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Testing Antibacterial Activity of Daun Kentut (*Paederia foetida* L.) Leaf Extract against *Escherichia coli* in Vitro

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Abstract

Escherichia coli is a pathogenic bacterium that causes infectious diseases in the digestive tract and can produce Lipopolysaccharide (LPS), including lipid A. Exposure to endotoxins in lipid A, can cause systemic effects, such as sepsis, which can lead to clinical manifestations and even death. Daun Kentut (*Paederia foetida* L.) is one of the herbal plants containing bioactive compounds that can inhibit the growth of *E. coli* bacteria. The aim of this research is to determine the effective concentration of daun kentut leaf extract to inhibit *E. coli* growth. This study is an experimental research using maceration extraction method and antibacterial disc diffusion method with 3 repetitions at concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, negative control (aquadest), and positive control (ciprofloxacin) conducted from March to April 2023 in the Microbiology Laboratory of Kadiri University. The observation results of inhibition zones in each treatment varied. The presence of these inhibition zones is due to the presence of secondary metabolite compounds in daun kentut leaf extract that have antibacterial activity, such as inhibiting protein synthesis mechanisms, causing damage to cell wall permeability, and ultimately leading to bacterial cell lysis and death. The statistical analysis using the One Way Anova test with a P-value of 0.05 shows that each variable has significant differences and effects. Thus, it can be concluded that the effective extract variation is the 100% concentration because, in this study, the 100% concentration has an inhibition zone of 26.72 mm and is classified as very strong in inhibiting *E. coli* bacteria.

Keywords: Antibacterial; *Eschericia coli*; Daun Kentut Leaf Extract.

Abbreviations: Antimicrobial resistance (AMR), LAF (Laminar Air Flow), Lipopolysaccharide (LPS), Nutrient Agar (NA).

INTRODUCTION

Escherichia coli is a pathogenic bacterium that causes infectious diseases in the digestive tract, including acute diarrhea that can affect anyone (Patel, 2017). *E. coli* bacteria produce toxins that can damage the mucosal cells of the small intestine, leading to clinical symptoms such as watery diarrhea, abdominal cramps, mild fever, nausea, and malaise (Tuntun, 2016). *E. coli* ATCC 25922 is a pathogenic microorganism that commonly infects humans, causing diseases such as diarrhea, meningitis, and others. E. coli causes up to 52% of infectious diseases. The spread of *E. coli* ATCC 25922 can occur through hand-to-mouth contact or passive transfer via food or drink intermediaries (Savitri et al., 2020).

Lipopolysaccharide (LPS) is a product produced by *E. coli* that forms the outer bacterial membrane, consisting of specific-O polysaccharides, core polysaccharides, and lipid A. The toxin activity of endotoxin occurs in lipid A. Exposure to endotoxin can cause systemic effects such as changes in blood pressure and body temperature, coagulation abnormalities, decreased circulating leukocyte, and platelet counts caused by hemagglutinin and hemolysin, which are virulence factors in the infection process leading to tissue damage (Divakara et al., 2017). Another role of endotoxin resulting in death is cardiovascular instability and septic shock leading to death from bacterial infection and each body's ability to respond to infection by gram-negative bacteria (Savitri et al., 2022). The systemic effects include bleeding, immune system disorders, sepsis, and death (Purwanto, 2018).

In 2017, there were 48.9 million cases related to sepsis and 11 million deaths worldwide, accounting for nearly 20% of global deaths (Rudd et al., 2020). Sepsis occurs annually in children under 5 years old at a rate of 44%, and a quarter of these cases are due to neonatal sepsis (Yustika et al., 2020). The incidence of sepsis in Indonesia remains high at 30.29%, with a mortality rate ranging from 11.56% to 49% (Batara et al., 2018).

The treatment of infectious diseases caused by bacteria typically involves synthetic antibiotics such as ampicillin, gentamicin, and metronidazole. The

excessive use of these antibiotics can lead to the rapid development of antimicrobial resistance (AMR) (Simmons et al., 2020). These drugs can cause side effects such as rash, diarrhea, abdominal cramps, and hypersensitivity. Synthetic antibiotic costs are also expensive for lower-income groups. Therefore, further research is needed on potential herbal materials to replace synthetic antibiotic drugs.

Some people have adopted the use of herbal plants for treatment because the materials are readily available and affordable for all segments of society. Over time, medicinal plants can be developed as traditional medicines, including daun kentut (*Paederia foetida* L). Daun kentut is a source of important natural products and chemical materials for health. It contains bioactive compounds such as flavonoids, terpenoids, paedolone, β-sitosterol, friedelin, campesterol, methyl mercaptan, and other active compounds (Patel, 2017).

