by pressing the cotton swab against itinner wall of the tube.
b. Wipe cotton wool containing the bacterial suspension on the surface of the media *Trypticase Soy Agar*, then cover the petri dish and let it sit for a while 3-5 minutes.
c. A solution of lime peel is made with concentration of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%. A 0.78% solution means that The solution consists of 0.078 ml of lime peel extract and 9.92 ml distilled water,

1.56% solution consisting of 0.156 ml of lime peel extract and 9.84 ml distilled water, 3.125% solution consisting of 0.312 ml orange peel extract thin and 9.68 ml distilled water, 6.25% solution consisting of 0.625 ml skin extractlime fruit and 9.375 ml of distilled water, 12.5% solution means that The solution consists of 1.25 ml of lime peel extract and 8.75 ml distilled water, 50% solution consisting of 5 ml of lime peel extract and 5 ml distilled water, 50% solution consisting of 5 ml of lime peel extract and 5 ml distilled water, as well as 100% solution means that the solution consists of 10ml lime peel extract and 0 ml distilled water. Fruit peel concentration 100% lime obtained from pure extract.

d. To obtain the Tube Incubation concentration, all tubes are incubated at 37°C for 24 hours anaerobically.
e. Observations were made on the clarity of all treatment tubes by comparing the negative control tube and the control tube positive. Then plant 0.05 ml in the media *Trypticase Soy Agar* with technique *spreading* and incubation for 2 x 24 hours an*aerobic*.
f. The resulting bacterial growth was observed *spreading*. Determination of MIC and KBM is determined by counting the number of colonies bacteria growing on the media *Trypticase Soy Agar*stated in *Colony Forming Units* (CFU) and compared with negative controls and positive control.
g. Interpretation of the bacteriostatic and bactericidal activity of the test extract by means compared the decrease in bacterial colonies exposed to antibacterial compounds of the test extract relative to the initial number of bacterial colonies.

RESULT

The samples in this study were bacterial colony preparations of *Lactobacillus Acidophilus* ATCC 4356.

Phytochemical Test Results of Lime Peel Extract (Citrus Aurantifolia)

Citrus aurantifolia lime peel extract (16 milligrams) was dissolved in 10 milliliters of 96% ethanol. After being left for two days and agitated every twelve

hours, the mixture was filtered. The acquired liquid extract was evaporated using a rotary evaporator until the alcohol content evaporated, producing a thick extract that could be utilized right away and kept refrigerated.

Table 1. Phytochemical test results of lime peel extract (*Citrus Aurantifolia*)

No.	Active Ingredients	Mark (%)
1.	Flavonoids	7.05%
2.	Tannin	1.05%
3.	Essential oil	0.51%
4.	Saponins	5.82%
5.	Triterpenoids	2.41%

Table 1. shows that in lime peel (*Citrus Aurantifolia*) contains active ingredients, namely 7.05% flavonoids, tannins 1.05%, essential oil 0.51%, saponin 5.82% and triterpenoids 2.41%.

Inhibitory and Killing Power Test Results Lactobacillus Acidophilus

In this study the samples used were bacteria *Lactobacillu Acidophilus* ATCC 4356 and tested on 8 extract treatment groups lime peel (*Citrus Aurantifolia*) with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78% and 2 control groups consisting of a positive control group using *Chlorhexidine 0.2%,* the negative control group used sterile distilled water.

Table 2. Inhibitory and killing power values of lime peel extract (n=3)

Pengulangan	K (+)	K (-)	100 %	50 %	25 %	12,5 %	6,25 %	3,125 %	1,56 %	0,78 %
1	-	161	-	-	-	-	-	-	10	29
2	-	154	-	-	-	-	-	-	11	39
3	-	167	-	-	-	-	-	-	13	31

Note: Value in Cfu/ml

The results of measuring the number of *Lactobacillus Acidophilus* colonies in repeats 1, 2, and 3 in Table 2 show that there are counted bacterial colonies as much as 10 Cfu/ml, 11 Cfu/ml, and 13 Cfu/ml present in the lime peel extract (*Citrus Aurantifolia*) at a concentration of 1.56%. At a concentration of 0.78%, colonies with 29 CFU/ml, 39 CFU/ml, and 31 CFU/ml were produced for repetitions 1, 2, and 3. *Lactobacillus Acidophilus* is a living

organism. adverse control cohort Three different counts of *Lactobacillus Acidophilus* colonies were observed using sterile distilled water: 161, 154, and 167 CFU/ml.

