



In Vitro Study of Antibacterial Activity of Snake Fruit Extract against Extended Spectrum Beta Lactamase (ESBL) Escherichia coli

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ABSTRACT

Snake fruit is a tropical fruit that is currently in great interest by the public. This fruit is known to have high antioxidants so it can prevent an increase in cholesterol and other plasma lipids. Moreover, it can also prevent diarrhea. This study investigated the effective doses (25%, 50%, and 100%) from snake fruit extract against the ESBL-*Escherichia coli* (*E. coli*). This study was an experimental study with a post-test-only control group design in Microbiology Laboratory, Universitas Prima Indonesia. To investigate the antibacterial activity of snake fruit extract, this study was used disc diffusion methods which used five different groups including positive control (meropenem), negative control (DMSO), 100%, 50%, and 25% of snake fruit extract. The antibacterial assay showed that it required 100% snake fruit extract to inhibit the growth of ESBL-*E. coli* with average inhibition zone diameter of 7.33 ± 0.58 mm. The lower concentration extract did not show any antibacterial activity like the negative control group. The positive control group showed the most potent antibacterial activity with an average inhibition zone diameter of 36.33 ± 0.58 mm. Thus, it can be concluded that the snake fruit has antibacterial activity against ESBL-*E. coli*, but it was not as good as the meropenem

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Kata kunci:

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ABSTRAK

Buah salak merupakan buah tropis yang saat ini banyak diminati oleh masyarakat. Buah ini dikenal memiliki antioksidan yang tinggi sehingga dapat mencegah peningkatan kolesterol dan berbagai lipid plasma. Selain itu, juga dapat mencegah diare. Penelitian ini menyelidiki dosis mana yang efektif (25%, 50%, dan 100%) dari ekstrak salak terhadap ESBL-*Escherichia coli* (*E. coli*). Penelitian ini merupakan penelitian eksperimen dengan desain post test only control group di Laboratorium Mikrobiologi Universitas Prima Indonesia. Untuk mengetahui aktivitas antibakteri ekstrak buah salak, penelitian ini menggunakan metode difusi cakram yang menggunakan lima kelompok berbeda yaitu kontrol positif (meropenem), kontrol negatif (DMSO), ekstrak buah salak 100%, 50%, dan 25%. Uji antibakteri menunjukkan bahwa diperlukan 100% ekstrak salak untuk menghambat pertumbuhan ESBL-*E. coli* dengan diameter zona hambat rata-rata 7.33 ± 0.58 mm. Ekstrak konsentrasi rendah tidak menunjukkan aktivitas antibakteri seperti pada kelompok kontrol negatif. Aktivitas antibakteri paling kuat ditunjukkan oleh kelompok kontrol positif dengan rata-rata diameter zona hambat 36.33 ± 0.58 mm. Dengan demikian dapat disimpulkan bahwa salak memiliki aktivitas antibakteri terhadap ESBL-*E. coli*, tetapi tidak sebaik meropenem.

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INTRODUCTION

Humans have various activities, lifestyles, diets, and direct contact with the environment. Hence, humans are prone to infect by various bacteria. One of these infections, diarrhea, was caused by bacteria that is also the most common disease in some developing countries, one of these, Indonesia. Moreover, diarrhea has become a global infectious health problem that causes relatively high mortality and morbidity in the short term (WL Ragil & PS Dyah, 2017).

Diarrhea is a disease characterized by more than three defecations in a day occupied by loose stool consistency with or without mucous or blood. Various factors like poor environmental sanitation, inadequate water supplies, poverty, and lower levels of education may affect the morbidity and mortality of diarrhea in some developing countries (Rahman et al., 2016).

Data from the local (Padang City) Health Institute in 2014 showed that diarrhea was the most common infectious disease in Padang, accounting for 80,272 cases in Padang Timur District. Moreover, this disease was more common among men (39,975 people) than women (40,927 people). It may be due to poor water quality contaminated by various bacteria, *Escherichia coli* (Zikra et al., 2018). This disease was found at a high rate not only in Padang but also in Medan. Badan Pusat Statistik (BPS) in North Sumatera (2017) reported that diarrhea cases were 10,225 cases. On the other hand, Tarigan et al. (2020) reported that around 26,025 people from 2,229,408 in Medan suffered from diarrhea diseases (Tarigan et al., 2020).

