JURNAL MULTIDISIPLIN MADANI (MUDIMA)
Homepage: https://journal.formosapublisher.org/index.php/mudima
ISSN: 2808-5639 (Online)
Research Article

Volume 4, No 8, August (2024)	DOI: https://doi.org/10.55927/mudima.v4i8.11133	Page: 1307-1326

Fraction Activities Rosella Flower (Hibiscus Sabdariffa L) as an Antibacterial and Antibiofilm Escherichia Coli ATCC 25922

Ratna Ika Yusuf[1*], Ana Indrayati[2], Ismi Rahmawati[3]
Department of Pharmaceutical Science, Faculty of Pharmacy, Setia Budi University, Surakarta
Corresponding Author: Ratna Ika Yususf ratnaikayusuf19@gmail.com

ARTICLE INFO

Keywords: Antibiofilm, Roselle Flowers, E.Coli, Hibiscus Sabdariffa L

Received : 18 June
Revised : 20 July
Accepted : 21 August

ABSTRACT

Rosella flowers (Hibiscus sabdariffa L) contain alkaloid, flavonoid, saponin and tannin compounds. The compounds contained in rosella flowers have activity as antibacterial and antibiofilm. Biofilm is the ability of microorganisms that stick to the surface of objects and form a matrix. This study aims to antibacterial and biofilm activity in the form of inhibition of biofilm formation and biofilm degradation of E.coli bacteria. Rosella flowers were extracted using maceration method with 96% ethanol solvent. The thick extract was then fractionated with n-hexane, ethyl acetate and water solvents. Antibacterial activity test using diffusion and dilution methods followed by observation of cell wall and membrane damage using SEM and ion leakage with AAS. Biofilm activity test was conducted using a microplate device and readings were taken at a wavelength of 595 nm. The absorbance results of the inhibition percentage to determine the IC50 and EC50 values. The average IC50 values of biofilm formation inhibition test on extract, n-hexane fraction, ethyl acetate fraction and water fraction against E. coli bacteria are 1.059; 1.070; 0.921; 0.863. The EC50 values of biofilm degradation test on extract, n-hexane fraction, ethyl The minimum killing concentration value of rosella flower water fraction against E. coli is 25 mg/ml. the effect of the active fraction of rosella flower on E. coli bacteria is on the cell wall and cell membrane

INTRODUCTION

Infectious diseases are a problem in the health sector that continues to grow over time, where infectious diseases can be transmitted from one person to another. Infections can be caused by various microorganisms such as viruses, bacteria, fungi and protozoa. One of the bacteria that causes infection that is most commonly found is bacteria Escherichia coli Excessive and inappropriate use of antibiotics can cause bacteria to develop resistance mechanisms. The increase in antibiotic resistance poses a global threat to human health and requires the discovery of effective antimicrobial alternatives. One of the bacteria that has become a major problem in resistance is bacteria E. coli pathogens that are the cause of various clinical syndromes such as diarrheal diseases, meningitis, and urinary tract infections. Bacteria E. coli The pathogen causes three clinical syndromes: urinary tract infections, enteric disease/diarrhea, and meningitis. The main mechanism where E. coli causing enteric disease includes adhesion and colonization of the intestinal mucosa, manipulation of the host cell cytoskeleton or evasion of host immune defenses, and production of toxins.

Plants are one of the alternative treatments, namely rosella (Hibiscus sabdariffa Us.) which has antimicrobial, antioxidant, anti-inflammatory, antidiabetic, antihypertensive and antifungal activity. BRosella flowers have very broad antibacterial activity, especially on planktonic bacteria, but very little research has been conducted on their biofilm activity. The process of biofilm formation begins when solitary microbes attach to a suitable surface, then attach and emit a QS signal. When communication occurs, bacteria will issue signals (autoinducers) to call bacteria, microorganisms, both gram-positive - gram-negative bacteria and fungi, which have different QS systems. Gram-negative bacteria produce AHL signals (Acyl homserine lactones), gram-positive bacteria produce signal peptides, while fungi produce farnesol or tyrosol compounds as a quorum sensing system between cells. After emitting a QS signal, the bacteria will secrete EPS (extracellular polymeric substance) as a sturdy protective matrix. Then the bacteria form microcolonies and will develop to form a biofilm. There are various approaches to inhibit biofilm formation, including the use of anti-biofilm agents

The research carried out was the ZOI test results of crude extract, N-hexane fraction, ethyl acetate fraction and water fraction E. coli It is known that the largest diameter of the clear zone is found in the water fraction disc. On E. coli the diameter formed is 9.67 ± 0.95mm (weak). Saponins act on bacterial cell walls by reducing surface tension and disrupting membrane permeability. Saponin then diffuses across the cytoplasmic membrane, disrupting the stability of the membrane, causing the cytoplasm to leak and exit the cell, which can cause the cell to die. Triterpenoids are antibacterial agents that interact with porins in the outer membrane of bacteria and then produce strong polymer bonds that can damage the porins.

Study conducted showed that the antibacterial activity test showed that roselle at concentrations of 20mg, 30mg and 40 mg could inhibit and kill bacteria well, but the most optimum for inhibiting bacteria was a concentration of 40 grams of roselle which produced an inhibition zone of 6.5 mm against bacteria. E. coli which is categorized as having moderate antibacterial activity.

Research conducted on rosella flowers can inhibit the growth of bacteria E. coli because it contains phenolic compounds consisting of flavonoids, tannins, anthocyanins and saponins. Flavonoid compounds are the largest phenolic compounds contained in rosella flowers. This compound produces red, purple, blue and yellow substances in natural plants. showed that rosella flowers at concentrations of 100% and 90% could inhibit bacterial growth E. coli The greater the concentration, the greater the inhibition zone formed, because the greater the concentration, the greater the active substance contained in it, thus causing the inhibitory power against bacterial growth to be greater.

