



Antibacterial potential of leaf and fruit of citrus extract (*Citrus aurantifolia*) against *klebsiella oxytoca*



Zuraidah , Widiyari, Wati Oviana

Universitas Islam Negeri Ar-Raniry, Indonesia

* Corresponding author: zuraidah23ibrahim@gmail.com

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ABSTRACT

Antimicrobial agents play a crucial role in decreasing the impact of infectious diseases worldwide. Many medicinal plants have been identified as rich sources of natural antimicrobial compounds that can be used as an alternative to traditional treatments for bacterial infections. Indonesia, particularly in the Aceh region, has a warm and humid climate that supports a diverse range of plants that have medicinal properties. One example is the lime (*Citrus aurantifolia*) which can be used for medicinal purposes. This study aimed to determine the inhibitory effects of leaves and fruit extracts of lime on the growth of *Klebsiella oxytoca*. The method used was an experimental method with a post-test only control group design. The results showed that both types of extracts contain flavonoid, alkaloid, and saponin compounds. However, triterpenoids were only found in the fruit extract, and steroids were only found in the leaf extract. The antimicrobial test of lime fruit and leaf extracts against *Klebsiella oxytoca* bacteria showed that both types of extracts were able to inhibit the growth of the test bacteria at all concentrations.

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INTRODUCTION

Klebsiella is a type of bacteria that is gram-negative, anaerobic, and rod-shaped. It is commonly found in the intestinal tract and is known to be an opportunistic pathogen, meaning it can cause infections in people with weakened immune systems (Echeverri & Catano, 2010). *Klebsiella* commonly lead to infections in the urinary tract, wounds and respiratory system (Neog, Phukan, Puzari, Sharma, & Chetia, 2021). They can easily spread resistance to antibiotics by exchanging plasmids with other bacteria, often causing outbreaks of infections acquired in

hospitals. *Klebsiella oxytoca*, previously known as *Bacterium oxytocum*, is typically found in the environment. It is often linked to infections in newborns that affect the blood, urinary tract, central nervous system, lungs, skin, and soft tissue (Minami, Okabe, Shiode, Hayashi, 1989). It is also known to be associated with people who have serious underlying health conditions. Additionally, *Klebsiella oxytoca* is known to produce toxins in the organs that it infects. Like other bacterial populations, *Klebsiella oxytoca* has also developed its resistance to a wide range of antibiotics (Yang et al., 2022). According to a study by Chakraborty et al., (2016), *Klebsiella oxytoca* demonstrated 100% resistance to ampicillin and resistance to other antibiotics such as amoxicillin, ceftriaxone, ciprofloxacin, cotrimoxazole, nalidixic acid, and tetracycline at 75%, 25%, 50%, 50%, 75%, and 50% respectively. The study found that gentamicin and ceftriaxone were the most effective antibiotics against the bacteria. Additionally, it reported that 50% of the isolates were resistant to multiple drugs.

Antimicrobial agents play a crucial role in decreasing the impact of infectious diseases worldwide. Many medicinal plants have been identified as rich sources of natural antimicrobial compounds that can be used as an alternative to traditional treatments for bacterial infections (Manandhar, Luitel, & Dahal, 2019). The World Health Organization (WHO) recognizes that medicinal plants are a valuable source for obtaining a wide range of drugs. Many plants have been used for their antimicrobial properties, which are due to the phytochemicals produced in the secondary metabolism of the plant.

Indonesia, particularly in the Aceh region, has a warm and humid climate that supports a diverse range of plants that have medicinal properties. One example is the lime (*Citrus aurantifolia*) which can be used for medicinal purposes (Mubarak & Soraya, 2018). Citrus fruits are a great source of important nutrients like vitamins, minerals, and dietary fibers that are necessary for the healthy growth and development of the body. Studies have shown that these fruits have various beneficial properties, such as anti-cancer properties, the ability to fight off microorganisms, antioxidant properties, the ability to prevent ulcers, anti-inflammatory properties, the ability to lower harmful lipids in the blood, the ability to fight off typhoid, and the ability to protect the liver (Enejoh et al., 2015; Fratianni, Cozzolino, & Feo, 2019).

