

# Inhibition Test of Lemon Juice (*Citrus aurantifolia*, Swingle) on the Growth of *Corynebacterium diphtheriae*

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Infectious diseases caused by bacteria are still a health problem in developing countries. One of them is the transmission of *Corynebacterium diphtheriae* which is the cause of the emergence of diphtheria. Countermeasures that are usually done on patients are using chemical antibiotic treatment. However, the use of chemical antibiotics can cause side effects for patients. Other treatment methods that can be applied are using natural ingredients, one of which is a lemon. The content of antibacterial compounds found in lime can inhibit the growth of *Corynebacterium diphtheriae* bacteria. The concentration of lime juice tested in this study were 60%, 70%, 80%, and 90%. Based on the results of the study showed that there is a zone of inhibition in the test of lime juice on the growth of *Corynebacterium diphtheriae* at a concentration of 70%, 80%, 90%, while at a concentration of 60% there is no inhibitory zone. Based on the test results, lime juice can be used to inhibit the growth of *Corynebacterium diphtheriae*.

**Key words:** *Lime juice, inhibitory power, Corynebacterium diphtheriae*

## Introduction

Diphtheria has become a health problem that must be considered as a remedy. Diphtheria is an acute bacterial disease that attacks the tonsils, pharynx, larynx, nose, and sometimes attacks the mucous membranes or skin and sometimes the conjunctiva or vagina (Chin, 2000; Deterding, 2007). This disease is a disease that has long emerged and is still endemic in developing countries, one of which is Indonesia. Indonesia is the second highest country with endemic cases of diphtheria, even increasing every year (Ministry of Health, 2013; Kartono, 2008). The spread of diphtheria is increasingly widespread in Indonesia since 2005, even the government has set the status of an extraordinary event (KLB) for its spread has claimed many lives (East Java Health Office, 2013; Ministry of Health, 2013).

The government has tried to tackle the spread of the disease. One of them is by treating patients. Currently, the use of synthetic drugs (chemistry) is widely used and developed to

overcome various diseases. However, treatment using drugs made from chemicals has side effects for body health, some of which can cause bacterial resistance to antibiotics and the emergence of hypersensitive symptoms. In addition, the costs involved in chemical treatment are quite expensive. To minimize this, currently treatments using natural ingredients have been developed and researched by scientists using plants and animals (Indrati, 2012). One of the plants that can be used for treatment is lime fruit.