METHODS

A. Plant Determination

Determination is used to determine the correctness of plants related to the morphological characteristics of rosella flowers. Morphological characterization of rosella flowers was carried out at the Research and Development Center for Medicinal Plants and Traditional Medicine (B2P2TOOT) Tawangmangu, Karanganyar. This determination was carried out to ensure that the samples used were genuine rosella flowers

B. Extraction

The ethanol extract of rosella flowers was made using a ratio of (1:9), by weighing 900 g of rosella flower powder, then soaking it by adding 10L of 96% ethanol solvent, putting it in a vessel and then closing it. The extract was soaked for 3x24 hours with occasional stirring, after which the extract was collected and then the filtrate was evaporated using rotary evaporator at 40theC until it becomes a thick extract.

C. Antibacterial Activity Test

1. Diffusion Method Antibacterial Activity Test

Antibacterial activity test E. coli using the paper disc diffusion method, prepare a sterile petri dish then pour 15 ml of MHA media into the sterile petri dish and let it sit until it solidifies. The media that has solidified is then smeared with the bacterial suspension on the surface of the media. The media is inserted into disc paper (blank disk) measuring 8 mm with an absorption capacity of 60 µL, each disc was dripped with the test solution (extract, fraction n-hexane, ethyl acetate fraction and water), ciprofloxacin as a positive control and DMSO 5% as a negative control, carried out 3 times, then incubated for 24 hours at 37°C.

2. Dilution Method Antibacterial Activity Test

The dilution method antibacterial activity test was carried out to determine the MIC and KBM of the most active fraction of rosella flowers, the test medium used was Brain Heart Infusion (BHI). This method uses 1 row of test tubes consisting of 10 sterile tubes containing concentrated active fractions of rosella flowers. MIC testing was carried out by making the concentration of the rosella flower fraction, namely 50 mg/ml; 25mg/ml; 12.5 mg/ml, 6.255 mg/ml; 3.1 mg/ml; 1.56 mg/m; 0.78 mg/ml; and 0.39 mg/ml; positive control (+) and negative control (-). Tube 1, which is a negative control, contains 2 ml of the active fraction of rosella flowers. Tubes 2 to 9 contain 1 ml of each concentration with graded dilutions then 1 ml of bacterial suspension is added aseptically. Tube 10 as a positive control only contains bacterial suspension, then incubated at 37ºC for 24 hours. The smallest concentration in the tube that looks clear without any growth of test bacteria is determined as the MIC value. The MIC value is expressed as the lowest concentration of the active fraction of rosella flowers that can still inhibit the test bacteria. Minimum Kill Concentration (KBM) is determined by inoculating a clear media tube by streaking onto selective media and incubating at a temperature of 37theC for 24-48 hours. Observe whether or not colonies grow on the surface of the plate media.

D. Optimization of Biofilm Formation Time

Research uses microtitier plate flat-bottom polystyrene 96 wells, carried out by adding 200 µL of bacterial suspension to each wells then optimize the incubation time. Aims to obtain optimal incubation time to form biofilm. The variations in incubation time used are 1, 2, 3 and 4 days (Ziega, 2021). After the incubation period, the microplate was washed using running water 3 times, then 200 µL of 1% crystal violet solution was added to each well and incubated at room temperature for 15 minutes. The microplate was washed again using running water 3 times. 200 µL of 96% ethanol solution was added to each well and incubated at room temperature for 15 minutes. Next, the biofilm growth (Absorbance OD595) was read using a tool iMark- Biorad Microplate Reader. Testing is carried out aseptically inside Laminar Air Flow (LAF) which has previously been cleaned with alcohol, then sterilized with UV turned on for approximately 2 hours before use. The result of the largest absorbance value is expressed as biofilm formation E. coli the optimal one.

E. Biofilm Formation Inhibitory Activity Assay

Biofilm inhibition testing was carried out with the aim of obtaining the activity of rosella flowers in growing biofilms against bacteria E. coli Biofilm formation inhibitory activity tests were carried out using microplate round bottom polystyrene 96 wells with BHI media. A total of 100 µL of sample in the media was added to each well then 100 µL of bacterial suspension in the media was equivalent to 1.5x108 CFU/mL was added to the well containing the sample, incubation was carried out at a temperature of ± 37theC for 72 hours. After incubation, the contents of the wells were discarded and the plate was washed with running water then dried for 15 minutes by turning it upside down microplate at room temperature. A total of 200 µL of 1% crystal violet solution was added to each well for a staining time of 15 minutes. The contents of the well are thrown away and the well is rinsed again with running water. The microplate was dried by turning it upside down at room temperature for one hour. Then 200 µL of 96% ethanol solution was given to each well on the plate optical density read at λ 595 nm. Each test was replicated 3 times. The control test (bacteria+media+fraction) is a test control for the activity of rosella flowers in inhibiting biofilm growth. The positive control used ciprofloxacin. The results of the formation of biofilm growth are calculated using the OD inhibition percentage formula as follows. Inhibition.

$$= \frac{Negative\ control\ OD - Treatment\ OD}{OD\ Negative\ Control} \text{ x } 100\%$$

After obtaining the average percent inhibition of biofilm formation from each fraction concentration, proceed with determining the IC value50 by using the linear regression line equation between the percent inhibition of biofilm formation and the concentration of the fractions to see the relationship between the concentration and the percent inhibition of the biofilm in inhibiting 50% of the biofilm.

