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Mechanism of Ethyl Acetate Extract (Isolate TE 325) Against *Escherichia Coli* with Atomic Absorption Spectrophotometry and UV-Vis Spectrophotometry Analysis

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Abstract. Infection is a disease with a high incidence, especially in Indonesia. Treatment of infections using Antibiotics but constrained cases of resistance. Exploration of microorganisms that can produce antibiotics is done, one of which is the exploration of Actinomycetes bacteria. The TE 325 is Actinomycetes bacterial isolate that produces Antibiotic compounds and inhibits the growth of Escherichia coli bacteria. Ethyl acetate extract of TE 325 isolate can inhibit the growth of *Escherichia coli* bacteria, but the analysis of the mechanism of action of ethyl acetate extract has never been done in inhibiting the growth of *Escherichia coli* bacteria. This study aims to analyze the mechanism of action of ethyl acetate extract in inhibiting the growth of *Escherichia coli* bacteria in the event of bacterial cell leakage. The instruments used were Atomic Absorption Spectrophotometry (AAS) and UV-Vis Spectrophotometry. The extract concentrations used were 0%, 2.5%, and 5%. The results obtained by cell leakage are shown by increasing the concentration of extract. There is an increase in metal ions K^+ and Ca^{2+}, as well as leakage of proteins and nucleic acids. Leakage of K^+ and Ca^{2+} metal ions showed the mechanism of action of antibiotics against *Escherichia coli* bacteria by damaging cell membrane permeability and damaging cell walls.

INTRODUCTION

Infection is a disease that often occurs in the community. Treatment of cases of infection is still constrained by cases of bacterial resistance [1]. One of the causes of resistance is due to the improper management of Antibiotic therapy. Improper therapy management causes drug activity is not in accordance with the mechanism of action expected to inhibit the growth of a bacterium that causes infection, thus triggering the occurrence of resistance [2]. Resistance can be prevented, namely, by using Antibiotics appropriately so that the expected mechanism of Antibiotic action is appropriate in inhibiting bacterial growth. There are five types the mechanism of action of a medicine in inhibiting bacterial growth, namely inhibiting cell wall synthesis, inhibiting bacterial cell metabolism, inhibiting bacterial protein synthesis, inhibiting nucleic acid synthesis, and disrupting integrity of cell membrane [3]. Mechanism of Antibiotic activity is inhibited bacterial growth by analyzing leak of bacterial cell [4].

Cell leakage analysis was carried out with KP13 samples using Atomic Absorbance Spectrophotometer (AAS) and UV / Vis Spectrophotometer to analyze cell leakage in *Escherichia coli* bacteria [5]. The results are contained by leakage of metal ions Ca^{2+} and K^+. The outer membrane of the cell consists of lipopolysaccharides, phospholipids, and lipoproteins[6]. The strength of the lipopolysaccharide layer is stabilized by its interaction with Ca^{2+} ions and other divalent ions. Potassium ion (K^+) is contained in the cytoplasm on growing cells and affect the stability of bacterial cell membrane permeability [7]. The results of the absorption spectrophotometer indicate the leakage of proteins and nucleic acids [8] [9]. The mechanism of cell leakage in *Klebsiella pneumonia* bacteria with the most active fraction sample of ethanol extract of Cayenne leaves causes leakage of *Klebsiella pneumonia* cells characterized by leakage of nucleic acids, proteins, DNA, RNA, and Ca^{2+} [10].

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Isolation of new microorganisms has been carried out and obtained TE 325 isolates that it can produce Antibiotics. The Antibiotic activity can inhibit the growth of *Escherichia coli* bacteria with a minimum inhibitory level of 2.5%. Antibiotic compounds from TE 325 isolates have not known mechanism action in inhibiting the growth of *Escherichia coli* bacteria, so it needs to be investigated the mechanism action of antibiotic compounds from TE 325 isolates. This research is a follow-up study to find out the mechanism of action of Antibiotic compounds from TE 325 isolates in inhibiting the growth of *Escherichia coli* bacteria by analyzing bacterial cell leakage using the Atomic Absorbance Spectrophotometer (AAS) instrument and UV/ Vis spectrophotometer.

