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Leakage Induced in *Eschericia coli* Cells by Secondary Metabolites of the J7 Bacterial Isolates from the Rhizosphere of *Zingiber officinale* Roscoe var. Rubrum

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ABSTRACT

Isolate J7 is a bacterial strain separated from the rhizosphere of *Zingiber officinale* Roscoe var. Rubrum that potentially produces antibacterial compounds against *Escherichia coli*. The study aimed to determine the antibacterial activity of the most active fraction of this isolate from its capacity to induce leakage in *E. coli* cells. The secondary metabolites were extracted from Isolate J7 using ethyl acetate solvent and then fractionated with different ratios of hexane and ethyl acetate solvents—1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:1 (v/v), ethyl acetate and methanol with 1:1 (v/v) ratio, and methanol 100%. Fractions were identified based on the spotting on the Thin-Layer Chromatography (TLC) plate. Only the most active fraction was tested to define its ability to cause leakage of cellular components like nucleic acid and protein. The leakage was scanned with a UV-Vis spectrophotometer at wavelengths of 260 and 280 nm. The results showed that F2 was the fraction that prevented the growth of *E. coli* most actively because it created a zone of inhibition sized 11.58±0.95 mm in diameter with the lowest MIC among the other fractions (10%). Also, based on the spectrophotometric analysis, the addition of F2 at concentrations of 10% and 20% resulted in higher levels of protein, DNA, and RNA than the negative control. In conclusion, F2 can cause plasma membrane leakage in *E. coli* at a concentration of 10%. Another fraction that exhibited antibacterial activity was F3. In the spotting analysis of the TLC plate, F3 appeared to have a spot profile and R_f that were similar to F2 but considerably different from the inactive fractions (i.e., showing no antibacterial activity). Observed with multiple wavelengths, the R_f values of F2 and F3 spots varied between 0.56-0.57 and 0.61-0.62 (254 nm) and 0.47-0.48 and 0.56-0.57 (366 nm), respectively. Because these spot profiles did not appear in the inactive fractions, compounds with this range of R_f values are, thereby, suspected as the active substances that inhibit the growth of *E. coli*.

Keywords: *Escherichia coli,* cell membrane leakage, secondary metabolite, Isolate J7

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INTRODUCTION

Infectious disease is a continuously growing problem in the field of medicines. One of its causes is exposure to bacteria, for example, *Escherichia coli.* Nowadays, many bacteria have developed antibiotic resistance whose prevalence is aided by the excessive and inappropriate use of antibiotics as anti-infectives (Rahayu, 2006). It refers to a condition when bacteria change over time and can even survive antibiotics.

Antibiotic resistance has triggered the exploration of natural resources as a countermeasure. For decades, secondary metabolites produced by microbes have become a major source of new antibiotics. Nevertheless, many of their potential compounds have yet to be included in the drug development (Genilloud *et al*., 2011) even though sources for the development come in various forms, for instance, soil. Soil, particularly the rhizosphere, is the habitat of many microbes. This layer surrounds the roots of plants and, therefore, its development is influenced by roots (Dakora and Donald, 2002).

Sulistyani *et al.* (2016) identified antibacterial activities in 12 out of 19 bacterial isolates. One of which was isolated from the rhizosphere of red ginger plants and coded with J7 (Isolate J7). Their study also extracted the broth cultures of the isolates using ethyl acetate solvent. Based on the antibacterial susceptibility testing of the extract, Isolate J7 had been proven to inhibit the growth of *E. coli* and *Staphylococcus aureus*. Therefore, to develop the most active fractions of Isolate J7 as alternative antibiotics against *E. coli*, further research must focus on fractionation, leakage of cellular components in the test microbes due to exposure to antibacterial substances, and the mechanism of the bacterial growth inhibition.

METHOD

Materials

The research used Isolate J7 from the rhizosphere of red ginger plants obtained in a previous study by Dr. Nanik Sulistyani, M.Si., Apt. The other research materials included Mueller Hinton (MH) medium, Starch Nitrate Broth (SNB) medium, NaCl 0.9%, Brain-Heart Infusion (BHI) broth, McFarland standards (10^8 CFU/mL), *Escherichia coli*, chloramphenicol, dimethyl sulfoxide (DMSO), ethyl acetate, N-hexane, methanol, distilled water, and chloroform.

