Majalah Kedokteran Sriwijaya
Th. 52 Nomor 3, Oktober 2020

ANTIBACTERIAL ACTIVITY OF KELUAK (PANGIUM EDULE) POWDER CRUDE EXTRACT USING THE MICROWAVE ASSISTED EXTRACTION (MAE) METHOD

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ABSTRACT

Keluak is commonly used by Indonesian people as a seasoning flavor. Information regarding the content of bioactive compounds and in fermented Pangi seeds (Keluak) and their activities is still little researched so that further research is needed. To extract bioactive compounds from Keluak, an extraction process is carried out using Microwave Assisted Extraction (MAE) which has the advantage of a shorter extraction time and good temperature control. The purpose of this study was to determine the use of the best material:solvent ratio and extraction time to obtain a crude extract of Keluak powder with the best antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The experimental design used in this study was a randomized block design (RBD) which was factorial arranged with two factors, the first factor being the ratio of material:solvent (1:5 (w/v), 1:10 (w/v), and 1:15 (w/v)) and a factor of two is the extraction time (10, 15, and 20 minutes). Each treatment was repeated three times in order to obtain 27 experimental units. Data were analyzed using analysis of variance (ANOVA) with a 95% confidence interval. The best treatment in this study was the use of a material:solvent ratio of 1: 5 (w/v) and an extraction time of 15 minutes with a yield value of 87.45%, total phenol of 11.41 mg GAE / g extract, total tannins of 5.20 mg TAE/g of extract, the clear zone diameter in *Escherichia coli* was 5.69 mm, and the clear zone diameter in *Staphylococcus aureus* was 7.10 mm. The antibacterial activity of the extract on *Escherichia coli* and *Staphylococcus aureus* is only bacteriostatic so that it can only inhibit the growth of the two bacteria for a certain period of time.

Keywords: Antibacterial, Extraction, Keluak, MAE, *Pangium edule*

1. INTRODUCTION

Keluak is a type of spice that is often used as a spice in Rawon recipe. Pangi seeds that have been fermented for 40 days (Keluak) contain total phenol content of about 26 mg/g of extract.[1] Phenolic compounds are known to have antibacterial activity,[2] which encouraged the research to determine the effectiveness of antibacterial compounds in Keluak against several types of bacteria. Although Keluak is often used in everyday life, it is rare to find instant seasoning in the form of Keluak powder. Instant spices containing Keluak are produced in the form of a paste which may be due to the difficulty of obtaining good powder

characteristics and the market demands that are not too high so that long shelf life is not really needed.

In this study, Keluak powdering process was carried out which aims to be used as a component of instant spices with a longer shelf life. If it is proven to contain high bioactive compounds, Keluak powder can also be used as a medicinal or supplement ingredient. To obtain bioactive compounds (especially those with antibacterial properties) from the Keluak and to test their antibacterial activity, an extraction process is necessary. The conventional extraction method is carried out in high temperatures and a long time so that it is prone to destruction of bioactive compounds in the extracted sample. Another method that can be applied for extraction is the microwave assisted extraction method (MAE), which is an extraction method using microwaves to extract dissolved components in the sample.

MAE has several advantages including shorter extraction time, good temperature control, less energy and solvent consumption, more yields, higher accuracy and precision, and simple array of equipment that combines the Soxhlet features and the advantages of microwave. Because of its advantages, it is expected that Keluak powder extract will be obtained with better results in shorter time. In carrying out the extraction using the MAE method, there are several factors that need to be considered, including the ratio of material:solvent and extraction time. The volume of the solvent must be sufficient to ensure that the entire plant matrix is immersed in the solvent during the irradiation time. Apart from the volume of the solvent, time is another parameter that affects the amount of analyte extracted.

This research was conducted to determine the effect of the material:solvent ratio and extraction time using the MAE method on the antibacterial activity of the crude extract of Keluak powder. The use of different material:solvent ratios and extraction time is presumed to result in different antibacterial activity. If the extract is proven to contain high amounts of bioactive compounds, it is possible to use the extract as a drug or supplement. In addition, the information generated from this study is expected to provide information on the content of antibacterial compounds in the Keluak powder and in turn, it is expected that it adds the selling value of instant spices containing Keluak with its functional properties.

