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## Antibacterial Activity Test Of Extract, N-Hexane Fraction, Ethyl Acetate And Water Of Kesum Leaves (*Polygonum Minus H*) Against *Escherichia Coli* ATCC 25922 Bacteria

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### Abstract

*Diarrheal disease caused by Escherichia coli is a serious health problem in Indonesia, especially in toddlers, with increasing antibiotic resistance. Kesum leaves (Polygonum minus H.) have antibacterial potential because they contain flavonoids, phenols, alkaloids, and saponins. To evaluate the antibacterial activity of 70% ethanol extract and fractions of n-hexane, ethyl acetate, and kesum leaf water against E. coli ATCC 25922, to identify effective concentrations, and to determine the MIC and MBC of the most active fraction. Laboratory experimental study with 70% ethanol maceration extraction and liquid-liquid fractionation. E. coli ATCC 25922 population, 7 kg of simplex samples from Sambas, West Kalimantan. Instruments include an incubator, SPSS 25. Analysis techniques: disc diffusion, liquid/solid dilution, ANOVA. The ethyl acetate fraction was the most effective (inhibition zone 12.36 mm, at a concentration of 40%, strong category), followed by the extract (inhibition zone 11.31 mm, at a concentration of 30%, medium category), n-hexane (10.46 mm), water (8.86 mm). MIC and MBC of the ethyl acetate fraction were 30%. The extract and fraction of kesum leaves have moderate to strong antibacterial activity against E. coli ATCC 25922, supporting the development of anti-diarrhea herbal medicine.*

**Keywords:** Antibacterial Activity, *Escherichia Coli*, Ethyl Acetate Fraction, Kesum Leaves, Minimum Inhibitory Concentration.

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### INTRODUCTION

Diarrheal disease is a major health problem in Indonesia, especially among children under five in developing regions, with prevalence decreasing from 18.5% in 2013 to 12.3% in 2018, but still causing significant mortality due to dehydration and pathogenic infections such as *Escherichia coli*. This gram-negative *E. coli* bacterium is often the main cause of acute diarrhea due to contamination of food and water contaminated with human or animal feces, with a national incidence reaching 270 per 1,000 population and extraordinary cases of 21 incidents in 2018 [Sihaloho, 2024].

The prevalence of diarrhea caused by *E. coli* remains high in children aged 0-5 years, reaching 29.53% of cases in Bima, West Nusa Tenggara, driven by poor sanitation and unhygienic lifestyles such as lack of handwashing [Fatonah et al., 2022]. According to the WHO (2021), diarrhea causes 525,000 global under-five deaths annually, with *E. coli* being a major contributor through its intestinal-damaging toxins [Zara Fitriany, 2021].

*E. coli* infections cause serious digestive disorders if the number exceeds normal limits, often found in food due to poor hygiene, thus requiring effective antimicrobial intervention [Sihaloho, 2024]. Increasing antibiotic resistance exacerbates this problem, encouraging the exploration of traditional medicinal plants such as kesum leaves (*Polygonum minus Huds.*) which are rich in flavonoids, phenols, alkaloids, and saponins as potential antibacterial agents [Kartikasari et al., 2022][Kartikasari, 2024].

The use of ethanol extract and fraction of kesum leaves has not been tested specifically against *E. coli* ATCC 25922, different from previous studies that focused on essential oils or single fractions against other bacteria such as *Salmonella* or *Staphylococcus* [Effan C. Junaedi et al., 2023].

This study aims to evaluate the antibacterial activity of 70% ethanol extract, n-hexane fraction, ethyl acetate, and water of kesum leaves against *E. coli* ATCC 25922 using the disc diffusion method for the inhibition zone, and liquid dilution to determine the MIC and MBC of the most active fraction. The urgency lies in the need for alternative natural medicines to face antibiotic resistance and the high prevalence of diarrhea in Indonesia, potentially supporting the development of new herbal medicines

[WHO, 2021]. The novelty is the comprehensive test of the four forms of extracts/fractions on standard strains of *E. coli* that cause diarrhea, which has not been done before [Kartikasari, 2024][Kartikasari et al., 2023].

## RESEARCH METHODS

### Types and Methods of Research

This research is a laboratory experimental study with a quantitative approach that aims to test the antibacterial activity of 70% ethanol extract of kesum leaves (*Polygonum minus* Huds.), and its fractions (n-hexane, ethyl acetate, and water) against *Escherichia coli* ATCC 25922 bacteria using the maceration method for extraction and liquid-liquid partition for fractionation [Sugiyono, 2021]. The quantitative approach was chosen because it allows objective measurement of variables such as inhibition zone diameter, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) through Kirby-Bauer disc diffusion tests and liquid and solid dilutions, according to a systematic pre-post test experimental design to assess the effect of independent variables on dependent variables [Sudaryono, 2021][Triyanti et al., 2025]. This method is comprehensive because it integrates phytochemical screening to confirm active compounds such as flavonoids and phenols, which support the validity of antibacterial results [Kartikasari et al., 2022][Creswell & Creswell, 2023].

### Data Analysis Instruments and Techniques

The main instruments include laboratory equipment such as autoclaves for sterilization, incubators, Petri dishes, antibiotic paper discs, and microscopes for antibacterial testing, with materials in the form of simple kesum leaves, 70% ethanol solvent, n-hexane, ethyl acetate, distilled water, Nutrient Agar (NA) media, Nutrient Broth (NB), 1% DMSO, and *E. coli* ATCC 25922 culture [Maslahah, 2024]. Data analysis techniques include qualitative measurements of the disc diffusion inhibition zone (interpretation: very strong >20 mm, strong 10-20 mm, moderate 5-10 mm, weak <5 mm), quantitative MIC/MBC via dilution, as well as the Shapiro-Wilk statistical test for normality, Levene for homogeneity, and One-Way ANOVA with SPSS 25 for significant differences between groups at a significance level of  $\alpha = 0.05$  [Emzir, 2022]. This approach ensures reliability through three-fold replication and validity with positive (ciprofloxacin) and negative (DMSO) controls, in line with the principles of multivariate analysis in experimental research [Sugiyono, 2021][Octifani et al., 2024].