METHODS

Instrumentation

The instruments are used by a Shimadzu UV-visible Spectrophotometer, with a double beam specification and Atomic Absorbance Spectrophotometer (AAS) to analyzed ion K^+ and Ca^{2+} (Perkin Elmer 3110)

Selection of Solvents

The Ethyl acetate is selected for dissolve sample and DMSO 10%.

Culture preparation

The culture is prepared by 5 mL starter isolates into 50 mL *Starch Nitrat Borth* (SNB) media, that has sterilized [11]. It is Incubated at room temperature for five days by agitation using a magnetic stirrer. After five days, 50 mL of culture was transferred to an Erlenmeyer containing 500 mL of SNB media, that has sterilized and incubated at room temperature for five days with agitation using a magnetic stirrer. After 5 days of incubation, multilevel culture has carried out, 300 ml of the liquid culture has put into a sterile Erlenmeyer containing 3 liters of SNB sterile [11]. Then, Culture is incubated at room temperature for 14 days by agitation using a magnetic stirrer. To minimize the occurrence of contamination, culture preparation is carried out in the Laminar Air Flow (LAF) room.

Antibiotic Extraction

The culture has filtered with Buchner funnel, then concentrated at 50°C. The filtrate has extracted using ethyl acetate (1: 1 v/v) until the color of the extracted was the same as the original solvent. The water phase and the ethyl acetate phase are separated. The ethyl acetate phase was taken, and the active substance is separated from the ethyl acetate solvent by evaporation using a rotary evaporator followed by evaporation in the water bath until ethyl acetate extract is obtained [5].

Cellular leakage is analyzed by AAS

Bacterial suspension 18-24 hours of 5 ml, it was centrifuged at a speed of 3500 rpm for 15-20 minutes to obtain bacterial cell biomass. Biomass cell of bacterial consists of these deposits with phosphate buffer pH 7.4 and repeated washing two times. The precipitate is then suspended in 4 ml phosphate buffer solution pH 7.4. Leakage cell is expressed by measured metal ions contained in the bacterial test after contact with Extract ethyl acetate extract of TE 325 isolates at concentrations of 1MIC (2,5%) and 2MIC (5%). Ca^{2+} and K^+ ion leakage was detected using AAS (Atomic Absorption Spectrometer). The solution contact with cell was taken, and then centrifuged at 3500 rpm for 15-20 minutes to obtain a reserve of ions. Potassium (K^+) uptake at wavelengths of 766.5 nm and calcium (Ca^{2+}) uptake at wavelengths of 422.7 nm [12].

Cellular leakage is analyzed by UV-Vis Spectrophotometer

Bacteria suspension aged 18-24 hours by 10 ml centrifuged at a speed of 3500 rpm for 15-20 minutes to obtain cell in the form of sediment. The deposition of cell biomass was washed with phosphate buffer pH 7.4 and repeated washing two times. The precipitate is then suspended in 4 ml phosphate buffer solution pH 7.4. Leakage was expressed

by measuring the metal ions contained in the supernatant of bacteria after contacting with the extract of ethyl acetate of TE 325 isolates with bacteria at concentrations of 1MIC (2,5%) and 2MIC (5%) which had been incubated for 24 hours at 37°C. The suspension was centrifuged for 15 minutes at 3500 rpm. Then the supernatant was separated and taken to determine protein and nucleic acid content using UV-Vis spectrophotometers at wavelengths of 260 and 280 nm.

RESULTS AND DISCUSSION

Result

TE 325 is a bacterial isolate that can produce Antibiotic compounds. TE 325 bacterial isolate was isolated from the rhizosphere of the Cane Plant (*Saccharum officinale*) in Madugondo, Sitimulyo, Piyungan, Bantul, (7°49'57.9 "S 110°26 '01 .1" E). Preparation of this test was carried out by 5 mL of starter culture fluid that had been incubated for five days into Erlenmeyer containing 50 mL of sterilized SNB media. Transfer of this culture was carried out at LAF to prevent contamination. Making this testing culture is done by comparing the starter culture with SNB (Starch Nitrate Broth) media by 1:10. Incubation was carried out for 14 days with stirring using Thermolyne [13] [14].

