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Effect of papaya flower ethanolic extract (*Carica papaya*) on the time kill of tetracyclin against *Escherichia coli*

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ABSTRACT

Papaya (*Carica papaya*) is found throughout Indonesia. Papaya flowers contain saponin, tannin, alkaloid, and flavonoid compounds which functions as antibacterial. *Escherichia coli* is a Gram-negative rod bacteria that could be found in the human large intestine as a cause of diarrhea. The study aimed to determine the effect of papaya flower extract and a combination of papaya flower extract with tetracycline antibiotics using the smallest concentration capable of inhibiting 50% of bacterial growth against *Escherichia coli* bacteria ATCC 25922 using the time-kill test method. This study used the method of *Minimum Inhibitory Concentration* (MIC) and *time-kill*. The MIC of the ethanol extract of papaya flower was 2 mg/mL. The *time-kill test* showed that the death phase was occurred at 4 - 24 hours. The bacteriostatic effect of the extract was obtained at 0 hours to 24 hours, while the antibiotic and its combination with the extracts had a bactericidal effect at 8 hours and 24 hours.

Keywords: Papaya flowers (*Carica papaya*), *Escherichia coli*, MIC, *Time-kill test*

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INTRODUCTION

Diarrheal disease is still a health problem in Indonesia, it can be seen by the increasing number of diarrheal diseases from year to year and lead to. The high mortality (Kementerian Kesehatan Republik Indonesia., 2011). The diarrhea is a condition of a person who is experiencing bowel disease with a liquid or soft consistency and the most frequency can be water (usually three times a day or more). Based on the Basic Health Research (2020), diarrhea is the second death contribution in the group of children aged 29 days–11 months (14.5%). Diarrhea is an endemic disease that causes Extraordinary Events, in Indonesia it is still a contributor to mortality, especially in children under five (Kementerian Kesehatan Republik Indonesia, 2021). Transmission of diseases caused by *E.coli* can be directly through contaminated food or water or direct contact with humans or animals. (Bonkoungou et al., 2013) found that children under five years of age, were discovered the pathogenic bacterium *E.coli* by second rank for the cause of diarrhea, which is 24% after *Rotavirus* 30% in the first rank, then the third rank is *Salmonella sp* 9%, the fourth rank is *Shigella sp* 6%, the fifth rank is *Adenovirus* 5% and the last rank is *Campylobacter* 2%.

Escherichia coli is a pathogenic bacterium found in the intestines of animals or humans in which the most important part of the human intestinal tract and it is harmless in general. *Escherichia coli* is a pathogen that causes diarrhea and the other intestinal tract diseases. *Escherichia coli* is a Gram-negative rod bacteria, 2-3 mm, circular, convex, and non-pigmented colonies on nutrient and *Blood Agar Plate* (BAP) media. It a form the cell is single, in pairs, and short chains (not encapsulated) like coccus. *E.coli* showed positive results in indole, lysine decarboxylase, and mannitol fermentation tests, and produced gas from glucose. The isolates from urine can be immediately identified as *E.coli* by looking at their hemolysis on *Blood Agar Plate* (BAP) media, a typical colony morphology with a rainbow color "glittering" on differential media such as *Eosin Methylene Blue Agar* (EMB agar) media and positive with indole test (Setiarto, 2020).

Indonesia is one of the tropical countries which has many type of plants that can be used as traditional medicine. The traditional medicine needs to be based on the utility of plants that have been trusted for generations (Andriani et al., 2016). According to (Agustina et al., 2016), plants that will be used for traditional medicine have secondary metabolite compounds such as alkaloids, flavonoids, steroids, saponins, tannins, and terpenoids. (Jamil, et al., 2015) reported that this is especially caused by a bacterial infection. In the last decade, the development of herbal plants has been used as a treatment for diseases caused by infection with microorganisms, especially those caused by bacterial infections. The types of plants used as medicine are easy to find or live naturally in the surrounding environment. Parts of plants that can be used as traditional medicine include leaves, fruit, fruit skins, water, sap, and seeds. Papaya flower is one of the plants that can be used as a traditional treatment. The test activity of papaya flower extract as antidiarrheal against *E.coli* consisted of three concentrations, namely 20%, 30%, and 40%. The concentration of 20%, 30%, and 40% have an average diameter of 2.03 cm, 2.07cm, and 2.6 cm respectively. According to three concentrations, the largest diameter of the inhibition zone against *E.coli* was 40% (Pudyawanti et al., 2021).