Escherichia coli (*E. coli*) is a coliform group and a member of the *Enterobacteriaceae* family. This bacterium was a normal intestinal resident from Homoiterm, including humans; thus, it can become an indicator for faecal contamination as water pollution. When this bacterium is found in a certain concentration, it may cause diarrhoea, and in severe cases, it may cause death. Due to this reason, it can be used to evaluate the quality and safety of the water (Cho et al., 2018). Recently, some strains of *E. coli* can secrete Extended-Spectrum Beta-Lactamase (ESBL), and it was identified as a major multidrug-resistant bacteria in hospital and community settings worldwide. On the other hand, this *ESBL-E. coli* expand high medical costs and minimize antibiotic choices (Mahmud et al., 2020).

Many pharmacologic modalities have been developed to treat diarrhoea. However, these modalities were insufficient to increase the quality outcome because the chemical-based drug has some side effects and becomes nephrotoxic. So, it is recommended to use herbs for diarrhoea management. Since the ancient period, the herb as traditional medicine has been used in Indonesia. It was due to affordability, cheapness, and efficiency. Moreover, traditional medicine also has safer and fewer side effects than chemical-based drugs (Alviana, 2019).

Indonesia, a tropical region, has an advantage riched by various natural tropical herbs used as traditional medicine. For a long time, Indonesian people have been used snake fruit to treat diarrhoea. However, these people have not yet known the active compound responsible for treating diarrhoea. Therefore, some studies have been performed to explore the antimicrobial activity from the snake fruit extract against the *ESBL-E. coli*, which caused diarrhoea. Hence, this study was aimed to look for the antimicrobial activity from various concentrations of snake fruit extract against *ESBL-E. coli*, which cause diarrhoea by the measurement diameter of inhibition zone as killed *ESBL-E. coli* zone.

METHOD

Study Design

This study was an experimental study with post-test only group control design in Microbiology Laboratory, Universitas Prima Indonesia at September 2021-November 2021. All study procedure has been approved by the Health Research Ethics Committee from Universitas Prima Indonesia.

Instruments

The instruments that were used in this study were Petri dishes, microliter pipette, sterile bottles, vials, analytic scales, inoculation loop, reaction tube, racks, and clamps, autoclaves, incubators, hotplates, water bath, beaker glass, volumetric flask, caliper, distillation apparatus, stirring rod, tweezers, Bunsen burner, glass jar, Laminar Air Flow Cabinet (LAFB), microscope, dropper pipette, sterile cotton swab sterile, object-glass, and cover glass.

Materials

The materials were used in this were the normal saline solution, dimethyl sulfoxide (DMSO), *ESBL-E. coli* bacteria, blank paper disc, snake fruits, MHA (Mueller Hinton Agar) media, methanol, ethyl acetate, paper filter, meropenem, safranin, crystal violet, lugol, 93% ethyl alcohol, immersion oil, EMBA (Eosin Methylene Blue Agar) media, SIM (Sulfide Indole Motility) media, Kovac reagent, MR-VP (Methyl Red-Voges Proskauer) media, Urease Broth media, MR reagent (Methyl Red), 40% KOH, alpha naphthol, Simmons Citrate Agar Media., and TSIA media.

Snake fruit extract process

At the beginning, a kilogram of snake fruit was peeled and cut into small pieces and weighed as much as 500-600 grams. This snake fruit could not be dried because it was wet and easily oxidized. Hence, this snake fruit was put into one-liter methanol as the solvent in a glass jar for one week, and it was stirred regularly every 8 hours by flipping the jar three to five times. After that, it was filtered by a paper filter and evaporated by hotplate and water bath to form a concentrated form of snake fruit extract.

Characterization of ESBL-E. coli

Characterization of *ESBL-E. coli* was performed by some methods including gram staining, Inoculation on EMBA media, and biochemical test.

Gram staining

Gram staining was performed to evaluate the morphology of *ESBL-E. coli* bacteria. At the initial, the object-glass was clean with alcohol. After that, the clean object glass was dropped with distilled water and the *ESBL-E. coli* was suspended into the distilled water. Then, it was fixed into a thin layer by heating the object-glass over the Bunsen burner. Fixed *ESBL-E. coli* was drop by gram staining reagent included crystal violet as primer stain (30 seconds), Lugol solution as mordant (1 minute), 93% ethyl alcohol as decolorizing agent (15-25 seconds), and safranin as a counter stain (45 seconds). Between these drops, the object-glass was rinsed with distilled water. After that, it was observed

under a microscope with 100x magnification, using immerse oil (Pelt et al., 2016).

