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Antibacterial Activity of Ethyl Acetat Fraction of Basil Leave (Ocimum Basilicum L) Toward Escherichia Coli Growth

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ABSTRACT

Antibiotic resistance to *Escherichia coli* is commonly found. Several studies have found that basil leaves contain chemical compounds: flavonoids, alkaloids, tannins, and saponins, which act as antibacterials. This research aimed to determine the inhibitory power of the ethyl acetate fraction of basil leaves (*Ocimum basilicum* L) on the growth of Escherichia coli. This study used a post-test-only control group design. This study contained five groups: one positive control group, one negative control group, and three treatment groups. The treatment group consisted of the ethyl acetate fraction of basil leaves at 12.5% µ, 25% µ, and 50% µ. The control group was a positive control K(+) with co-trimoxazole and a negative control K(-) with 10% Dimethyl Sulfoxide (DMSO). Kruskal walls test was chosen, followed by the Mann-Whitney difference test. In the Kruskal Walls test, a significant difference was found (p-value < 0.050) for basil leaf ethyl acetate fraction treatment. The largest average was obtained, namely the ethyl acetate fraction with a concentration of 12.5%. The Mann-Whitney test was carried out to determine fundamental differences between groups. The results of ethyl acetate fraction showed that the Minimum inhibitory concentration (MIC) for Escherichia coli was 12.5%.

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1. Introduction

The problem of infectious diseases continues to grow in the health sector and is the main cause of death throughout the world, including in Indonesia (Milani et al., 2011). Infections can be caused by bacteria, fungi, viruses, and parasites (Roni et al., 2018). The prevalence of infectious diseases in Indonesia is relatively high because Indonesia is one of the countries with a tropical climate with dusty conditions and warm and humid temperatures, which supports microbes to continue to reproduce and ultimately cause infection (World Health Organization, 2014). The bacteria that currently can cause health problems, especially in Indonesia, is Escherichia coli. Escherichia coli often causes infections in the urinary tract and bile duct and causes diarrhea (Sunarno et al., 2023). Diarrhea is the second cause of death in children under five years old and is the cause of death in about 760,000 children every year. Diarrhea cases are increasing every year; there are 1.7 billion cases of diarrhea (Mosisa et al., 2021). Efforts are made to control infectious diseases by administering antibiotics.

Antibiotics are drugs that can kill bacteria. The antibiotics often used are ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, and gentamicin. Antibiotics, which have been widely used for Escherichia coli bacteria, have increased in the last few decades, causing organ damage, immunosensitivity, and also causing resistance (Vranic & Uzunovic, 2016). Previous research results showed that *Escherichia coli* was 100% resistant to ampicillin, 96.2% resistant to erythromycin, 94.3% resistant to nalidixic acid, 92.3% resistant to tetracycline and 92.3% resistant to oxytetracycline (Hadi et al., 2013). A total of 781 patients treated in the hospital showed that 81% of Escherichia coli has the highest resistance to ampicillin (73%) and low resistance to gentamicin (18%).8To reduce this problem, use active bacteria-killing substances contained in medicinal plants one of which is basil leaves (*Ocimum basilicum* L).

The secondary metabolite content of basil leaves includes essential oils, alkaloids, steroids, saponins, tannins, flavonoids, triterpenoids, and phenols (Kumalasari & Andiarna, 2020). Several groups of chemical compounds, including alkaloids, flavonoids, and essential oils, can inhibit *Escherichia coli* bacterial growth (Kumalasari & Andiarna, 2020). The use of active substances from plants has several advantages; namely, no is toxic, does not change easily under drastic pressure, temperature, and pH, and at low concentrations, it can function well, so basil leaves can be an alternative treatment that is considered quite effective and safe to use (Rachmawati et al., 2021) In various studies, such as research conducted by Ali and Dixit (2012) reported that the flavonoid content of basil leaves could provide antibacterial effects against E. coli, S. aureus, and K. pneumonia. This research also shows that the combination of the two basil leaf flavonoid compounds, namely orientin and visenin, provides a synergistic antibacterial effect compared to the use of one of the two flavonoid compounds (Soesetyaningsih & Azizah, 2020).

The chemical compounds contained in basil leaf extract have the potential to be a source of active antibacterial ingredients. Therefore, this study tested the antibacterial activity of basil leaf extract (*Ocimum sanctum* L.) with various concentrations to determine the most effective concentration in inhibiting antibacterial activity against the growth of Escherichia coli.