Instruments

The instruments used in this research were Laminar Air Flow (LAF; Monmouth Scientific), Biological Safety Cabinet (BSC), UV-Vis spectrophotometer (Shimadzu 1800), CAMAG TLC Scanner 4, and glassware.

Procedure

Preparation of broth culture of isolate J7

The culture was prepared by pouring 10 mL of bacterial starter into an Erlenmeyer flask containing 100 mL of sterile SNB (Wang *et al*., 2010). Then, the flask was placed on a magnetic stirrer and incubated at room temperature for five (5) days. After incubation, 50 mL of the suspension culture was transferred into an Erlenmeyer flask containing 500 mL of SNB medium. Then, the flask was placed on a magnetic stirrer and incubated at room temperature for five (5) days. After five days of incubation, 300 mL of the incubated broth culture was poured into a sterile Erlenmeyer flask containing 3 liters of sterile SNB medium. Then, this flask was agitated with a magnetic stirrer and incubated at room temperature for 14 days.

Extraction, fractionation, and thin layer chromatography

The broth cultures that had been incubated for 14 days was filtered using a Büchner and vacuum funnel, then concentrated at a temperature of lower than 50°C. The filtrate was extracted with ethyl acetate with a ratio of 1:1 (v/v). The ethyl acetate extract was fractionated in a separatory funnel according to its polarity. The solvent in the fractionation was a mixture of n-hexane and ethyl acetate with different ratios, namely 1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:1 (v/v), ethyl acetate-methanol 1:1 (v/v), and methanol 100%. The result of fractionation was evaporated at room temperature inside a fume hood. Each of the fractions was then eluted by Thin Layer Chromatography using the combination of chloroform, ethyl acetate, methanol with the ratio of 4:1:0.5 (v/v) as the mobile phase. Afterward, fractions with the same spot and R_f profile were categorized into one group.

Preparation of Escherichia *coli* culture

A total of 100 µL of the bacterial stock was grown in 1 mL BHI broth and incubated at 37.5°C for 18-24 hours. Afterward, 100 µL of the culture was placed in 1 mL BHI broth and incubated for 3-5 hours. Then, 100 µL of the culture was taken and diluted in NaCl 0.9% until the turbidity was equal to the McFarland standards, i.e., comparable to the density of a bacterial suspension of 10^8 CFU/mL (Sulistiyani and Mulyadi, 2013).

Antibacterial assay of the ethyl acetate extract and fraction of the broth culture of isolate J7

The medium used for this test was Mueller Hinton Agar (MHA) that had been inoculated with the suspension of *Escherichia coli*. Furthermore, each well of the plate was filled with 50 µL of the ethyl acetate extract of the Isolate J7's liquid culture at a concentration of 40% (b/v), allowed to absorb, and then incubated at 37ºC for 18-24 hours. The growth-inhibitory activity of this extract is indicated by the existence of a clear zone around the well (Alimuddin *et al*., 2011).

The same method was used to test the antibacterial activity of the other fractions. The Minimum Inhibitory Concentration (MIC) was determined using a serial dilution of 5%, 10%, 15%, and 20%. MIC is the lowest concentration of the test substance that prevents the growth of targeted bacteria.

Leakage assay based on protein and nucleic acid level

Suspension of the test bacteria aged 18-24 hours was centrifuged at 3500 rpm for 15-20 minutes to obtain microbial biomass. The biomass was washed with phosphate buffer (pH 7.4), and the washing process was repeated two (2) times. The cell was then suspended in 4 mL of phosphate buffer solution (pH 7.4). The presence of protein and nucleic acid content were identified after the active fraction was allowed direct contact with the test bacteria for 24 hours at 37°C in the incubator. The suspension was centrifuged for 15 minutes at 3500 rpm, and then the supernatant was separated and taken for analysis using a UV-Vis spectrophotometer with multiple wavelengths, namely 260 and 280 nm, to determine the protein and nucleic acid content (Jamal *et al*., 2013).