Inoculation on EMBA media

Fifteen milliliters of EMBA (Eosin Methylene Blue Agar) was poured into a petri dish. An inoculum of ESBL-E. Coli was streaked into the surface of EMBA, and it was incubated for 24-48 hours at 37°C. The metallic green colour indicated positive test results. (Sari et al., 2019)

Biochemical assay

The biochemical assay was performed to identify the biochemical reaction from the metabolism process of *ESBL-E. coli* as family of Enterobacteriaceae. This assay included indole, methyl red, voges Proskauer, simmon citrate, urease, triple sugar iron, and motility test.

- a. Indole Test: Indole test is very useful to identify the group of bacteria that can secrete tryptophanase enzyme. An inoculum of ESBL-E.coli was inoculated into Sulfide Indole Motility (SIM) Media and it was incubated for 24-48 hours at 37°C. After that, the inoculated ESBL-E. coli in SIM Media was dropped with Kovac reagent. When the surface of SIM Media formed a red ring, it indicated tryptophanase enzyme in the inoculated bacteria (positive indole test) (Shoaib et al., 2020).
- b. Methyl Red Test: This test was used to identify the glucose fermentation activity by determining pH medium. An inoculum of ESBL-E. coli was inoculated into MR-VP (Methyl Red-Voges Proskauer) media and incubated at 37°C for 24 hours. After that, five drops of MR reagent were dropped into the medium to identify the color changes. The red color indicates an acid condition in the medium (positive result), and the yellow indicates a negative result (Sari et al., 2019).
- c. Voges Proskauer Test: This test was used to identify the glucose fermentation activity by determining pH medium. An inoculum of ESBL-E. coli was inoculated into MR-VP (Methyl Red-Voges Proskauer) media and incubated at 37°C for 24 hours. After that, five drops of 40% KOH solvent were dropped into the medium to identify the color changes. Pink-red color indicated an alkyl condition in medium (positive result), and lack of pink-red color indicated a negative result (Sari et al., 2019).
- d. Simmon Citrate Test: An inoculum of ESBL-E. coli was inoculated into Simmons Citrate Agar media and incubated for 24 hours at 37°C. When the medium changed from green to bright blue, it indicated a positive result. This test is useful to identify the utilization of citrate as the sole of carbon from Enterobactericea (Sari et al., 2019).
- e. Urease Test: An inoculum of ESBL-E.coli was inoculated into Urease Broth Media, and it was incubated for 24-48 hours at 37°C. The media color that changes from yellow to pink (phenol red) indicates the bacteria's urease activity in media (Prasetya et al., 2019).
- f. Triple Sugar Iron Test: An inoculum of ESBL-E. coli was inoculated into Triple Sugar Iron Agar (TSIA) media by stabbing the inoculum into the center to the butt of the tube that filled by the TSIA media, and then the inoculum loop was streaked the surface of agar slant. After that, these media were incubated for 24-48 hours at 37°C. This test may indicate some glucose fermentation activity group included: (a) alkaline/ acid (red slant/ yellow butt) indicated a dextrose

fermentation bacteria; (b) acid/ acid (yellow slant/ yellow butt) indicate dextrose, lactose, and/ or sucrose bacteria; (c) alkaline/ alkaline (red slant/ red butt) indicated absence of carbohydrate fermentation activity (Sari et al., 2019).

- g. Motility Test: An inoculum from ESBL-E. coli colony in the slant agar was inoculated into the middle part of SIM media in a tube reaction. After that, it was incubated for 24 hours at 37°C. When the media became fog-like turbidity, it indicated the bacteria movement (Pelt et al., 2016).

Serial dilution of extract

The amount of 5 grams of a concentrated form of snake fruit extract was dissolved into 5 ml DMSO by volumetric flask, and it was labeled as 100% snake fruit extract. After that, 2.5 ml of 100% snake fruit extract was pipetted by microliter-pipette to dissolve into 5 ml DMSO by volumetric, and it was labeled as 50% snake fruit extract. Finally, 2.5 ml of 50% snake fruit extract was dissolved in the same way to form a 25% snake fruit extract.