One of the methods used to determine the effect of bactericidal or fungicidal is *time-kill test*. The *Time-kill test* is a method used to determine the effectiveness of antimicrobials using plate count, analysis based on log reduction and percent (Oladosu et al., 2013). There was an increase in growth control, form 0 hour the number of bacteria was 4.278 ± 0.01, to 4, 8, and 24 hours the number of bacteria were 6.471 ± 0.024, 7.286 ± 0.021 and 8.202 ± 0.023 Natasya's research 2019 (Natasya, 2019). In the treatment of kirinyuh leaf (*Chromolaena odorata* L.) ethanol extract, there was an increase in the 4 hours with the number of bacteria 5.402 ± 0.009, the 8 hours with the number of bacteria 5.653 ± 0.010, the 24 hours with the number of bacteria 7.403 ± 0.047.

The purpose of this study was to determine the effect of papaya flower ethanol extract (*Carica papaya*) and its combination with tetracycline on the growth of *E.coli* using the *time-kill test*.

MATERIALS AND METHOD

Materials

The materials used in this research included papaya flowers (Kedungrawan Village, Krembung Sidoarjo), *Escherichia coli* ATCC 25922 (Surabaya Health Laboratory Center), tetracycline (Novapharin®), 96% ethanol (Brataco®), tissue (Nice®), filter paper, NA media (*Nutrient Agar*) (Merck®), MHA media (*Mueller-Hinton Agar*) (Merck®), 0,9% NaCl (Merck®), distilled water (Brataco®), 100% Dimethyl sulfoxide (Emsure®), 0,5 McFarland (Merck®).

Methods

The use of concentration in inhibiting bacterial growth is based on the *Minimum Inhibitory Concentration* (MIC) value with the percentage of inhibitor formula. The bacterial inhibitor evaluation was based on the $\log_{10}$ CFU/mL *Time Kill-Test* data curve; which was seen on the growth phase of bacteria.

Raw material preparation

Criteria of the papaya flowers were green and white flowers, perfectly shaped into flowers, small and large circles, contains mucus, and do not contains harmful or toxic substances. Papaya flowers that have been collected as much as 2 kg and then washed with tap water until clean then dried in the sun and obtained a papaya flower weight of 306 gram, grinded then mashed by pounding it into powder simplicia, obtained weight of papaya flower powder simplicia of 275 gram. The simplicial was then stored in the closed and labeled container, at room temperature (Hamida et al., 2022).

Plant extraction

Papaya flower extract was made by maceration method. Weigh the simplicial powder as much as 200 gram with an analytical balance, then the simplicia is mixed with 600 mL of 96% ethanol in a dark container, then homogenized by stirring for 6 hours and let stand for 18 hours. The top layer obtained is a mixture of solvents and active compounds. The remainder from the first maceration is then remaceration twice. Every day the papaya flower extract is stirred evenly (Hamida et al., 2022).

Papaya flower powder phytochemical test

Papaya flower powder weighed as much as 5 gram. Add 50 mL 96% ethanol and let stand for 15 minutes. Samples were taken as much as 1 mL and put in each test tube for phytochemical tests (Hasibuan et al., 2020).

Identification of flavonoids

Papaya flower extract was taken as much as 1 mL then mixed with 3 mL of 70% ethanol after that homogenized and heated for 2-3 minutes. Then filtered and the filtrate obtained was added with 0.1 gram of Mg and 2 drops of concentrated HCl. The result is said to be positive if the color changes to red (Hasibuan et al., 2020).