### Population and Sample

The study population was a strain of pathogenic bacteria that causes diarrhea, specifically *E. coli* ATCC 25922 as a representative of normal intestinal flora that is pathogenic when excessive, obtained from an accredited laboratory collection with a certificate of authenticity [Sihaloho, 2024]. Samples were taken by purposive sampling with one pure strain of *E. coli* ATCC 25922, suspended in McFarland standard 0.5 ( $1.5 \times 10^8$  CFU/mL) for testing, as well as simplex of kesum leaves from Teluk Keramat, Sambas, West Kalimantan, determined at the Faculty of Mathematics and Natural Sciences, Tanjungpura University, Pontianak, resulting in 1,075 kg of dry simplex for extraction [Kartikasari, 2024]. The purposive technique is suitable for laboratory experiments because it focuses on the specific characteristics of the sample, minimizes bias and maximizes the generalization of antibacterial results [Sudaryono, 2021][Creswell & Creswell, 2023].

### Research Procedures

The procedure begins with plant determination and collection of 7 kg of fresh kesum leaves, followed by the preparation of simplicia through sorting, washing, chopping, oven drying at 40-50°C, and grinding, with organoleptic standardization, water content (<10%), and drying loss [Maslahah, 2024]. Extraction of 70% ethanol maceration (1:10) for 5 days, rotary evaporation, produced a thick extract (the yield was calculated), followed by phytochemical screening (alkaloids, flavonoids, saponins, triterpenoids, phenols) and liquid-liquid fractionation (n-hexane, ethyl acetate, water) with

solvent-free standardization [Raveilliani et al., 2021]. Antibacterial testing: autoclave sterilization at 121°C/15 minutes, bacterial culture rejuvenation at 37°C/24 hours, Gram staining, bacterial suspension, disc diffusion (20%, 30%, and 40% concentration), incubation at 37°C/24 hours for inhibition zone, liquid dilution (MIC) and solid dilution (MBC) of 30%, 15%, 7.5%, and 3.75%, and statistical analysis [Nurul et al., 2023]. All steps followed the standard protocol flow for reproducibility [Emzir, 2022][Syamsul et al., 2020].

## RESULTS AND DISCUSSION

### Determination of Kesum Leaves (*Polygonum minus* H)

Based on the results of the determination of kesum leaves carried out at the Head of the Laboratory of FMIPA Tanjungpura University Pontianak West Kalimantan, it can be seen that the plants used in this study are truly kesum leaves (*Polygonum minus* H) from the species *Persicaria minor* (Huds.) Opiz, Synonym *Polygonum minus* Huds, Family Polygonaceae. Determination aims to find out the type of plant to be used is appropriate and there are no errors in sampling.

### Sampling

Sampling of kesum leaves (*Polygonum minus* H) was taken from Teluk Keramat District, Sambas Regency, West Kalimantan Province.

### Making Simple Drugs

In the manufacture of kesum leaf simplicia, 7 kg of fresh plants were obtained and then wet sorted. The washed kesum leaves were then drained, then dried in the sun covered with a black cloth which aims to avoid direct exposure that could potentially damage the active substance content in the simplicia. According to (A. Bani, et al., 2023), the purpose of drying is to reduce the water content in the simplicia so as to prevent damage, inhibit microbial growth and allow storage of the simplicia for a longer time. Next, dry sorting was carried out to separate the dry sample from unwanted impurities during the drying process, then the dry and clean simplicia was ground using a blender and sieved to obtain simplicia powder with a uniform size. The simplicia powder obtained was 1.075 kg with a percentage of 15.35%. The results of the calculation of the simplicia weight can be seen in Table 1.

**Table 1. Calculation of the Percentage of Dry Weight of Kesum Leaf Simplex**

Wet Weight (g)	Dry Weight (g)	Percentage (%b/b)
7000	1,075	15.35%

### Standardization of Simple Drugs

#### Organoleptic Test

Organoleptic testing is an initial test carried out to determine the physical characteristics of a material based on sensory observations, including color, smell and shape. In this research, organoleptic tests were carried out on kesum leaf powder (*Polygonum minus* H.) to ensure the quality and consistency of a material before further testing was carried out.

**Table 2. Results of Organoleptic Test of Powder**

Observation Parameters	Observation result
Color	Brownish green
Smell	The distinctive aromatic properties of kesum leaves
Form	Powder
Flavor	A bit bitter and a bit spicy

#### Drying Shrinkage Test

The drying shrinkage test was conducted by heating an empty crucible at room temperature for 30 minutes. Then, 2 grams of the crude drug was weighed and placed into the previously heated and tared crucible. After weighing, the crucible containing the crude drug powder was placed back in the oven at room temperature for 30 minutes until the weight remained constant. Allow the crucible to cool in a desiccator to room temperature. Then, it was weighed and the percentage drying shrinkage was calculated. 105°C/105°C

**Table 3. Results of Determination of Drying Loss of Kesum Leaf Powder**

Replication	Sample Weight (g)	Percentage (%)
I	2	4.55
II	2	5.4
III	2	4.45
<b>Average</b>		<b>4.8 ± 0.52</b>

### Water Content Test

The moisture content test was performed by weighing 2 grams of powdered simplicia. The drying loss was measured using a Moisture Balance device at a set temperature, and the device beeped to indicate the analysis was complete. 105°C

**Table 4. Results of Kesum Leaf Powder Water Content Test**

Replication	Sample Weight (g)	Percentage (%)
I	2	8.78
II	2	8.45
III	2	8.30
<b>Average</b>		<b>8.51 ± 0.24</b>

Moisture content was determined to determine the remaining water content after the drying process. Test results showed that the moisture content of the kesum leaf powder was 8.51%.