2. METHOD

The research was carried out in the Food Processing Engineering and Technology Laboratory, the Food Chemistry and Biochemistry Laboratory, and the Food Microbiology Laboratory, Department of Agricultural Product Technology, as well as the Central Laboratory of Biological Sciences, University of Brawijaya. The research took place from January 2018 to June 2018.

The tools used in the produce of Keluak powder were spoon, glassware, suction ball, aluminum foil, baking pan, vacuum dryer, blender, and 20 mesh siever. The tools used for the extraction were Microwave Assisted Extractor, glassware, filter cloth, analytical scales and measuring pipettes. The tools used for analysis are analytical scales, oven (Memmert), glassware, suction ball, UV-Vis spectrophotometer (Thermo Scientyfic Genesis 20), Laminar Air Flow, microplate (Costar 96), microplate reader (Labtech), loop needle, bunsen, parchment paper, cotton, rubber, microtip, micropipette, incubator (Binder), vortex (LW Scientific Turbo Mixer TM 2000) and autoclave (GEA).

The material used in this study was Keluak seeds (fermented Pangi (*Pangium edule*) seeds) obtained from Madyopuro Market, Malang. To obtain good quality Keluak powder, additional ingredients such as tween 80 and maltodextrin are needed. In addition, distilled water wasused as a solvent in the extraction process. To perform the analysis, materials such as distilled water, Folin Ciocalteu, Na_2CO_3, ethanol, methanol,

gallic acid, Nutrient Agar (Merck) media, and Nutrient Broth (Merck) media were needed. *Escherichia coli* bacteria cultures obtained from the Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya, while isolates of *Staphylococcus aureus*, Salmonella typhimurium, and Bacillus cereus were obtained from the Biotechnology Laboratory, Department of Agricultural Product Technology, University of Brawijaya.

The research design used was factorial randomized block design (RBD) with 2 factors, namely the ratio of material:solvent and extraction time, each of which had 3 levels. The first factor is the ratio of the material: solvent used is 1: 5, 1:10 and 1:15. Factor 2, namely the extraction time used was 10, 15 and 20 minutes. From this design, 9 combinations were obtained and 3 repetitions were carried out, in order to obtain 27 experimental units.

Factor 1: Material:solvent ratio (R)
R1 =KeluakPowder (1):Distilled water (5)
R2= KeluakPowder (1):Distilled water(10)
R3 = KeluakPowder (1):Distilled water (15)
Factor 2: Extraction time (W)
W1 = 10 minutes
W2 = 15 minutes
W3 = 20 minutes

From these two factors, 9 treatment combinations were obtained which can be seen in table 1.

Table1.Combination of material:solvent ratio treatment and extraction time

No	Sample	Treatment Combination
1	R1W1	KeluakPowder (1):Distilled water (5), 10 min
2	R1W2	Keluak Powder (1):Distilled water (5), 15 min
3	R1W3	Keluak Powder (1):Distilled water (5), 20 min
4	R2W1	Keluak Powder (1):Distilled water (10), 10 min
5	R2W2	Keluak Powder (1):Distilled water (10), 15 min
6	R2W3	Keluak Powder (1):Distilled water (10), 20 min
7	R3W1	Keluak Powder (1):Distilled water (15), 10 min
8	R3W2	Keluak Powder (1):Distilled water (15), 15 min
9	R3W3	Keluak Powder (1):Distilled water (15), 20 min

Keluak Powdering Process[4]

Keluak seeds were sorted, washed and crushed for its flesh. The seed flesh used is brown to black in color, does not smell sweet (indicating the presence of microbial activity),not infected by fungi and not bitter. Tween 80 and maltodextrin were added, then mixed using blender, and flattened on baking pan. The mixture was dried in a vacuum dryer at 60^{o}C for 6 hours. After drying, the Keluakmixturewas grounded, sieved with 80 mesh sieves then proceeded to the MAE extraction process.