RESULTS AND DISCUSSION

The culture was prepared by combining starter culture and SNB media with a ratio of 1:10 to obtain a concentration of 10% (v/v) (Wang *et al*., 2010). The liquid SNB media is a suitable growth medium, provided that the nutrients needed for bacterial growth such as the source of carbon and other minerals are available (Todar, 2007). The carbon source in SNB media comes from a C-containing soluble starch, which varies between, for instance, tapioca starch and glycerol (Alimuddin *et al*., 2011). A total of 10 mL of bacterial starter was poured into an Erlenmeyer flask containing 100 mL of sterilized SNB medium first. This flask was placed on a magnetic stirrer then incubated at room temperature for five days. Stirring may affect the mixing of nutrients in the medium of fermentation and increase the resulted metabolites (Agustine *et al*., 2004). The starter was poured into a new

medium, and a continuous culture was conducted to maintain bacterial growth in the exponential phase.

Then with multilevel culture, 300 mL of incubated liquid culture was put into sterile erlenmeyer containing 3 liters of sterile SNB.

After 5 days of fermentation, a multilevel culture was carried out. A total of 50 mL of the culture broth was poured into an Erlenmeyer flask containing 500 mL of sterile SNB medium, agitated with a magnetic stirrer, and incubated at room temperature for five days. In the multilevel culture, 300 mL of liquid culture produced in the incubation was poured into a sterile Erlenmeyer flask containing 3L of sterile SNB medium.

The incubation lasted for 14 days, the production of secondary metabolites (antibiotics) started on Day 5 of incubation, increased, and achieved the highest yield on Day 14. The color change during the incubation period indicated that the bacteria secreted (color) pigments that were either capable of diffusing in the media or not (Ambarwati and Gama, 2009). Therefore, the color change in the culture broth was due to the ability of Isolate J7 to produce pigment (Figure 1).

(a) (b) (c) (d)

Figure 1. Color change in the culture broth of Isolate J7 after incubation for (a) one day, (b) five days, (c) ten days, and (d) fourteen days

Antibacterial Activity of the Ethyl Acetate Extracts of the Culture Broth of Isolate J7

The antibacterial susceptibility testing employed the diffusion method. The ethyl acetate extract of Isolate J7 was prepared at a concentration of 40% (w/v) using 10% DMSO solvent, then incubated at 37°C for 18-24 hours. Before use, the Mueller Hinton (MH) medium was first inoculated with the *Escherichia coli* suspension of 10^8 CFU/mL using a sterile cotton lid. Afterward, the extract was poured into the hole or well with a diameter of 6 mm. At the concentration of 40%, the extract created a zone of inhibition with an average diameter of 17.6±1.35 mm (Figure 2), while the negative control (DMSO 10%) showed no inhibitory activity. The positive control (Chloramphenicol 0.1%) produced a zone of inhibition with an average size of 45.2±3.48 mm.

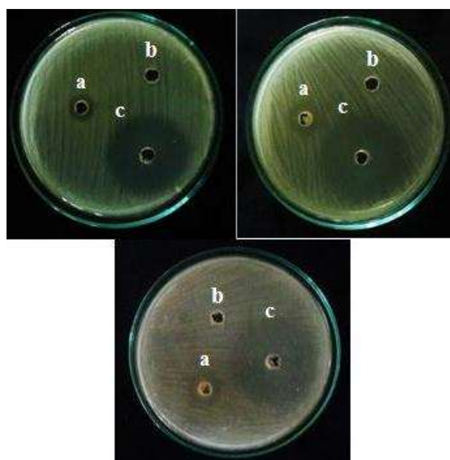

Figure 2. The Antibacterial Activities of (a) the Ethyl Acetate Extract of the Broth Culture of Isolate J7 (40% w/v), (b) Negative Control (DMSO 10%), and (c) Positive Control (Chloramphenicol 0.1%) against Escherichia coli

Fractionation of the Ethyl Acetate Extract of the Broth Culture of Isolate J7

The fractionation of the ethyl acetate extract employed a gradient solvent concentration. The solvents, namely n-hexane, ethyl acetate, and methanol, were mixed with different ratios to create a total volume of 100 mL (Table I). The mixture was replicated three times and stirred for one hour at 7,000 rpm.

Table I. The Solvent Ratios

Fractions	Solvents (v/v)		
	n-hexane (%)	Ethyl acetate (%)	Methanol (%)
1	100	-	-
2	90	10	-
3	80	20	-
4	70	30	-
5	60	40	-
6	50	50	-
7	40	60	-
8	30	70	-
9	20	80	-
10	10	90	-
11	-	100	-
12	-	100	100
13	-	-	100

The Thin Layer Chromatography of Fraction 1-13 produced chromatograms with a various pattern. Fractions with similar or exact profiles were combined into one group. Therefore, eight (8) groups of fractions were obtained (Table II).