F. Biofilm Degradation Activity Assay

This test is carried out as in the biofilm inhibition test, but in the biofilm degradation test each fraction is added when the biofilm has formed. Biofilm degradation activity tests were carried out using microplate round bottom polystyrene 96 wells with BHI media. Biofilms were formed by adding 70 µL of media to each well and then adding 70 µL of bacterial suspension in BHI which was equivalent to 1.5x108 CFU/mL. The microplate was then incubated at a temperature of ± 37theC for 72 hours. The contents of the well are then discarded and the well is washed using running water. Each well was then filled with 200 µL of sample in media microplate back to incubation at a temperature of ± 37theC for 24 hours. To determine biofilm, the same method as the biofilm formation inhibitory activity test can be applied. The control in this test is a negative control (bacteria + media) which is a control for biofilm formation where bacterial growth is not disturbed. The sample control (bacteria + media + fraction) is a control test for the activity of rosella flowers in degrading biofilm. Biofilm degradation results can be calculated using the following degradation percentages. Degradation.

$$= \frac{Negative\ control\ OD - Treatment\ OD}{OD\ Negative\ Control} \text{ x } 100\%$$

Biofilm destruction activity assay E. coli expressed by EC parameters50 (Effective Concentration) namely the concentration of the test compound that destroys the biofilm by 50%. EC value50 determined from the linear regression equation between sample concentration and the percentage of biofilm destruction. Linear regression equation, r table value with a confidence level of 0.95. EC Price50 Inversely proportional to the biofilm destruction activity, the greater the EC value50 hence the biofilm destruction activity E. coli The smaller it is, the greater the concentration required to destroy the biofilm by 50%.

G. Analysis Scanning Electron Microscopy (SEM)

In this research, SEM was used to observe the surface characterization of the biomaterial structure and also changes in bacterial morphology. Characterization analysis is expressed by the MIC results and pure suspension of bacteria as a control in SEM sample preparation. The MIC results and control cells were incubated for 24 hours and shaken at a temperature of 22-25theC. The results are centrifuged, then put the cell biomass into a prefixative solution (2.5% glutaraldehyde in phosphate buffer and cocodylate buffer), for several 2 hours. The results were centrifuged and the precipitate was then washed again with phosphate buffer solution and 2% tannic acid was added for further fixation. The results were centrifuged and the precipitate was then washed again with phosphate buffer and cocodylate buffer, vortexed, left for 10 minutes and this step was repeated 2 times. Next, the cells were centrifuged again and cell biomass was taken, 1% osmium tetra oxide was added, vortexed and left for 1 hour at cold temperature. Cells were dehydrated by adding a series of ethanols (50%, 70%, 80%, 95%, and absolute ethanol) and then suspended in t-butanol and dried by freeze drying. The sample is then placed on the carbon tip which has been attached to the stube. The cells were then coated with gold metal for 10 seconds using an ion sputter and observed with a JEOL JSM 5310 LV SEM scanning electron analysis microscope.

H. Analysis Atomik Absorption Spectrophotometer AAS

AAS was used to analyze cell leakage in this study. Ion leak analysis was carried out on tubes which was determined by calculating one KBM and 2 times the KBM in the dilution method antibacterial activity test. AAS is used to detect leaks of ions such as K+ and Ca2+. The results from 1 KBM and 2 times KBM were then taken in 1 mL and then diluted in a 100 mL measuring flask. Results were measured by wet digestion using HNO3 (Park, 1996). Leakage is expressed by measuring the metal ions present in the test bacteria after contact with the active fraction at concentrations 0 (control), 1 and 2. The supernatant fluid is analyzed using AAS Thermo Elemental tipe Solar MS. The cell solution resulting from centrifugation with fractions is taken to measure the ion content.

RESULTS AND DISCUSSION

A. Plant Determination

Plant determination is the first step taken to determine the correctness of the plants taken, adapt to the morphological characteristics of the plant, and avoid errors in collecting material correctly and in accordance with the classification system of living things. Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT) Tawangmangu, Karanganyar. Based on the results of the determination, it shows that the samples used in this study are indeed rosella flowers which have the Latin name (Hibiscus sabdariffa L.) can be seen on letter no. TL.02.04/D.XI.6/5434.409/2024

B. Extraction

Rosella flowers were extracted using 96% ethanol solvent. According to Trifani (2012), ethanol is used as a solvent because it is universal, polar and easy to obtain. 96% ethanol was chosen because it is selective, non-toxic, has good absorption and high filtering ability so that it can filter out non-polar, semi-polar and polar compounds. The 96% ethanol solvent penetrates more easily into the cell walls of the sample than the ethanol solvent with a lower concentration, resulting in a concentrated extract.

Table 1. Table of Viscous Extract Yield Results

Rosella Flower Simplicia Powder (g)	**Rosella flower thick extract (g)**	**Yield (%)**
900 g	221 g	24,5 %

C. **Fractionation**

The n-hexane solvent is a non-polar compound which has the chemical formula C6H14, the n-hexane solvent can attract compounds found in plants such as wax, volatile fats and oils. Nonpolar compounds found in rosella flower extract will be attracted to the n-hexane solvent. The second solvent is ethyl acetate, which is a colorless clear solution, ethyl acetate is a semi-polar solvent and can attract semi-polar compounds in plant cell walls. The polar solvent used in this research is water. The water solvent was chosen because it is affordable, easy to obtain, stable, non-toxic, non-flammable. Water solvents can dissolve polar compounds.

Table 2. Table of Results Percent Fractionation Yield

Extract weight (grams)	**Faction**	**Fraction weight (grams)**	**Yield (w/w) (%)**
60	hexane	10	16,6
	acetate	16	26,6
	Air	30	50

D. Phytochemical Screening

Phytochemical screening aims to find out compound contained in the ethanol extract, water fraction, ethyl acetate fraction and n-hexane fraction of rosella flowers which have potential as anti-biofilm and antibacterial. Test results for the chemical content of rosella flower extracts and fractions using the color reaction method.