In traditional medicine, *C. aurantifolia* is used for a variety of purposes, including repelling mosquitoes, treating worms and infections, relieving coughs and sore throats, stimulating digestion and appetite, reducing pain from arthritis and headaches, increasing urine production, tightening tissues, preventing scurvy, treating stomach issues and improving overall health (Tavallali, Bahmanzadegan, Rowshan, & Tavallali, 2021). Previous research has identified certain compounds in *C. aurantifolia*, such as terpenoids, coumarins, and flavonoids, that may contribute to these effects (Apraj, Thakur, Bhagwat, Mallya, & Sawant, 2011). Research has shown that extracts from the root of *C. aurantifolia* are effective in inhibiting the growth of various types of bacteria, including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Beta-haemolytic streptococci*, *Escherichia coli*, and *Neisseria gonorrhoeae*. Additionally, the fruit of the plant was found to inhibit the growth of anaerobic facultative bacteria (Rahman, Parvez, Islam, & Khan, 2011).

Many different types of plants have been found to have medicinal properties that can be used to treat a variety of illnesses. Studies have shown that various parts of these plants, such as flowers, leaves, roots, stem-bark, and fruits, possess therapeutic properties that can be used to treat different diseases. Historically, herbs have been used for medicinal purposes, particularly in rural areas of developing countries. Traditional medicine practitioners also use herbs to treat a range of illnesses. Some of the potential benefits of herbs have been confirmed by scientific research, while others require further study.

RESEARCH METHODS

Research Design

This research is a true experimental laboratory research with a post-test only controlled group design. The experimental method examines the effect of giving different concentrations of extra lime leaves and fruit on the growth of *Klebsiella oxytoca*. Antibacterial testing of lime leaf and fruit extracts using the disc diffusion method (Kirby-Bauer test), which is a method for testing antibacterials by placing a disk containing antibacterial compounds on the surface of media inoculated with test bacteria.

Population and Samples

The samples in this study were *Klebsiella oxytoca* B24 bacterial isolate obtained from Indonesia Culture Collection (InaCC) LIPI. *K. oxytoca* bacterial colonies were rejuvenated in NA media. The materials used to prepare the extracts were 300 g lime fruit and 300 g dried lime leaves.

Instruments

The tools used in this study were an autoclave, incubator, sample bottle, ose needle, test tube, test tube rack, vacuum distillation, laminar air flow, measuring cup, micropipette, aise, analytical balance, label paper, opaque paper, microscope. Bunsen lamp, drying oven, dark bottle, vacuum pump, round bottom flask, petri dish, rotary evaporator, glass bottle, watertight container, and tweezers.

Procedures

Extraction of lime leaves

Extraction was done by maceration method. Lime (*Citrus aurantifolia*) leaves were separated from the stalk and cleaned, dried without exposure to direct sunlight for 2 weeks, cut into small sizes, then weighed as much as 300 grams. The sample is put in a dark bottle, ethanol distillate is added until all samples are submerged, then stored in a place protected from light for 5 days while stirring frequently. After 5 days the leaves were separated by filtration which produced a macerate. This process was carried out for 3 times 5 days. All the macerates were combined and then evaporated the solvent by vacuum distillation and continued with a rotary evaporator to get a thick extract of lime leaves. The resulting sample extract of 30 ml.

Extraction of lime fruit

Lime fruits as much as 300 grams were washed thoroughly, cut crosswise with a width of approximately 0.5 cm. The fruit pieces were placed in a container and put into a drying oven at 60°C for 2 days until dry and discolored. The dried fruit was pulverized using a blender. Samples of lime fruit that have been mashed as much as 150 grams are then macerated twice using 98% methanol for 3 days and allowed to stand in an airtight container. Between soaking times the simplisia was stirred. The results of maceration were filtered with filter paper assisted by a vacuum pump. The residue of the first liquid extract was macerated again with 98% methanol for a day. Then filtered and the second liquid extract was obtained. Next, the first and second liquid extracts were combined. After that, the liquid extract was evaporated and produced a thick extract of 30 ml.

Phytochemical assay of lime leaf and fruit extracts

Flavonoid assay

The sample was weighed first and then extracted with methanol, filtered with cotton and transferred to another tube. Tests that use concentrated HCl reagent, the sample is added to concentrated HCl as much as 2 drops. Shake the extract vigorously then add Mg powder and shake vigorously once again. The sample is positive for flavonoids with concentrated HCl reagent if there is froth and the solution turns orange. Tests using 2N H₂SO₄ reagent, 2 drops of 2N H₂SO₄



are added to the sample and then shaken vigorously. The sample is positive for flavonoids when there is a very noticeable yellow, red or brown color change.

Alkaloid assay

The sample was weighed first and then extracted with ammoniacal chloroform. Filter with cotton and transfer to tubes A and B. In each tube A and B add Dragendorff reagent and Wagner reagent. The sample in tube A is positive for alkaloids if there is a reddish precipitate and in tube B there is a brownish precipitate.