Table II. The Fractions Grouping

Fraction	1	2	3	4	5	6	7	8	9	10	11	12	13
Groups of Fraction	F1	F2		F3		F4		F5	F6	F7		F8	

Antibacterial Activity of Fractions F1-F8 against *Escherichia coli*

Each of the eight groups of fractions (F1, F2, F3, F4, F5, F6, F7, and F8) was tested. The results revealed that only F2 and F3 exhibited inhibitory activity against *E. coli*. The MICs of F2 and F3 were 10%, which produced zones of inhibition with diameters of 11.58±0.95 mm and 9.25±0.87 mm, respectively (Table III).

Table III. The antibacterial activity of fractions F1-F8 against *E. Coli*

Fraction No.	**Zone of Bacterial Growth Inhibition (diameter; mm)**			
	5%	**10%**	**15%**	**20%**
F1	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
F2	6.00±0.00	11.58±0.95*	12.42±1.66*	12.67±1.44*
F3	6.00±0.00	9.25±0.87*	9.75±0.50*	13.08±0.52*
F4	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
F5	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
F6	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
F7	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
F8	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00
Chloramphenicol 0.1%	32.42±1.01*			
DMSO 10%	6.00±0.00			

Description: *shows significant difference (p value <0.05) from negative control and sample with 5% concentration

F2, the most active fraction, was then tested for its capacity to cause leakage of cellular components in *E. coli*. The addition of F2 at concentrations of 10% and 20% increased the release of substances from bacterial cells. The elements that leaked out of the cells and were measurable at a wavelength of 260 nm were parts of DNA, namely purines, pyrimidines, and ribonucleotides, while the ones measurable at 280 nm were tyrosine and tryptophan (Park and Park., 2003).

Table IV. Leakage of Cellular Components from *Escherichia coli* after the Addition of Fraction F2

Parameters	Values (After the Addition of Fraction F2)		
	F2 0%	F2 10%	F2 20%
OD280/260	0.872±0.002	1.007±0.007	1.008±0.002
Nucleic Acid (%)	5.3±0.289	3.5±0.000	3.5±0.000
Correction Factor	0.67±0.012	0.780±0.000	0.78±0.000
Protein Level (µg/mL)	0.410±0.005	1.797±0.009*	1.806±0.003*
DNA Level (µg/mL)	35.27±0.301	114.32±0.275*	114.87±0.275*
RNA Level (µg/mL)	28.21±0.241	91.45±0.220*	91.89±0.220*
OD260/280	1.146±0.002	0.993±0.007	0.992±0.002

Description= *shows significant difference (p value< 0.05) from F2 with 0% concentration

Leakage caused by exposure to antimicrobial compounds can be detected by observing excessive intracellular fluid discharges, such as ions, nucleic acids, and proteins (Miksusanti *et al*., 2008). The content of proteins, nucleic acids, and uracil in buffers is referred to as leakage of proteins, nucleic acid, and uracil (Bergmans *et al*., 2005). The release of substances in the form of nucleic acids and proteins showed that the cells leaked or the membrane permeability was damaged (Giacometti, 2000). According to Engelberg-Kulka *et al.* (2004), leakage of proteins and nucleic acids can occur through an intercalation mechanism between active compounds and DNA/RNA, which can inhibit the process of transcription and translation.

Almost all antibiotics work by damaging the cytoplasmic membrane. Because the fraction used in the study is hydrophobic, the initial mechanism involves attaching the compounds of the extract to phospholipids and lipoproteins in the outer cell membrane. Fractions also possibly bind to hydrophobic peptidoglycan and disrupt membrane permeability. As a result, the fractions enter the cell's cytosol and interfere with its entire metabolic process (Nurkanto *et al*., 2010). Miksusanti *et al.* (2008) referred to studies conducted by Sikkema *et al.* (1994) and Ultee *et al.* (2002) to explain that the accumulation of fractions in the cytoplasm can cause membranes to swell and alter their permeability and fluidity. Consequently, not only does the membrane potential decrease, but the enzyme working in the metabolic process also diminishes and eventually leads to the leakage of cellular components.