TABLE 1. Incubation discoloration for 14 days

Day	Color	Day	Color
1	Yellowish white	8	Turbid yellow
2	Yellowish white	9	yellow
3	Yellowish white	10	yellow
4	Yellowish white	11	yellow
5	Yellowish white	12	yellow
6	Yellowish white	13	yellow
7	Turbid yellow	14	yellow

(a) Day 1 (b) Day 5 (c) Day 10 (d) Day 14

FIGURE 1. Incubation discoloration for 14 days

The mechanism of Antibiotics is divided into several mechanisms. One mechanism of action is to inhibit bacterial growth, namely by causing damage to the cytoplasmic membrane, which is observed for cytoplasmic leakage shown by leakage such as K^+ ions. Membrane permeability that does not function properly can result in cell leakage and cell component discharge [15]. Increasing the concentration of K^+ outside the cell is an indicator of damage to the permeability of the membrane cell [16]. Another mechanism of growth inhibition by stability of bacterial cell walls. The affect the balance of the wall, namely the release of Ca^{2+} ions that cause bacterial death [17].

Gram-negative bacteria, namely *Escherichia coli* has a wall structure consisting of three layers, namely the cytoplasmic membrane, the outer membrane, and peptidoglycan [18]. The outer membrane of the cell consists of lipopolysaccharides, phospholipids, and lipoproteins. The cohesiveness of the lipopolysaccharide (LPS) layer is stabilized by interaction with Ca^{2+} ions and other divalent ions. The stability of permeability membrane cell is also influenced by the concentration of K^+ ions in cytoplasmic fluid [19].

TABLE 2. The results of AAS analysis for the leakage test of Ca^{2+} and K^{+} *Escherichia coli* due to the addition of Ethyl acetate extract of TE 325 isolates

Concentration	Parameter	Measurement results (Ppm)			Average±SD (Ppm)
		I	II	III	
0%	Ca^{2+}	0,985	0,954	1,016	0,985±0,031
	K^{+}	5347,602	5269,397	5328,051	5315,017±40,699
2,5%	Ca^{2+}	1,358	1,327	1,389	1,358±0,031
	K^{+}	3783.495	3803,046	3744,392	3776,978±29,865
5%	Ca^{2+}	2,258	2,227	2,258	2,247±0,017
	K^{+}	4878,370	4878,370	4956,575	4904,438±45,151