### Making Kesum Leaf Extract

The process of making ethanol extract from kesum leaves is carried out by weighing 700 grams of powdered herbal medicine. Then, the first maceration process is carried out using 70% ethanol solvent with a ratio of 1:7, amounting to 4,900 mL for 3 x 24 hours, with occasional stirring. The purpose of stirring is to accelerate the achievement of uniform concentration of the extracted substance in the solvent.

Next, after 3 days, the mixture was filtered using flannel cloth to produce the first macerate. The residue was then re-soaked with solvent (remaceration). Remaceration was carried out in a 1:3 ratio, adding 2,100 mL of solvent to the remaining simplex (residue) for 2 x 24 hours to optimize the extraction process for active compounds.

The maceration process was carried out for 5 days. All the macerates were first filtered using flannel to separate the dregs, then filtered again using a Buchner funnel lined with filter paper to obtain a clear macerate.

The macerate is evaporated to remove any remaining ethanol solvent remaining in the extracted macerate. Evaporation is performed using a rotary evaporator at a temperature of 100°F to obtain a thick extract, extend the shelf life of the material, and reduce the risk of spoilage. 50°C

Based on the test results, the yield of the ethanol extract of kesum leaves was 35.57%. The higher the yield, the greater the amount of extract obtained (A. Bani et al., 2023). The calculation results can be seen in Table 5.

**Table 5. Results of Thick Extract of Kesum Leaves**

Powder Weight (g)	Weight of Thick Extract (g)	Extract Yield (%)
700 gr	249 gr	35.57%

### Extract Standardization

#### Ethanol Free Test

**Table 6. Ethanol-Free Results**

Ethanol Free Test	Observation result	Library(Priamsari et al., 2020)
Kesum leaf extract + Concentrated H <sub>2</sub> SO <sub>4</sub> + CH <sub>3</sub> COOH 1%, heated	(+) No ester odor	No ester odor

An ethanol-free test was conducted to ensure that the ethanol solvent did not affect the antibacterial activity of the kesum leaf extract, as ethanol has disinfectant properties. The test results

showed that the kesum leaf extract was free from 70% ethanol solvent, as indicated by the absence of a distinctive ester odor, thus concluding that the obtained extract could be used for further testing (Achmad et al., 2024).

### Drying Shrinkage Test

The drying shrinkage test was carried out by heating an empty crucible at 105°C for 30 minutes. Then, 2 grams of the simplicia was weighed and placed into the previously heated and tared crucible. After weighing, the crucible containing the simplicia powder was put back into the oven at 105°C for 30 minutes until the weight remained constant, allowing the crucible to cool in a desiccator to room temperature. Then, it was weighed and the percentage of drying shrinkage was calculated.

**Table 7. Results of Determination of Drying Loss of Condensed Extract**

Replication	Sample Weight (gr)	Percentage (%)
I	2	8
II	2	9.8
III	2	6.2
<b>Average</b>		<b>8 ± 1.8</b>

Drying loss is a parameter used to determine the amount of substance remaining after a drying process at 105°C for 30 minutes or until a constant weight is reached. The purpose of determining the drying loss value is to establish the maximum limit for compound loss that can occur during the drying process. The drying loss value obtained from the kesum leaf powder was 8%.

### Water Content Test

The moisture content test was performed by weighing 2 grams of powdered simplicia. The drying loss was measured using a Moisture Balancer at 105°C, and the instrument beeped to indicate the analysis was complete.

**Table 8. Results of the Water Content Test of Kesum Leaf Extract**

Replication	Sample Weight (gr)	Percentage (%)
I	2	8.33
II	2	6.94
III	2	5.85
<b>Average</b>		<b>7.04 ± 1.24</b>

Determination of water content was carried out to determine the remaining water content after the drying process. The test results showed that the water content of the kesum leaf powder was approximately 8.51%.

### Organoleptic Test

Organoleptic testing is an initial test carried out to determine the physical characteristics of a material based on sensory observations, including color, smell and shape. In this research, organoleptic tests were carried out on kesum leaf powder (*Polygonum minus* Huds.) to ensure the quality of the extract and determine changes in the characteristics of the material after the extraction process.

**Table 9. Results of Organoleptic Test of Extract**

Observation parameters	Observation result
Color	Dark brown
Smell	Aromatic Characteristics
Form	Thick Extract

### Identify the Chemical Content of Kesum Leaves

Identification of chemical compounds aims to determine the types of compounds contained in the extract of kesum leaves (*Polygonum minus* Huds.). Phytochemical screening is carried out qualitatively to detect the presence of secondary metabolites, including flavonoids, tannins, saponins, alkaloids, and terpenoids.