Keluak Powder Extraction Process

Keluak powder that is ready to be extracted was then dissolved in distilled water with variation of the ratio of material:solvent 1: 5, 1:10, and 1:15 with the same amount of solvent used in each combination ratio, which waas as much as 45 ml (respectively, for ratio of 1:5 (w/v), 1:10 (w/v),and 1:15 (w/v), Keluak powder was used as much as 9 g, 4.5 g, and 3 g). This is performed to maximize the sample capacity in the MAE vessel. Afterwards the Keluak powder mixture was put into the MAE with varying lengths of time, which were 10, 15 and 20 minutes. The power

used is 1300 W with temperature of 55°C. The extracted Keluakmixture is then filtered with filter cloth so that the final result is a crude extract of Keluakpowder and would be tested on predetermined parameters.

Testing the Antibacterial Activity of Keluak Powder Extract

Antibacterial activity testing was carried out using the well method. In the agar solid, seed medium that has been mixed with the tested bacteria, a well is made then filled with antibacterial substance (Keluak powder extract) and incubated at 37ºC for 18-24 hours.[5] Furthermore, there is a clear zone around the well which indicates the presence or absence of bacterial growth. The clear zone formed is then measured.

Observation

Observations made on the crude extract of Keluak include chemical content analysis. The parameters observed included: water content,[6] extract yields, total phenolcontent[7], tannin content[8], antibacterial activity.

Data Analysis

Observation data using factorial randomized block design (RBD) were analyzed statistically using ANOVA (Analysis of Variance) in Minitab 17 software. If it showed significant effect on both treatments,5% DMRT (Duncan Multiple Range Test) was performed. If there is no real effect, the LSD (Least Significant Difference)test 5% would be carried out.

3. Result

Raw Material Analysis

Keluak powder is used as raw material for extraction. Preparation of the powder before the extraction process is intended so that the results of all test parameters reflect the actual condition when the Keluak powder is applied as an instant seasoning. The results of raw material testing on 3 test parameters (moisture content, total phenol) can be seen in table 2.

Table2.Results of the characteristic test of Keluak powder before extracting

Parameter	Results
Water content (%)	4,08 ± 0,03
Total phenol (mg GAE/g)	0,52 ± 0,15
Total tannins (mg TAE/g)	0,38 ± 0,01

Extract yield

Extraction yield is the ratio of mass before and after the extraction process. The ratio of material:solvent (w/v) and the extraction time used are thought to have an impact on the final yield of the extract because these two factors are important factors in carrying out extraction with the microwave assisted method (MAE). The yield of extraction results from various extraction treatments carried out in this study can be observed in Figure 1.

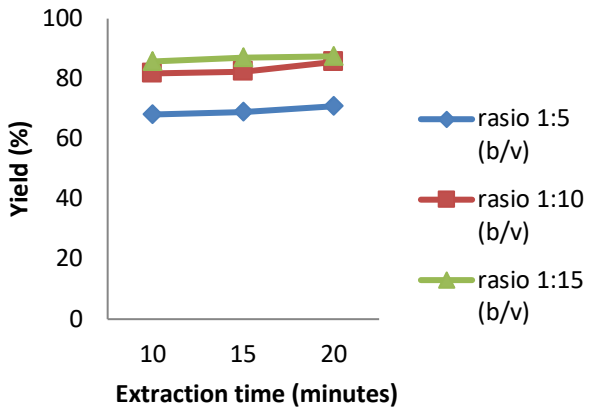

Figure 1.The average yield of crude extract from all treatment combinationsgiven

From the analysis of ANOVA variance with a 95% confidence interval (α = 0.05), it is known that both the interaction of the two factors used in the extraction process, the factor of the ratio of material:solvent and the length of time of extraction have a significant effect on the yield produced so that further tests Duncan Multiple Rate Test (DMRT 5%) was conducted. The average yield that has been tested for 5% DMRT can be observed in Table 3