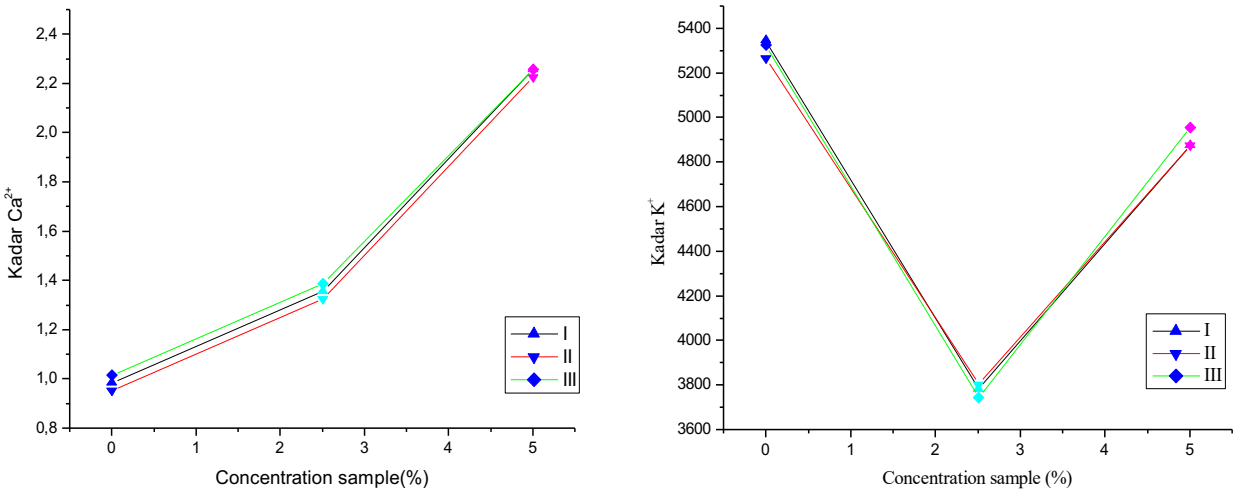

FIGURE 2. The results of AAS analysis for the leakage test of Ca^{2+} and K^{+}

Cell leakage testing can also be done using analysis using the UV / Vis spectrophotometry instrument. Microbes are sensitive when the bacterial growth are inhibited and the microbial was death.

TABLE 3. The results of UV / Vis Spectrophotometer Analysis of 260 and 280 nm

Concentration (%)	Parameter (nm)	Measurement results of absorbance			Average±SD
		I	II	III	
0	260	0,3994	0,3075	0,2775	0,2083±0,1466
	280	0,3539	0,2614	0,3291	0,3148±0,0478
2,5	260	0,1436	0,1648	0,0856	0,7846±0,000
	280	0,5758	0,4666	0,3997	0,6973±0,1737
5	260	0,7846	0,7846	0,7847	0,1313±0,0410
	280	0,7976	0,7976	0,4967	0,4807±0,0888

TABLE 4. The results of protein and nucleic acid analysis

Parameter	Rata-rata ±SD		
	0%	2,5%	5%
OD 280/260	0,9740±0,184	0,7819±0,067	0,8111±0,193
Nucleic Acid (%)	4,333±1,607	7,50±2,291	8,00±5,567
Correction Factor	0,73±0,104	0,577±0,09	0,5933±0,215
Protein (µg/ ml)	0,231±0,054	0,231±0,103	0,365±0,245
DNA concentration (µg/ ml)	16,406±3,175	24,615±5,388	35,535±6,404
RNA concentration (µg/ ml)	13,125±2,540	19,692±4,310	28,428±5,123

Discussion

The 14th days incubation period, the color changes from turbid white to yellowish. This change in the shade of culture fluid is due to Actinomycetes release color pigments that can diffuse or not on the media during the incubation period [20]. Multilevel culture aims to condition TE 325 isolates entering the log phase (exponential) [21]. The difference in the time of the growth phase can occur because Actinomycetes have a very different growth time [11]. Incubation for 14 days resulted in a change in the color of culture fluid TABLE 1 and FIGURE 1. It shows that the color change that occurred in the culture liquid TE 325 isolates was caused by TE 325 isolates secreting color pigments. The color pigment can also indicate the presence of secondary metabolites [22], one of which is Antibiotics [23]. The TE 325 isolates have activity as an Antibiotic against *Escherichia coli* bacteria with a certain mechanism action.