**Table 10. Chemical Content Test of Kesum Leaf Extract**

Chemical content	Test	Results	Note	Library (D. Kartikasari et al., 2022)
Flavonoid	Extract + mg powder + HCL 3 drops	Red	+	Red color formed
Phenol	Extract + FeCl <sub>3</sub>	Blackish Blue	+	Formation of purple or blackish blue color
Saponin	Aquadest	Formation of foam	+	Formation of foam
Alkaloid	Extract + Hcl 0.5ml + Reiaagein Drageindrof	Orange precipitate forms	+	Forms an orange or brick red color
Terpenoid	Extract + Liebermen Bourchard	Orange color is formed	-	Formed blue, green colors
Steroid	Extract + 1 ml Chloroform + 1 ml concentrated sulfuric acid	turquoise	+	Red, orange or blue-green color is formed

Before testing the antibacterial activity against *Escherichia coli* bacteria, phytochemical screening was first carried out to determine the presence of flavonoids, tannins, saponins, alkaloids, and terpenoids. The results of phytochemical screening showed that the 70% ethanol extract of kesum leaves contained active compounds in the form of flavonoids, tannins, saponins, and alkaloids, while terpenoid compounds were not detected. According to research by D. Kartikasari et al. (2022), phytochemical screening of the ethanol extract of kesum leaves (*Polygonum minus* Huds.) using 70% ethanol solvent showed phytochemical contents namely flavonoids, tannins, alkaloids, phenols, saponins, and terpenoids, with negative results for steroids. The same study using 96% ethanol solvent showed phytochemical contents namely flavonoids, phenols, saponins, and tannins, with negative results for alkaloids, terpenoids, and steroids.

The results of the phytochemical screening test on the 70% ethanol extract of kesum leaves (*Polygonum minus* Huds.) in this study showed that the extract positively contained several secondary metabolite compounds in the form of flavonoids, phenols, alkaloids, saponins, terpenoids, and steroids. The flavonoid content was identified through a color change to red after the addition of Mg powder and concentrated HCl. The addition of Mg powder and HCl in the flavonoid test aims to reduce the benzopyrone core in the flavonoid structure so that flavylum salts are formed, and HCl plays a role in triggering an oxidation-reduction reaction with Mg metal acting as a reducing agent for flavonoid compounds (Ulfah et al., 2024). According to Taufik et al. (2023), the presence of tannins is evidenced by the appearance of a blue-black color on the addition of FeCl<sub>3</sub>, which can occur because the Fe<sup>3+</sup> ion interacts with the ketone group in the polyphenol compound which acts as a metal binder. Alkaloid content is indicated by the formation of an orange/orange precipitate upon application of Dragendorff's reagent because the nitrogen atom in the alkaloid compound forms a coordinate covalent bond with the K<sup>+</sup> ion as a metal ion (Ulfah et al., 2024). Meanwhile, saponin compounds are detected by the formation of stable foam due to the presence of glycoside compounds that are able to produce foam when mixed with water and can be hydrolyzed into glucose and other compounds (Taufik et al., 2023). In the terpenoid-steroid test using the Liebermann-Burchard reaction, the terpenoid test shows a negative result because no brown ring is formed, while the steroid test gives a positive result which is indicated by the formation of a blue-green color. The difference in results occurs because terpenoid and steroid compounds will produce certain colors when reacting with concentrated sulfuric acid (Ulfah et al., 2024).

#### **Fractionation of n-Hexane, Ethyl Acetate and Water**

Fractionation was carried out using n-hexane, ethyl acetate, and water solvents, and replicated three times. The results of the calculation of the fractionation yield percentage of the n-hexane fraction of kesum leaves were 54%, in the ethyl acetate fraction the yield percentage was 61%, while the water fraction obtained a yield percentage of 90%.

**Table 11. Results of Fractionation Yield of n-hexane, Ethyl acetate and Kesum Leaf Water**

Solvent	Extract Weight (g)	Fraction Weight (g)	Percentage (%)
n-Hexane	10 g	5.4 g	54%
Ethyl Acetate	10 g	6.1 g	61%
Water	10 g	9 g	90%

The use of liquid-liquid extraction techniques aims to maximize the extraction process, where compounds will react based on their polarity. This extraction is carried out first by using a non-polar n-hexane solvent to extract non-polar compounds with water that previously contained dissolved extracts (Herdiana, I and Aji, 2020). The n-hexane fraction is taken and then added again with n-hexane up to 3 replications. The second extraction is carried out using a semi-polar tending to non-polar solvent ethyl acetate to extract semi-polar compounds in the aqueous phase that is fractionated with 3 replications.

The solvent addition was carried out repeatedly in n-hexane, ethyl acetate, and water solvents, the extraction process was stopped when the solvent used had become clear or colorless. The highest yield was in the water fraction because water is a polar solvent so it can be concluded that the compounds contained in the kesum leaves are dominated by polar compounds. The second highest yield was in the ethyl acetate fraction because the semi-polar nature of the ethyl acetate and ethanol solvents so that the withdrawal of compounds from the extract was large. The smallest yield was in the n-hexane extract, this was due to the small non-polar compounds in the kesum leaves. This separation process aims to separate the active compound components from the extract that has been obtained, so that the target compound can be produced in higher quantities and produce active compounds with the most optimal activity (Nainggolan et al., 2024).

The yield of the extract fraction is calculated based on the ratio between the number of fractions obtained and the amount of initial extract used. The higher the yield of the extract fraction, the greater the number of compounds successfully reacted with each solvent (Herdiana, I, and Aji, 2020).

### Fractionation Standardization

#### n-Hexane Free Test

This n-hexane-free test aims to determine the presence or absence of residual solvent in the extract, as evidenced by observing the changes that occur after the test. The test results showed that the obtained n-hexane fraction did not produce flames or smoke during the combustion process over a flame, thus concluding that the sample extract was completely free of residual n-hexane solvent (Pakaya et al., 2024). The results can be seen in Figure 1.