Identification of saponins

Papaya flower extract was taken as much as 1 mL and added with 10 mL of distilled water later heated for 15 minutes after that homogenized waited for 5 minutes. The formation of stable foam indicates presence of saponin. (Hasibuan et al., 2020).

Identification of steroids

Papaya flower extract was taken as much 1 mL then added 3 mL of 70% ethanol, 2 mL of concentrated sulfuric acid, and 2 mL of anhydrous acetic acid. A positive result is if there is a color change from purple to blue or green (Hasibuan et al., 2020).

Identification of tannins

A total of 1 mL of papaya flower extract was taken and heated for 2 minutes after that added 2-3 drops of 1% FeCl3. The occurrence of a color change with the formation of brown or blackish blue indicates the presence of tannins (Hasibuan et al., 2020).

Identification of phenolic

A samples of papaya flower extract that have been allowed to stand can then be taken in amounts of up to 1 mL. After that, add 1% NaCl solution and 10% gelatin solution, each in an amount of 1 mL. Homogenize, then let stand. Observe the presence of white precipitate in the sample (Hasibuan et al., 2020).

Preparation of 0.5 *mac farland* standard solution

The composition of the *McFarland* 0.5 solution was 9.95 mL of 1% H_2SO_4 and 0.05 mL of 1% $BaCl_2$ solution. Making 1 mL of 1% H_2SO_4 and then dissolving it with 10 mL of distilled water, while making 1% $BaCl_2$ solution by weighing 0.1 $BaCl_2$ then dissolved in 10 mL of distilled water. The *McFarland* solution was made by taking 9.95 mL of H_2SO_4 and then mixed with 0.05 mL of $BaCl_2$ solution (Sari et al., 2021).

Media preparation

Preparation of *Mueller Hinton Agar* (MHA) Media

MHA media weighed as much 38 grams and dissolved with 1 liter of aquadest, the mouth of the erlenmeyer covered with a cotton swab, and then heated until completely dissolved. The MHA medium was sterilized in an autoclave at 121°C for 15 minutes and cooled to 45°C. The cooled media was put into a sterile Petri dish and allowed to solidify (Lintang et al., 2020).

Inoculation of the Test Bacterial

Escherichia coli were inoculated on *nutrient agar slant* tubes (NAS) and *nutrient agar* plates (NA) by scratching 1 ose containing *E.coli* aseptically, by which the needle was to bring the mouth of the test tube and the edge of the petri dish closer to the Bunsen flame when the needle was scratched. The test tube was closed again with cotton and the petri dish was closed again and then incubated in an incubator at 37°C for 24 hours (Suhaillah & Santoso, 2018).

MIC Determination

MHB medium which had contained a bacterial culture by equalizing it with 0,5 *Mcfarland's* medium. The papaya flower extract were dissolved by using 1 ml of 100% DMSO and the antibiotic solubles by adding sterile aquadest. The second series of concentrations were diluted using a 10-*fold* dilution. The 24 cuvettes added as much as 110 µL of MHB medium contained *E.coli* bacteria culture with 90 µL of series of antibiotic concentrations (8,4,2, and 1 µg/mL) and extracts (2.1,0.5, and 0.25 mg/mL) placed in 4 cuvettes sequentially. The volume of each cuvette was 200 µL, each treatment was added to the cuvette and repeated 3 times with the volume according to the concentration (Natasya, 2019).

The sample in the cuvette was measured using a UV-Vis Spectrophotometer with a wavelength of 595 nm absorbance. Sample calculations were performed before incubation and after incubation for 24 hours. The formula for determining percentage of inhibition can use (Pratiwi et al., 2010):

$$\%inhibitor = \left(1\text{-}\left(\frac{ODt24-ODt0}{ODgc24-ODgc0}\right)\right) X\ 100\% \qquad (1)$$

Information :

- ODt24 = after incubation
- ODt0 = before incubation
- ODgc24 = growth check after incubation
- ODgc0 = growth check before incubation

In this research, the determination of MIC was based on the lowest concentration that could inhibit the growth of the test bacteria, namely > 50% (Nurkanto, 2012).