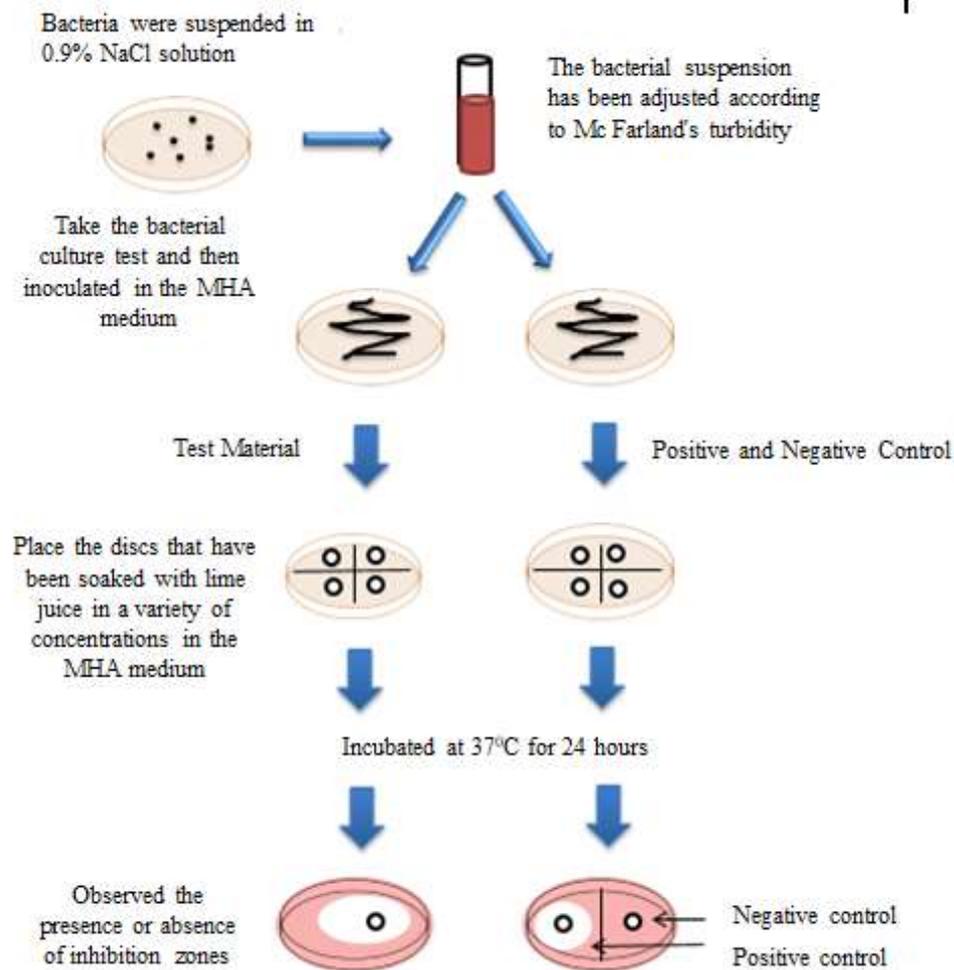
In Indonesia, lime (*Citrus aurantifolia*) is a plant that is not difficult to find because of its very wide spread and trees can grow in almost all types of soil (Sarwono, 2001). Lime has a variety of useful chemical compounds such as citric acid, amino acids (tryptophan and lysine), essential oils (citral, limonene, camphor lemon, geranyl acetate, linalyl acetate, felandren, cadinen, actildehyd, nonildehyd), glycosides, fats, resin, citric acid, calcium, phosphorus, iron, sulfur vitamins B1 and C, vitamin A, saponins and flavonoids (hisperidin, naringin, tangeretin, eriocotrin and eriocitrocid) (Adindaputri, et al., 2013; Dalimartha, 2000; Hariana, 2006). Of all the contents of the above compounds, compounds that play an important role as an antibacteri namely, flavonoids, citric acid, limonene (Hilmi, 2016). Research conducted by Razak et al (2013) has proven that lime juice (*Citrus aurantifolia*, Swingle) has antibacterial activity against *Sthapylococcus aureus* with various concentrations of 25%, 50%, 75%, and 100%. In addition, lime juice also has antibacterial activity that can inhibit the growth of *Enterococcus faecalis* bacteria (Yahya, 2016). Christina's research results (2017) also showed that lime juice water (*Citrus aurantifolia*, Swingle) can inhibit the growth of *Stapylococcus epidermidis* bacteria with concentrations of 25%, 50%, 75%, and 100% and there is an influence of contact duration on the growth of bacteria where bacteria are not grow after contact for the first 5 minutes and followed by the following minutes with a squeeze of 100% lime juice. So, the higher the concentration of lime juice and the longer contact with the bacterium *Sthapylococcus epidermidis*, the better the inhibition. From the results of these studies, it can be seen the potential of lime fruit as an antibacterial is very large.

## Research Method

This research is an experimental research, namely research that deliberately gives treatment to the object of research with the aim of studying its effects (Kasjono and Yasril, 2009). In this case, the treatment is carried out to directly observe the antibacterial activity of lime juice and Minimum Inhibitory Concentration (MIC) of the *Corynebacterium diphtheriae* bacteria. The complete design of the research work can be seen in Figure 1. The sample used is a suspension of *Corynebacterium diphtheriae* with 106 CFU / mL bacteria and lime fruit (*Citrus aurantifolia*, Swingle) 1 kg. In general, the steps taken in this study can be seen in Figure 1.

### *Sterilization Equipment*

Sterilization of equipment is needed to avoid contamination. The equipment to be sterilized is washed thoroughly and dried. The steriliation process is carried out using an oven for dry sterilization at 160°C - 180°C, and an autoclave for wet sterilization at 121°C.



**Picture 1** Working Scheme of Inhibition Test for Lemon Juice Against the Growth of *Corynebacterium Diphtheriae* Bacteria

#### *Making lime juice (Citrus aurantifolia, Swingle)*

Making lime juice is carried out in laminar air flow to reduce the risk of contamination. Lime fruit (*Citrus aurantifolia*, Swingle) of 2 kilograms was washed clean using sterile aquades. Lime is cut into 4 parts, each piece of lime squeezed. The lime juice is collected in a beaker and separated into Erlenmeyer while filtered using a sterile filter cloth 1 time. The resulting juice is the juice of lime juice with a concentration of 100%. The juice is a mother liquor prepared to make lime juice with a concentration of 25%, 50%, 75%, 100%. How to make the solution can be seen in Table 1.