**Figure 1. n-Hexane free test**

#### Ethyl Acetate Free Test

The ethyl acetate-free test was conducted to ensure that the resulting extract was free from residual ethyl acetate solvent used during the fractionation process, as the presence of residual solvent in the extract can affect subsequent test results and potentially cause toxic effects on the biological activity being tested (Pakaya et al., 2023). Based on the test results, the obtained ethyl acetate extract did not exhibit an ester odor. The extract was said to be free from ethyl solvent if no ester odor was detected.

Therefore, it can be concluded that the sample extract was completely free from residual ethyl acetate solvent.



**Figure 2. Ethyl Acetate Free Test**

### **Antibacterial Activity Test of Escherichia coli ATCC 25922**

#### **Gram staining**

Gram staining is a method for classifying bacteria into Gram-positive and Gram-negative bacteria based on differences in chemical composition and the strength of their cell wall structure. This classification is determined by the response of the bacterial cell wall to the dyes crystal violet and safranin. The dyes used can be either basic or acidic; basic stains have a positively charged chromophore that plays a role in color formation, while negatively charged acid stains produce color through the interaction of opposite charges (Damayanti et al., 2024).

The results of Gram staining identification of Escherichia coli ATCC 25922 bacteria show red cells with a rod shape (bacilli). The visible red color indicates that Escherichia coli bacteria are classified as Gram-negative bacteria. Because the cell wall of Gram-negative bacteria has a relatively thin peptidoglycan layer, it cannot retain the main dye, namely crystal violet and then absorbs the second dye, namely safranin, which results in the cells appearing red (Safitri et al., 2024). This observation was carried out using a microscope with 100x magnification. Gram staining can be seen in the image.



**Figure 3. Gram Staining of Escherichia coli ATCC 25922 Bacteria**

#### **Antibacterial Activity Testing by Diffusion Method**

Antibacterial activity testing of extracts, n-hexane fractions, ethyl acetate, and keisum leaf water was carried out to determine their effects on the growth of Escherichia coli ATCC 25922 bacteria. The method used in this test was the disk diffusion method (disk/paper disc) to determine the Minimum Inhibitory Concentration. This method was chosen because it has several advantages, namely it is easy to do, makes it easier to observe the inhibition zone formed, does not require special equipment, and is relatively economical.

This study used Nutrient Agar (NA) media. The test was conducted using the disc diffusion method, where the surface of the agar media was inoculated with 100  $\mu$ l of bacterial suspension, then spread evenly using a glass spreader. Paper discs were used as the carrier media for the antibacterial compounds, which were first saturated with the test material at concentrations of 20%, 30%, and 40%, respectively. Ciprofloxacin 5  $\mu$ g was used as a positive control, while DMSO 1% was used as a negative control. Next, the paper discs were placed on the surface of the agar media that had been inoculated with the test bacterial culture, then incubated for 1  $\times$  24 hours at 37°C. The presence of

antibacterial activity was indicated by the formation of a clear zone around the paper discs, which indicated inhibition of bacterial growth. The results of the antibacterial activity test of the extract, n-hexane fraction, and ethyl acetate can be seen in Table 12.

**Table 12. Antibacterial Test Results Using the Diffusion Method**

Test Materials	Concentration	Inhibitory Power			Mean ± SD	Inhibitory Power Category
		R1	R2	R3		
Extract	20%	8.25	8.35	9.26	8.62±0.55	Currently
	30%	9.40	10.15	11.39	9.77±0.53	Currently
	40%	10.40	11.29	12.25	11.31±0.93	Strong
n-Hexane	20%	7.25	7.35	8.24	7.79±0.62	Currently
	30%	8.25	8.46	9.68	8.35±0.14	Currently
	40%	10.30	10.50	10.60	10.46±0.15	Currently
Ethyl	20%	9.70	8.68	10	9.85±0.21	Currently
	30%	10.29	11.43	12.30	11.35±1.00	Strong
	40%	11.47	12.33	13.29	12.36±0.91	Strong
Water	20%	7.1	7.4	8.12	7.54±0.52	Currently
	30%	8.15	8.45	9.11	8.57±0.49	Currently
	40%	8.39	8.86	9.34	8.86±0.47	Currently
Cipro	5µl	25.35	25.40	26.00	25.58±0.36	Very strong
DMSO	1%	0	0	0	0±0	

Based on the results of the antibacterial activity test, the kesum leaf extract at a concentration of 40% produced an inhibition zone of 11.31 mm. The ethyl acetate fraction at a concentration of 40% showed the highest inhibition zone of 12.36 mm. Meanwhile, the n-hexane fraction at a concentration of 40% produced an inhibition zone of 10.46 mm, and the water fraction produced an inhibition zone of 8.86 mm. These results indicate that the keisum leaf extract and fraction are able to inhibit the growth of Escherichia coli ATCC 25922 bacteria, which is indicated by the formation of a clear zone around the disc. Based on the data presented in Table 4.12, it can be seen that the increase in the concentration of the test material is proportional to the increase in the inhibition power produced in each treatment group.

#### Determination of Minimum Inhibitory Concentration (MIC) Value

The determination of the Minimum Inhibitory Concentration (MIC) value was carried out with the aim of knowing the smallest amount of antibacterial active substance that can inhibit the growth of Escherichia coli ATCC 25922 bacteria. The determination of the MIC value was carried out with various concentrations, the aim was to find out the smallest amount of antibacterial active substance needed to inhibit the growth of the tested bacterial organism. The results of the antibacterial test at a concentration of 30% of the solution looked clear and no lumps or bacteria were seen growing. Thus, the sample solution in the test solution with a concentration of 30% can be stated as the Minimum Inhibitory Concentration (MIC) value.