Bacteria Suspension

An inoculum of *ESBL-E. coli* was suspended into 3-5 ml of normal saline solution in the sterile test tube at LAFC. After that, it was homogenized and incubated at room temperature for five minutes (show a specific turbidity colour) and compared the turbidity of the suspension to 0.5 McFarland standard (total of colon number 1.5×10^8 CFU/mL).

Antibacterial Assay

ESBL-E. coli in bacterial suspension was obtained by sterile cotton swab to be spread into the surface of MHA media. After that, each petri dish was divided into four quadrants, and a paper disc was placed in each quadrant that represented a different group of treatment. Each group of treatments was repeated three times. These paper discs have been soaked into 100%, 50%, and 35% snake fruit extract. Meanwhile, the positive and negative control was used Meropenem and DMSO, respectively. Moreover, these Petri dishes were incubated for 24 hours; then, the form inhibition zones were measured by caliper in millimeter. Furthermore, *ESBL-E. coli* also underwent antibiotic resistance assay against some antibiotics including amikacin, amoxicillin, ampicillin, ampicillin+clavulanic acid, Ceftriaxone, Cefotaxime, Cefepime, Chloramphenicol, Gentamycin, Levofloxacin, Meropenem, Piperacillin-Tazobactam, and Tetracycline by disc diffusion methods as described above. The formed inhibition zone in antibiotic resistance assay was measured and interpreted as resistance or sensitive based on the CLSI (Clinical and Laboratory Standards Institute) protocol.

RESULTS AND DISCUSSION

Characteristic of ESBL-E. coli

At the beginning, the used *ESBL-E. coli* from slant agar was identified by some methods included gram staining, EMBA media culture, and biochemical test.

Gram staining

An inoculum of *ESBL-E. Coli* was stained by gram staining to ensure the *ESBL-E. coli* morphology under the light microscope. The microscopic view of these colony was shown in Figure 1.

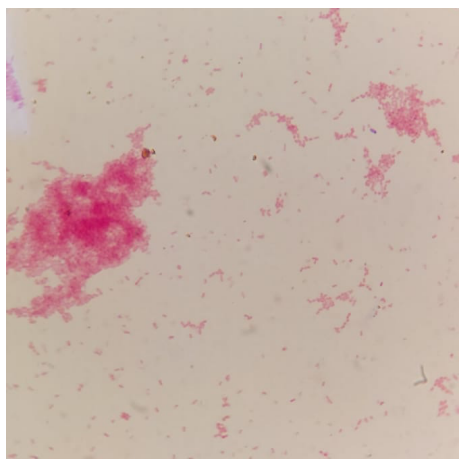


Figure 1. *ESBL-E. Coli* Stain: Gram staining; Magnification: 1000x

Based on the Fig 1, the *ESBL-E. coli* colony was a coccobacilli form bacteria with red color in gram staining. According to this gram staining, this *ESBL-E. coli* colony was a gram-negative bacterium. When this colony was stained by the primer stain (crystal violet), this primer stain was decolorized by the ethyl alcohol because this colony does not have any peptidoglycan layer which keep the primer stain inside. Moreover, this colony was stain by counter-stain (safranin) that has red color and it caused the colony look red under the microscope (Pelt et al., 2016).

Bacterial Inoculation on EMBA Media

An inoculum of *ESBL-E. coli* was culture into EMBA Media by streaking it at the surface of EMBA media. The growth colony in the EMBA media was shown by Figure 2.

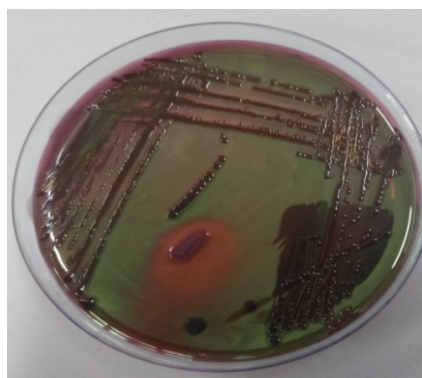


Figure 2. *ESBL-E. coli* colony on EMBA Media

Fig 2 showed that the colony looks metallic green color on EMBA media. Before the *ESBL-E. coli* was cultured into EMBA media, and this media had light purple color. This EMBA media color becomes metallic green color after *ESBL-E. coli* was cultured that indicated lactose fermenter bacteria. EMBA media was used to differentiate *Enterobacteriaceae*,

especially *E. coli*. EMBA media was selective and differential media, which was used to differentiate lactose fermenters from non-fermenter, while the *E. coli* was lactose fermenter bacteria. The color change of EMBA media was used to differentiate the fermentation activity from the colony. When the EMBA media become metallic green color, it indicates the colony is lactose fermenter bacteria. EMBA media contains some compounds included contains lactose, sucrose, peptone, eosin Y, and methylene blue; methylene blue inhibits the growth of gram-positive bacteria (Harijani et al., 2013; Juwita et al., 2014).

