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Research Article

ANTIMICROBIC ON KAFFIR LIME (*CITRUS HYSTRIX*) LEAVES AGAINST *BACILLUS CEREUS* : TIME-KILL TEST METHOD

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ABSTRACT

Kaffir lime (Citrus hystrix) is a horticultural plant that is widely used as a natural flavor in various food and beverage products. The content in lime leaves is useful for health, including flavonoids, minerals, vitamin C, limonoids, and carotenoids. Bacillus cereus causes a diarrheal disease that attacks the intestines. This study aims to determine the kaffir lime leaf extract in inhibiting the growth of Bacillus cereus bacteria using the Time-Kill Test method. This study used the Minimum Inhibitory Concentration (MIC) and Time-Kill Test methods. The MIC results in kaffir lime leaf extract that can inhibit bacteria at 2 mg/mL, while the Time-Kill Test shows a death phase at 24 hours. Kaffir lime leaf extract has a bacteriostatic effect at 0, 4, 8, and 24 hours, while amoxicillin and the combination of amoxicillin and extract have a bacteriostatic effect at 4, 8, and 24 hours. Based on the results of the One Way ANOVA test with a 95% confidence level (p <0,05) showed a significant effect of kaffir lime leaf extract on the growth of Bacillus cereus.

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Keywords: *Bacillus cereus, kaffir lime leaves (citrus hystrix), MIC, time-kill tets*

INTRODUCTION

Diarrhea is one of the endemic diseases in Indonesia, characterized by loose stools and a many bowel movements per day. The type of diarrhea that occurs in children is bacillary dysentery. Baciller dysentery is an acute infectious disease that attacks intestinal epithelial cells and causes serious patient problems if not followed up properly (Williams & Berkley, 2018). The cause of diarrhea occurs due to bacteria found in food and drinks contaminated with bacteria that cause diarrhea, including *Escherichia coli, Shigella, Staphylococcus aureus, Bacillus cereus*, and others.

Bacillus cereus is a gram-positive bacterium that triggers poisoning accompanied by indications of vomiting and diarrhea, with spores that are more sensitive to the environment than vegetative cells (Bottone, 2010).

Indonesia occupies the second highest level in the world regarding biodiversity after Brazil, where 7000 types of plants areefficacious and a medicine. Indonesians have long used plants as alternative medicine in curing, prevention, recovery, and health promotion. Plants that are useful as medicine usually contain phytochemical compounds that are efficacious as a treatment. Phytochemical compounds in plants can include flavonoids, alkaloids, steroids, and essential oils that will give a very characteristic aroma, taste, and smell to the original plant (Rizky et al., 2020).

Purut orange (*Citrus hystrix*) is a fruit plant that can be grown by the whole community both in the garden and farm. *Purut* orange also has a pharmacological effect beneficial as an antiseptic and antioxidant (Mifthahendrawati, 2014). Ethanol extract from *purut* orange leaves slows the growth of *Bacillus cereus* using the good diffusion sensitivity test method and obtained moderate antibacterial sensitivity results of 6,255%, 12,5%, and 25%, as well as 505 and 100%, including strong antibacterial sensitivity (Nugraheni, R, Noorhamdani & Hanif, 2021).

The methods that are often used for sensitivity tests are diffusion and dilution, but there are the most appropriate methods to determine the bactericidal effect, namely the MIC and *Time-Kill Test* methods. *Minimum Inhibitory Concentration* (MIC) is the lowest concentration of antibiotics or antimicrobials that can inhibit the growth of specific microbes (Golan & Wilkins, 2008). *Time-Kill 2esta t* a method to measure the inhibition and killing of bacteria (Eliopoulos, G. M., and R. C. Moellering, 1996).