#### Determination of Minimum Lethal Concentration (MBC) Value

The determination of the Minimum Killing Concentration value in this study was carried out using all test solutions. It is expected that one concentration that is known to have the ability to inhibit the growth of Escherichia coli ATCC 25922 bacteria in the media to remain clear, which means that the test solution with a concentration of 30% given can kill Escherichia coli ATCC 25922 bacteria.



**Figure 4. Antibacterial Testing by Dilution Method**

The observation results showed that in Petri dishes containing NA media and inoculated with test solutions at various concentrations, Escherichia coli ATCC 25922 bacteria did not grow at a concentration of 30%, which was indicated by the media remaining clear. In contrast, at concentrations of 15%, 7.5%, and 3.75%, bacterial growth was still visible, indicated by the appearance of colony spots and turbidity in the media. At a concentration of 30%, Escherichia coli ATCC 25922 bacteria were unable to adapt and balance, so that concentration was declared effective in killing bacteria. Thus, the Minimum Killing Concentration (MBC) value was set at a concentration of 30%. Kesum leaf extract (*Polygonum minus* Huds.) can kill Escherichia coli ATCC 25922 bacteria, it is estimated to contain active compounds in plants that are effective as antibacterials, namely flavonoids and phenols.

### Data Analysis Using SPSS

The data obtained were then analyzed using the SPSS version 25 program with the One Way Anova (Analysis of Variance) method and continued with the Post Hoc Test using the Tukey method. This test aims to determine the significant differences between the extract, n-hexane fraction, ethyl acetate fraction, and water fraction in inhibiting the growth of Escherichia coli ATCC 25922 bacteria, compared to the positive control antibiotic ciprofloxacin and the negative control DMSO 1%.

**Table 13. Normality Test Results**

Concentration	Statistics	Sig
20% Extract	.823	.172
30% Extract	.980	.730
40% Extract	1,000	.958
20% n-Hexane	.825	.175
30% n-Hexane	.857	.261
40% n-Hexane	.964	.637
20% Ethyl Acetate	.910	.417
30% Ethyl Acetate	.994	.852
40% Ethyl Acetate	.999	.939
20% Water	.947	.554
30% Water	.955	.593
40% Water	1,000	.988
K+	.807	.132

The initial test conducted was a normality test to determine whether the research data was normally distributed or not. The normality test used was the Shapiro-Wilk test because the number of samples analyzed was less than 50. If the test results show a significance value of  $p < 0.05$ , then  $H_0$  is rejected and the data is declared not normally distributed. Based on Table 4.13, the results of the normality test show that all treatment groups have a significance value of  $p > 0.05$ , which means it can be concluded that all research data are normally distributed because they meet the normality assumption. Therefore, the next analysis can be carried out, namely the homogeneity of variances test.

**Table 14. Results of Homogeneity Test**

Variables	Levene Statistics	Sig
Based on Mean	.867	.588

Next, a homogeneity of variance test was performed to determine whether the samples used had the same variance. This test was conducted to assess the variance differences between two or more data groups as a prerequisite for further statistical analysis. The results of the homogeneity test showed a significance value  $> 0.05$ , which means the data variance is homogeneous. The results of the homogeneity of variance test were  $0.588 > 0.05$ , so it can be concluded that the data have the same variance or are homogeneous. Next, all data were analyzed using a one-way hypothesis test (One-Way ANOVA).

**Table 15. One Way Test (ANOVA) Results**

Variables	Sum of Squares	df	Mean Square	F	Sig
Between Groups	785,839	12	65,487	135,375	.000

The test results using the One Way ANOVA test showed a Sig value of 0.000 ( $p < 0.05$ ). Therefore, it can be concluded that  $H_0$  is rejected so that the research hypothesis is proven, namely there is a difference in the effectiveness of antibacterial power in the extract, n-hexane fraction, ethyl acetate fraction, and water fraction. The One Way ANOVA test was used to determine the difference in average values between treatment groups. Furthermore, all data were analyzed using the Duncan Error Rate Difference test.

Further testing using the Tukey method showed statistically significant differences between treatment groups, indicated by a Sig value  $< 0.05$ . All extract concentrations (20%, 30%, and 40%) were significantly different compared to the positive control (Sig = 0.000). Comparisons between the extract and the n-hexane fraction, ethyl acetate fraction, and water fraction also showed significant differences, confirming that the type of solvent and concentration level influenced the test results, with a tendency for greater effects at high concentrations. In addition, the ethyl acetate fraction and water fraction at all concentrations showed significant differences compared to the other treatment groups and the positive control, indicating variations in activity between the fractions tested. Overall, the Tukey post hoc test confirmed that each group gave a statistically different response, thus strengthening the results of the ANOVA test that there were significant differences between treatment groups.

## DISCUSSION

This study aims to identify the antibacterial activity of extracts, n-hexane fractions, ethyl acetate of kesum leaves (*Polygonum minus* Huds.) against *Escherichia coli* ATCC 25922 bacteria. The stages of this study began with determining the plants which aimed to determine whether the type of plant to be used was appropriate and there were no errors in sampling.

After the determination was carried out, the collection of 7000 g of kesum leaves was carried out and wet sorting was carried out then dried in the sun covered with a black cloth which aims to avoid direct exposure which has the potential to damage the active substance content in the simplicia. Next, dry sorting was carried out to separate the dry sample from unwanted impurities during the drying process, then the dry and clean simplicia was ground using a blender and sieved so that the simplicia powder obtained was 1075 g with a percentage of 15.35% as seen in Table 1. The yield of simplicia is said to be good if the value is more than 10% (Selviana et al., 2024).