Table 3. Table Results of Phytochemical Screening of Extracts and Fractions

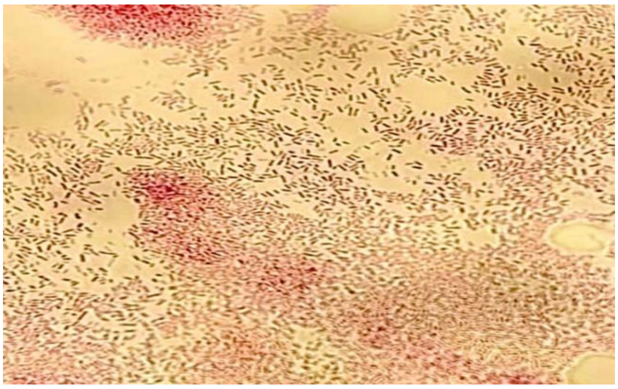
Metabolites Seconds	**Extract**	**hexane**	**Ethyl Acetate**	**Air**
Alkaliod	+	+	+	+
Flavonoid	+	+	+	+
Saponin	+	-	-	+
Tannin	+	-	+	+

The results of phytochemical screening showed that the ethanol extract contained alkaloids, flavonoids, saponins and tannins. The results obtained from the n-hexane fraction contained alkaloid compounds, flavonoids. The ethyl acetate fraction contains alkaloids, flavonoids, saponins, and the water fraction contains alkaloids, flavonoids, saponins and tannins.

E. Antibacterial Activity Test

Bacterial Gram Staining Method

Test bacteria used in this study is E. coli. Identification was carried out to ensure that the bacteria used in this study were correct E.coli pure without any other bacterial contamination. The identification carried out includes gram staining, which can be seen in Figure 5 below.

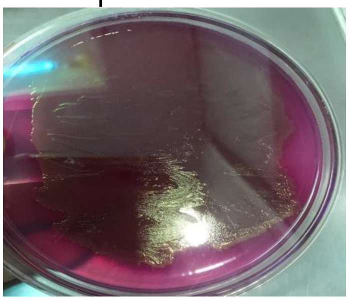


Picture 1. Image of Microscopic Identification Results

The purpose of Gram staining is to differentiate bacteria based on Gram characteristics. Gram staining uses 4 types of staining, Gram staining begins with the application of a basic dye, crystal violet (Gram A), which functions to color the entire surface of bacterial cells, iodine solution (Gram B) which functions as a mordant, forming a bond between crystal violet and iodine which will increase the affinity, increasing the dye by bacterial cells, alcohol (Gram C) produces pores in gram negatives which have many layers of fat, so that the crystal violet iodine complex does not stick to the bacterial cell walls (fades out). This causes the Gram negative cells to become clear in color. Safranin (Gram D) staining was dropped because E.coli It has a cell wall composition containing more lipopolysaccharide than Gram positive so that the bacteria do not retain crystal violet substances so that when stained with safarin the bacteria will maintain the safarin color to light red. the results of staining bacterial cells red are bacteria E.coli. Test bacteria E.coli shows rod-shaped bacteria, single, paired, no spores, and no capsule-shaped bacterial cells.

Method Differential

Differential media is selective media that functions to identify certain types of microorganisms. Media identification can generally be seen from the form of microorganisms, for example the color of the colony or the presence of precipitation. Endo Agar media is a selective media and differential media used to isolate gram-negative rod bacteria based on the ability of the bacteria to ferment lactose or not. The Endo Agar media test showed that the metallic flash colored colonies on the media were red due to bacteria E.coli and coliforms metabolize lactose into aldehydes and acids, which then release aldehydes from the compound fuxin sulfate, which makes the colonies red like a metallic flash.



Picture 2. Picture E. Coli on Endo Agar Media

Biochemical Methods

The aim of the bacterial biochemical test is to determine the physiological characteristics of pure cultures. Bacteria are grown in media Sulfida Indol Motility (SIM), Klinger Iron Agar (KIA), Lysine Iron Agar (HIS), Simon Citrate then incubated at 37theC for 24 hours.

Table 3. Table of Results of Biochemical Identification of Bacteria E. Coli

Testing	Results	Identification Results

Sim	-++	-++	Red
KIA	A/AG S(-)	A/AG S(-)	Yellow
HIS	K/K S()-	K/K S(-)-	Purple
citric			Green

To determine the formation of indole sulfide and motility, identification was carried out on SIM media (Sulfida Indol Motility). Microorganism testing E.coli The SIM shows the result (-++) and the sulfide result is negative which is indicated by the absence of black color on the media. This shows that microorganisms E.coli cannot reduce thiosulfate to produce hydrogen sulfide. After addition Erlich A and B contain dimethylaminobenzaldehyde to form red rosindole. A positive indole test is caused by bacteria E. coli which produces indole from tryptophan as a source for motility tests, positive results were obtained indicating the spread of bacteria E. coli on SIM media.

Dilution Method

Antibacterial activity testing is carried out using the dilution method or dilution series test. This method aims to determine the MIC value in the antibacterial activity test by looking at the turbidity of the bacterial suspension which has been incubated for 3 days so that the bacteria have a biofilm and then treated with water fraction. The MIC results are observed using see turbidity in the tube. Turbidity in the tube is due to the growth of test bacteria.

Table 4. Table of MIC Results Using the Dilution Method

Concentration mg/ml	**Replication 1**	**Replication 2**	**Replication 3**
100	-	-	-
75	-	-	-
50	-	-	-
25	-	-	-
12,5	+	+	+
6,25	+	+	+
3,13	+	+	+
1,56	+	+	+
0,781	+	+	+
0,390	+	+	+
Ciprofloxacin	-	-	-
Water fraction (-)	-	-	-

Information:
(-) : No bacterial growth
(+) : There is bacterial growth

The dilution method is useful for determining the minimum dose of bacteriostatic and bactericidal drugs. Minimum Kill Concentration (KM) can be determined from the lowest concentration which indicates the absence of growth of bacterial colonies in the media. Observation results showed turbidity at a concentration of 0.390; 0.781; 1.56; 3.13; 6.25; 12.5 mg/ml KBM results for the water fraction are shown at a concentration of 25 mg/ml. This shows that the minimum concentration in the water fraction can kill bacteria. And .coli. Negative control in this study was used to determine the presence of bacteria that grew in the media used. The positive control in the antibacterial test is the antibiotic ciprofloxacin. Ciprofloxacin is used as a comparison in testing antibacterial activity because it is an antibiotic with a broad spectrum and works by inhibiting the action of DNA gyrase in bacteria.