Research by Natasya (2019) on the antibacterial effect of *kirinyu* leaf ethanol extract (*Chromolaena adorata*) on *S.aureus* bacteria using the *Time-Kill Test* method. The results of the *Time-Kill Test* of kirinyu leaf ethanol extract showed a bacteriostatic effect. In contrast, tetracycline and a combination of (tetracycline and *kirinyu* leaf ethanol extract) showed a bactericidal effect where there was a decrease in *S.aureus* bacteria at the 8 and 24 hours. Based on the description above, this study aims to determine the extract of citrus leaves (*Citrus hystrix*) in inhibiting the growth of *B.cereus* bacteria with the *Time-Kill Test* method (Natasya, 2019).

MATERIAL AND METHODS

The study was conducted from March to May 2022. Phytochemical test research was conducted at the Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Surabaya State University, and the evaporation 2esta t the Biological Laboratory of the Faculty of Mathematics and Natural Sciences, Surabaya State University. The MIC reading was done in Molecular Biology Laboratory, and the *Time-Kill Test* test was conducted at the Clinical Bacteriology Laboratory, Faculty of Health Sciences, University Muhammadiyah Sidoarjo.

The ingredients used in this study were *purut* orange leaves from the Porong market, *aquadest*, amoxicillin, *Nutrient Agar* (NA), *Mueller Hinton Agar* (MHA), *Mueller Hinton Broth* (MHB), *Mac Farland* 0,5, DMSO 100%, aluminum foil, cling wrap, and *Bacillus cereus* bacteria. The tools used in this study are analytical scales, autoclaves, ovens, cuvets, *petri* dishes glass cups, *erlenmeyer*, bunsen, three legs racks, gauze, volume pipettes, *spiritus*, tube racks, drip pipettes, blenders, UV-Vis Spectrophotometers, *Laminar Air Flow* (LAF), colony counters, incubators.

Extract Making. *Citrus hystrix* leaves weighing 2000 grams are cleaned and dried. Dried leaves weighing 300 grams are then mashed using a blender and made dry *simplicia*, then the powder obtained is 200 grams.

Maceration. *Simplicia* orange leaves are weighed as much as 200 grams, put in a container, mixed with 600 mL of 96% ethanol solvent, and stirred for 30 minutes. Then soak it a full day until it settles at the time of filtration, stirred in advance so that the precipitate can be dissolved. Filtering was conducted using layered filter paper, placed on a funnel, and filtered everything until it was pulp. The rest of the filtration was resoaked with 96% ethanol of 600 mL. This was done until the last day of maceration, the third day. The top layer was a mixture of solvents. This layer was taken and repeated three times. The results were put into the evaporating flask and installed it on the evaporator.

Phytochemical Test. Phytochemical tests were conducted to determine the content contained in the plants to be tested. Phytochemical tests include flavonoid, phenol, Tannin, and saponin tests. One phytochemical test was conducted on the leaves of *purut* oranges. **Flavonoid Test.** The 1 mL sample was added 3 mL of 70% ethanol, homogenized, heated and shake again, and then strained. The filtering results were added Mg 0,1 grams and two drops of concentrated HCl. **Phenol Test.** A 1 mL sample was inserted into a test tube and then add 1 mL of 1% NaCl solution and 1 mL of 10% gelatin solution were. **Tannin Test.** The 1 mL sample was heated with 20 mL of water over the water reservoir, then filtered. The filtering results and a few drops of FeCl 1% were obtained. **Saponin Test.** A 1 mL sample was heated using 10 mL of water in a reservoir. The filtering results are homogenized and left at room temperature for 15 minutes.

Breeding of bacterial cultures. A bacterial suspension was taken from several bacteria from the supply culture with an *ose* needle, then planted in a saucer containing *Chocolate Agar Plate* (CAP) media. The bacterial culture was then incubated at 37℃ for 24 hours.

Data Analysis. The data obtained were statistically analyzed with the SPSS version 26 program, and then the normality of the data was viewed with the Shapiro-Wilk test with data variants using the Levene test. When the data distribution is normal, it meets the parametric statistics requirement. Then the One Way ANOVA parametric statistical test and the Tamhane Post Hoc test were conducted. From the test, it can be identified whether there is a significant difference ($p \leq 0.05$) in the *Time-Kill Test* results.