Next, the standardization of the medicinal plants was carried out. The drying shrinkage test for the medicinal plants, as shown in Table 3, yielded a result of 4.8%; this result meets the requirements of the Indonesian Herbal Pharmacopoeia, which requires a drying shrinkage of no more than 10%. The water content test yielded a result of 8.51%. According to Maryam (2020), a good water content is less than 10%, as an extract with too high a water content can reduce the quality of the medicinal plants, making them susceptible to mold and mildew growth.

After standardization, extraction was carried out using 70% ethanol with a ratio of 1:7 as much as 4900 mL for 3 x 24 hours with occasional stirring. The purpose of stirring is to accelerate the achievement of uniformity of the concentration of the extracted substance in the solvent. Furthermore, after 3 days, filtration was carried out using flannel cloth to produce the first macerate. The residue was then soaked again with the solvent (remaceration) carried out with a ratio of 1:3 by adding 2100 mL of solvent for 2 x 24 hours.

The maceration process was carried out for 5 days with the results of all macerates being filtered first using flannel cloth to separate the dregs, then filtered again using a Buchner funnel that has been coated with filter paper to obtain a clear macerate. Then the macerate results were evaporated using a rotary evaporator at a temperature of 50°C with the aim of evaporating the remaining ethanol

solvent still present in the extracted macerate. Based on the results obtained in Table 5, namely 249 g of thick extract with a yield of 35.57%; the higher the yield, the more the amount of extract obtained (A. Bani et al., 2023).

Next, the extract was standardized. The ethanol-free test revealed no distinctive ether odor, thus concluding that the kesum leaf extract was free from 70% ethanol. The ethanol-free test was conducted to ensure that the ethanol solvent did not affect the antibacterial activity of the kesum leaves, as ethanol has disinfectant properties (Achmad et al., 2024).

The drying loss test for the extract, as shown in Table 7, yielded a result of 8%; this result meets the requirements of the Indonesian Herbal Pharmacopoeia, which requires a drying loss of no more than 10%. The moisture content test yielded a result of 7.04%. According to Maryam (2020), moisture content plays a crucial role in determining extract stability. A moisture content greater than 10% can reduce its stability and increase the risk of mold and mildew growth.

Before testing the antibacterial activity against *Escherichia coli* bacteria, a phytochemical screening test was first carried out to determine the compounds contained in the kesum leaves (*Polygonum minus* Huds.). The results obtained showed that the extract positively contained several secondary metabolite compounds. Flavonoids were identified through a color change to red after the addition of concentrated Mg and HCl aimed to react with the benzopyrone core in the flavonoid structure so that the formation of flavylum salts and HCl plays a role in triggering oxidation, reduction, with Mg metal acting as a reducing agent for flavonoid compounds (Ulfah A. et al., 2024).

According to (Taufik et al., 2023) the presence of phenol is evidenced by the appearance of a blackish blue color on the addition of FeCl<sub>3</sub>. Changes in Fe<sup>3+</sup> ions can occur when they interact with the ketone group in polyphenol compounds which act as metal chelators. The alkaloid content is indicated by the formation of an orange/orange precipitate on the administration of Dragendorff's reagent because the nitrogen atom in the alkaloid compound forms a coordinate covalent bond with the K<sup>+</sup> ion as a metal ion (Ulfah A. et al., 2024). Meanwhile, saponin compounds are detected by the formation of stable foam due to the presence of glycoside compounds that are able to produce foam when mixed with water and can be hydrolyzed into other glucose (Taufik et al., 2023).

In the terpenoid-steroid test using the Liebermann-Burchard reagent, the terpenoid test showed a negative result because no brown ring formed, while the steroid test gave a positive result, indicated by the formation of a blue-green color. This difference in results occurs because terpenoid and steroid compounds produce specific colors when reacting with concentrated sulfuric acid (Ulfah A. et al., 2024).

The extract was then fractionated using a liquid-liquid extraction technique to maximize the extraction process, where compounds were extracted based on their polarity. The first extraction used a non-polar n-hexane solvent to extract non-polar compounds with water that already contained the dissolved extract (Herdiana I. and Aji, 2020). The n-hexane fraction was taken and then added with n-hexane up to three replicates.

The second extraction was carried out using a semi-polar, tending to non-polar ethyl acetate solvent to extract semi-polar compounds in the aqueous phase fractionated with 3 replications. The third extraction was carried out using a polar aqueous solvent fractionated with 3 replications. The addition of solvents was carried out repeatedly in n-hexane, ethyl acetate, and water; the extraction process was stopped when the solvent used had become clear.

The highest yield was found in the water fraction with a percentage of 90% because water is a polar solvent, so it can be concluded that the compounds contained in the kesum leaves are dominated by polar compounds. The second largest yield was the ethyl acetate fraction with a percentage of 61% because the semi-polar nature of the ethyl acetate and ethanol solvents so that the withdrawal of compounds from the extract was large. The smallest yield was found in the n-hexane extract with a percentage of 54%; this is due to the small non-polar compounds in the kesum leaves. This separation aims to separate the active compound components from the extract that has been obtained, so that

target compounds can be produced in higher quantities and produce active compounds with the most optimal activity (Nainggolan et al., 2024).

The yield of the extract fraction is calculated based on the comparison between the number of fractions obtained and the amount of initial extract used; the higher the yield value of the extract fraction, the greater the number of compounds successfully extracted by each solvent (Herdiana I. and Aji, 2020).

Next, fractionation standardization was carried out using the n-hexane-free test. This test aimed to determine the presence or absence of residual solvent in the extract. The test results showed that the obtained n-hexane fraction did not produce flames or smoke during the combustion process, thus concluding that the sample extract was completely free of residual n-hexane solvent (Pakaya et al., 2024).