The results of research using the dilution method showed that the water fraction had Minimum Kill Concentration (KBM) activity. This is because the water fraction contains alkaloids, flavonoids, tannins and saponins. Saponins act on bacterial cell walls by reducing surface tension and disrupting membrane permeability. Saponin then diffuses across the cytoplasmic membrane, disrupting the stability of the membrane, causing the cytoplasm to leak and leaving the cell, which can cause the cell to die. Saponin then diffuses across the cytoplasmic membrane, disrupting the stability of the membrane, causing the cytoplasm to leak and exit the cell, which can cause the cell to die (Pratama et al., 2023 .).

F. Biofilm Activity Test Results

Optimization of Biofilm Formation Time

Optimization time Biofilm formation aims to determine the optimum time for bacteria E. coli ATCC 25922 in forming the best biofilm. Optimization time variants used are 24 hours, 48 hours, 72 hours. Before optimizing the time for biofilm formation, the turbidity of the bacterial suspension was adjusted to the turbidity Mc Farland 0,5 (1,5 x 108 CFU/mL).

Table 5. Table Results of Optimization of Biofilm Formation Time

No	Absorbance		
	24 hours	48 hours	72 hours
1	0,221	0,549	0,686
2	0,266	0,594	0,542
3	0,368	0,515	0,542
4	0,327	0,516	0,623
5	0,373	0,596	0,656
6	0,218	0,424	0,613
7	0,231	0,499	0,693
8	0,218	0,553	0,615
9	0,364	0,522	0,684
10	0,367	0,542	0,688
Rate-rate	0,295 ± 0,066	0,531 ± 0,047	0,634 ± 0,054

Optimizing the time for biofilm formation, the wavelength used was 595 nm. Wavelength the is the most optimal wavelength in reading biofilm. Results of optimization of bacterial biofilm formation time E. coli It is known that at varying times there are different levels of turbidity of the bacterial suspension. The level of turbidity of the bacterial suspension is proportional to the bacterial growth phase that occurs during the incubation time.

The degree of turbidity of the bacterial suspensions was comparable with the bacterial growth phase that occurs during the incubation time. The bacterial growth phase consists of 4 phases. The bacterial growth phase consists of 4 phases, namely the lag phase, log phase, stationary phase and death phase. In this study, the optimum incubation time was three days. This is the incubation time that reaches the stationary phase of biofilm formation on the third day. Biofilm formation in this study used a microplate tool made from plastic, because bacteria are more likely to stick to plastic materials. Biofilm formation can occur on various types of surfaces and various environmental conditions. Bacteria are more likely to adhere to hydrophobic surfaces and nonpolar surfaces such as plastic than to hydrophilic surfaces such as metal or glass (Jamal et al., 2018)

The attachment of microbes to the surface of objects is influenced by the characteristics of the liquid media used, for example pH, temperature, nutrient levels and ionic strength. Temperature control in this study was carried out during the incubation process, namely at a temperature of 37°C. Hydrodynamic properties can also influence the level and extent of bacterial attachment, for example the fluid velocity characteristics. Apart from that, the level of bacterial attachment is influenced by the condition of the bacterial cell surface, for example the production of extracellular polymers (EPS), cell surface hydrophobicity, and the presence of fimbriae and flagella.

Biofilm Formation Inhibition Test

The biofilm inhibition test was carried out by inserting extracts and fractions of rosella flowers with various concentrations into a microplate, then adding media and bacterial suspension to each well (wells) then incubated. Microplate which has been incubated and then added to the solution crystal violet into the well containing the biofilm, a purple color will form on the part of the biofilm that is still attached. This makes it possible to detect the biofilm quantitatively through the formation of a purple ring microplate. Crystal violet is the main dye that can bind negatively charged molecules such as chloride ions, sulfate ions, nitrate ions, carbonate ions, and homoserine lactone. It plays an important role in the structure and formation of biofilms in bacteria. E. coli. Negatively charged molecules and polysaccharides in the extracellular matrix can color live and dead cells, and color the biofilm matrix (Rollando, 2017). The violet crystals added to the wells are rinsed with running water. The crystal violet color will remain attached to the part where the biofilm is present, for example the formation of a purple ring microplate. The absorbance value of biofilm inhibition can be seen in appendix 20. Table 15 shows the results of the absorbance numbers obtained and then the percentage of biofilm inhibition is calculated.

Table 6. Table Results for the Percentage of Biofilm Formation

Mean Inhibition of Biofilm Formation (%) ± SD				
Concentration	**Extract**	**hexane**	**Ethyl acetate**	**Water Fraction**
50 mg/ml	76,54±0,58	75,69±1,06	77,65±0,07	79,66±0,25
25 mg/ml	62,73±0,63	64,19±0,28	66,95±0,47	67,91±0,12
12.5 mg/ml	48,22±0,19	47,76±0,26	49,37±0,68	52,74±0,28
6.25 mg/ml	41,89±0,51	40,68±0,19	49,42±3,26	50,68±0,26
Control (+) 87.23 ± 0.20				

The results of the percent inhibition of biofilm formation obtained in this study for each test sample can be seen in table 15, that the greater the concentration of the extract or fraction from rosella flowers, the higher the results of the percentage inhibition of bacterial biofilm formation. E. coli. The greater the test concentration, the greater the secondary metabolite content contained in the extract or fraction, so the greater the potential to inhibit biofilm formation. Meanwhile, for this inhibition, the best value is in the water fraction, where at a concentration of 50 mg/ml the value is 79. .66%.