Saponin assay

The sample was weighed first and then extracted with ammoniacal chloroform. Filter with cotton wool and transfer to another tube. Shake the sample vigorously and let stand for 2 minutes, then add 2 drops of 2N HCl. Shake vigorously and observe whether bubbles form after standing for 10 minutes. The sample is positive for saponins if there are bubbles with a lot of intensity and consistent for 10 minutes.

Triterpenoid and steroid assay

The sample was weighed first and then extracted with ethanol. Filter using cotton and then heat to dry. Extract again with chloroform and water (1:1). The chloroform extract is dripped on a drip plate as much as 2 drops and leave to dry. Add concentrated sulfuric acid as much as 1 drop and anhydrous acetic acid as much as 1 drop. Positive samples contain triterpenoids when experiencing red or brown color changes and positive containing steroids when experiencing blue, purple or green color changes.

Antibacterial activity assay of lime leaves and fruits

Antibacterial activity assay using paper discs with Mueller Hinton Agar (MHA) media. The positive control uses Meropenem, a Carbapenem class antibiotic and the negative control uses CMC, a food additive that functions as a thickener and is believed not to form a clear zone on bacterial growth. Antibacterial tests were carried out by preparing 6 disks each that had been dripped with lime leaf and fruit extracts with concentrations of 40%, 60%, 80%, 100%, negative control, and positive control which were placed on MHA media that had been inoculated with test pathogenic bacteria *Klebsiella oxytoca* InaCC B24, then incubated at 37°C for 17-24 hours. The clear zone formed around the disk was measured with a caliper and recorded.

Data Analysis

The data obtained after the assays were then analyzed for each zone of inhibition and categorized based on the strength of the inhibition produced. The following formula for the average zone of inhibition was used:

$$\frac{D_v + D_h}{2} - D_c$$

Description:

D_v = Vertical diameter

D_h = Horizontal diameter

D_c = Disc diameter

RESULTS

Extraction of lime leaves by maceration method produces macerate with a liquid texture. After being evaporated, it produces a concentrated green extract with a thick texture (Figure 1), and then the extract was subjected to phytochemical assay.



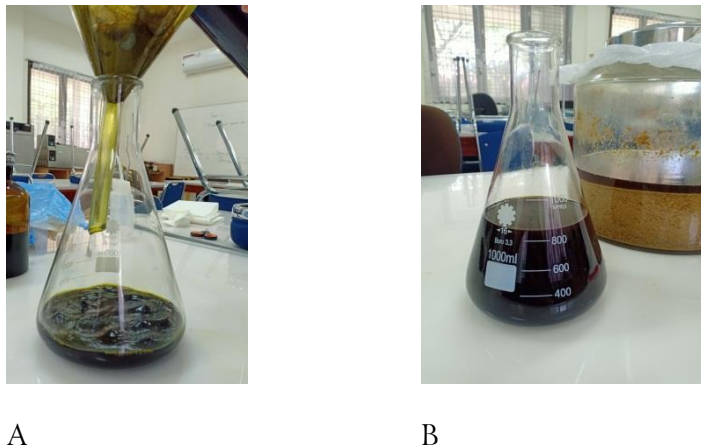


Figure I. (A) Lime leaf extract (B) Lime fruit extract

The results of the phytochemical test on the extracts of lime fruit and leaves showed that both types of extracts contain flavonoid, alkaloid, and saponin compounds (Table I). However, triterpenoids were only found in the fruit extract, and steroids were only found in the leaf extract.

Table I. Phytochemical assay results of lime leaf extract

Extract	Assay	Positive	Negative	Description
Lime leaf	Flavonoid	√		Red solution formed
	Alkaloid:			
	a. Dragendrof	√		Formed orange brown precipitate
	b. Mayer	√		Formed brownish red color
	c. Wagner	√		Formed reddish color
	Saponin	√		Bubbles formed
	Triterpenoid		√	No red solution formed
	Steroid	√		Green solution formed
Lime fruit	Flavonoid	√		Red solution formed
	Alkaloid:			
	a. Dragendrof	√		Formed orange brown precipitate
	b. Mayer	√		Formed brownish red color
	c. Wagner	√		Formed reddish color
	Saponin	√		Bubbles formed
	Triterpenoid	√		Red solution formed
	Steroid		√	No green solution formed

The antimicrobial test of lime fruit and leaf extracts against *K. oxytoca* bacteria showed that both types of extracts were able to inhibit the growth of the test bacteria at all concentrations (Figure 2). The inhibitory category was in the range of weak to moderate, while the positive control, meropenem, was still better at inhibiting with a strong inhibitory category (Table 2).

