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Liquid Smoke Antimicrobial Test of Cocoa Fruit Peel Against *Eschericia Coli* and *Staphylococcus Aureus* Bacteria

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Abstract. Cocoa fruit peel is one of the natural wastes that has not been widely used. Cocoa fruit peel is thought to have secondary metabolites that can kill bacteria. The purpose of this study is to determine the antibacterial properties of liquid cocoa peel smoke and, determining minimum inhibitory concentration as well as concentration growth of *Escherichia coli* and *Staphylococcus aureus* pathogenic bacteria. This research had been conducted in April to May 2019. Testing of antibacterial activity was Well method. The results of study showed that the liquid smoke of cocoa peel at 10% moisture content was able to inhibit the growth of *E.coli* and, *S.aureus* bacteria at the highest level compared to the liquid smoke of brown skin moisture content of 15%, 20% and 25%. The inhibitory zone value in Cocoa fruit peel liquid smoke with moisture content of 10% against *E.coli* bacteria was 25.1 mm, while *S.aureus* bacteria had an inhibition zone value of 32.6 mm. *E.coli* bacteria showed stronger resistance compared to *S.aureus*. This was indicated by the *E.coli* inhibition zone was smaller than the *S. Aureus* inhibition zone. The minimum inhibitory concentration and, the minimum kill concentration in a row were at concentrations of 3.125% and 12.5% for *E.coli* bacteria.

1. Introduction

Cocoa (*Theobroma cacao* L) is one of the most important commodities in the plantation sector in Indonesia. Indonesia is the world's second largest cocoa producer after Ivory Coast. Cocoa (*Theobroma cacao L*), one of Indonesia's most important export commodities, is planted extensively with a total area of 1.6 million hectares, producing 500,000 tons of dry beans in 2011. Beside seeds as a main product, fruit peels are also produced at harvest time with volumes that are almost the same as seeds [1].

Cocoa farmers generally only use cocoa seeds during harvesting, while cocoa peel is usually only partially used as animal feed ingredients and the rest is left becoming garbage or agricultural waste until rot around the plantation. Cocoa peel waste that produced in large quantities will be a problem if it is not handled properly because this solid waste production reaches more than 60% of the total fruit production, this will be a great potential to pollute the surrounding environment [2]

Cocoa fruit skin has many benefits if further examined. Some reports say that the content of cacao fruit skin has active compounds which are beneficial to humans. The peel of Cacao fruits contains phenolic compounds and flavonoids [3]. The polyphenol contents include cinamic acid, tannin, pyrogallol, quercetin, resorcinol and epicatecin-3-error [4]. The major compounds contained in Cocoa peel are (-) epicatecin and phidroxybenzoic acid [5], where these compounds can be used as antimicrobials. Cocoa polyphenols are functioned as antimicrobial against several pathogenic bacteria and cariogenic bacteria [6] [7]. Previous studies have reported that Cocoa fruit peel has antibacterial compounds to inhibit bacterial growth, including research [8] which extracts bioactive components from cocoa peel waste and, its effects on antioxidant and antimicrobial activity, [1] conducting an antibacterial activity test for cocoa peel extract (*Theobroma cacao* L) against *Escherichia coli, Bacillus subtilis,* and, *Staphylococcus aureus* [9] analyzed the liquid smoke chemical content of Cacao peel by GC-MS method. Seeing the potential of the

cocoa fruit skin, this study utilizes Cocoa fruit peels to produce liquid smoke. Liquid smoke is the result of condensation or steam condensation resulting from combustion directly or indirectly using materials that contain a lot of lignin, cellulose, hemicellulose, and hydrocarbon compounds [10]. The liquid smoke that produced will be observed antibacterial properties against the *Escherichia coli* and, *Staphylococcus aureus* bacteria and, knowing the Minimum Inhibitory Concentration (MIC) and Minimum Killer Concentration (KBM) growth of pathogenic bacteria that have the smallest antibacterial activity which is indicated by the small clear zone produced.