The ethyl acetate-free test was conducted to ensure that the resulting extract was free from residual ethyl acetate solvent used during the fractionation process, because the presence of residual solvent in the extract can affect the results of subsequent tests, as well as potentially cause toxic effects on the biological activity being tested (Pakaya et al., 2023). Based on the test results, the ethyl acetate extract obtained did not smell of ether; the extract was said to be free from ethyl acetate solvent if no ether odor was detected, so it can be concluded that the sample extract was completely free from residual ethyl acetate solvent.

Before testing antibacterial activity, Gram staining was first performed to classify bacteria into Gram-positive and Gram-negative based on differences in chemical composition and the strength of their cell wall structure. The Gram stain identification results for *Escherichia coli* bacteria showed red cells with a rod shape (bacilli); the visible red color indicates that *Escherichia coli* bacteria are classified as Gram-negative bacteria. Because the cell wall of Gram-negative bacteria has a relatively thin peptidoglycan layer, it cannot retain the main dye, namely crystal violet, and then absorbs the second dye, namely safranin, which results in a red appearance (Safitri et al., 2024).

Furthermore, antibacterial activity testing of extracts, n-hexane fractions, ethyl acetate, and kesum leaf water (*Polygonum minus* Huds.) was carried out to determine their effect on the growth of *Escherichia coli* ATCC 25922 bacteria using the diffusion method by pouring NA media into a sterile Petri dish and allowing it to solidify, then adding 100  $\mu$ L of bacterial suspension evenly using a glass spreader. The bacterial suspension was left for 5 minutes to absorb into the agar medium, then taking the disc paper using sterile tweezers that had been previously dipped with the test material at each concentration of 20%, 30%, and 40%. Ciprofloxacin 5  $\mu$ g was used as a positive control, while DMSO 1% was used as a negative control.

The disc paper was then placed on the surface of the agar medium inoculated with the bacterial suspension and incubated for 1 x 24 hours at 37°C. The negative control test, using 1% DMSO, did not show any inhibition zone formation. This is because the DMSO solvent has neither bactericidal nor bacteriostatic activity against the test bacteria.

A positive control test was conducted to compare the diameter of the inhibition zone produced by the extract, n-hexane fraction, ethyl acetate, and kesum leaf water with the antibiotic ciprofloxacin. The results obtained showed that ciprofloxacin against *Escherichia coli* ATCC 25922 bacteria produced an inhibition zone with an average value ranging from 10-20 mm; this indicates that ciprofloxacin has very strong antibacterial activity due to the mechanism of action of ciprofloxacin which plays a role in inhibiting the activity of the DNA gyrase enzyme in bacteria (Sofyana et al., 2024).

Based on the test results using the diffusion method, the antibacterial activity of kesum leaf extract at a concentration of 40% produced an inhibition zone of 11.31 mm. The ethyl acetate fraction at a concentration of 40% showed the highest inhibition zone of 12.36 mm because its main antibacterial compound is semipolar; ethyl acetate is able to dissolve and concentrate active compounds such as flavonoids, phenols which play an important role in inhibiting bacterial growth. Where flavonoids are basically known to be polar; flavonoids that play the most antibacterial role are

generally semipolar (aglycones) do not have sugar groups so they more easily penetrate bacterial cell membranes. Therefore, semipolar solvents such as ethyl acetate are able to extract active compounds optimally, so that the ethyl acetate fraction shows the strongest antibacterial activity. In contrast, the water fraction contains flavonoids which are very polar but its antibacterial activity is relatively lower because flavonoid glycosides have difficulty interacting and penetrating bacterial cell membranes so that their effectiveness is reduced (Eiden, Katamang et al., 2023).

Meanwhile, the n-hexane fraction at a concentration of 40% produced an inhibition zone of 10.46 mm and the water fraction produced an inhibition zone of 8.86 mm as seen in Table 12. These results indicate that the extract and fraction of kesum leaves are able to inhibit the growth of *Escherichia coli* ATCC 25922 bacteria. The antibacterial ability to inhibit the growth of microorganisms is influenced by the type and concentration used. This is because the higher the concentration used, the larger the inhibition zone obtained.

The next antibacterial test was to determine the MIC (Minimum Inhibitory Concentration) with the aim of knowing the smallest amount of active antibacterial substance. The results obtained at a concentration of 30% solution looked clear and no clumps or bacteria were seen growing, thus the sample solution in the test solution with a concentration of 30% can be stated as the MIC value. Furthermore, using the solid dilution method, the results obtained did not experience growth at a concentration of 30% which was indicated by the media remaining clear. In contrast, at concentrations of 15%, 7.5%, and 3.75%, bacterial growth was still visible. Thus, the Minimum Bactericidal Concentration (MBC) value was set at a concentration of 30%.

## CONCLUSION

This study showed that 70% ethanol extract of kesum leaves (*Polygonum minus* Huds.) and its fractions (n-hexane, ethyl acetate, water) have moderate to strong antibacterial activity against *E. coli* ATCC 25922, with the ethyl acetate fraction being the most effective (average inhibition zone of 12.36 mm at 40% w/v, strong category), followed by extract (11.31 mm), n-hexane (10.46 mm), and water (8.86 mm), confirmed by statistically significant analysis. Phytochemical screening confirmed flavonoids, phenols, saponins, and alkaloids as the main contributors, while MIC and MBC were both 30% w/v in the ethyl acetate fraction, indicating bactericidal potential. However, limitations include the use of a single bacterial strain, limited replication and the absence of isolation of specific compounds, which limits generalization to resistant clinical strains.

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