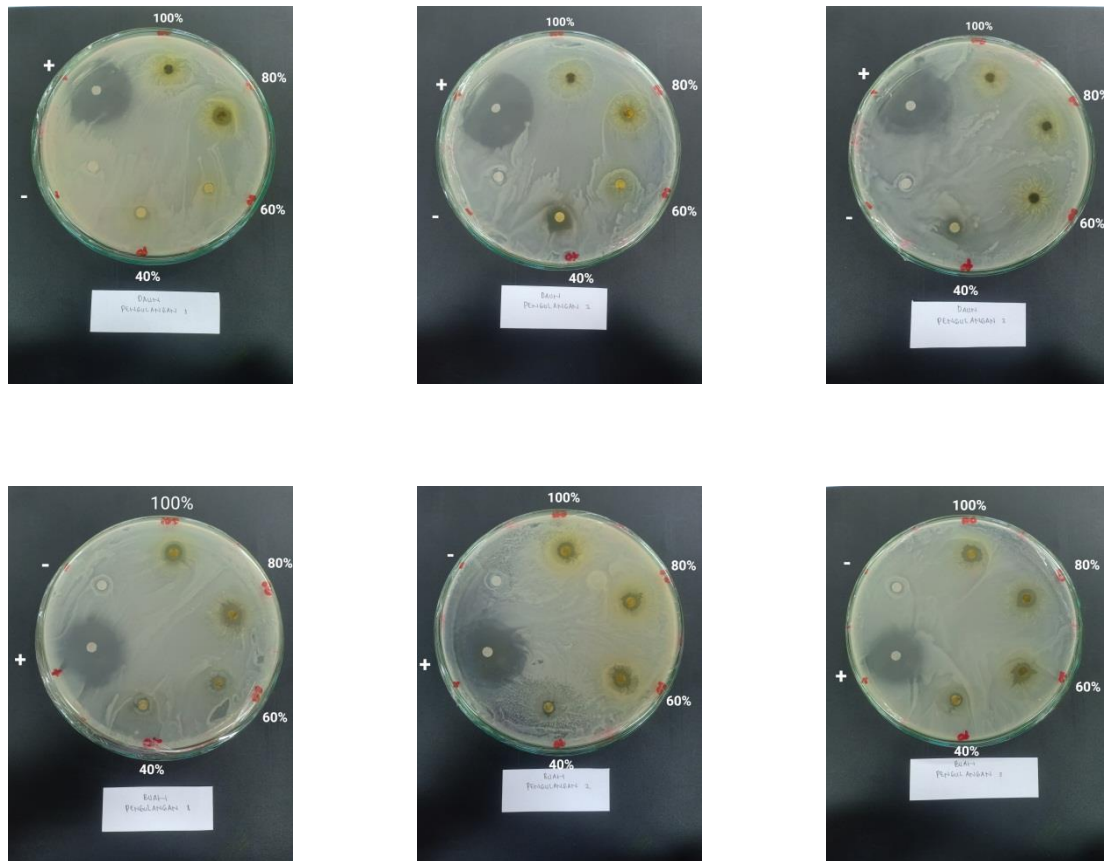


Figure 2. Clear zone of lime leaf extract (above) and lime fruit extract (below)

Table 2. Inhibition of lime fruit and leaf extract against the growth of *K. oxytoca*

No	Concentration (%)	Repetition (mm)			Average
		I	II	III	
Lime leaf	40	5.25	16.28	7.35	9.63
	60	1.27	3.57	3.85	2.89
	80	10.28	4.2	4.27	6.25
	100	0	2.85	3.85	2.23
	Meropenem (K +)	53.68	55.1	54.98	54.58
	CMC (K -)	0.00	0.00	0.00	0.00
Lime fruit	40	3.35	5.25	4.32	4.31
	60	5.77	10.23	12.7	9.56
	80	14.95	9.17	10.08	11.4
	100	9.22	9.07	10.18	9.49
	Meropenem (K.+)	49.28	43.33	41.08	44.56
	CMC (K. -)	0	0	0	0

DISCUSSION

Herbal medicines are becoming increasingly popular and in demand. The World Health Organization (WHO) encourages and recommends the use of traditional herbs or remedies in the healthcare sector as a large amount of raw material is readily available. Plants are complex in nature

and their therapeutic effects can vary based on species, location, and harvesting methods. Ensuring proper identification and purity of herbs is crucial, as improper authentication and contamination by microorganisms or pesticides can make standardization of herbal drugs necessary (Apraj et al., 2011). The WHO states that describing a medicinal plant using macroscopic and microscopic techniques is the initial step in establishing the identity and degree of purity of such materials and should be performed before any further testing is conducted.