Table 7. Table of IC Value Results50 Inhibition of Biofilm Formation

Sample	Nilai IC_{50} Inhibition of Biofilm Formation (mg/ml)			IC $rate_{50}$
	Replication 1	Replication 2	Replication 3	
Additional	11,16	11,80	11,42	11,46
hexane	11,72	11,93	11,64	11,76
ethyl	9,246	6,918	9,057	8,407
acetate	7,533	7,638	7,328	7,323
air				

The inhibition value of biofilm formation shows that the IC value50 The smallest is shown by the water fraction, namely 7,500 ± 0.111 mg/ml. The smaller the IC value50 the greater the inhibitory power of biofilm formation. Inhibition of biofilm formation with IC value50 smallest to largest is shown by the water fraction, then the rosella flower ethyl acetate fraction, extract and n-hexane fraction.

Hasil IC50 The results obtained were analyzed using statistical analysis one way anova to see significant differences between rosella flower extracts and fractions. The requirement in the normality test is that the data must be homogeneous with a sig value greater than alpha > 0.05 so that H0 is accepted and the IC data50 declared to be normally distributed. Normally distributed data is then continued with parametric tests, namely one way anova. Significant value of test results one way anova The size is 0.000 < 0.05 so this proves that the data obtained is homogeneous. Data obtained from the Tukey test shows a sign (*) in the column mean difference which means IC results50 shows significant differences. Statistical tests can be seen in attachment 23.

Rosella flowers contain flavonoid compounds. Flavonoids, saponins and tannins are secondary metabolites that are soluble in polar solvents. N-hexane is a nonpolar solvent and will attract secondary metabolites that are soluble in nonpolar solvents. The water fraction shows the IC value50 the lowest because in the water fraction there are secondary metabolites of alkaloids, flavonoids, saponins and tannins which have activity in inhibiting bacterial biofilm formation.

The process of forming a biofilm begins with bacterial cells sticking to the surface of an object, then the bacteria form an extracellular matrix (EPS), which is an adhesive compound that makes the bacteria stick more firmly permanently and forms microcolonies and a biofilm begins to form. The mechanism of action of a secondary metabolite contained in plants provides an anti-biofilm effect by inhibiting the process of attachment (adhesion) of bacteria to the surface of objects, disrupting the regulation of quorum sensing and reduces the growth of extracellular polymers (EPS).

Biofilm Degradation Test

Biofilm degradation tests start with bacteria E. coli made to produce a biofilm first. Then the solution in the plate was discarded and washed, then treated with extracts and roselle flower fractions with various concentrations. The absorbance value of biofilm degradation can be seen in Appendix 21. The results of the absorbance value obtained and then calculated the percentage of biofilm degradation can be seen in the table.

Table 8. Table 17 Percentage Results of Biofilm Degradation

Mean Biofilm Degradation (%) ± SD				
Concentration	**Extract**	**Hexane**	**Ethyl Acetate**	**Air**
50 mg/ml	73,75±0,16	77,25±0,16	76,08±0,28	79,25±0,67
25 mg/ml	67,70±0,36	69,37±0,36	68,87±0,27	71,64±0,64
12.5 mg/ml	56,16±0,44	63,10±0,75	58,10±1,61	63,82±0,08
6.25 mg/ml	41,90±0,85	44,51±0,28	43,78±1,85	46,45±0,16
Control (+) 88.50 ± 0.30				

The percent of biofilm degradation results are used to determine the EC value50 Biofilm degradation from ethanol extract, n-hexane fraction, ethyl acetate fraction and water fraction. The positive control in this study was the antibiotic ciprofloxacin at a dose of 0.076 mg/ml. The positive control on biofilm degradation showed an average percentage of 88.50%. The water fraction had the highest value of percent biofilm degradation with a value of 79.25%, followed by the n-hexane fraction with a value of 77.25%, the ethyl acetate fraction with a value of 76.08% and the extract with a value of 73.75%. Calculation of percent biofilm degradation using a linear equation between the percent concentration of the extract or fraction and the percentage value of biofilm degradation.

Table 9. Table of EC Value Results50 Biofilm Degradation

Sample	EC value$_{50}$ Inhibition of Biofilm Formation (mg/ml)	EC installment-installment$_{50}$

	Replication 1	Replication 2	Replication 3	
Extract	8,912	9,183	9,817	9,304
N hexane	7,261	7,079	7,194	7,178
ethyl	8,279	9,268	7,328	8,291
acetate	6,653	6,456	6,591	6,657
air				

EC value results50 showed that the water fraction had the lowest value, namely 6.657 mg/ml, followed by the n-hexane fraction 7.178 mg/ml, the ethyl acetate fraction 8.291 mg/ml and the extract 9.304 mg/ml. The smaller the EC value50 obtained, the greater the activity of the fraction in degrading bacterial biofilms E.coli. EC Results50 The results obtained were analyzed using statistical analysis one way anova to see significant differences between rosella flower extracts and fractions. The requirement in the normality test is that the data must be homogeneous with a sig value greater than alpha > 0.05 so that H0 is accepted and the EC data50 declared to be normally distributed. Normally distributed data is then continued with parametric tests, namely one way anova. Significant value of test results one way anova The size is 0.000 < 0.05 so this proves that the data obtained is homogeneous. Data obtained from the Tukey test shows a sign (*) in the column mean difference which means IC results50 shows significant differences. Statistical tests can be seen in the attachment.

Saponin is the most effective compound in biofilm degradation. Saponins can penetrate bacterial cell walls and destroy the polymer matrix that forms biofilms, thereby reducing the size and density of the biofilm. Saponins work in a more direct and aggressive way, such as damaging cell membranes and the biofilm matrix through physical mechanisms. Saponin is a compound glycosides which consists of an aglycone (sapogenin) and one or more sugars, has amphiphilic properties (hydrophobic and hydrophilic). Due to its amphiphilic nature, saponins have the ability to interact with cell membranes and lipids in the biofilm matrix, causing physical disruption, and making it quickly reduce the existing biofilm. has been formed.