Time-kill Test Method

Treatment of *time-kill test*

Bacterial culture on MHB medium was prepared and then equilibrated to 10^6 CFU/mL (1:150 dilution of 0.5 *McFarland*). Each test tube contains 9 mL of MHB which has been added with papaya flower extract, tetracycline antibiotics along with a combination of papaya flower extract and tetracycline antibiotics (1/4 MIC : 1/4 MIC) then added with 1 mL of *E.coli* bacteria (Figure 1). Incubation for 24 hours at 37°C. There are 3 controls used in this test, namely growth, media and NaCl sterility. Each sample was repeated 3 times (April et al., 2016).
Information :

- T.1 : 9 mL of MHB and Extract + 1 mL of Bacterial Culture
- T.2 : 9 mL of MHB and Antibiotics + 1 mL of Bacterial Culture
- T.3 : 9 mL of MHB and a combination of extracts and antibiotics + 1 mL of bacterial culture
- T.4 : 9 mL MHB + 1 mL Bacterial Culture (Growth Control/Negative Control)

Calculation of bacterial colonies

Each sample at 0, 4, 8 and 24 hours was taken as much as 100 µL and diluted 10-*fold* with 0.9% NaCl. 100 µL sample was taken then spread inoculated in a cup containing MHA media and incubated at 37°C for 24 hours. Calculation of bacterial colonies using Colony Counter (Natasya, 2019).

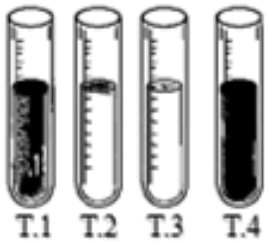


Figure 1. Time-Kill test treatment in each test tube

In each petri dish test that contained bacterial colonies, it was carried out 3 times and counted between 30-300 CFU/mL (Figure 2) (Basri & Khairon, 2012). The number of bacterial colonies can be calculated using the formula:

$$\frac{\text{CFU}}{\text{mL}} = \text{bacterial colonies} : \frac{1}{\text{concentration}} \times \text{volume sample inoculation} \quad (2)$$

(Natasya, 2019).

Time-kill Curve

Time-kill curve are constructed with "X" as the sample time and "Y" for the number of bacterial colonies in log10 CFU/mL. Curve data contains growth control data, papaya flower extract, tetracycline antibiotics and a combination of papaya flower extract and tetracycline antibiotics. The purpose of the *time-kill curve* is also to determine the growth phase of bacteria, including the lag phase/bacterial adaptation phase, the log phase, the stationary phase (experiencing a fixed amount of bacterial growth) and the Death/death phase (Sharah et al., 2015). According to (Silva et al., 2011), the effect on bacteristatic had a decrease of < 3 log10 CFU/mL and bactericidal had a decrease of > 3 log10 CFU/mL.

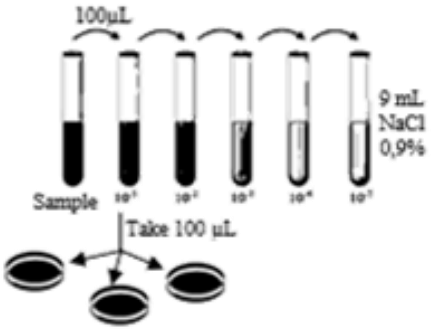


Figure 2. Dilution 10-fold

Data Analysis

The data obtained were analyzed statistically with the SPSS version 26.0 program and then the normality of the data was seen using the Shapiro-Wilk test and the variance of the data using the *Levene test*. If the data distribution is normal, then the analysis is continued by using the *One Way ANOVA* and *Post Hoc Tukey* parametric statistical tests. If the data is not normal, then a non-parametric test is performed using *Kruskal-Wallis*. From this test, it can be identified whether there is a significant difference in the results of the *time-kill test*.