Based on the description above to optimize the utilization of Cocoa peel as a raw material for making liquid smoke that has antibacterial activity, the authors are interested in conducting research on the liquid smoke antimicrobial test of cocoa fruit peel on *Escherichia coli* and *Staphylococcus aureus.*

2. Methodology

Research has been carried out at the Agricultural Technology Laboratory, Ekasakti University and the Laboratory of Microbiology and Biotechnology at Andalas University in April - May 2019. This study used a pre-experimental design using *Eschechia coli* bacterial isolates and Staphylococcus aureus collections in the Laboratory of Microbiology and Agricultural Biotechnology Agricultural Technology Faculty, Andalas University. Liquid smoke was obtained from the pyrolysis method by using different levels of Cocoa peel moisture content.

The materials used in this study was cocoa skin obtained from Lubuk Minturun Village, Padang City, which had been regulated water content according to the treatment, and the pyrolysis process was carried out to obtain liquid smoke from Cocoa peel *nutrient agar* (NA) and, *nutrient broth* (NB) as growth media, amoxilin, aquades, H_2SO_4, $BaCl_2.2H_2O$, physiological salts,

The equipments used were autoclave, oven, petri dish, ose needle, Bunsen lamp, Erlenmeyer 250 ml, test tube, micro pipette, vortex, incubator, hot plate stirrer, calipers, cotton, plastic wrap, aluminum foil and, tweezers

2.1 Testing of Inhibitory Zone Activity against *E. Coli* and, *S. aureus* Bacteria [11].

2.1.1 Making Media

The sloping agar media was made by dissolving 2 grams of Nutrient Agar (NA) in 100 ml of aquades using erlenmeyer. After that, it is homogenized with a *stirer* ver the *hot plate stirrer* until boiling. 5 ml was poured each in 3 sterile test tubes and covered with *aluminum foil,* The media was sterilized in outoclave at 121°C for 15 minutes, then left at room temperature for ± 30 minutes until the media solidified at a slope of 30°. The sloping agar media was used for bacterial inoculation.

The basic media was made by weighing 6 grams of Nutrient Agar (NA), then dissolved in 300 ml of distilled water using erlenmeyer. After that, the media is homogenized with the *stirrer o*n top of the *hot plate stirrer* until it boiled. These homogeneous media were sterilized in an autoclave at 121oC for 15 minutes, then cooled to a temperature of ± 45-50°C. This media is eady to be used by including 0.2 ml of bacteria in every 00 ml of media.

2.1.2 Making Standard Solution Turbidity (McFarland Solution)

The 99.5 ml 0.36 N H_2SO_4 solution was mixed with 1.175% $BaCl_2.2H_2O$ solution as much as 0.5 ml in erlenmeyer. Then, shake it until a cloudy solution was formed. This turbidity was used as a standard bacterial suspensios turbidity test.

2.1.3 Making Test Bacterial Suspensions
The inoculated test bacteria were taken with sterile ose wire and, then suspended into a tube containing 2 ml of 0.9% NaCl solution until the turbidity obtained was the same as the standard turbidity of *Mc. Farland S*olution. The same treatment was carried out for each type of test bacteria

2.1.4 Making Testing Media
The base layer was made by pouring 75 ml NA each and added with bacterial suspense. Left it until the media solidified *laminar air flow*. Furthermore, wells are made based on number of samples using the base of a sterile pipette to form wells that will be used in the antibacterial test.

2.1.5. In-vitro Antibacterial Activity Test
Liquid smoke test solutions with various concentrations (10%, 15%, 20% and 25%); aquades solution as a negative control; Amoxilin solution as a positive control where each was drop in a different well as much as 0.2 ml. Then the petri dishes were incubated at 37oC for 24 hours.