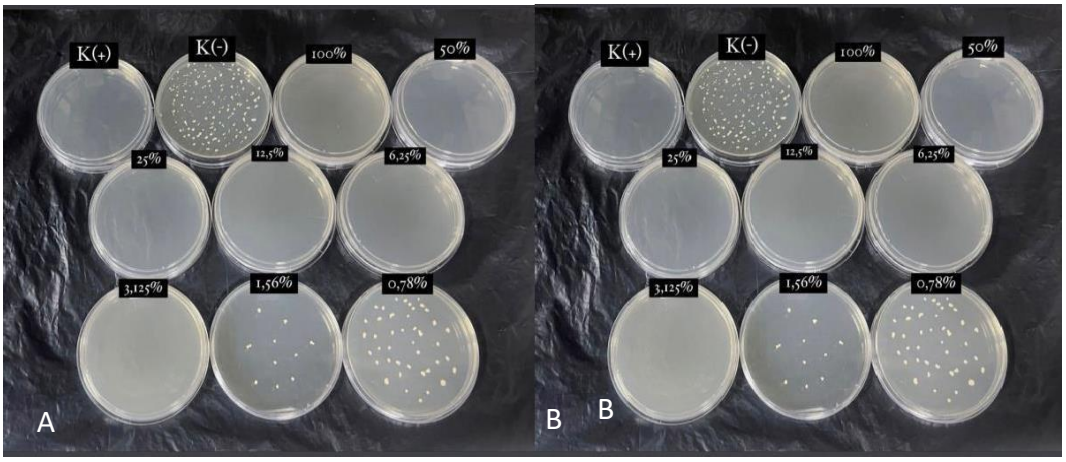

Figure 1.A. Repetition 1.B. Repetition of 2 test results *Colony Count* bacteria *Lactobacillus Acidophilus* on the concentration of lime peel extract (*Citrus Aurantifolia*) 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78% as well as negative control and positive control.

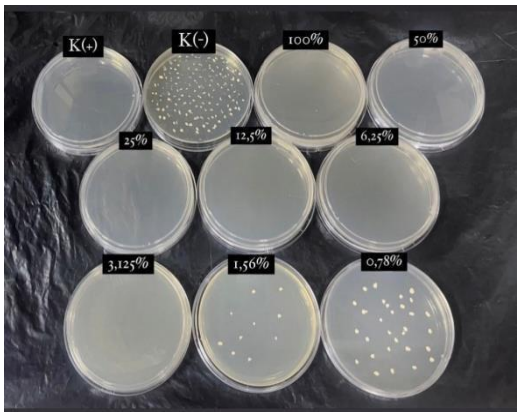

Figure 1.C. Repetition of 3 test results *Colony Count* bacteria *Lactobacillus Acidophilus* on the concentration of lime peel extract (*Citrus Aurantifolia*) 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78% as well as negative control and positive control.

In contrast, the positive control group demonstrated that either every colony of *Lactobacillus Acidophilus* had been enumerated or that none had been The results of the resistance and power tests utilizing the dilution method with colony count on the media Muller Hilton Broth for lime fruit peel extract (*Citrus Aurantifolia*) at 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and

0.78% are displayed in Figures 1. A, B, and C. Based on test findings that were repeated three times for each sample, the concentration of *citrus aurantifolia* (lime peel extract) was found to be 0.78% and 1.56%, respectively, and the presence of *lactobacillus acidophilus* bacteria was still detectable in the negative control, which was distilled water.

Repeating concentrations 3.125%, 6.25%, 12.5%, 25%, 50%, 100%, and using chorhexidine 0.2% as a positive control indicates no development of *Lactobacillus Acidophilus* bacteria on three separate occasions. Consequently, the ultimate outcome is attained The kill level minimum (KBM) is 3.125%, and the minimum inhibitory level (MIC) is 1.56%.

Descriptive Analysis

The table presents the findings of a descriptive study that was conducted to determine the average resistance and power kill values for each treatment group, as well as whether the data was distributed normally. The data is normally distributed if the table's results indicate that the standard deviation is less than the average. The maximum average bacterial growth, measured in Table 3, was 160.67 CFU/ml for *Lactobacillus Acidophilus* in the negative control group (sterile distilled water). The lowest average bacterial growth was seen at an extract concentration of 1.56%, or 11.33 CFU/ml. The data is regularly distributed in the negative control group when the standard deviation < mean, or 6,506 Cfu/ml < 160.67 Cfu/ml, is less than the mean.