2. Methods

2.1. Tools and Materials

The research instrument is used as a blender (National), filter paper, vacuum rotary evaporator, rag, evaporator cup, Erlenmeyer (Pyrex), spatula, tube, measuring cup (Pyrex), test tube rack, test tube (Pyrex), stirring rod, funnel, petri dish, Bunsen burner, object glass, deck glass, microscope (Olympus), dropper pipette, micropipete, incubator, laminar air flow, digital scale, autoclave, vortex, and colony counter.

Basil leaves taken from the area around the house, Nutrient Broth, Agar Media, Co-trimoxazole, distilled water, Dimethyl Sulfoxide (DMSO), 96% ethanol, McFarland standard, and Escherichia coli suspension. coli in the microbiology laboratory at Swadaya Gunung Jati University were used in this research.

2.2. Research Procedure

2.2.1. Sterilization of tools

Physical sterilization methods carry out sterilization, generally carried out by heating at high temperatures. The sterilization method used an autoclave filled with NaCl as high as the attack limit, setting a temperature of 121^0 C with a pressure of 1.5 kg/cm (15 pounds) or around 2 atm for 15-20 minutes. Before being sterilized, all tools are wrapped in HVS paper and then put in plastic. The loop needle is sterilized over a Bunsen flame.

2.2.2. Making Nutrient Broth Media

Weigh the nutrient broth (NB) media according to the procedure on the packaging. Add distilled water and stir until evenly mixed with a stir stick. Heat carefully using a bath/heating element until the media is mixed homogeneously (indicated by a clear yellow color). Before autoclaving, pour the NB into a test tube. Close the test tube with cotton wool or a tube cap, and do not close it too tightly. Sterilize all media in the test tube using an autoclave for 2 hours at a pressure of 1 atm 121OC. After being autoclaved, the NB media in the test tube is allowed to cool.

2.2.3. Plant Determination

The first stage of research was to determine basil plants. Plants are determined by showing them and determining their correctness according to morphological characteristics.

2.2.4. Making Ethanol Extract from Basil Leaves

Basil leaf extract was made using 96% ethanol solvent using the maceration method. The maceration method was chosen because it is easy to do and cheap. A total of 10 kg of basil leaves was cleaned of dirt, washed with running water until clean, and dried in the sun for 48 hours. Then, basil leaves are crushed using a blender. After that, the fine basil leaves were macerated with 3 L of 96% ethanol in a vessel, then covered flat and soaked for three days, stirring occasionally. After three days, the basil leaf extract solution was filtered to obtain phytrate and dregs. After that, all the filtrate was collected and put into a vacuum rotary evaporator at a temperature of 40^0 C, and then the remaining filtrate was evaporated using an evaporator cup in a water bath to obtain a thick extract.

2.2.5. Fractionation

Fractionation was carried out using the method (Liquid-Liquid Fractionation) with ethyl acetate solvent. A total of 90 grams of basil leaf extract was dissolved in 1.8L of distilled water, put into a separating funnel with the tap closed, then added with 1.8L of ethyl acetate, shaken slowly until homogeneous. After a separate layer of solution was visible, the ethyl acetate fraction was separated from the water fraction by opening the separating funnel tap and collected in an Erlenmeyer. The ethyl acetate fraction was then evaporated using a rotary evaporator at a temperature of 40^0 C to obtain a thick ethyl acetate fraction.

2.2.6. Cultivation of Escherichia coli

Escherichia coli bacteria was obtained from the Research Laboratory of Universitas Swadaya Gunung Jati. The bacterial breeding method used is the pour plate method on agar media, and the bacteria can be grown at 370 C in an aerobic atmosphere.

2.2.7. Preparation of Escherichia coli suspension

The isolate on the agar stock was grown using agar plate media and placed in an anaerobic jar containing a gespack, then closed tightly and placed in the incubator for 2 x 24 hours. After growing, take the colony and put it in 0.5 Mac Farland brain heart infusion (BHI) fluid.

2.2.8. Preparation of Basil Leaf Fraction Concentration

The extract that has been obtained from the extraction process is carried out extract purification process with a rotary evaporator at 45^{o}C until a thick extract is obtained. The thick ethanolic extract of basil leaves was fractionated using ethyl acetate as solvent. The basil leaf fraction formed (100% concentration level) will be diluted using DMSO with a concentration level of 12.5%, 25%, and 50%.