RESULT AND DISCUSSION

Carica papaya flower based on the results of phytochemical tests, the data in Table 1 showed the characters of the extract.

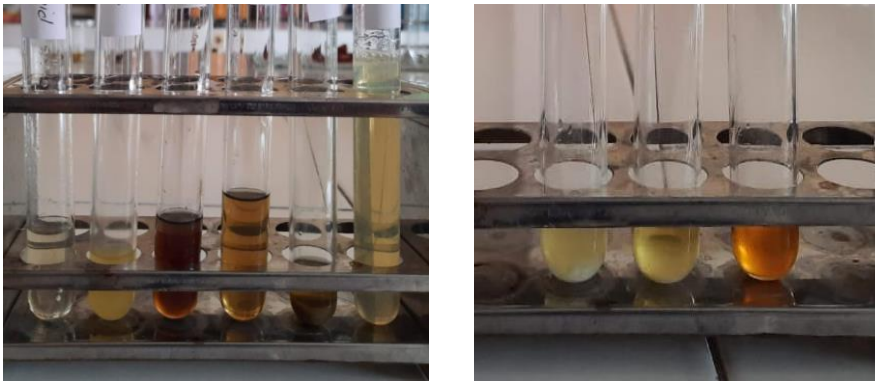
Table 1. Phytochemical test results of papaya flower extract (*Carica papaya*)

Phytochemical Tests	Reagents	Result	Conclusion
	Mayer	Orange precipitate	+++
Alkaloids	Wagner	Chocolate precipitate	+++
	Dragendorf	White precipitate	+++
Flavonoids	Mg + HCl $_{concentrated}$ + Ethanol	Red	++
Saponins	-	Presence of stable foam	++
Steroid	Libermann-Burchard	Purple to blue or green	-
Triterpenoid	Kloroform + H_2SO_4 $_{concentrated}$	Brownish red	+++
Phenolic	NaCl 10% + Gelatin 1%	White precipitate	+++
Tannins	$FeCl_3$ 1%	Greenish brown	++

Description :
(-): The tested compound was absence
(+) : A small amount of the tested compound
(++): Enough amount of the tested compound
(+++): Abundance of the tested compound

Carica papaya flower the results of phytochemical tests, it was found that the of papaya flowers (*Carica papaya*) contained alkaloids, flavonoids, saponins, triterpenoids, phenolic compounds and tannins (Table 1 and Figure 3). The phytochemical characters was affected by environmental growth factors, by which the physiological and biochemical properties of papaya flowers were dependence (Venkatachalam, 2019). *Minimum Inhibitory Concentration* is a dilution method in which the growth of test microorganism is inhibited by a minimum concentration based on the time before and after incubation. The MIC value was determined based on the percentage value of inhibitors of papaya flower

extract and tetracycline, which was the lowest concentration obtained a percentage of inhibitors > 50% of the bacterial growth (Nurkanto, 2012).



(a) (b) (c) (d) (e) (f) (g) (h) (i)

Figure 3. Result of phytochemical screening: (a) flavonoid; (b) phenolic; (c) triterpenoid; (d) steroid; (e) tannins; (f) saponins; (g) alkaloid; (h) dragendorf; (i) wagner

Based on data on the percentage of bacterial inhibition ability in Table 2 using MIC_{50}, which is the concentration used to inhibit 50% of bacterial growth (Solanki et al., 2015), it was found that papaya flower extract and tetracycline antibiotics have inhibitory activity on E. coli bacteria. The concentrations used for testing using the time-kill test method were MIC concentrations of 2 mg/mL in extracts and 1 g/mL in antibiotics.