#### *Making Mueller Hinton Broth (MHB) media*

As many as 38 grams of Mueller Hinton Broth were dissolved in 1000 ml of distilled water and heated while stirring until dissolved. The finished solution is then sterilized in an autoclave for 15 minutes at 121°C and cooled to 45-50°C. The final stage is pouring into a sterile petri dish and stored in the temperature 2-8 °C (Kambang, et al., 2015).

**Table 1** Variation of Lime Juice Concentration Variation

Concentration Variation	Volume of lime juice (mL)	Volume of sterile distilled water (mL)
10%	1,0	9,0
20%	2,0	8,0
30%	3,0	7,0
40%	4,0	6,0
50%	5,0	5,0
60%	6,0	4,0
70%	7,0	3,0
80%	8,0	2,0
90%	9,0	1,0
100%	10,0	0,0

#### *Making media Tullurite Blood Agar*

A total of 6.3 grams of tullurite so that the base is dissolved in 200 mL of sterile aquades while homogenized and heated. The solution was then sterilized using an autoclave for 15 minutes at 121°C and cooled to 45-50°C. After that, add 10 ml of blood and 2 ml of potassium tullurite and stir until homogeneous. The final stage is pouring it into the petri dish aseptically and stored at 2-80 C (Kambang, et al., 2015).

#### *Making positive and negative controls*

Making positive control is done by weighing 1 gram of erythromycin and dissolving it in 1 ml of distilled water. After homogeneous, pipette as much as 20 µl and put into paper discs and then placed on Muller Hinton Agar (MHA) medium and incubated for 24 hours at 37°C.

For negative control, pipette as much as 20 µl of distilled water is then put into paper discs and affixed to the Muller Hinton Agar (MHA) medium. The incubation process is carried out for 24 hours at a temperature of 37°C.

#### *Making McFarland Turbidity Standard 0.5%*

9.95 mL of 0.36 N H<sub>2</sub>SO<sub>4</sub> solution or 1% sulfuric acid was mixed with 0.5 mL of Barium chloride BaCl<sub>2</sub>.2H<sub>2</sub>O 1.175% solution in Erlenmeyer. The solution is homogenized to form a turbid solution. This turbidity is used as a standard for bacterial suspension turbidity.

#### *Making a bacterial suspension*

Hadioetomo (1990) explains that suspension can be carried out by taking one end of a bacterial colony from the subculture media, then suspending it into a tube containing 0.9% NaCl until the turbidity is the same as McFarland's standard. One way to compare it is to hold 2 tubes together, one standard tube and one bacterial suspension tube. Then look and compare the turbidity against the background of white paper with thick lines and colored markers. If it's less turbid, add the colony, whereas if it's more turbid, add 0.9% NaCl.

#### *Antibacterial Testing*

Antibacterial activity test was carried out using the paper disc diffusion method (Setiabudy, 2001). After the *Corynebacterium Diphtheriae* suspension is standardized to the

McFarland 0.5% standard, dip the swab into the suspension. Turn the swab several times pressed firmly inside the tube wall above the fluid level to remove the excess inoculum. After that, it is inoculated on the Muler Hinton Agar growth medium by scraping the entire surface vertically and around the edge of the slope to ensure equitable growth across the surface of the medium. Sterile paper discs (from filter paper) placed with a diameter of 0.5 cm were immersed in different concentrations of lime juice for 2 hours. Paper discs are taken aseptically with an enten needle or tweezers by applying them to the wall of the test tube and then settling for a while. This is done so that the remnants of the solution do not drip and spread on the surface of the agar. Sliced paper discs are taken using tweezers and placed on media containing test bacteria by gently pressing using tweezers so that they are really attached to the surface of the media that has been planted with test bacteria. The antibacterial test was incubated for 24 hours at room temperature. The detailed step of the antibacterial test can be seen in Figure 1.