This study explores the use of lime fruit and leaves (*Citrus aurantifolia*), phytochemical screening and antibacterial activity tests of ethanol extracts from the fruit and leaves were performed as an initial study on the utilization of lime as traditional medicine. The results of the phytochemical screening showed that the extracts contain flavonoid, alkaloid, saponin, triterpenoid, and steroid compounds. The variation in phytochemical composition of *C. aurantifolia* can be attributed to factors such as the chemical-physical properties of the plant, the composition of the soil it is grown in, and other factors such as exposure to sunlight and geographical location. This variation is reflected in the biological properties of *C. aurantifolia* extracts. Our findings suggest that the peel and leaves of this plant can be used as a source of bioactive compounds for use in food supplements or nutraceutical products. Specifically, flavonoids found in the extracts of *C. aurantifolia* (rutin, apigenin, quercetin, kaempferol, nobiletin, tangeretin, and hesperidin) are known for their antioxidant and anti-inflammatory properties. Citrus fruits are abundant in flavonoids, and the most prevalent flavonoids found in lime extracts include apigenin, rutin, quercetin, kaempferol, nobiletin, hesperidin, hesperitin, and neohesperidin (Jayaprakasha et al., 2008; Loizzo et al., 2012). These flavonoids possess powerful properties that can affect the body's response to allergens, viruses, and carcinogens, displaying anti-allergic, anti-inflammatory, antimicrobial, and anticancer abilities (Okwu, 2005).

The antibacterial activity assay of lime fruit and leaf extracts in this study shows that the extracts have the ability to inhibit the growth of test bacteria. This can be seen from the formation of inhibition zones at all extract concentrations. According to research by Afroja, Falgune, Jahan, & Akanda (2018), citrus ethanolic extract at a concentration of 2.125-20 mg/ml has been found to have significant effects against certain bacterial strains such as *Shigella*, *Salmonella typhi*, and *Klebsiella*. The study showed that *Shigella* was particularly sensitive to the extract with an average zone of inhibition of 14.90 mm, followed by *Klebsiella* (14.49 mm), *E. coli* (13.77 mm) and *S. typhi* (12.01 mm). Additionally, citrus peel methanolic extract was found to be effective against *S. aureus* at a concentration of 31.25 µg/ml. On the other hand, citrus ethyl acetate extract was found to be effective at higher concentrations between 250-750 µg/ml. Similar study conducted by Wardani, Jekti, & Sedijani (2018) on the antibacterial activity of lime peel against *S. epidermidis*, *P. aeruginosa*, and *K. pneumonia*, using two types of solvents, ethyl acetate and ethanol, found that the ethyl acetate solvent was more effective in inhibiting and providing a significant effect on the growth of the test bacteria.

Inhibition zone in the positive control, meropenem, is still greater than the extract. It could be due to the method of extraction, purification, and preparation of the plant extract antimicrobial compound may have reduced its efficacy. More over, several factors have been found to influence the pattern of antimicrobial susceptibility of plant extracts. Some of these factors include factors like environmental conditions, the solvent used, the origin of the microorganisms, the biochemistry, physiology, metabolism, and adaptation of the microbes, the species of plant, the biochemistry, age, and parts of the plant used, the concentration of the extract, and the time of extraction (Izah, 2018)

The presence of phytochemicals in lime extracts were such an important factor that played role in antibacterial activity. In general, the antibacterial properties of plant phytochemicals work by disrupting the bacterial cell membrane or inhibiting several virulence factors such as enzymes

and toxins (Ramona, Erika, Anna, Maria, & Sobarzo-s, 2016). Moreover, the ability of *C. aurantifolia* to inhibit bacterial growth is thought to be caused by certain plant chemicals, including 5, 8-dimethoxy-psoralen, 5-geranyloxy-psoralen, palmitic acid, linoleic acid, oleic acid, 4-hexan-3-one and citral (Sandoval-montemayor et al., 2012).

CONCLUSION

In this research, phytochemical screening and antibacterial testing were conducted on lime (*Citrus aurantifolia*) leaf and fruit extracts. The results showed that the extract contains bioactive compounds and is able to inhibit the growth of *Klebsiella oxytoca*. Further investigation is needed to evaluate other extraction methods for isolating active compounds to enhance antimicrobial activity. Additionally, other parts of the plant need to be studied to evaluate the plant extract as a potential antimicrobial agent.

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