Ethyl acetate extract of TE 325 isolate has shown Antibiotic activity against *Escherichia coli* bacteria. The mechanism of action of these Antibiotic activities against *Escherichia coli* bacteria can be determined by analyzing the *Escherichia coli* bacterial cell leakage using an AAS (*Atomic Absorption Spectrophotometer*). The presence of K^+ and Ca^{2+} ions which are read in AAS shows that the release of K^+ and Ca^{2+} ions from bacterial cells on the effect of TE 325 ethyl acetate extract samples on *Escherichia coli* bacteria. The addition of ethyl acetate extract to test bacteria used 3 concentrations, namely 0%, 2.5% and 5%. The results of ion leakage are shown in TABLE 2 and FIGURE 2. In the test, there was a decrease in K^+ levels when the TE 325 Ethyl acetate extract concentration was increased, at 0% at 5315.017ppm, at 2.5% at 3776.978 ppm, and 5% at 4904, 438 ppm. K^+ ion levels decreased with the addition of extract concentrations of TE 325 due to active compounds contained compounds that could bind and precipitate K^+ ions, namely alkaloids. Alkaloid compounds contain nitrogen atoms which have lone pairs so they can be used to form coordinate covalent bonds with metal ions [24]. As an approach by the results of the K^+ ion is inversely proportional to the concentration of the sample added, that is, in the test of alkaloids with major reagents, it is estimated that nitrogen in the alkaloids will react with metal K^+ ions to form potassium-alkaloid complexes then precipitate [25]. The formation of the precipitating potassium-alkaloid complex also increased between group 1 fraction and K^+ metal ions, so that the K^+ metal ions in the supernatant of the sample read by Atomic Absorption Spectrophotometry also decreased. Potassium (K^+) is the main cation contained in the cytoplasm in growing cells and affects the stability of bacterial cell membrane permeability. Ion K^+ has an important role in activating cytoplasmic enzymes to maintain turgor pressure and regulate pH stability in the cytoplasm. Ion K^+ in bacteria has an important role in its function to maintain the unity of the ribosome. The presence of K^+ metal ions in cells has an important role in RNA stabilization. Another test is to analyze the leakage of Ca^{2+} metal ions in Escherichia coli bacteria. The results showed an increase in Ca^{2+} levels when the concentration of ethyl acetate extract TE 325 was also increased. This shows the leakage in the cell wall. Ca^{2+} metal ions are found in the cytosol, the cytoplasmic fluid and are also found in cell walls that play a role in enzyme activity [26]. The Ca^{2+} ion has the function of connecting lipopolysaccharides to the cell wall of gram negatives bacteria so that it serves to maintain the stability of the bacterial wall. The release of ions from cells caused by exposure to other compounds from the outside can affect the stability of the cell wall, which can then result in bacterial death. The results of the study can be concluded that an increase in K^+ metal content outside the cell indicates a membrane permeability damage. In contrast, increase in Ca^{2+} metal ions indicates damage to the cell wall [27]. Research conducted on KP 13 isolates showed that K^+ ion leakage decreased, namely 789,149; 774,764; and 737,361 Ppm. Ca^{2+} ion cell leakage has increased, which is 0.155; 0.466; and 0.281 Ppm [5].

The mechanism of inhibition or death of microbes to exposure to active compounds can be known by cell leakage, metabolic disorders, and changes in cell morphology. An increase in the absorbance value of the measured cell indicates an increase in the number of cell contents released from the cell. Components of cell contents that leak out of cells can be measured at a wavelength of 260 nm. DNA includes purines, pyrimidines, and ribonucleotides, while at wavelengths of 280 nm, it can measure tyrosine and tryptophan. The results of the calculation of bacterial cell leakage are shown in TABLE 4. Methods for correcting the presence of nucleic acids can be developed by measuring the absorbance ratios at 280 and 260 nm. This ratio can be used to estimate the amount of protein that also contains nucleic acids. The results showed an increase in levels of Protein, Nucleic Acid, DNA and RNA. Pure protein has a ratio (A280/260) greater than 1.7, while nucleic acids have a ratio value (A280/260) less than 0.5. Based on the ratio data, the addition of fraction groups of 0%, 2.5% and 5% is not fulfilling these criteria. The suspected leak is a mixture of protein and nucleic acid [28]. DNA quality can be determined by calculating the ratio between OD260/280 values in samples that are measured using a spectrophotometer. DNA is said to be pure if the OD260/280 ratio values range in the range of 1.8-2.0 [29]. The test results of each extract concentration produced OD260 / 280 values less than 1.8. OD260/280 ratio value is less than 1.8; there, is phenol or protein contamination [30]. If the OD260/280 ratio value is

more than 2.0, DNA has been contaminated by RNA [31]. These results indicate that DNA was contaminated with phenols and proteins.

CONCLUSION

The mechanism of the antibiotic activity in TE 325 isolates was done by interfering membrane permeability. It is shown through by increasing K^+ ions and damaging cell walls with an increase in Ca^{2+} ions as well as the presence of protein and nucleic acid leakage.

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