Table 5 Preparation of basil leaf fraction concentration (*Ocimum basillicum* L)

Concentration	**Fraction**	**DMSO**
12,5%	0,125gr	0,875ml
25%	0,25gr	0,75ml
50%	0,50gr	0,50ml

To make a fraction of 12.5%, 0.125 grams of extract are added and then dissolved with 0.875 ml DMSO. For a fraction with a concentration of 25 %, 0.25 grams of extract are added and then dissolved with 0.75 ml DMSO. For a fraction with a concentration of 12.5%, 0.50 grams of extract are added and then dissolved with 0.50 ml DMSO.

2.2.9. Cotrimoxazole preparation

One co-trimoxazole capsule (400 mg) was dissolved in 100 ml distilled water until homogeneous. This dilution is the first dilution. 400 mg in 100ml = 4000 ml. Then, with the following formula:

$$V1 \text{ x } C1 = V2 \text{ x } C2$$
$$1 \text{ x } 4000 = 100 \text{ x } C2$$
$$4000 = C2 \text{ x } 100$$
$$C2 = 40$$

2.2.10. Antibacterial activity examination (dilution method)

Prepare a petri dish and prepare five sterile test tubes. The concentration of ethyl acetate fraction of basil leaves used was three concentrations, namely 12.5%, 25%, and 50%. One control group (K-) was not given fractions, and 1 control group (+) was given co-trimoxazole. A suspension of the Escherichia coli bacteria being tested was provided, 1 ml of Escherichia coli isolate was taken, and Inoculation was carried out in a test tube containing 5 mL of NB media as a control. Provide 3 test tubes containing basil leaf fractions of various concentrations, and each concentration tube is given 1 mL of bacterial suspension. Then incubated at 37^0 C for 24 hours.

Perform 10-8 dilutions of the bacterial suspension using a test tube, which is then homogenized using a vortex mixer. The last two dilutions are planted on agar media using the spread plate method in duplicate. Incubate for 24 hours at 370 C. Observe the number of negative control colony growth and treatment with various doses of basil leaf fractions using a colony counter. Colony growth is calculated using the Total Plate Count method with the formula:

Number of colonies $\left(\frac{CFU}{Ml}\right) = a\ x \frac{1}{n} x\ Fk\ kj$

a = number of colonies
n = dilution
Correction factor (Fk) = 10

Calculation of percent (%) inhibition of colony growth

$$a = \frac{b-c}{d} \text{ x } 100\%$$

Information :

a. % inhibition: the ability of the ethyl acetate fraction of basil leaves to inhibit the growth of *Escherichia coli*
b. The number of colonies in the 0% treatment: the number of *Escherichia coli* colonies was not given the concentration of the ethyl acetate fraction of basil leaves
c. Number of colonies for each concentration:
d. Number of *Escherichia coli* colonies after treatment with basil leaf ethyl acetate fraction with concentrations of 12.5%, 25%, and 50%.

2.3. Data Analysis

2.3.1. Univariate Analysis

Description of the research results carried out testing of mean, median, maximum data, and minimum data.

2.3.2. Bivariate Analysis

Data distribution was analyzed statistically using the Shapiro-Wilk test. This test shows abnormal data distribution ($p<0.05$). Next, data transformation was carried out, but the distribution was still abnormal. Then, a homogeneity test was carried out to obtain homogeneity. The following analysis was carried out using the non-parametric Kruskal walls test, which was continued with the Mann-Whitney difference test.

3. Results and Discussion

3.1. Results

Testing the inhibition zone of the ethyl acetate fraction of basil leaves (*Ocimum basilicum* L) against *Escherichia coli* was carried out at 12.5%, 25%, and 50% and Co-trimoxazole five times repetition. Table 1 shows the inhibition zones of the ethyl acetate fraction of basil leaves (*Ocimum basilicum* L) against *Escherichia coli* bacteria.

Table 1 Results of treatment with five repetitions of the ethyl acetate fraction of basil leaves with three concentrations

Treatment	**Repetition to-**				
	1	**2**	**3**	**4**	**5**
Control (+)	0	0	0	0	0
Control (-)	450x 10^9	329 x 10^9	420 x 10^9	372x 10^9	465x 10^9
12,5%	296x 10^9	302 x 10^9	310x 10^9	310x 10^9	260x 10^9
25%	206x 10^9	251x 10^9	230x 10^9	230x 10^9	185x 10^9
50%	112x 10^9	80 x 10^9	76 x 10^9	76 x 10^9	104x 10^9

Based on Table 1, it can be seen that the five samples (positive control, negative control, 12.5% concentration, 25% concentration, and 50% concentration) showed the highest number of *Escherichia coli*, namely a concentration of 12.5% with an average value of 290x10^9 and the lowest number of *E.Coli* bacteria was concentration 50% with an average value of 94 x 10^9. At a concentration of 25%, the average number of *Escherichia coli* was 213 x 109. In the negative control, the number of E.Coli bacteria The average is 407 x 10^9, while in the positive control, it is 0.