Alkaloid compounds can interfere with the process of bacterial attachment to surfaces, which is the initial step in biofilm formation. This can change the strong biofilm (strong biofilm former) becomes a weak biofilm. The alkaloids in rosella flowers can interfere with communication signals between bacteria Quorum sensing, which plays a role in biofilm formation. This can inhibit the growth of biofilm by bacteria E. coli. Alkaloids can damage the biofilm structure, including the glycosidic bonds in the biofilm polysaccharide chains. Alkaloids can inactivate genes that trigger the synthesis of extracted polysaccharides (EPS), which is an important component in biofilm formation. This can reduce the ability of bacteria E. coli to form a strong biofilm.

CONCLUSION

Rosella flower water fraction (Hibiscus sabdariffa L) has antibacterial activity to bacteria E. coli ATCC 2522 with a KBM value of 25 mg/ml and an MIC value of 25 mg/ml. The most active fraction of rosella flowers (Hibiscus sabdariffa L) in inhibiting formation bacterial biofilm E. coli ATCC 25922 is a water fraction with an IC value50 biofilm inhibition of 7,500 ± 0.111 mg/ml and EC value50 Biofilm degradation was 6.657 ± 0.082 mg/ml. Effect of action of water fractions of rosella flowers (Hibiscus sabdariffa L) against bacteria E. coli ATCC 25922 is in the cell membrane and cell wall of bacteria.

REFERENCES

A.Amalia Dahlia, Asni Amin, R.L., & Fakultas. (2012). Identification of Morphology and Specific Parameters of Simplicia and Rosella (Hibiscus Sabdariffa L.) Leaf Extract from Kab. Enrekang (South Sulawesi). 04(02), 159–175.

Alhede, M., Alhede, M., Qvortrup, K., Kragh, K. N., Jensen, P. Ø., Stewart, P. S., & Bjarnsholt, T. (2020). The Origin Of Extracellular Dna In Bacterial Biofilm Infections In Vivo. Pathogens And Disease, 78(2), 1–15.

Ali, K., Yuziani, & Mulyati Sri Rahayu. (2022). Activity Test of Ethanol Extract of Breadfruit Leaves (Artocarpus Altilis) Against Escherichia Coli Bacteria. Human and Health Scientific Journal, 5(2), 265–271.

Alvita, L. R., Falah, S., & Nurhidayat, N. (2017). Water Extract Activity Of Papaya Leaf As Antibiofilm Against Escherichia Coli. Current Biochemistry, 2(3), 164–175. Https://Doi.Org/10.29244/Cb.2.3. 164-175

Andayani, N., Nurhayati, D., & Saing, M. D. (2022). Optimization of Bacterial Growth E. Coli and Bacillus Subtilis in Edamame Media to Optimize the Growth of E. Coli And Bacillus Subtilis. 1(1), 45–53.

Arjuna, A., Pratama, W. S., Sartini, S., & Mufidah, M. (2018). Anti-Biofilm Preliminary Test of Green Tea and Black Tea Extracts in Streptococcus Mutans Via the Microtiter Plate Method. Galenika Journal Of Pharmacy (E-Journal), 4(1), 44–49.

Basavaraju, M. (2022). Escherichia Coli: An Overview Of Main Characteristics. November.

Brooks, G. F., Butel, J. S., & Ornston, L. N. (2008). Jawetz, Melnick And Adelberg Medical Microbiology

(Translation). EGC Medical Books, Jakarta, 627–629.

Chofidah, A. I., Danu, M. D., & Rosyidah, I. H. (2019). Test Antibacterial Activity of Roselle Kombucha (Hibiscus Sabdariffa L.) Against the bacteria Escherichia Coli and Staphylococcus Aureus, S1 Pharmacy Study Program, Anwar Medika Hospital Stickers, Sidoarjo, East Java, D3 Pharmacy Study Program, Hospital Stickers. 2(1), 43–47.

Estri, M. R., & Anggarbeni, S. R. (2015). Test of the Inhibitory Power of Water Boiled from Rosella Flowers (Hibiscus Sabdariffa L.) Against the Growth of Esherichia Coli Bacteria. Wiyata Journal, 2, 9–13.

Fathoni, M. M., Isnaeni, I., & Darmawati, A. (2021). Anti-Bacterial Activity Of Rosela Flower Extract (Hibiscus Sabdariffa L.) Against Extended-Spectrum Beta-Lactamase (Esbl) Eschericia Coli. Scientific Periodicals in Pharmaceutical Chemistry, 8(1), 7.

Fiana, F. M., Kiromah, N. Z. W., & Purwanti, E. (2020). Antibacterial Activity of Ethanol Extract of Breadfruit Leaves (Artocarpus Altilis) Against Bacteria Staphylococcus Aureus And Escherichia Coli. Pharmacon: Indonesian Pharmacy Journal, 10–20.

Fink, R., & Filip, S. (2023). Surface-Active Natural Saponins . Properties , Safety , And Efficacy. International Journal Of Environmental Health Research, 33(7), 639–648.

Fitriana, Y. A. N., Fatimah, V. A. N., & Fitri, A. S. (2020). Anti-Bacterial Activity of Betel Leaves: Extract Test for Khm (Minimum Inhibitory Level) and Kbm (Minimum Bactericidal Level). Saintex, 16(2), 101–108.

Hardyanti Eka Putri. (2016). Uji TAcute toxicity of Ethanol Extract of Bidara Leaves (Ziziphus Spina-Christi L.) TThe Morphology and Histology of Mice Liver (Mus Musculus) Male. X, 1–21.

Hermawati, A. H., Surtini, S., Arohman, A. L., & Hariyanto, H. (2023). Ciprofloxacin Antibiotic Test on Growth Escherichia Coli In Vitro. Scholarly Journal, 10(3), 181–188.

Isnaini, L. (2010). Extraction of Natural Liquid Red Colorant Containing Antioxidant From Rosella (Hibiscus Sabdariffa L) Flower Petals and Its Application in Food Products The Extraction of Natural Liquid Red Colorant Containing Antioxidant From Rosella (Hibiscus Sabdariffa L) Calyx And. Journal of Agricultural Technology, 11, 18–27.