Table 3.The average yield of Keluak extract was due to variations in the treatment of the ratio of material:solvent and extraction time

ratio of material:solvent	Length of Time (minutes)	Yield	DMRT 5%
1:5	10	68,10 ± 0,64 a	1,12
1:5	15	68,96 ± 0,80 a	1,18
1:5	20	70,85 ± 0,91 b	1,21
1:10	10	81,77 ± 0,85 c	1,24
1:10	15	82,41 ± 0,54 c	1,25
1:10	20	85,60 ± 0,68 d	1,27
1:15	10	85,75 ± 0,56 d	1,28
1:15	15	87,10 ± 0,13 e	1,28
1:15	20	87,45 ± 0,23 e	

Total Phenol of Keluak Crude Extract

The results of total phenol testing in all treatment combinations can be seen in Figure 2.

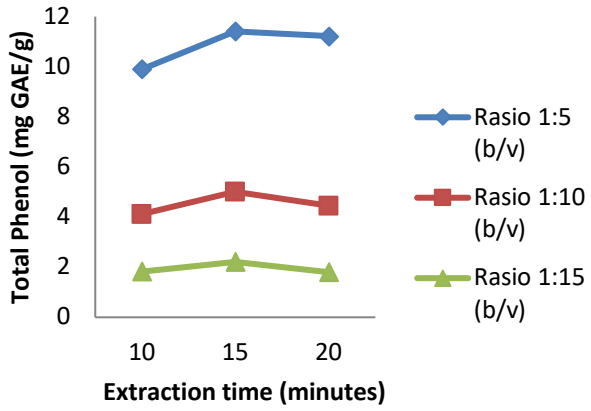

Figure 2.The average of total phenol ofKeluak crudeextract from all combinations of treatments given

Total Tanninsof Keluak Crude Extract

The results of total tannin testing in all treatment combinations can be seen in Figure 3.

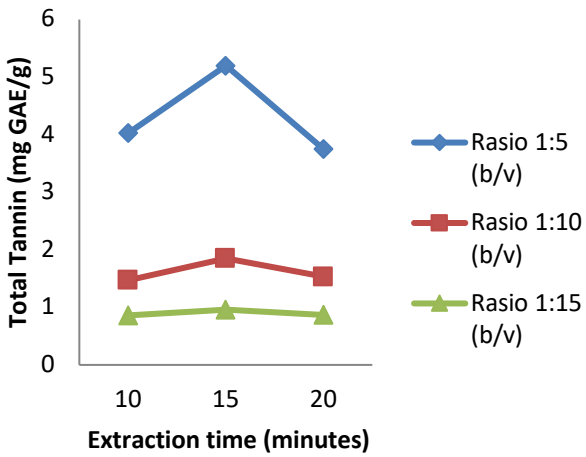

Figure 3. The average of total tannins ofKeluak crudeextract from all combinations of treatments given

Antibacterial Activity Test of KeluakCrude Extract Against *Escherichia coli* and *Staphylococcus aureus* Bacteria

The results of the test for the antibacterial activity of the Keluak crude extract against *Escherichia coli* can be observed in Figure 4.

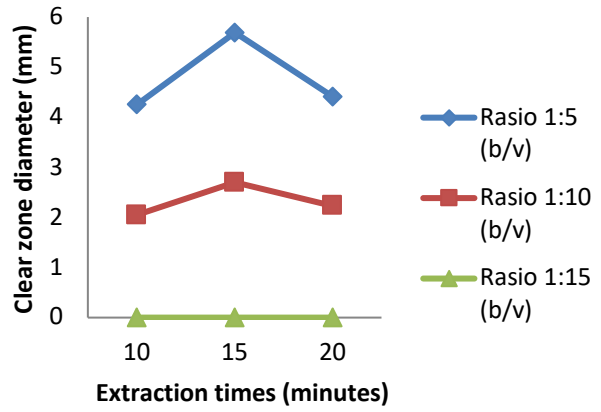

Figure 4. Antibacterial activity of Keluak powder crude extract against *Escherichia coli*

The results of testing the antibacterial activity of the Keluak crude extract against *Staphylococcus aureus* can be seen in Figure 5.