Table 2 Average number TPC (*Total Plate Count*)

Treatment	**N**	**Mean**	**Std.Deviation**	***P Value***
K (+)	5	0		
K (-)	5	40720000000	5632672545	
P1	5	28960000000	1986957473	0,000
P2	5	21340000000	2687563953	
P3	5	4370600000	5876028914	
Total	25	19078120000	15899194180	

The Kruskal-Wallis test obtained significant results in the control and treatment groups with a p-value of 0.000 (<0.050). The minimum inhibitory concentration was calculated to determine the lowest concentration of the ethyl acetate fraction of basil leaves, which inhibited the growth of Escherichia coli bacteria.

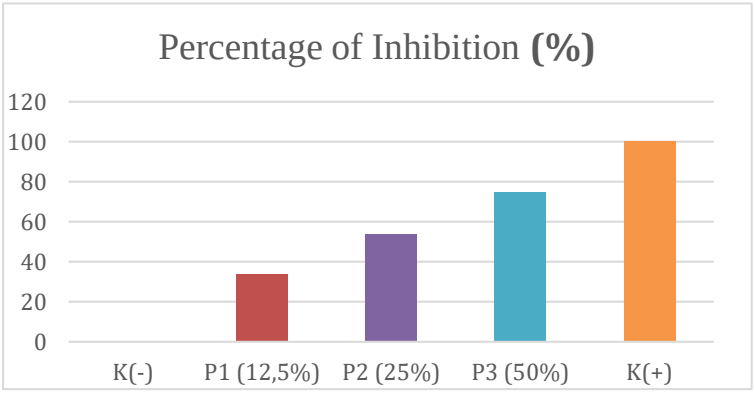

Figure 1 Percentage of Inhibition of *Escherichia Coli* Bacteria

Figure 1 shows that the Minimum Inhibitory Content (MIC) is 12.5% because the concentration of the fraction with the highest number of remaining bacteria among the other concentrations is 28960000000. The next test was carried out by Mann-Whitney analysis to see the differences in each concentration of the ethyl acetate fraction of basil leaves on the growth of Escherichia coli bacteria, as seen in Table 3.

Table 3 Difference test between groups of treatment of ethyl acetate fraction of basil leaves on the growth of *Escherichia coli*

Concentration	Concentration	Z	p value
K(+)	K(-)	-2.785	0.008
	P1	-2.785	0.008
	P2	-2.785	0.005
	P3	-2.785	0.005
K(-)	K(+)	-2.785	0.008
	P1	-2.611	0.009
	P2	-2.611	0.008
	P3	-2.611	0.008
P1	K(+)	-2.785	0.008
	K(-)	-2.611	0.009
	P2	-2.611	0.009
	P3	-2.611	0.009
P2	K(+)	-2.785	0.005
	K(-)	-2.611	0.008
	P1	-2.611	0.009
	P3	-2.611	0.009
P3	K (+)	-2.785	0.005
	K (-)	-2.611	0.008
	P1	-2.661	0.009
	P2	-2.611	0.009

Table 3 shows that there are variations in results between concentrations. Significant results between treatment groups are all smaller than 0.050. So, there is a significant difference in concentration between treatments.

3.2. Discussion

Comparative results of testing various concentrations of the ethyl acetate fraction of basil leaves (*Ocimum basillicum* L) to inhibit the growth of *Escherichia coli* bacteria using the liquid dilution technique. To determine the comparative effect of administering various doses of the ethyl acetate fraction of basil leaves (*Ocimum basillicum* L), the sample was diluted four times (up to (10-8) by taking 1 ml of each bacteria sample and then mixed in 9 ml of sterile distilled water and homogenized with a vortex mixer. The results of the last two dilutions were then planted using the spread plate technique on nutrient agar media and incubated for 24 hours at 37^0C, then the number of colonies was counted using a colony counter. The colony count was done using the "Standard Plate Count" by counting from a plate and selecting the number of colonies in the sample (Fruin et al., 1977). An increase in the concentration of the ethyl acetate fraction of basil leaves was followed by an increase in the inhibitory power against the growth of Escherichia coli, which can be seen in Figure 1. Based on the results of this research (Figure 1), it was found that the ethyl acetate fraction of basil leaves can inhibit antibacterial activity. It has been proven that at concentrations of 12.5%, 25%, and 50%, it has potential as an antibacterial that can inhibit Escherichia coli bacteria. The higher the concentration, the higher the inhibition. This inhibitory ability is due to secondary metabolites.