RESULTS AND DISCUSSION

Table 1. *Purut* Orange Leaf Phytochemical Test Results

Compound	Results
Mayer Alkaloids	+++
Wagner Alkaloids	+++
Dragendorf Alkaloids	+++
Flavonoids	+++
Saponins	+++
Steroids	-
Triterpenoids	+++
Phenolic	+++
Tannins	+++

Information :
(-) : No. compound content
(+) : Low compound content
(++) : Medium compound content
(+++) : High compound content

The phytochemical test showed that the 96% ethanol extract *simplicia* powder from *purut* orange leaves contains secondary metabolites of flavonoids, alkaloids, tannins, saponins, triterpenoids, and phenolics (Table 1). The phytochemical test results do not contain steroids. This happens because

of growing environmental factors that can change the physiological and biochemistry contained in the leaves of *purut* orange (Venkatachalam & Thani, 2019) .

One of the dilution methods, namely MIC, can inhibit microorganisms with a minimum concentration based on the time before and after incubation. Based on the MIC value determined by the inhibitor of *purut* orange leaf extract and amoxicillin, which is the lowest concentration that produces a barrier percent of 50%, which is to inhibit 50% of the growth of test bacteria (Nurkanto, 2012).

Based on the inhibitor percentage data in Table 2. Purut orange leaf extract and amoxicillin are known to have different antibacterial activity against Bacillus cereus bacteria with different concentrations and different percent inhibitors at each concentration. The concentration value used for testing using the Time-Kill Test method is a MIC concentration of 2 mg/mL in the extract and 2 µg/mL in antibiotics.

Table 2. Percent Inhibition of *Purut* Orange Leaf Extract and Amoxicillin

Treatment (mg/mL) and (µg/mL)	Retardant (%)
EDJP 2 (mg/mL)	50,30
EDJP 1 (mg/mL)	35,51
EDJP 0,50 (mg/mL)	19,11
EDJP 0,25 (mg/mL)	8,00
AMC 8 (*µ*g/mL)	11,48
AMC 4 (*µ*g/mL)	9,572
AMC 2 (*µ*g/mL)	89,69
AMC 1 (*µ*g/mL)	1,510

Description: EDJP = orange leaf extract *purut*; AMC = amoxicillin

Concentrations viewed from the lowest concentration of MIC can be inhibiting, i.e., in orange leaf extract *purut* of 2 mg/mL, amoxicillin of 2 µg/mL, while concentration on the combination is taken from 1/4:1/4 of the MIC value. So that the concentration of the combination on Testing using the time-kill method test, i.e., 0.5 µg/mL for amoxicillin and 0.5 mg/mL for *purut* orange leaf extract.

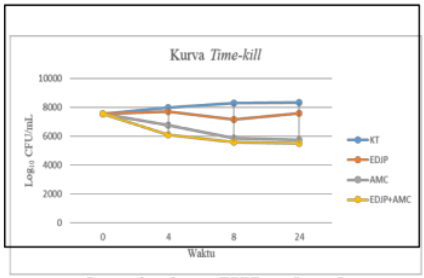

Figure 1. Time-Kill Test curve, description: KK = developmental control; EDJP = *purut* orange leaf extract; AMC = amoxicillin; EDJP+AMC = combination *purut* orange leaf extract and amoxicillin

Figure 1, indicates that the entire control has bacteria in a number almost the same at the beginning (0^{th} hour). Control growth has increased in numbers bacteria at the 4^{th} to the 24^{th} hour with the highest colony. Treatment on control EDJP experienced an increase in the number of bacteria at the 0^{th} – 24^{th} hour, but not as much as on growth control. Treatment on AMC control, the number of bacteria shows a decrease in the number of bacteria, while at the 24^{th} hour the reduction of the number of bacteria is higher. This shows that amoxicillin can inhibits the growth of *Bacillus cereus* bacteria. On combination control treatment EDJP+AMC, the number of bacteria shows no different decrease in the 0^{th} to the 24^{th} hour with amoxicillin.