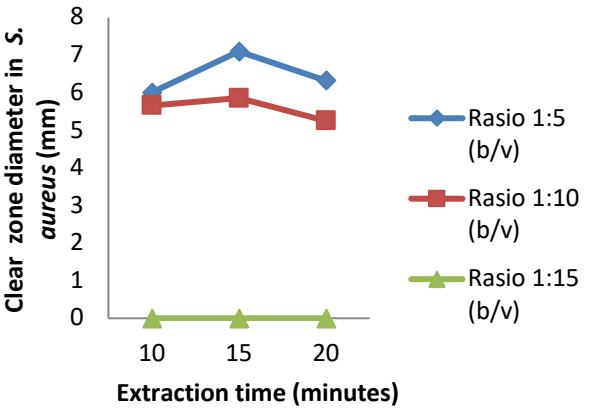

Figure 5.Antibacterial activity of Keluakcrude extract against *Staphylococcus aureus*

Determination of the Best Treatment by Zeleny Method

Determination of the best treatment is carried out by the Zeleny method, [10] with the best expected treatment is that which has the maximum (highest) value for each tested parameter. From determining the best treatment for each parameter obtained the results of the combination treatment using the ratio of 1:5 (w/v) material:solvent and 10 minutes of extraction time as the best treatment. The R1W2 extract was then tested for its minimum inhibitory concentration (MIC) and minimum lethal concentration against *Escherichia coli* and *Staphylococcus aureus*.

Minimum Inhibitory Concentration Test of Keluak Crude Extract from The Best Extraction Treatment Against *Escherichia coli* and *Staphylococcus aureus* Bacteria

Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration for an antibacterial compound needed to inhibit the growth of certain microorganisms after incubation.[11] The Minimum Inhibitory Concentration Test was a continuation test of the former antibacterial activity test conducted, so the test were using bacteria which reacted to the extract, which were*Escherichia coli* and *Staphylococcus aureus*.The minimum inhibitory concentration can be determined by measuring the turbidity of the bacterial suspension before and after incubation with an antibacterial compound, which in this study was Keluak crude extract. The measurement of turbidity is carried out with a spectrophotometer, where the cloudier the suspension is, the greater the absorbance value will be read. This indicates the high number of bacteria in the suspension. If the extract has antibacterial activity, the difference in absorbance of the suspension before and after incubation will be 0 or below 0.

The results of the best treatment combination in this study were extraction with

a solvent: material ratio of 1: 5 (w/v) and a long extraction time of 15 minutes (R1W2) and then tested the minimum inhibitory concentration. The observation time was determined according to the time of appearance of the maximum clear zone diameter, which were respectively 5 hour for *Staphylococcus aureus* and 6 hour for *Escherichia coli*. The results of the MIC test against *Escherichia coli* and *Staphylococcus aureus* can be observed in table 4 and table 5.

Table 4. Minimum Inhibitory Concentration of Keluak crude extract against *Escherichia coli*

Bacteria	Concentration	Absorbance Value		ΔOD
		Before Incubation	After Incubation	
E. coli	0%	0,02	0,26	0,24
	10%	0,03	0,14	0,11
	20%	0,05	0,15	0,10
	30%	0,09	0,18	0,09
	40%	0,10	0,19	0,09
	50%	0,14	0,22	0,08
	60%	0,15	0,22	0,07
	70%	0,16	0,23	0,07
	80%	0,17	0,23	0,06
	90%	0,28	0,33	0,05
	100%	0,36	0,35	-0,01
Amoxicillin	1%	0,04	0,02	-0,02

Table5. Minimum Inhibitory Concentration of Keluak Crude Extract against *Staphylococcus aureus*