Jaddoa, N. T. M., & Gharb, L. A. (2021). The Antibiofilm Activity Of Hibiscus Sabdriffa L. Against Methicillin-Resistant Staphylococcus Aureus. Iraqi Journal Of Agricultural Sciences, 52(3), 626–631.

Miquel, S., Lagrafeuille, R., Souweine, B., & Forestier, C. (2016). Anti-Biofilm Activity As A Health Issue. Frontiers In Microbiology, 7(Apr), 1–14.

Muslihin, A. M., & Budiyanto, A. B. (N.D.). Determination of water soluble essence content, ethanol soluble essence content and identification of alkaloids in 96% ethanol extract of cherry leaves (Muntingia Calabura L.).

Nahor, E. M., Rumagit, B. I., & Tou, H. Y. (N. D.). Comparison of the Yield of Ethanol Extract of Andong Leaves (Cordyline Futicosa L.) Using Maceration and Sochletation Extraction Methods 40–44.

Nopiyanti, V., & Aisiyah, S. (2020). Test for Determining the Spf Value (Sun Protection Factor) of Roselle Flower Fractions (Hibiscus Sabdariffa L.) As an Active Sunscreen Substance D. Journal Of Pharmacy, 9(1), 2656–8950.

Novita, W. (2016a). Against the Growth of Streptococcus Mutans Willia Novita Bacteria. jmj, 4.

Nurhayati, L. S., Yahdiyani, N., & Hidayatulloh, A. (2020). Comparison of Yogurt Starter Antibacterial Activity Testing Using the Well Diffusion Method and the Disc Diffusion Method. Journal of Animal Products Technology, 1(2), 41.

P, D. P. S., Nur, Y., Fadraersada, J., Farmasi, F., & Mulawarman, U. (2018). Stability Profile of Rosella (Hibiscus Sabdariffa) Flower Extract as a Lipstick Colorant Candidate. November, 20–21.

Purba, H., Simanjuntak, H. A., & Situmorang, R. (2020). Phytochemical Screening Of Rosella Flowers (Hibiscus Sabdariffa L) And Antimicrobial Activity Test. Journal of Chemical Education, 12(2), 70–78.

Putri, F. E., Diharmi, A., & Karnila, R. (2023). Identification of Secondary Metabolite Compounds in Brown Seaweed (Sargassum Plagyophyllum) Using the Fractionation Method. Journal of Indonesian Agricultural Technology and Industry, 15(1), 40–46.

Ridwan, M., Risandiansyah, R., Yahya, A., Ridwan, M., Risandiansyah, R., & Yahya, A. (N.D.). Antibacterial and Antibiofilm Activity of Rosella (Hibiscus Sabdariffa) Petal Extract Against Streptococcus Mutans 1–11.

Rollando. (2017). Isolation, Identification, Characterization, and Testing of Gallic Acid Derivative Antibiofilm. 7(2), 105–111.

Samrot, A. V., Mohamed, A. A., Faradjeva, E., Jie, L. S., Sze, C. H., Arif, A., Sean, T. C., Michael, E. N., Mun, C. Y., Qi, N. X., Mok, P. L., & Kumar, S. S. (2021). Mechanisms And Impact Of Biofilms And Targeting Of Biofilms Using Bioactive Compounds—A Review. Medicine (Lithuania), 57(8), 1–28.

Soegianto, L., Hertiani, T., & Pramono, S. (2016). Isolation and Identification of Antibacterial and Antiquorum Sensing Substances in Roselle Flower Petal Extract (Hibiscus Sabdariffa L). Indonesian Journal of Pharmaceutical Sciences, 14(2), 212–218.

Sudarwati Dwi, & Sumarni Woro. (2016). Test of Antibacterial Compound Activity in Moringa Leaf and Rosella Flower Extracts. Indonesian Journal Of Chemical Science, 5(1), 1–4.

Sugito, S., & Marliyana, S. D. (2021). Performance Test of the Thermo Ice 3000 Atomic Absorption Spectrophotometer on Pb Metal Using Crm 500 and Crm 697 at the Uns Integrated Laboratory. Indonesian Journal Of Laboratory, 4(2), 67.

Suwadi, P., Fauzan, R. D., Yulianto, A., Usman, A. N., & Fauzi, A. (2021). Diversification of Rosella Plants (Hibiscus Sadbariffa L.) as an Effort to Improve the Welfare and Economy of the Community of Sumberdem Village, Wonosari, Malang. Semar (Journal of Science, Technology and Arts for Society), 10(1), 22.

Syarifuddin, A., Sulistyani, N., & Kintoko, K. (2018). Activity Of Antibiotic Bacterial Isolate Kp13 And Cell Leakage Analysis Of Escherichia Coli Bacteria. Indonesian Journal of Pharmaceutical Sciences, 16(2), 137.

Widarta, I. W. R. (2020). Patron Prof. Dr. Ir. I Ketut Satriawan, Mt Responsible Prof. Ir. I Nyoman Semadi Antara, Ph. D Chief Editor Gede Arda, Stp., M. Sc. Internal Reviewer Dr. Ir. Dewa Gde Mayun Permana, Mp. Dr. I Wayan Arnata, S. But ., M . The External Reviewer Managing Editor N . L . Ari Yusasrini, Stp., Mp. Production and Distribution I Kadek Adiguna, Se Ni Kadek Pindari, S. Comm. 5(2).

Widnyani, I. A. P. A., Yoga, W. K. Y., Ariani, N. K. S., & Sintyadewi, P. R. (2023). Processing of Rosella (Hibiscus Sabdariffa) Flowers as a Syrup Product with Antioxidant Content at SMA Negeri 1 Rooftop Lembongan. Widya Laksana Journal, 12(2), 204–211.