Biochemical Tests

ESBL-E. coli colony used in this study also underwent some biochemical tests, including IMViC test (Indole, Methyl Red, Voges Proskauer, Simmon Citrate, and Motility), Urease test, and TSIA test. This test observed color-changed media due to the reaction between the bacteria and some substrate in the media. The biochemical tests result was showed by the Figure 3.

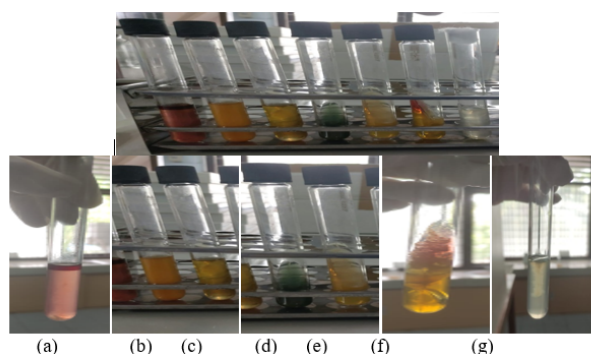


Figure 3. Biochemical assay of *ESBL-E. coli*. Indole (a), Methyl Red (b), Voges Proskauer (c), Simmon Citrate (d), Urease (e), TSIA (f), and Motility (g)

Table 1
 Biochemical Assay of *ESBL-E. coli*

Tests	Results	Description
Indole	+	Red-color ring on the surface of the tube
MR	-	No color change from yellow to blood red
VP	-	No color change from yellow to brick red
Simmon Citrate	-	No color change from green to blue
Urease	-	No color change from yellow to pink
TSIA	+	Acid/ Acid; H ₂ S (-); O ₂ (+)
Motility	+	Motile Bacteria

Based on Table 1, Indole, TSIA, and motility test indicated this colony has *E. coli* characteristics. Although other biochemical tests did not show any *E. coli* characteristics, other tests included gram staining and EMBA media culture indicated the *E. coli* characteristics in this colony.

The indole test aimed to identify a bacteria's ability to degrade a tryptophan to indole. Fig 3 showed that the colony formed a red color due to the reaction between the Kovac reagent against the formed indole.

MR-VP (Methyl red-Voges-Proskauer) test aimed to identify a bacteria's ability to ferment glucose. MR-VP media contents peptone, glucose, and buffer phosphate. These tests qualitatively determined the degree of acidity in the media.

Fig 3 showed that both MR and VP tests indicated the negative results, which means that this colony could not produce acid properly from glucose fermentation reaction and was unable to react with alpha-naphthol and potassium hydroxide (KOH) (Hemraj et al., 2013).

Simmon Citrate Test used Simmon Citrate media to evaluate the ability of bacteria to convert nitrate to nitrite. Fig 3 showed that this colony did not have any ability to convert nitrate to nitrite because there was no color change in the Simmon citrate media (green) (Sari et al., 2019).

The urease test was aimed to evaluate the bacteria's ability to secrete urease enzyme. When the bacteria in this media had urease enzyme, this bacteria would break the carbon and nitrogen bond in the urea to form ammonia that changed the acid condition to alkaline condition in the urease broth media and colored the media into pink color. Fig 3 above did not show any color changes in urease broth media and it indicates that the colony did not have any urease enzymes (Wahyuni et al., 2018).

The TSIA test was done by using TSIA (Triple Sugar Iron Agar) media in determining carbohydrate fermentation and producing H₂S gas. The TSIA test can distinguish bacteria based on their ability to ferment lactose, glucose, and sucrose as well as the ability of the bacteria to produce H₂S gas. (Amelia et al., 2016)

TSIA test used TSIA media to identify carbohydrate fermentation activity and formation of H₂S gas. Fig 3 above showed that the colony formed a yellow color in both the slant and butt of the test tube, which means that the colony could ferment lactose, glucose and sucrose in TSIA media; it was labeled acid/ acid. However, this colony did not form any precipitations, which means that this colony did not form any hydrogen sulfide gas. Moreover, this colony showed the formation of oxygen in the TSIA media. This oxygen formed a gap between the TSIA agar and the butt tube.