Bacteria	Concentration	Absorbance Value		ΔOD
		Before Incubation	After Incubation	
S. aureus	0%	0,01	0,23	0,22
	10%	0,04	0,15	0,11
	20%	0,06	0,14	0,08
	30%	0,07	0,13	0,06
	40%	0,08	0,14	0,06
	50%	0,09	0,11	0,05
	60%	0,12	0,16	0,04
	70%	0,20	0,23	0,03
	80%	0,22	0,24	0,02
	90%	0,25	0,27	0,02
	100%	0,38	0,34	-0,04
Amoxicillin	1%	0,03	0,015	-0,015

Minimum Lethal Concentration Test of Keluak Crude Extract from Best Extraction Treatment Against *Escherichia coli* and *Staphylococcus aureus* Bacteria

The Minimum Lethal Concentration Test (MLC) is a test conducted to determine the lowest concentration of an antibacterial compound in killing 99.9% of target bacteria.[12] The Minimum Lethal Concentration Test (MLC) is related to the Minimum Inhibitory Concentration (MIC) test. Through the Minimum Lethal Concentration test, the results of the Minimum Inhibitory Concentration test can be confirmed because there is still the possibility of the presence of very small numbers of bacteria during the

turbidity reading. The concentration that showed the best effectiveness in inhibiting bacterial growth was tested for its ability to kill bacteria. The results of the test can be seen in table 6.

Table 6.Minimum Lethal Concentration of Keluak crude extract against *Escherichia coli* and *Staphylococcus aureus* bacteria

Bacteria	The concentration of MIC Test Results (%)	Growth Test Results / Not
E. coli	100	Growth
S. aureus	100	Growth

From the test results, it is known that the extract of Keluak powder does not have the ability to kill the two types of tested bacteria which are gram-positive and negative bacteria. The extract only has growth inhibiting (bacteriostatic) but not killing (bactericidal) activity. This proves that the Keluak extract is not effective when used as a primary source of antibacterial compounds because it has weak antibacterial activity. The extract is only able to inhibit bacterial growth for a certain period of time (under 24 hours) before finally the inhibitory effect weakens and disappears.

4. DISCUSSION

Raw Material Analysis

The total phenol of Keluak powder made using maltodextrin as a filler is 19.19 ppm (the best treatment result) or 0.01919 mg/g.[4] The difference in the total phenol of kluwak powder obtained in the Estiasih and Sofia[4] research with this study was caused by differences the source of the raw materials used. The difference in raw material intake can have an impact on the differences in the Keluak varieties used, in addition to the cultivation location and fermentation process of the Keluak that are used cannot be uniform. This can lead to differences in the content of bioactive compounds in the materials used.

The total tannins in Pangi powder were 0.841% or 8.41 mg/g.[13] The total tannins in Pangi powder were different from the research conducted by Prishandono[13] due to differences in the conditions of the Pangi used. In this study, fermented Pangi powder was used or commonly known as Keluak, while Prishandono[13] used unfermented seeds of the Pangi plant.

Extract Yield

Research conducted by Farida and Nisa[14] regarding anthocyanin extraction from mangosteen peel using the MAE method states that the percentage of extract yield will increase along with the increase in the amount of solvent used. The increased yield was caused by the extraction time which was not long enough to evaporate the solvent. The yield results obtained in this study are in accordance with the literature. The highest extract yield was produced by the material:solvent ratio of 1:15 (w/v).

Extraction time has an influence on the final extract yield. The maximum extraction yield was obtained in the extraction time of 20 minutes. The heating efficiency of the solvent during microwave exposure must also be considered because its ability to evaporate depends on how quickly the temperature rises.[15] The extraction time of 20 minutes produces less than optimal heat in extracts with higher material:solvent ratio so that solvent evaporation occurs in minimal amounts. On the other hand, when the extraction time is 10 minutes with the ratio of material:solvent less heat is maximized, so that a larger amount of evaporation occurs. This results in a higher amount of yield.