In this study, the leakage was detected by high levels of protein and nucleic acid. When leaked in low concentrations, these two components were also detectable even without the addition of Fraction F2. It might be possible because cells naturally die after 24 hours of incubation and nutritional deficiency. Signs of death include broken bits of membrane and the release of intracellular materials like protein and nucleic acid to the buffer solution, making them detectable by spectrophotometer.

Rf Values of TLC Spots of Active Fractions

Chromatography is a physical method of compounds separation based on differences in the partitioning of two phases, namely the stationary phase and the mobile phase (Kristanti *et al*., 2008). In

this study, all groups of fractions were treated as samples. Each of them was bottled at a concentration of 40% of 10 µL; each bottle contained 4 mg of the fraction. The mobile phase was based on the results of optimization, namely the combination of chloroform, ethyl acetate, and methanol with a ratio of 4:1:0.5, while the stationary phase was silica gel F_{254}. The chromatogram showing the separation of the compounds in each group of fractions is presented in Figure 3.

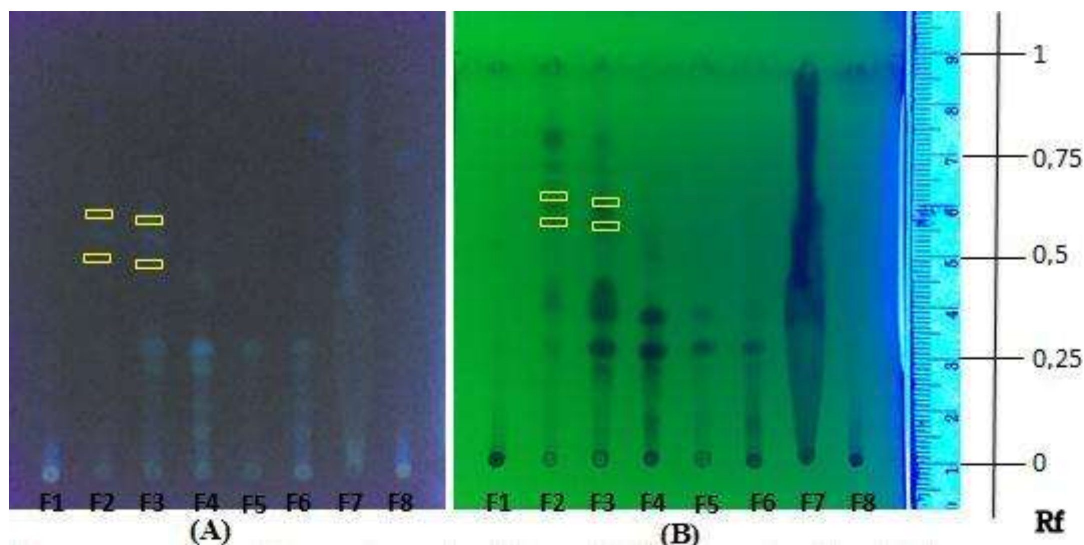

Figure 3. The chromatogram profile (TLC) of each fraction visualized under UV light: 366 nm (A) and 254 nm (B). The box marks the spot that is predicted to have antibacterial activity

The eluted spots were scanned with CAMAG TLC Scanner 4 at multiple wavelengths, namely 254 nm and 366 nm. The spot profiles of the fractions were compared to identify the differences between fractions that showed inhibitory activity against the growth of *Escherichia coli* (F2 and F3) and the ones that had no inhibitory properties against the bacteria (F1, F4, F5, F6, F7, and F8).

Based on Table V, the spots of the active and inactive fractions can be distinguished at a wavelength of 254 nm. The R_f values of F2 spots are 0.57 and 0.62, while the ones of F3 are 0.56 and 0.61. These spots are suspected as the active substances that are responsible for the inhibitory activity against *Escherichia coli* because they were found only in the elution of the active fractions (none was identified in the others). Furthermore, based on the area (%), F2 (5.53-8.82%) is believed to contain greater active matters than F3 (2.53-6.59%). This finding is consistent with the results of the antibacterial susceptibility test, i.e., F2 is more active than F3.