2.1.6. Observation and Measurement
Observations were made after 24 hours of incubation period. Clear areas are indications of bacterial sensitivity to antibacterial materials used as test materials which were expressed by the diameter of the inhibitory zone. The diameter of the inhibitory zone was measured in units of millimeters (mm) using a sliding length by means of the overall diameter minus the diameter of the well. Then the inhibition zone diameter was categorized as antibacterial power strength based on Davis and Stout classification.

2.2. Testing the Minimum Inhibitory Concentration (MIC) and Minimum Concentration Test (KBM) [12].
Determination of KHM and KBM values using liquid dilution method with the provisions of 5 tubes as treatment, 1 tube as positive control, 1 tube as negative control, and 1 tube as media control.

2.2.1 Test treatment
The treatment tube (5 tubes) was filled with 5 ml of NB medium. Tube 1 was added with 5 ml of liquid smoke, then homogenized using *vortex)* A mixture of liquid smoke and media in tube 1 was taken 5 ml and, moved to in the second tube and homogenized. The mixture in tube 2 was taken 5 ml and moved in tube then, homogenized. The mixture in tube 3 was taken 5 ml transferred in tube 4 then, homogenized. The mixture in tube 4 was taken 5 ml transferred in tube 5 then, homogenized. The mixture in tube 5 was taken 5 ml and discarded. Each tube (tube 1-5) was taken 0.1 ml of solution and then discarded after that 0.1 ml of bacterial suspension 1 x 10^8 CFU/ml is added so that the total volume of each tube was 5 ml.

2.2.2. Negative controls
Tube 6 is filled with NB of 4.9 ml then 0.1 ml of bacterial suspension was added to 10^8 CFU / ml, after that it was homogenized.

2.2.3. Positive controls
Tube 7 is filled with 5 ml of NB then 5 ml of 1% amoxilin solution is added and homogenized 0.1 ml of the stock solution was removed then 0.1 ml of bacterial suspension 108 CFU / ml was added and, homogenized again.

2.2.4. Media control
Tube 8 was a media control containing 1 ml of NB media. Furthermore, all tubes (tubes 1-8) were incubated on the shaker waterbath at 40°C for 24 hours. Then the presence or absence of turbidity in the

culture media was observed and compared to the control. The first smallest concentration that shows clarity is determined as the KHM liquid smoke value of the Cocoa fruit peel of each test bacterium. KBM was determined by means of culture media in each tube that planted in NA media. Incubate at 37°C for 24 hours then seen whether or not there was a growing bacterial colony and, comparing it to the control.

3. Results and Discussion

3.1 Testing of Inhibitory Zone Activity of E. coli and, S. aureus Bacteria

The antimicrobial activity test in liquid smoke of Cocoa fruit peel on the growth of pathogenic bacteria was carried out by usingthe well diffusion method. The well diffusion method is a method used to determine the liquid smoke activity of Cocoa fruit peel smoke in inhibiting the growth of *Escherichia coli* and, *Staphylococcus aureus* by observing the clear zone formed.

Antibacterial testing using the well diffusion method was carried out by using some of the content of the water content of cocoa pods, 10, 15, 20 and 25%. While the positive controls used were amoxilin and, negative controls in the form of sterile aquades. Positive control is served as a comparison whether liquid smoke extract used is feasible or not used while negative control is functioned as a control to ensure that liquid smoke extract has inhibitory activity against E. coli and S. aureus bacteria

The liquid smoke antibacterial activity of Cacao fruit peel shows the presence of inhibitory zones with different diameters of each percentage of water content in the Cocoa fruit peel. In the treatment of 10% moisture content, the inhibition zone produced for *E. coli* bacteria is 25.1 mm and S. aureus was 32.6 mm. This inhibitory zone value was included in the very strong category for both types of bacteria. Treatment of 15% water content in inhibition zone produced for *E. coli* bacteria was 22.3 mm and, *S. aureus* bacteria was 26.9 mm. This inhibitory zone value belongs of the very strong category for both types of bacteria. In the treatment of water content of 20%, the inhibitory zone that produced for *E. coli* bacteria was 19.7 mm and, *S. aureus* bacteria was 24.7 mm. This inhibitory zone value is included in the strong category for E. coli bacteria and, very strong for *S. aureus* bacteria. In the treatment of 25% moisture content, the inhibition zone produced for E. coli bacteria was 16.5 mm and S. aureus bacteria was 21 mm. This inhibitory zone value is included in the strong category for E. coli bacteria and is very strong for S. aures bacteria.