This is due to a pattern of growth for 24 hours of incubation. At 0^{th} hour does not indicate the time generation. This is due to bacterial cells not splitting yet. The 4^{th} hour shows the highest generation because the bacteria are still in the phase Adjustment. At the 8^{th} hour, bacteria enter the exponential phase due to bacteria having divided cells by many (Hogg S., 2005), while at the 24^{th}-hour experience a phase of death. Balance the total number of bacteria caused by a decrease in the degree of cell division. It is caused by low levels of nutrients and the accumulation of toxic substances that interferes with cell division. It is characterized by increased mortality that exceeds the growth rate resulting in a decrease in bacterial populations Overall. While in this phase, the death rate of bacteria is greater mortality.

The combination of extracts with Antibiotics Can Inhibit Bacteria from Gram-positive and negative pathogens. Some large combinations are synergistic (Aiyegoro et al, 2008). It is caused because it has a content of phytochemicals, including alkaloids, flavonoids, steroids, and tannins, that are effective against positive and negative bacteria with inhibit cell wall synthesis (peptidoglycan) and the making of murein. In addition, amoxicillin may inhibit the cross-relationship between polymer rings and linear peptidoglycan that becomes the main components of the bacterial cell wall gram-positive. How to leaf extract works citrus *purut* and the antibiotic amoxicillin can become an important factor in improving bactericidal efficacy observed when combined.

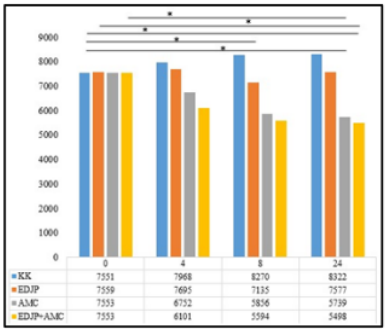

Figure 2. Log Number of Bacteria *Time-Kill Test*, Description: * = p < 0.05; EDJP = *purut* orange leaf extract; AMC = amoxicillin; KK = growth control; EDJP+AMC = leaf extract combination citrus *purut* and amoxicillin

Figure 2 indicates that the number of acquired bacteria in the time-kill test can be determined from bacteriostatic and bacteriocidal in every treatment. Log values were obtained from the differentiation between growth controls with each treatment. Treatment antibiotics and combinations at the 4th, 8th hour, and 24 exhibited bactericidal effects (an effect of an agent that can kill bacterial

growth). The extract treatment includes bacteriostatic (an effect that leads to the death of bacteria). It is called bactericidal if the log value is < 3 log10 CFU/mL and bacteriostatic if it is > 3 log10 CFU/mL (Viganor et al., 2011). On testing One Way ANOVA statistics (Figure 2), the number of colonies bacteria in the time-kill test obtained results significantly different in the treatment of KK 0 with AMC 24, KK 0 with EDJP 8, KK 0 with EDJP+AMC 24, EDJP 0 with EDJP+AMC 24, and EDJP+AMC 0 with AMC 24.

CONCLUSION AND SUGGESTION

Based on the research conducted, it can be concluded that *purut* orange leaf extract (*Citrus hystrix*) can inhibit the growth of *B.cereus* using the *Time-Kill Test* method by showing curve results on the *purut* orange leaf extract treatment at the 4^{th}, 8^{th}, and 24^{th} hours in the death phase, while in the *purut* orange leaf extract treatment, the results of the *Time-Kill Test* log data clock to 0^{th}, 4^{th}, 8^{th}, and 24^{th} hours have a bacteriostatic effect. Further studies can use different concentrations and antibiotics from the *Time-Kill Test* method to observe the effects and interactions between *purut* orange leaf extract and amoxicillin and whether it is resistant to bacteria.

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