#### *Barrier Zone Diameter Reading*

The reading is done using a dark colored paper mat or a slightly dark background (see Figure 1). Observations were carried out using the naked eye without loops by measuring the diameter of the resistance zone that occurs in the saucer so that MH uses a ruler, dial caliper or zone reader. The inhibition zone dimension measured is the clear area around the disc (no bacterial growth). Measurements are made from one end to the other through the center of the disc.

### **Results and Discussion**

In this study, the test material used was lime juice (*Citrus aurantifolia*, Swingle). Lime juice (*Citrus aurantifolia*, Swingle) has the ability to inhibit the bacteria *Corynebacterium diphtheriae*. The antibacterial activity of lime juice occurs due to the presence of antibacterial compounds such as citric acid, flavonoids and limonene. Lime fruit has the highest content of citric acid (Kurnia, 2004). After 24-hour incubation, there is the result of the formation of inhibitory zones around the disk which contains lime juice. The inhibition zone is then measured using a calipers. The results obtained are found inhibition zones around the disk at a concentration of 70%, 80%, 90%, and 100% with an average diameter of inhibition zones respectively of 8.2 mm, 9.8 mm, 12.9 mm, 18.9 mm, and at a concentration of 60% there is no inhibitory zone (see Table 2). In this study, the antibiotic used as a positive control was erythromycin which is a reference for the antibacterial activity of lime juice. Erythromycin was chosen because it works to inhibit bacterial protein synthesis by interfering with translocation and formation of *Corynebacterium diphtheriae* (Jawetz, 2007) and at 0% concentration there is no inhibitory zone because aquadest has no active substances that can inhibit the growth of *Corynebacterium diphtheriae* bacteria. Treatment with a concentration of 0% is used as a negative control to ensure that the aquadest used as diluent of lime juice concentration does not contain anti-microbial substances.

**Table 2** Research Results of Lemon Juice (*Citrus aurantifolia*, Swingle) on the Growth of *Corynebacterium diphtheriae* Bacteria

Concentration test (%)	Obstacles zone (mm)		
	Repetition 1	Repetition 2	Average (mm)
60	0	0	0
70	8,8	7,6	8,2
80	9,3	10,2	9,8
90	12,7	13,1	12,9
100 (KP)	19,2	18,5	18,9
Aquades (KN)	0	0	0

Information :

Positive Control (KP)

Negative Control (KN)

The difference in inhibition zones formed is thought to be the presence of compounds in lime juice (*Citrus aurantifolia*, Swingle) which are antibacterial such as citric acid, flavonoids and limonene, lime fruit has the highest content of citric acid (Kurnia, 2004). The content of citric acid in lime juice causes lime juice to have a low pH. Lemon juice 100% has a pH of 2.3 (Razak, 2013). At that pH *Corynebacterium diphtheriae* cannot grow because *Corynebacterium diphtheriae* cannot grow at acidic pH (Setiabudy, 2001). The low acidity of lime juice (*Citrus aurantifolia*, Swingle) will change the pH of bacterial cells. Changes in the pH of these bacterial cells will inhibit the process of sending amino acids from RNA thereby inhibiting bacterial growth (Irianto, 2014). Limonene can destroy the cytoplasmic membrane and control the internal composition of cells and will cause bacterial cell death.

The content of citric acid, flavonoids, and limonene contained in lime juice (*Citrus aurantifolia*, Swingle) works synergistically in inhibiting the growth of *Corynebacterium diphtheriae* bacteria, so based on the results of this study prove that the juice of lime juice (*Citrus aurantifolia*, Swingle) works synergistically in inhibiting the growth of *Corynebacterium diphtheriae* bacteria, so based on the results of this study proves that the juice of lime juice (*Citrus aurantifolia*, Swingle) growth of *Corynebacterium diphtheriae*. The higher the concentration of lime juice (*Citrus aurantifolia*, Swingle), the greater the diameter of the inhibition zone formed. Conversely, the lower the concentration of lime juice (*Citrus aurantifolia*, Swingle), the smaller the diameter of the inhibition zone formed.

## Conclusion

Based on research that has been done, it can be concluded that the juice of lime juice (*Citrus aurantifolia*, Swingle) has an antibacterial activity that can inhibit the growth of *Corynebacterium diphtheriae* bacteria. The minimum concentration that can inhibit bacterial growth is 70% with inhibition zones formed of (8.2 mm).

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