Table V. The TLC analysis results of the fraction groups using CAMAG TLC Scanner 4 at a Wavelength of 254 nm

F1			F2			F3			F4			F5			F6			F7			F8		
Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)
1	0,02	56,37	1	0,02	7,84	1	0,01	5,97	1	0,02	10,17	1	0,02	16,88	1	0,02	20,72	1	0,02	3,65	1	0,02	50,15
									2	0,04	12,57				2	0,04	20,76	2	0,04	2,40			
						2	0,08	3,02				2	0,07	10,06									
																		3	0,09	13,12			
						3	0,14	10,15	3	0,13	5,75				3	0,13	8,51						
									4	0,17	9,98	3	0,17	6,37	4	0,18	4,34						
			2	0,20	5,81										5	0,20	5,67						
						4	0,23	17,64	5	0,22	24,80	4	0,22	28,92	6	0,23	16,64				2	0,23	2,80
																		4	0,26	10,29			
			3	0,31	13,84	5	0,30	29,50	6	0,30	22,82	5	0,31	15,04	7	0,31	5,86						
2	0,33	1,73																					
																		5	0,36	18,92			
									7	0,40	5,37				8	0,40	0,78						
															9	0,43	1,99						
3	0,47	3,81	4	0,48	12,10	6	0,48	9,53															
												6	0,49	2,14									
									8	0,50	2,25							6	0,50	22,91			
			5	0,57	8,82	7	0,56	6,59															
			6	0,62	5,53	8	0,61	2,53															
			7	0,66	6,91	9	0,65	2,48										7	0,67	16,20			
4	0,70	3,21	8	0,70	22,49	10	0,70	5,52															
																					3	0,79	47,06
						11	0,82	7,08	9	0,82	6,28	7	0,82	20,58	10	0,81	14,73	8	0,82	12,52			
5	0,83	34,88	9	0,84	16,66																		

Table VI. The TLC analysis results of the fraction groups using CAMAG TLC scanner 4 at a Wavelength of 366 nm

F1			F2			F3			F4			F5			F6			F7			F8		
Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)	Peak	Rf	Area (%)
1	-0,02	27,99	1	0,02	13,82	1	0,02	7,93	1	0,02	26,19	1	0,02	57,06	1	0,03	57,02	1	0,02	26,93	1	0,02	68,87
2	-0,00	59,65	2	0,01	16,96	2	0,00	14,67															
									2	0,07	8,29	2	0,07	13,84	2	0,07	12,09	2	0,07	36,87			
						3	0,12	26,31													2	0,11	5,84
									3	0,13	13,38												
															3	0,15	15,28						
									4	0,18	13,23												
									5	0,23	7,70												
						4	0,28	12,28	6	0,27	19,14	3	0,27	9,23	4	0,28	5,58						
																		3	0,29	9,53			
																		4	0,38	18,75			
									7	0,42	5,00												
			3	0,48	14,96	5	0,47	15,79															
									8	0,50	2,90												
			4	0,57	29,38	6	0,56	18,34															
			5	0,77	4,47																		
			6	0,80	9,12																3	0,81	4,29
																		5	0,84	7,91			
3	0,87	12,36	7	0,87	11,29	7	0,87	4,68	9	0,86	4,16	4	0,87	19,87	5	0,87	10,04				4	0,86	21,00

At a wavelength of 366 nm, the spots of the active and inactive fractions are also distinguishable (Table VI). The R_f values of F2 spots are 0.48 and 0.57, while the ones of F3 are 0.47 and 0.56. Because these spots were only found in the active fractions (none was identified in the others), they are suspected as the active substances that induce the inhibitory response against the growth of *Escherichia coli*. When observed from the area (%) and associated with the activity of each fraction, F2 has more extensive area than F3, implying that the former may inhibit the growth of *Escherichia coli* better than the latter. This finding is in line with the zone of inhibition formed during the antibacterial susceptibility test of each group of fractions, i.e., F2 produced a larger zone of inhibition than F3 when applied at the same MIC.

CONCLUSION

The results of the antibacterial activity test showed that F2 and F3 had antibacterial properties against *Escherichia coli*. F2 is the most active fraction that, at the concentration of 10%, created a zone of inhibition with a diameter of 11.58±0.95 mm. F2 caused leakage of cellular components, as indicated by the increase of nucleic acid and protein observed at wavelengths of 260 and 280 nm. When analyzed at a wavelength of 254 nm, the R_f values of the spots of F2 and F3 were 0.56-0.57 and 0.61-0.62, respectively. Meanwhile, at a wavelength of 260, the R_f values were 0.47-0.48 and 0.56-0.57. These spots are predicted as the agents behind the inhibitory properties of the ethyl acetate extract of Isolate J7 against *Escherichia coli.*

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