The data is regularly distributed at a concentration of 1.56% Std. Deviation < average, or 1,528 Cfu/ml < 11.33 Cfu/ml.The data is regularly distributed at a concentration of 0.78% Std. Deviation < average, or 5,292 Cfu/ml < 33.00 Cfu/ml. Overall, the data were determined to be normally distributed by descriptive analysis.

Table 3. Descriptive analysis of the inhibitory and killing power of lime peel

K(-)	3	154	167	160,67	6.506
1,56%	3	10	13	11,33	1.528
0,78%	3	29	39	33,00	5.292

Note: Value in Cfu/ml

Normality test Shapiro-Wilk

The purpose of this is to ascertain whether or not the data is regularly distributed. This study used the Shapiro-Wilk normality test on a sample of 30 people (10 groups multiplied by 3) because it was smaller than 50. Use additional tests if the sample size is 50 or greater. Lillie fors and Smirnov Kolmogorov. If the value p>0.05, the Shapiro-Wilk is normality test indicated that the data was normally distributed. Table 4 is normality examination of the control group's negative data distribution is deemed to be normally distributed, as indicated by the graph's tes tρ=0.915 > 0.05 value. The number on the test graph, ρ = 0.637 > 0.05, indicates that the concentration is 1.56% and is said to be regularly distributed.

Table 4. Normality test results (*Shapiro-Wilk*)

Control Group	Df	*ρ* (Sig.)
K (-)	3	0,915
1,56%	3	0,637
0,78%	3	0,363

Homogeneity test

Test Levene performs the homogeneity test. Test to evaluate The sample data group is drawn from a homogeneous (i.e., similar variance) population. Testing-related data is considered homogeneous based on its significant value (p). If the test Shapiro-Wilk indicates that the data is normally distributed, then the data is homogenous (marked p>0.05); if the test p<0.05 indicates that the data is not homogeneous. Table 5. p=0.001 < 0.05 in the results, indicating that the data is not homogeneous.

Table 5. Homogeneity test results with the levene test

Test of Homogeneity of Variances			
Levene Statistic	Df1	Df2	Sig.(*ρ)*
5.403	9	20	0,001

Kruskall-Wallis test

The results of the homogeneity test indicate that the data are not homogeneous, and the non-parametric Kruskall-Wallis tests come next. In the event that the value of ρ is less than 0.05, a significant difference exists among the ten treatment groups; otherwise, there is no significant difference between the ten groups' treatments. Table 6's Kruskall-Wallis test findings indicate a value of p=0.001 < 0.05, indicating that there is a difference between each group. Owing to the variations amongst every group, the Mann-Whitney test was subsequently conducted. to ascertain with certainty which groups differ noticeably.

Table 6. Results of Kruskall-Wallis test

Testof *Kruskall Wallis*		
Kruskall-Wallis	Df	Sig.(*ρ)*
28,882	9	0,001

Mann-Whitney test

Utilize non-parametric statistical test data analysis to determine post hoc from the Kruskall-Wallis test as well.Whitney-Mann.If the significance value (ρ) is greater than 0.05, it means that there is a significant difference in concentration; if it is less than 0.05, there is no significant difference in concentration. Mann-Whitney test findings on Appendix 5 indicate that there is a significant difference (ρ=1) between the positive control group and concentrations of 100%, 50%, 25%, 12.5%, 6.25%, and 3.125%. At 1.56% concentration, mark ρ =0.02 is shown, while at 0.78% concentration, value ρ = 0 indicates that there was no significant change observed. According to the test results, the minimum kill concentration (KBM) of lime peel extract against the bacteria *Lactobacillus Acidophilus* is 3.125%, whereas the minimum inhibitory concentration (MIC) is 1.56%.

DISCUSSION

Based on the research findings, an average value of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100% is found in the treatment of lime peel extract (Citrus Aurantifolia) concentration, and the positive control group, which underwent testing for the bacteria *Lactobacillus Acidophilus*, indicates the absence of its colonies counted on the media. *Lactobacillus Acidophilus* bacteria can be killed at concentrations as high as 100% extract concentration, while the lowest concentration that can do so is 3.125% extract concentration.