The inhibitory zone values produced in this study fall into the strong and very strong category because most of the inhibitory zone values produced are above 20 mm. This result is also greater when compared with the study [1], which conducted an antibacterial activity research of Cocoa peel extract to inhibit the growth of *E. coli* with a mean diameter of 8.83 mm. whereas for *S. aureus* inhibition zones, the average diameter is 10 mm. Previous research in testing the antimicrobial activity of Forastero variety cocoa fruit concentrate skin extract showed that Cocoa fruit peel extract as antibacterial activity against *E. coli* and, *S. aureus. E. coli* bacteria are more sensitive to extract active compounds. Bacterial inhibition occurs at all concentrations of the extract [8]. The liquid smoke inhibition zone of Cocoa fruit peel is found in Figure 1.

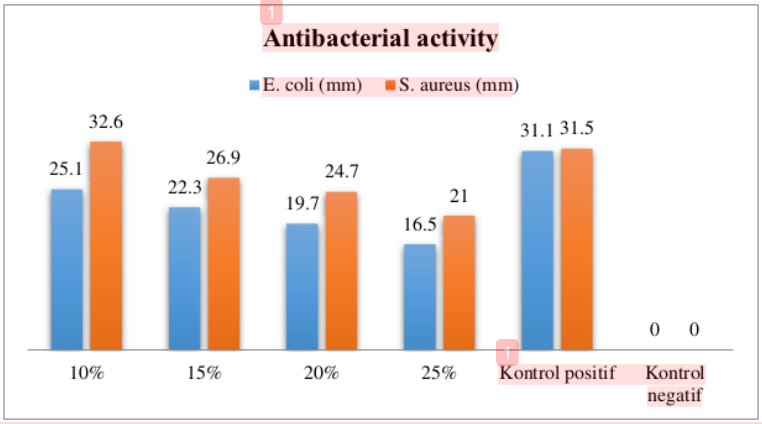

Figure 1. Comparison diagram of the liquid smoke inhibition zone of Cocoa fruit peel on the growth of *E. coli* and, *S. aureus* bacteria.

The classification of antibacterial power is based on the opinion [13], the criterias for antibacterial power strength are the diameter of the inhibition zone of 5 mm or less categorized as weak, the inhibition zone 5 - 10 mm which is categorized as medium. The inhibition zones of 10-20 mm are categorized as strong and the inhibition zone of 20 mm or more is categorized as very strong. Data obtained that liquid smoke of Cocoa fruit peel can inhibit the antibacterial activity of *S. aureus* with 10-25% Cocoa fruit peel moisture content and, categorized as very strong, whereas for *E. coli* bacteria, the percentage of 20 and 25% cocoa peel moisture content is classified as having strong antibacterial activity.

Qualitative test results of the active components which are carried out by [14], Cocoa fruit peel turned out to contain tannins, polyphenols, flavonoids, alkaloids and steroids which are active components that can be utilized in the world of food industry, food, pharmaceutical, dental and oral health industries. The mechanism of action of an antimicrobial compound is to inhibit the synthesis of the cell wall of microorganisms which causes lysis, changing the permeability of the cytoplasmic membrane and, causing nutrient leakage from within the cell, causing cell protein denaturation and, inhibiting enzyme action in cells. Alkaloid compounds are organic compounds that have nitrogen atoms and, alkaline as well as can cause coagulation of bacterial cell proteins, causing inhibition of bacterial growth.