Research on the antibacterial activity test of aloe vera extract against *E. coli* proved that aloe vera extract has no antibacterial effect due to the type of plant and the amount of antibacterial substance content in aloe vera extract, rendering it ineffective against *E. coli* bacteria (Suryati et al., 2017). Various herbal medicines tested by Rahmawati et al. (2017) showed that the concentration of extract with the highest inhibitory effect was turmeric extract at 5.64 mm. However, this antibacterial activity was still moderate.

In Utary's study (2016), an extract of daun kentut was tested against bacteria Sigella sonnei and Salmonella typhi, and chemical analysis of the leaf and stem of the kentut plant was also conducted by Handrianto (2018). However, research on the antibacterial activity test of daun kentut extract against *E. coli* is still rare. Therefore, the researcher aims to investigate further the effectiveness of daun kentut in inhibiting the growth of *E. coli* bacteria.

MATERIALS AND METHODS

Research Design

The type of research used is quantitative experimental with the disc method.

Population and Sample

The population in this study is *Escherichia coli* bacteria. The sample used in this study is wild-type *Escherichia coli* ATCC 25922 bacteria obtained from Nano Lab Laboratory with number R4607050.

Research Variables

The independent variable in this study is the sembukan leaf extract with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% (Juliastuti et al., 2020). The dependent variable in this study is the growth of *E. coli* bacteria on agar media, which is then measured by the inhibition zones visible from the clear zones formed. Control variables include: incubation temperature, medium content, and incubation duration.

Tools and Materials

The tools used in this study include digital balance (SS-A 2000), disc paper, glassware (pyrex), incubator (FCD-3000 Series), autoclave (B-ONE AA 18 litres), Vernier calliper, and LAF (Laminar Air Flow). This research uses materials such as sembukan leaves obtained from Melaten Hamlet, Ngrami Village, Sukomoro District, Nganjuk Regency, East Java, 96% ethanol, aquades as a solvent for media and extracts, label paper, wool thread, cover paper, for bacterial growth media, Nutrient Agar is used, wild-type *Escherichia coli* ATCC 25922 bacteria with a dose of 10^5 CFU/mL from Nano Lab Laboratory, and ciprofloxacin as a positive control.

Procedures

Extract Preparation

The preparation of sembukan leaf extract was carried out using the maceration method. 1 kg of sembukan leaves (simplisia) was placed in a maceration container and then added with 3 L of 96% ethanol in a ratio of 1:3. The mixture was left in a place protected from sunlight for 3 days, stirred every 12 hours, and then filtered. The residue was remacerated (re-maceration) once with the same solvent as before. The maceration and filtration process from maceration and remaceration were collected and evaporated to obtain a concentrated extract.

Medium Preparation

It is known that 2.8 grams of Nutrient Agar (NA) is used for 100 mL of aquades. Since each Petri dish will be filled with 25 mL of media (15 mL for the first layer, 10 mL for the second layer) and 12 Petri dishes will be prepared, 8.4 grams of NA is required in 300 mL of aquadest.

Bacterial Inoculation

Escherichia coli bacteria were inoculated into the medium using the pour plate method (tube or pour method).

Preparation of Daun Kentut Leaf Extract Concentrations

Different concentrations of the extract were prepared as follows:

a. 10% concentration: by mixing 0.2 mL of extract with aquades up to the 2 mL mark.
b. 20% concentration: by adding 0.4 mL of extract with aquadest up to 2 mL.
c. 30% concentration: by adding 0.6 mL of extract with aquadest up to 2 mL.
d. 40% concentration: by adding 0.8 mL of extract with aquadest up to 2 mL.
e. 50% concentration: by adding 1 mL of extract with aquadest up to 2 mL.

f. 60% concentration: by adding 1.2 mL of extract with aquadest up to 2 mL, and so on.
g. 100% concentration: 2 mL of extract without adding aquadest.
h. Positive control preparation involved weighing 100 mg of ciprofloxacin dissolved in 2 mL of aquadest to make a 5% solution.