Based on Table 1, it can be seen that the five samples (positive control, negative control, 12.5% concentration, 25% concentration, and 50% concentration) showed the highest number of *Escherichia coli*, namely a concentration of 12.5% with an average value of $290x10^9$ and the lowest number of *Escherichia coli* bacteria was concentration 50% with an average value of 94 x 109. At a concentration of 25%, the average number of *Escherichia coli* was 213 x 109. In the negative control, the number of *Escherichia coli* bacteria The average is 407 x 109, while in the positive control, it is 0. The aim of knowing the average number of TPCs is to determine the potential of various concentrations of basil leaves in inhibiting the growth of *Escherichia coli* bacteria. The average results of TPC calculations and Kruskal-Wallis test analysis in the control and treatment groups can be seen in Table 2.

The research results (Table 2) show that the average number of *Escherichia coli* bacteria at a concentration of 12.5% was higher than the number of bacteria at a concentration of 50%. Based on the results of this research, it was found that the ethyl acetate fraction of basil leaves can inhibit antibacterial activity. It has been proven that at concentrations of 12.5%, 25%, and 50%, it has potential as an antibacterial that can inhibit Escherichia coli bacteria. The higher the concentration, the higher the inhibitory power for bacterial growth. This inhibitory ability is due to the chemical compounds contained in basil leaves. Inhibition of *Escherichia coli* bacteria growth is caused by the active substances contained in the Ethyl Acetate Fraction of basil leaves, such as alkaloid compounds and flavonoid compounds (Mustika, 2014). Inhibitory activity against bacteria Pseudomonas aeruginosa and Staphylococcus aureus is better produced by the ethyl acetate fraction of basil leaves, where the ethyl acetate fraction of basil leaves can provide inhibition in the thick fraction, 100% concentration (Annisa, 2021).

The ability of flavonoid compounds to act as antibacterial by denaturing and damaging bacterial cell proteins. Bacterial and bacterial cell membranes release their intracellular compounds so that damage occurs and the damage cannot be repaired. Flavonoids are derivatives of phenolic compounds that can interact with bacterial cells by absorption, which involves hydrogen bonds. Phenol forms protein complexes with weak bonds at low levels, followed by penetration of phenol into cells, which can cause protein deposition and denaturation. Apart from that, phenol can inhibit the activity of bacterial enzymes, which ultimately disrupts the metabolism and survival process of the bacteria. 15 The mechanism of action of flavonoids can increase membrane permeability and loss of bacterial cell membrane potential (Croppi et al., 2020). Phenolic compounds that bind to bacteria will disrupt

membrane permeability, resulting in cations and macromolecules from cells being lost and causing disruption of cell growth and death (Larasati & Apriliana, 2016). In addition, phenolic compounds have been studied for their antibacterial. Phenolic compounds have a dual mechanism in inhibiting bacterial growth, namely by interacting with proteins and bacterial walls, causing damage to the cytoplasmic membrane, inhibiting nucleic acid synthesis, reducing membrane fluidity, wall synthesis or cell energy metabolism (Slobodníková et al., 2016). The positive control that the researchers used was co-trimoxazole. Co-trimoxazole is an antibiotic that contains two compositions, namely trimethoprim, and sulfamethoxazole, which work to inhibit obligate enzymatic reactions in two consecutive stages in microbes so that the combination of these two drugs provides a synergistic effect (Mogi et al., 2016).

4. Conclusion

The ethyl acetate fraction of basil leaves has the potential to inhibit the growth of Escherichia coli bacteria. There is a significant difference in each concentration of the ethyl acetate fraction of basil leaves on the growth of Escherichia coli bacteria with a p-value < 0.050. The antibacterial test results showed it shows that the ethyl acetate fraction of basil leaves has a MIC value of 12.5% against *Escherichia coli* bacteria.

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