The motility test is the most common method to differentiate each type of bacteria from the Enterobacteriaceae family. This test used semi-solid media or SIM (Sulfur, Indole, and Motility) media to identify the bacteria movement. Fig 3 showed that the media become fog-like turbidity along the puncture area of the inoculum loop, which indicated the movement of the bacteria from the inoculum colony. This turbidity was caused by reducing concentration in media from 1.5% to 0.4% when the bacteria was moving to keep its shape (Leboffe & Pierce, 2011).

Antibiotic Resistance Assay

Antibiotic resistance test was aimed to identify the resistance of *ESBL-E. coli* colony against some antibiotics including amikacin, amoxicillin, ampicillin, ampicillin+clavulanic acid, Ceftriaxone, Cefotaxime, Cefepime, Chloramphenicol, Gentamycin, Levofloxacin, Meropenem, Piperacillin-Tazobactam, and Tetracycline. The result of the antibiotic resistance test is described in Table 2.

Based on Table 2, the *ESBL-E. coli* was resistance to amikacin, amoxicillin, ampicillin, amoxicillin (clavulanic acid), ceftriaxone, cefotaxime, cefepime, chloramphenicol, gentamycin, levofloxacin, piperacillin-tazobactam, and tetracycline. However, this *ESBL-E. coli* was only sensitive to meropenem. This result was similar to the data from the Clinical and Laboratory Standards Institute 2018 (CLSI 2018). On the other hand, Normaliska et al. (2019) also reported *ESBL-E. coli* had some resistances properties against some antibiotics, including penicillins, cephalosporins, aztreonam,

aminoglycosides, trimethoprim-sulfamethoxazole (TMP-SMZ), and quinolones (Normaliska et al., 2019).

Table 2
Antibiotic Resistance Test Results against ESBL-E. coli

Antibiotic	Description
Amikacin	R
Amoxicillin	R
Ampicillin	R
Amoxicillin/Clavulanic acid	R
Ceftriaxone	R
Cefotaxime	R
Cefepime	R
Chloramphenicol	R
Gentamycin	R
Levofloxacin	R
Meropenem	S
Piperacillin-Tazobactam	R
Tetracycline	R

Abbreviation: R = Resistance; S = Sensitive

Antimicrobial Assay

Finally, some concentrations of snake fruit extract underwent an antimicrobial activity assay against *ESBL-E. coli* that has been characterized before. The result of antimicrobial assay was described in Table 3.

Table 3
Antimicrobial Assay on Snake fruit Extract against ESBL-E. coli

Groups	Diameter of Inhibition Zone (mm), Mean ± SD
100 % snake fruit extract	7.33 ± 0.58 mm
50 % snake fruit extract	6.00 ± 0.00 mm
25 % snake fruit extract	6.00 ± 0.00 mm
Positive control	36.33 ± 0.58 mm
Negative control	6.00 ± 0.00 mm

Based on Table 3, only the highest concentration of snake fruit extract showed antibacterial activity against the *ESBL-E. coli*. This antibacterial activity was shown by the average inhibition zone, which was 7.3 ± 0.58 mm. Meanwhile, the lower concentration snake fruit extract did not show any antibacterial activity because of an absence of formed inhibition zone like the negative control group, the average diameter inhibition zone of 6.00 mm was the diameter of paper disc without inhibition zone. However, the antibacterial activity of meropenem as a positive group still had the most potent antibacterial than other groups with an average inhibition zone of 36.3 ± 0.58 mm. Furthermore, the average diameter inhibition zone from 100% snake fruit extract indicated the moderate antibacterial activity against the *ESBL-E. coli*.

CONCLUSIONS

It can be concluded that the highest concentration of snake fruit extract (100%) had a moderate antibacterial activity for *ESBL-E. coli*. However, the inhibition zone formed in the control positive (Meropenem) was wider (36.33 ± 0.58 mm) than the highest concentration of snake fruit extract (7.33 ± 0.58 mm).

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