Total Phenol and Tanin of Keluak Crude Extract

In carrying out the extraction, the temperature and power of the tool are equalized so that the exposure to microwaves in various extraction treatments given also depends on the extraction time carried out. The more solvent used, the longer the extraction

time needed to maximize the amount of analyte extracted. If it is seen from the data trend, the highest total phenol value was obtained at the extraction time of 15 minutes, and there was a decrease in the total phenol value at 20 minute. The decrease was caused by the destruction of the phenol components at high temperatures. It should be noted that the solvent used in this extraction process is water. Water has a high dielectric constant which causes a shorter time to heat it in microwave exposure.[16]

In 15 minute, the heating that occurs in the solvent is perfect to remove the phenol component in the Keluak powder so that when the extraction process is continued, the phenol component that has come out of the powder matrix will suffer damage due to contact with high temperatures. The temperature increase will occur faster when using the ratio of 1:5 (w/v), especially because the amount of solvent used is less than the other ratios.

The longer the time taken to extract the sample using the MAE method, there will be a significant increase in the total extracted tannins, but the measurable decrease in total tannins also occurs when the irradiation time is extended, possibly due to compound decomposition.[17] The decrease occurred due to longer exposure to microwaves, which resulted in the solvent experiencing an increase in temperature and damaging the extracted tannins. Research on the effect of pH, temperature, and time on tannin inactivation in Acioa barteri leaves, total tannins decreased with increasing pH, increasing storage period and increasing temperature.[18]

Research on the effect of temperature treatment on total tannins and antioxidant activity of Quercus cerris extract states that high temperature treatment causes a decrease in total tannins from 11.69% to 8.55%.[19] The measured change in total tannin extract in this study is in accordance with the results of studies because the total tannin increased at 10 minutes of extraction and decreased after 15 minutes. The extraction process has reached its maximum point in 15 minutes so that when the process is continued, no more compounds can be extracted from the material. The decrease can also occur due to the prolonged contact between the tannins and the solvent that has been exposed to microwaves and has experienced an increase in temperature.

Minimum Inhibitory Concentration Test of Keluak Crude Extract from Best Extraction Treatment Against Escherecia coli and *Staphylococcus aureus* Bacteria

Antibacterial compounds are classified into bacteriostatic and bactericidal. Classification as bacteriostatic or bactericidal is based on the ratio of the minimum lethal concentration and the minimum inhibitory concentration of the extract to bacteria. Antibacterial compounds can have both properties depending on the dose used.[20] From the tests carried out, it is known that the extract concentration with a value of ΔOD 0 or below 0 is the extract concentration of 100%, therefore it can be concluded that the minimum concentration required to inhibit the growth of *Escherichia coli* bacteria and *Staphylococcus aureus* with Keluak powder extract was 100%. The use of extract concentrations below 100% will not have an effect that can inhibit the growth of the two tested bacteria.

The results of the Minimum Inhibitory Concentration test showed that the difference in absorbance values before and after incubation was greater in *Escherichia coli*. The effectiveness of the extract as an antibacterial compound can be observed through the absorption of absorbance. The lower the absorbance of the suspension, the higher the effectiveness of the extract in inhibiting bacterial growth. This is related to the characteristics of *Escherichia coli* as gram-negative bacteria. Gram-negative organisms have a more complex cell wall structure than gram-positive organisms. The cell wall of gram-negative bacteria has a capsule, a peptidoglycan layer, and 2 cell membranes (outer membrane and inner membrane). This

difference makes the recovery of infections caused by gram-negative bacteria more difficult to cure. Antibacterial compound molecules will find it difficult to penetrate cells with more complex cell wall structures.[21]

5. CONCLUSION

The ratio of material:solvent used in the extraction process had a significant effect (α = 0.05) on the formation of clear zones in *Staphylococcus aureus* and *Escherichia coli* bacteria. The extraction time used in the extraction process had a significant effect (α = 0.05) on the formation of clear zones in *Staphylococcus aureus* and *Escherichia coli* bacteria. The interaction of the two factors had a significant effect (α = 0.05) on the formation of clear zones in *Staphylococcus aureus* and *Escherichia coli* bacteria.

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