Treatment

Inoculated *E. coli* bacteria onto agar media before solidification using the pour plate technique. After the media solidified, circular filter papers with a diameter of 0.6 cm were soaked in each concentration of the extract. The filter papers were then placed on the solidified NA media in 4 different Petri dishes. Each dish was labelled as follows:

a. P1: 10% Concentration
b. P2: 20% Concentration
c. P3: 30% Concentration
d. P4: 40% Concentration
e. P5: 50% Concentration
f. P6: 60% Concentration
g. P7: 70% Concentration
h. P8: 80% Concentration
i. P9: 90% Concentration
j. P10: 100% Concentration
k. K-: Aquadest (Negative Control)
l. K+: Ciprofloxacin (Positive Control)

Data Analysis

After 24 hours of bacterial incubation and disc placement, observations and calculations of the area of transparent zones were performed by measuring the clear area formed at the edges of the placed paper disks twice, vertically and horizontally. The obtained results were averaged using the following formula (Dwi, 2019):

Zone of inhibition calculation formula = (AB) + (CD) / 2. Subsequently, the obtained data were processed and analyzed using Statistical Package for the Social Sciences (SPSS) version 26.0 for Windows for ANOVA testing. Normality and homogeneity tests were conducted with a p-value > 0.005. If the data were significant, One-Way ANOVA testing was performed with a significance value of p < 0.005. After obtaining significant differences in the data, further testing was conducted using Post Hoc Test LSD.

RESULTS AND DISCUSSION

Zone of Inhibition Diameter

The clear zones around the discs were measured using a calliper. The total diameter was subtracted by the disc diameter of 6 mm to obtain the average zone of inhibition as shown in Table 1.

Table 1. Results of Inhibition Test.

Replication	Inhibition Zone Hambat (mm)											
	K+	K-	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
1	37.10	0.00	4.35	7.60	8.30	8.00	8.55	9.35	10.55	16.70	19.15	26.00
2	35.00	0.00	5.65	5.35	8.30	6.70	10.90	10.40	14.45	16.40	18.40	26.45
3	37.30	0.00	5.60	6.70	3.70	6.20	5.90	11.45	9.25	19.90	21.30	27.70
Average	**36.47**	**0.00**	**5.20**	**6.55**	**6.77**	**6.97**	**8.45**	**10.40**	**11.42**	**17.67**	**19.62**	**26.72**

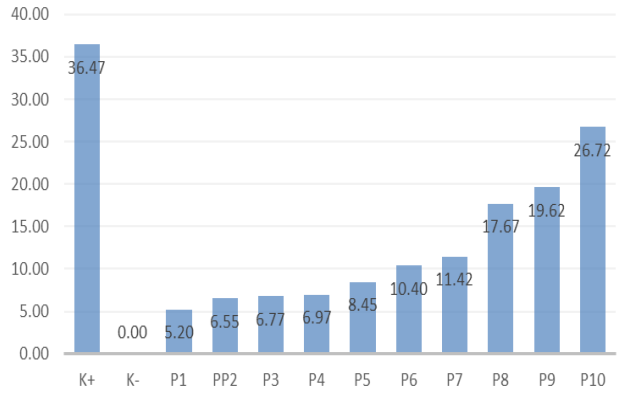

Figure 2. Average Diameter of Inhibition Zones Diagram.

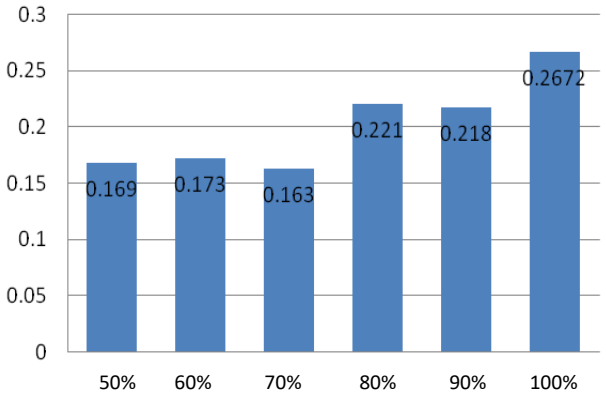

Figure 3. Extract Performance Effectiveness Comparison Diagram.

Normality Test

The data obtained were analyzed using SPSS 26.0 for Windows. The Shapiro-Wilk normality test results can be seen in Table 2, indicating that the significance value is > a (0.05), thus confirming that the data are normally distributed.

Table 2. Normality Test Results.