Based on research, it has been determined that the presence of flavonoid components, tannins, and saponins in lime peel extract (*Citrus aurantifolia*) at a 100% concentration is most effective in eliminating the bacteria *streptococcus mutans*. The flavonoid content is an antibacterial substance that damages the bacterial membrane by binding, forming complexes with extracellular and soluble proteins, and forming complexes with bacterial cell walls. It also possesses lipophilic qualities.[9,12] *Citrus aurantifolia*, or fruit skin lime, has been found in other studies to possess antibacterial action. Clear zones are formed at concentrations of 5%, 10%, 15%, 20%, and 30% of *Lactobacillus acidophilus* bacteria. Therefore, lime (*Citrus Aurantifolia*) concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100% were found to be effective in eliminating the *Lactobacillus Acidophilus* bacteria in this study when fruit peel extract was used.[10.]

In studies on citrus aurantifolia (lime peel extract), concentrations of 1.56% were found to yield as many as 10, 11, and 13 CFU/ml colonies that were still alive after repetitions 1, 2, and 3. These findings demonstrate that the bacterial development of *Lactobacillus Acidophilus* can be inhibited by a concentration of 1.56% lime peel extract (*Citrus Aurantifolia*). Lime fruit peel extract (*Citrus aurantifolia*) at 0.78% concentration suggests the existence Each repetition had viable colonies with 29, 39, and 31 CFU/ml.

According to the study's findings, the *Lactobacillus Acidophilus* bacteria could be inhibited by extracts at concentrations of 1.56% and 0.78%, although this was ineffective because some bacteria remained. backed by the study hypothesis of *streptococcus mutans* bacteria at concentrations of 100%, 50%, 25%, 12.5%, and 6.25% in lime peel extract (*Citrus Aurantifolia*). According

to the results, bacterial growth is fruitful at concentrations of 12.5% and 6.25%. The concentration of the extract determines the amount of active chemicals that contribute to its antibacterial properties, and vice versa. Reduced concentration of the extract will result in a decreased level of active component content, which will lower the extract's antibacterial activity.[13,14].

This study uses 0.2% chlorhexidine as a positive control, and the fact that the positive control group performed well suggests that the existence of bacteria that are regarded as living in the media is a positive control. Because of the interaction between positive charges and chlorhexidine molecules with bacteria's cell walls, at normal pH, chlorhexidine can bind bacteria on the oral cavity's surface, causing the bacteria to penetrate into the cytoplasm and ultimately kill the germs. The mechanism by which chlorhexidine works is the presence of bonds or contacts between the positive charge of the molecule and the negative charge of the phosphate particles in the bacterial walls. This interaction allows the molecule to enter the bacterial body and produce harmful effects.[3]

The control group was negative in this study that uses sterile *Aquadest*, sterile *Aquadest* is used as a solvent and has no effect as an antibacterial so that it does not affect the results of the antibacterial test, the results of the control test is negative in this study that did not show any change. This proves that Sterile *Aquadest* does not affect bacterial growth *Lactobacillus Acidophilus*.[15]

Concentration of lime peel extract (*Citrus Aurantifolia*) 100% is a pure extract from lime peel without additional solvents, which means the natural chemical content in that concentration is very high. The chemical content contained in lime peel extract based on phytochemical analysis tests, namely flavonoids, tannins, essential oils, saponins and triterpenoids. High content of flavonoids, tannins, essential oils and saponins work by changing the composition of the amino acid chain in the DNA, so that the DNA of the bacteria will be damaged. By damaging the DNA, the cell's nucleus will encourage lysis of the cell's nucleus. Thus the bacteria will become inactive.[16]

CONCLUSION

Based on this research testing the inhibitory and killing power of lime peel extract (*Citrus Aurantifolia*) against bacteria *Lactobacilus Acidophilus*on children's dental caries can be concluded *that in the concentration of lime peel extract (Citrus Aurantifolia*) 1.56% was determined as the inhibitory concentration minimum (MIC) in inhibiting bacterial growth *Lactobacillus Acidophilus*. In the treatment the extract concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100% indicates that there are no living colonies in the media or concentration has killed the bacteria *Lactobacillus Acidophilus* over all. Therefore the concentration of 3.125% was determined as minimum kill concentration (KBM). Based on these results, extract lime peel (*Citrus Aurantifolia*) is stated to have inhibitory power and killing power against bacteria *Lactobacilus Acidophilus* in children's dental caries.

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