Table 2. Percentage of inhibitors of Papaya flower extract and tetracycline antibiotics

Treatment	Concentration	Inhibitor (%) ± SD
papaya flower extract	2 mg/mL	61.43 ± 0.086
papaya flower extract	1 mg/mL	40.48 ± 0.077
papaya flower extract	0.5 mg/mL	42.22 ± 0.129
papaya flower extract	0.25 mg/mL	47.30 ± 0.061
tetracycline antibiotics	8 µg/mL	87.29 ± 0.328
tetracycline antibiotics	4 µg/mL	74.38 ± 0.431
tetracycline antibiotics	2 µg/mL	73.90 ± 0.107
tetracycline antibiotics	1 µg/mL	82.04 ± 0.033

Time-kill test is the most appropriate method in determining bactericidal or fungicidal effect. This method is a powerful means of obtaining dynamic information between microbial strains and antimicrobial agents. The *time kill test* proves antimicrobial effect is concentration or time dependent (Rodríguez-Hernández, 2017). Maintenance *time-kill* was determined by sampling control and tube media containing antimicrobial agents in the incubation intervals (0, 4, 8, 10, 12 and 24 hours) and growth of colonies that spread on plate media. Interpretation of *time-kill* data was carried out on a *time-kill curve* to measure decrease or increase in number of bacteria between combinations compared to the most active single antimicrobial agent (Pfaller et al., 2004).

Bacteristatic and bactericidal effects, as well as the growth phase of papaya flower extract and tetracycline antibiotics; the combination of papaya flower extract and tetracycline antibiotics with *E.coli* bacteria were tested by using the *time-kill test* method. The concentration would be used was seen from the lowest concentration of MIC which inhibits papaya flowers by 2 mg/mL, tetracycline antibiotics by 1 µg/mL while the concentration for the combination used was 1/4 : 1/4 MIC value. Based on the research of (Shi et al., 2017), 1/4 MIC was used to determine the inhibitory mechanism between extracts

and antibiotics in *E.coli* bacteria. So that the concentration of the combination used by testing the *time-kill test* method for papaya flower extract was 0.5 mg/mL and for tetracycline antibiotics was 0.25 µg/mL.

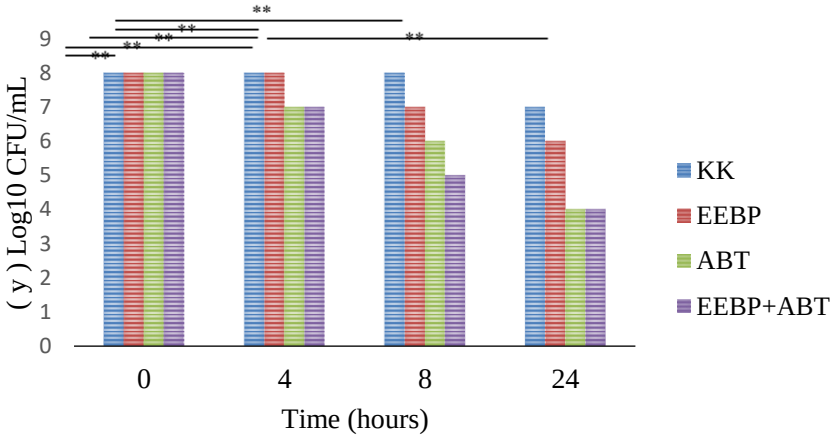


Figure 4. Number of Bacterial Colonies in the *Time-Kill Test* Log, Description: ** = p > 0.05, KK = growt control (negative control); EEBP = papaya flower ethanolic extract; ABT = tetracycline antibiotics; EEBP + ABT = combination of papaya flower extract and tetracycline antibiotics

Based on the results of the log time-kill test data contained in (Figure 4), the growth phase of the bacteria could be determined. In the KK treatment, from 0 to 8 hours there was a lag phase or adaptation phase, meanwhile at 24 hours there was a growth phase with a relatively short time. It was characterized by a gradual increase in the number of bacterial colonies. In the EBP treatment, there was a lag phase at 0 hour, and then there was at 4 hours to 24 hours there was a death phase which was marked by a decrease in the number of bacterial colonies. This should that papaya flower extract could inhibit the growth of *E.coli* bacteria. There was a lag phase at 0 hour, and then the death phase occurred at 4 hours to 24 hours which was marked by a decrease in the number of bacterial colonies every hour in ABT treatment. This shows that tetracycline antibiotics could inhibit the growth of *E.coli* bacteria. For EBP+ABT combination there was a lag phase at 0 hour, then at 4 to 24 hours the number of bacterial colonies showed a decrease so that it can be seen that the combination treatment occurred in. This is because the mechanism of action between papaya flower extract and tetracycline antibiotics inhibits the growth of *E.coli* bacteria and the extract has similarities with antibiotics.