Tests of Normality							
		Kolmogorov-Smirnov[a]			*Shapiro-Wilk*		
	Group	***Statistic***	***df***	***Sig.***	***Statistic***	***Df***	***Sig.***
Inhibitation zone	Concentration of 10%	.357	3	.	.815	3	.150
	Concentration of 20%	.	3	.	.	3	.
	Concentration of 30%	.373	3	.	.779	3	.065
	Concentration of 40%	.280	3	.	.938	3	.520
	Concentration of 50%	.385	3	.	.750	3	<.001
	Concentration of 60%	.183	3	.	.999	3	.934
	Concentration of 70%	.219	3	.	.987	3	.780
	Concentration of 80%	.175	3	.	1.000	3	1.000
	Concentration of 90%	.324	3	.	.877	3	.315
	Concentration of 100%	.385	3	.	.750	3	<.001
	Positive Control	.288	3	.	.928	3	.481
	Negative Control	.286	3	.	.931	3	.493

a. *Lilliefors Significance Correction*

Homogeneity Test

Next, a homogeneity test was conducted. From Table 3, the significance value obtained was 0.054, which is greater than the chosen value (0.05), indicating that the data are homogeneously distributed.

Table 3. Homogeneity Test Results.

Tests of Homogeneity of Variances					
		Levene Statistic	***df1***	***df2***	***Sig.***
Inhibiton zone	*Based on Mean*	2.180	11	24	.054
	Based on Median	.397	11	24	.944
	Based on Median and with adjusted df	.397	11	9.780	.927
	Based on trimmed mean	1.964	11	24	.081

ANOVA Test

An ANOVA test was performed to determine whether the independent variable influences the independent variable. Table 4 below demonstrates this.

Table 4. ANOVA Test Results.

ANOVA					
Inhibiton zone					
	Sum of Squares	*Df*	*Mean Square*	*F*	*Sig.*
Between Groups	35.708	11	3.246	141.675	<.001
Within Groups	.550	24	.023		
Total	36.258	35			

Discussion

Antibacterial Activity Test in this study aims to determine the effective concentration of *Paederia foetida* leaf extract to inhibit the growth of *E. coli* ATCC 25922, as indicated by the inhibition zones at various extract concentrations using the disc diffusion method. This method was chosen because disc diffusion testing can be performed more quickly, and the materials used are readily available. Research conducted by Ratnasari et al. (2020) demonstrated that the disc diffusion method yielded larger inhibition zones compared to the well method. The well method often produces smaller inhibition zones due to difficulties during execution, such as the potential for media cracking or fracturing around the wells, which can interfere with the extract diffusion process and affect the clarity of the formed zones. In contrast, the disc diffusion method allows for better absorption of *Paederia foetida* leaf extract into the media, minimizing execution difficulties or errors.

Extraction in this study was conducted using the maceration method, where dried *Paederia foetida* leaves were blended after air-drying. Maceration involved soaking the leaves in 96% ethanol at a ratio of 1:3 for three intervals of 24 hours each. The extract was then filtered and evaporated using a water bath to obtain a concentrated extract. The extract was applied by soaking filter paper discs with a diameter of approximately 6 mm in the extract for about 15 minutes. One of the critical variables that can influence antibacterial activity testing is the duration of filter paper disc immersion. With a 15-minute immersion period, the filter paper discs adequately absorbed the extract, resulting in maximal inhibition zone diameters, as depicted in Figure 5.1. The discs were then placed on the surface of agar plates inoculated with bacteria and incubated at 37°C for 24 hours. This temperature is suitable for the growth of *E. coli* ATCC 25922, with 37°C being the optimal temperature, according to Kurniati et al. (2020). Temperatures above 44°C can lead to *E. coli* inactivation.

After 24 hours of incubation, the inhibition zones were observed and measured using a calliper for accurate millimetre measurements. The obtained results showed that the formed inhibition zones were not entirely clear, indicating the presence of other bacteria around the filter paper discs. This occurrence could be due to some non-sterile tools and materials used, especially in handling the filter paper discs and forceps used to place them. The measured results revealed the average inhibition zone diameters, where K+ (ciprofloxacin) exhibited a 36.47 mm inhibition zone. K- showed slight clarity around the disc during the second replication due to contamination during disc placement. P1 (10% concentration) exhibited a 5.20 mm inhibition zone, categorized as moderate. P2 (20% concentration) had a 6.55 mm inhibition zone, also categorized as moderate. The results of this study differed from those of Dewi et al. (2019), where the 40% concentration yielded a 14.05 mm inhibition zone categorized as strong. This difference could be attributed to the different extraction solvents used; Dewi used ethanol, likely contributing to bacterial growth inhibition. However, this study's findings were consistent with Nurhalimah et al. (2019), where a 90% concentration yielded a 10.20 mm inhibition zone, categorized as strong.