Protein coagulation will interfere with the constituent components of peptidoglycan in bacterial cells which cause the cell wall layer are not formed intact, thus causing bacterial cell death. Flavonoids in cocoa peel Extract belong to a group of phenolic compounds that have glycoside bonds. Phenolic compounds will interact with bacterial cell membrane proteins through an adsorption process by binding to the hydrophilic part of the cell membrane. Further, phenolic compounds will enter to the Cell membrane and, causing cell protein precipitation. This disturbs the permeability of the cell membrane, so that the cell membrane can undergo lysis [1]. The liquid smoke antibacterial inhibition zone of Cacao fruit peel is shown in Figure 2.

Phenol compounds inhibit bacterial population growth by extending the lag phase proportionally in the body or in the product while the growth rate in the exponential phase remains unchanged unless the phenol concentration which is very high [10]. [15], adding compounds that play an important role as antimicrobials in liquid smoke are phenol and acetic acid compounds, antimicrobial properties will increase if there are organic acids together with phenol compounds. In addition to these two compounds,

aldehyde compounds, acetone and ketones also have bacteriostatic and bactericidal power in smoke products

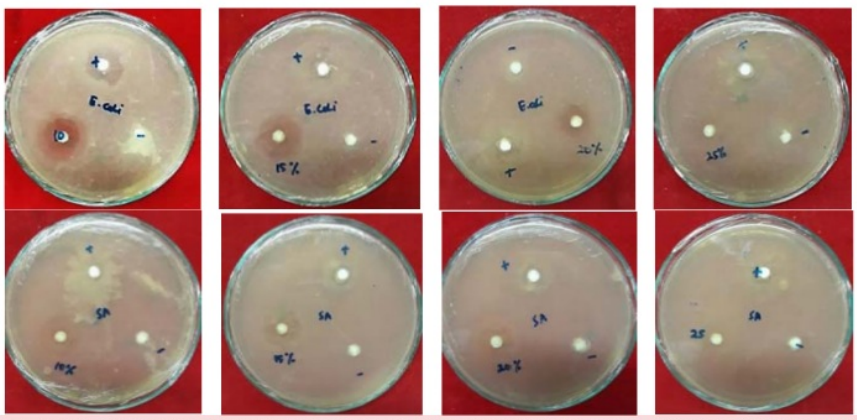

Figure 2. The results of the antimicrobial activity test with the well diffusion method at several percent moisture content (10, 15, 20 and 25%), negative control (-) and positive (+) control on the growth of *E. coli* and, *S. aureus* bacteria.

E. coli is a gram negative bacterium that is more likely to be resistant to active compounds. This is what is likely to cause the bioactive compounds present in the liquid smoke of the cocoa peel cannot penetrate the cell membrane larger than the *S. aureus* bacteria, so that there is no growth inhibition. Whereas S. aureus is a gram positive bacterium whose cell wall structure is simpler. These bacterium tends to be more susceptible to the activity of antibacterial components such as phenolic compounds and penicillin. The simple structure of cell walls causes antibacterial compounds that easily to enter the cell and, finding the target to work. Gram-negative bacteria are generally more resistant to antibacterial and, disinfectants than Gram-positive bacteria that are not sporadic because Gram-negative bacteria have a lipopolysaccharide layer on their cell membranes that can inhibit the entry of antibacterial and into cells [16].

3.2 Minimum Inhibitory Concentration (MIC)

The observation results of minimum inhibitory concentration (MIC) are through the turbidimetric method (visual observation). In this observation that was observed is that the turbidity rate of growth media that had been incubated for 24 hours. The test results are negative (-) if the growth media solution does not occur turbidity, the test results are positive (+) if the growth media solution becomes cloudy. The smallest content of the extract which did not show turbidity after being compared with the control (test solution which did not contain bacterial suspence) was the MIC value.