In the research of (Prasetya et al., 2019), the growth curve of *E.coli* bacteria in time (t1), the growth phase of *E.coli* bacteria consists of lag phase, log phase and stationary phase. The first phase is lag phase which occurs at 0 to 8 hours. In the lag phase, the bacteria experience a process of adaptation to environmental conditions, namely pH, temperature, and nutrition, and an increase in the number of bacterial cells occurs slowly. In this phase, the bacterial isolates consisting of 4 isolates had rapid growth from the 9th to the 24th hour. Bacteria have not reached the stationary phase or equilibrium period. In this phase, metabolic activity slows down. Nutrient deficiencies, accumulation of waste products, and changes in pH that are toxic to cells are considered to be the causes of the cessation of cell exponential growth. In addition, the bacteria have not yet reached the death phase, so further incubation is needed for more than 24 hours.Papaya flower extract has antibacterial compounds including alkaloids, flavonoids, saponins, triterpenoids, phenolics, and tannins. Each compound has a different mechanism of action, including inhibition of the synthesis of cell walls, inhibition of protein synthesis, inhibition of nucleic acid (DNA or RNA) synthesis, or inhibition of the synthesis of essential metabolites (Bauman, 2012).

The mechanism of action of tetracyclines involves inhibiting protein synthesis in bacterial ribosomes. Tetracyclines enter bacteria through two processes, namely passive diffusion and active transport.

Furthermore, tetracycline enters the ribosome and binds reversibly to the 30S ribosome and prevents the tRNA-aminoacyl binding to the ribosomal mRNA complex, which prevents the formation of new amino acids in the peptide from being formed. This can prevent elongation between new peptide chains and stop protein synthesis (Koto et al., 2010).

In Figure 4, a bactericidal effect can be determined with a log value > 3 log10 CFU/mL and a log bacteristatic value of < 3 log10 CFU/mL after 24 hours of incubation (Silva et al., 2011). Antibacterial compounds are compounds that can interfere with the growth of bacteria. There are two properties of antibacterial toxicity, including bactericidal, which is antibacterial and can kill bacteria, and bacteriostatic, which is antibacterial and inhibits bacterial growth (Purnamaningsih et al., 2017). The log kill value is obtained from the difference between development control and each treatment. The bactericidal effect can be observed between 8 and 24 hours after the antibiotic treatment or combination treatment. While the extract treatment at 0, 4, 8, and 24 hours was included in the bacteristatic.

After the *One Way ANOVA* statistical test was carried out based on the number of bacterial colonies in log *time-kill test*, the results were not significantly different in the treatment of KK 0 with EEBP 0, KK 0 with EEBP 4, EEBP 0 with KK 0, EEBP 0 with EEBP 4, EEBP 4 with KK 0, EEBP 4 with EEBP 0, EEBP 4 with ABT 0, EEBP 8 with ABT 0, EEBP 24 with ABT 4, ABT 0 with EEBP 4, and ABT 4 with EEBP 24 (Figure 4).

CONCLUSION

Based on this research, it can be concluded that papaya flower and a combination of papaya flower extract and tetracycline antibiotics can inhibit the growth of *E. coli* bacteria by using the time-kill test method, which is indicated by the curve results in the combination treatment and papaya flower extract experiencing a death phase at 4, 8, and 24 hours, which were marked by a decrease in *E. coli* bacterial colonies. The research of the log time-kill test data showed a bacteriostatic effect on papaya flower extract treatment at 0, 4, 8, and 24 hours, and a bactericidal effect at combined treatment at 8 and 24 hours.

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