For P3 (30% concentration), the inhibition zone was 6.77 mm, categorized as moderate. P4 (40% concentration) had a 6.97 mm inhibition zone, also categorized as moderate. P5 (50% concentration) exhibited an 8.45 mm inhibition zone, categorized as moderate. P6 (60% concentration) had a 10.4 mm inhibition zone, categorized as strong. P7 (70% concentration) exhibited an 11.42 mm inhibition zone, categorized as strong. P8 (80% concentration) showed a 17.67 mm inhibition zone, categorized as strong. P9 (90% concentration) had a 19.62 mm inhibition zone, categorized as strong. Meanwhile, P10 (100% concentration) exhibited a 26.72 mm inhibition zone, categorized as very strong. Adha et al. (2021) stated that increasing extract usage corresponds to an increase in secondary metabolites contained within the extract. This assertion is supported by Javed et al. (2020), highlighting that higher extract concentrations significantly affect antibacterial compound content, allowing these compounds to diffuse into bacterial cells. Compared to previous studies, this research yielded significantly larger inhibition zones.

This inhibition is attributed to the secondary metabolites in the extract damaging cell structures by inhibiting protein synthesis mechanisms, such as saponins, leading to reduced surface tension and increased permeability, causing intracellular compounds to exit and bacterial cell lysis. Additionally, tannins can disrupt polypeptide synthesis, resulting in imperfect cell wall formation. Flavonoids inhibit bacterial growth by affecting nucleic acid synthesis, membrane function, and energy metabolism, leading to permeability damage in cell walls, microsomes, and lysosomes. Other compounds like phenols, alkaloids, and steroids also contribute to this antibacterial activity process, resulting in the formation of clear zones around the discs. According to Torar et al. (2017), gram-negative bacteria have thinner peptidoglycan layers compared to gram-positive bacteria. Additionally, gram-negative bacteria have cell walls containing a high amount of lipopolysaccharides (LPS). Compounds present in *Paederia foetida* leaf extract damage the cell walls by penetrating LPS (Vitalia, 2019).

From the results obtained, it is known that all concentrations ranging from 10% to 100% exhibit inhibitory activity. The strongest inhibition was observed at the 100% concentration. In this study, the spacing between the discs was 2 cm with a 1.5 cm edge distance, resulting in overlapping at the 100% concentration. This can also be due to the excessive concentration of the extract. The inhibition zone at the 100% concentration could be larger if the discs were placed according to guidelines, with a 3 cm distance between discs and a 2 cm edge distance (Windiyanti, et al., 2023). Nevertheless, the inhibition zone diameter at 100% concentration was not larger than that of ciprofloxacin, which is a synthetic antibiotic with potent antibacterial activity against both gram-negative and gram-positive bacteria.

After obtaining the data, further analysis was conducted using SPSS 26.0 for Windows. The homogeneity test of the three data samples showed a homogeneous distribution with a value of $p<0.05$. Subsequently, a normality test was performed, which indicated a value of $p<0.05$, confirming a normal distribution of the samples. In the ANOVA test, it was found that the significance value was <0.05, indicating that H0 was rejected and H1 was accepted, meaning there is an influence between the independent variables on the dependent variable simultaneously. There were

significant differences in the average values of each treatment, as described in the post hoc test table. The LSD post hoc test results showed significant differences in each treatment. However, some concentrations had significance values >0.05 compared to other concentrations, specifically the 30% concentration compared to the 40%, 50%, and 60% concentrations. This indicates that the comparison of these concentrations was not statistically significant.

These significant differences were reinforced by the comparison of extract performance effectiveness at concentrations categorized as strong to very strong, specifically the 50%, 60%, 70%, 80%, 90%, and 100% concentrations as shown in Figure 5.3. The graph illustrates that concentrations with high-performance effectiveness are the 80% and 100% concentrations. The 90% concentration has a larger average inhibition zone than the 80% concentration, but the 80% concentration demonstrates greater extract performance effectiveness.

CONCLUSIONS

Based on the research results on the antibacterial activity test of *Paederia foetida* leaf extract against *E. coli*, it can be concluded that *Paederia foetida* leaf extract affects the growth of *E. coli* ATCC 25922. Various concentrations exhibited different levels of inhibitory activity, where higher concentrations showed greater ability to inhibit the growth of *E. coli* ATCC 25922. This is evidenced by the data showing that in treatments P1-P10, the inhibition zones were as follows: P1= 5.20 mm, P2= 6.55 mm, P3= 6.77 mm, P4= 6.97 mm, P5= 8.45 mm, P6= 10.40 mm, P7= 11.42 mm, P8= 17.67 mm, P9= 19.62 mm, P10= 26.72 mm. From the average inhibition zone sizes, it is concluded that *Paederia foetida* leaf extract can be considered a candidate drug for preventing diseases caused by *E. coli*. However further research with various developments is needed to obtain an effective treatment for diseases caused by *E. coli*.

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