The study results of the minimum inhibitory concentration (MIC) of liquid cocoa peel smoke are shown in Table 1. In this study, the results were obtained for the Minimum Inhibitory Concentration (MIC) value of 6.25%, where at this concentration, the growth media do noccur turbidity which indicated that E. coli bacteria were unable to develop during incubation as well as at concentrations of 12.5 - 50% there is no a change in solution or remain clear on the growth medium, this indicates that E. coli bacteria are not able to develop during incubation. But at a concentration of 3.125% the growth medium turns cloudy, this means that at this concentration *E. coli* bacteria can grow during incubation. In the negative

control (-) there is a clear level of turbidity from the media solution, because in the negative control (-) there was no addition of liquid smoke from the Cocoa fruit peel so that bacteria can develop during incubation. In the positive control (+) the growth medium also undergoes a change, which is slightly cloudy, which signifies *E. coli* bacteria from being able to grow at a concentration of 1% solution. Changes in the turbidity level of growth media are shown in Figure 3.

Table 1. The analysis results of minimum inhibitory concentrations of Cocoa fruit peel liquid smoke

Tube Number	Concentration	Test result	Information
1	50%	-	Clear
2	25%	-	Clear
3	12,5%	-	Clear
4	6,25%	-	Clear
5	3,125%	+	Slightly cloudy
Negative control	0	++	Murky
Positive control	1%	+	Slight cloudy
Media control	0	-	Clear

Sign (+): the solution in the tube looks cloudy, meaning there is bacterial growth. Sign (-): the solution in the tube starts to clear, which means the growth of bacteria begins to be blocked. Positive control contains amoxilin and, bacterial suspension equivalent to *McFarland*, negative control contains NB media and, bacterial suspension. Media control is only NB media.

From the results of the research above, liquid smoke from cocoa pods can inhibit the growth of *E. coli* bacteria with a concentration of 6.25%. In this study, it can directly determine the KHM value, which is at a concentration of 6.25%, but because this method is qualitative so that subjectively influenced by researchers and, the color of liquid smoke solution is darker than the media, it makes it difficult to determine and, KHM observation. According to [17], testing visually has a weakness that is the human eye when observing turbidity can not distinguish between bacterial cells that live with dead bacterial cells and, solutions can achieve concentrated color, therefore the results of the observation are less accurate. To clarify the antibacterial activity of Cocoa fruit peel liquid smoke, further testing is needed to determine which bacteria are still alive and dead, one of which is a test of minimum kill concentration (KBM).

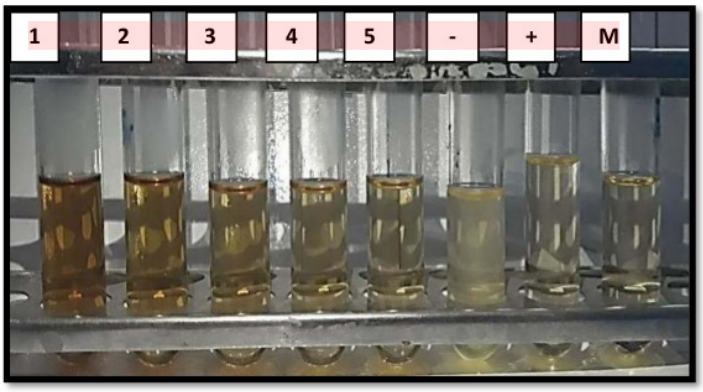

Figure 3. The minimum turbidity level of test inhibition

3.3 Minimum Killer Concentration (KBM)

Minimum Killer Concentration (KBM) is the lowest level content of antimicrobial which can kill bacteria characterized by no microbial growth in solid medium or growth of its colonies are less than o,1% of the initial inoculum amount on the solid medium that has been planted. The results of the KBM analysis using liquid smoke from Cocoa fruit peel as an antibacterial show that liquid smoke has bacteriostatic activity. This can be seen from the results of tests that are carried out after being planted on solid media which characterized by the growth of colonies or not. The KBM analysis is shown in Table 2.

Table 2. Test results for the minimum killer concentration of cocoa fruit peel liquid smoke

Tube Number	Concentration	Number of Colonies	SPC (CFU/mL)	Concentration
1	50%	0	0	50%
2	25%	0	0	25%
3	12,5%	0	0	12,5%
4	6,25%	11	$1,1 \times 10^7$	6,25%
5	3,125%	26	$2,6 \times 10^7$	3,125%
Negative control	0	285	$2,9 \times 10^9$	-
Positive control	1%	6	$6,0 \times 10^6$	-
Media control	0	0	0	-

The table above shows the growth of *E. coli* bacterial colonies at several levels of the concentration of Cocoa fruit peel liquid smoke decreased. At concentrations of 12.5 - 50% *E. coli* bacteria cannot grow on solid media, whereas at concentrations of 3.125 - 6.25% *E. coli* bacteria grow on solid media with amounts of 1.1 x 107 CFU / mL for concentrations of 6, 25% and, 2.6 x 107 CFU / mL for a concentration of 3.125%. In the negative control, the number of colonies that grow far more than the others is 2.9 x 109 CFU / mL while in the positive control the concentration of amoxilin 1% tablet colony of *E. coli* bacteria still grow with the amount of 6.0 x 106 CFU / mL .

Based on the data in Table 3, it can be seen that the minimum kill concentration of *E. coli* bacteria is at a concentration of 12.5% which at this concentration, *E. coli* bacteria cannot grow after being planted into solid media. The growth of *E. coli* colonies bacteria is found in Figure 4.

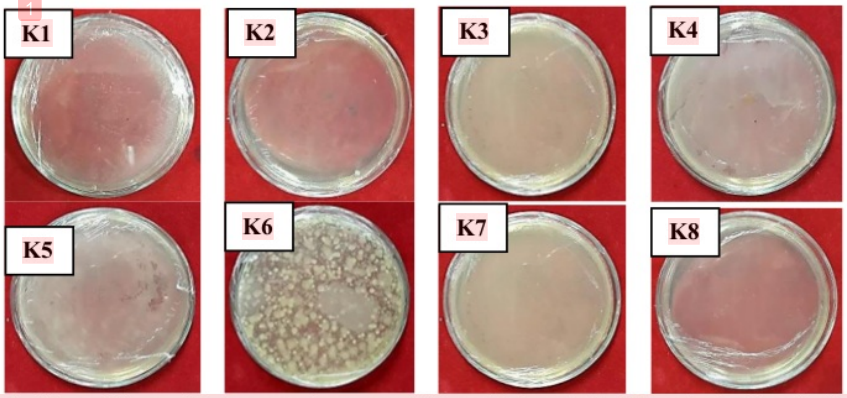

Figure 4. The minimum kill concentration test of Cocoa fruit peel liquid smoke in *E. coli* bacteria. K1 = Concentration 1 (50%); K2 = Concentration 2 (25%); K3 = Concentration 3 (12.5%); K4 = Concentration 4 (6.25%); K5 = Concentration 5 (3.125%); K6 = negative control; K7 = Positive control; K8 = NB media control

At a concentration of 3,125 - 6,25%, the number of colonies of bacteria grow in small amounts, whereas at this concentration the growth of colonies is less than 0.1% of the initial amount. This means that this concentration can be said to be the minimum kill concentration.

4. Conclusion

Liquid smoke from Cocoa fruit peel has the potential as an antibacterial agent against *E. coli* and, *S. aureus*. The most effective liquid smoke from Cocoa fruit peel inhibits the growth of E. coli and S. aureus with a moisture content of 10%. The inhibition zone value of Cocoa fruit peel liquid smoke water content of 10% against *E. coli* bacteria was 25.1 mm, while *S. aureus* bacteria have an inhibition zone value of 32.6 mm. *E. coli* bacteria show stronger resistance compared to *S. aureus*. The minimum inhibitory concentration and, the minimum kill concentration in a row are at concentrations of 3.125% and 12.5% for *E. coli* bacteria.

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