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THE EFFECT OF EXTRACTION TIME ON ANTIOXIDANT ACTIVITY IN ETHANOL EXTRACT BAY LEAF (Syzygizum polyanthum) WITH ULTRASONIC METHOD

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Abstract: Free radicals are molecules that have one or more unpaired electrons. Many natural ingredients can be a source of antioxidants, one of which is bay leaves. The extraction of secondary metabolites in bay leaves can be done by ultrasonic extraction method, also known as sonication. Extraction by sonication technique is influenced by several factors, one of which is the length of time. To determine the effect of extraction time on the antioxidant activity of bay leaf extract by ultrasonic method and to determine the optimal time in ultrasonic bay leaf extraction. This research uses experimental quantitative method. The extraction method used was sonication or ultrasonic extraction with varying extraction times of 15 minutes, 25 minutes and 35 minutes. Antioxidant activity testing was done with DPPH method. The results showed that the extraction time with sonication method had a significant effect (sig<0,05) on antioxidant activity. The best antioxidant activity test results at 35 minutes resulted in an IC_{50} value of 42.25 ppm.

1. INTODUCTION

Free radicals are molecules that have one or more unpaired electrons, making them highly reactive and potentially damaging cells. In the face of this threat, antioxidants play an important role by donating hydrogen atoms to free radicals, thereby halting the chain reaction and stabilizing the molecule. Several natural ingredients have been known to contain antioxidants, one of which is bay leaf (Syzygium pxolyanthum).

Bay leaves are well-known as a spice for their rich aroma, but they have also long been utilized in traditional medicine due to their bioactive compound content. The results of the study showed that bay leaves contain secondary metabolites such as phenolic compounds, steroids, saponins, alkaloids, and flavonoids. Among these compounds, flavonoids—specifically quercetin—are the main components that act as antioxidants (Bhadreswara, 2023).

Scientific evidence regarding the antioxidant activity of bay leaves is getting stronger. A study by Bhadreswara (2023) reported that bay leaf extract showed an IC_{50} value of 11,001 ppm, which falls under the category of very potent antioxidant activity. Another study by Zebua et al. (2023) also supports this finding, with an IC_{50} value of 38.34 ppm in bay leaf ethanol extract obtained through the maceration method. An IC_{50} value of less than 50 ppm indicates a high antioxidant potential (Zebzua et al., 2019; Haryantxo, 2023).

One popular method used to measure antioxidant activity is the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. This method is popular because it is simple, fast, and efficient in the use of reagents, and is very suitable for initial screening tests of various plant extracts (Devitria et al., 2020).

In the process of extracting active compounds from bay leaves, the sonication method (ultrasonic) is becoming a modern alternative that is increasingly being used. This technique utilizes high-frequency acoustic waves (>20 kHz) to speed up the extraction process. Compared to conventional methods, sonication offers several advantages, including more concentrated extract results, higher active ingredient content, and shorter processing time (Rifkia, 2020; Szusilxoningrzum, 2023).

One of the factors that affects extraction is the extraction time. If the extraction time is too long or exceeds the ideal limit, oxidation will occur, causing the loss of compounds in the solution.

Conversely, if the extraction time is too short, the active compounds of the material are not fully extracted. Research by Rifkia Via et al (2020) stated that moringa leaf extract using the UAE method at 70°C for 20 minutes produced the highest yield value of 25.64%. Research by Kristina et al (2022) stated that the highest yield value was 30.24%, and high antioxidant levels with a value of 99.74 ppm were obtained during 30-minute extraction.

Based on this description, it is necessary to do research on the right extraction time to determine the effect of extraction time on antioxidant activity in bay leaf extract (Syzygium pxolyanthum) and the extraction time that can produce the highest antioxidant activity by the ultrasonic method.

2. RESEARCH METHODS

2.1 Tools and Ingredients

2.1.1 Tools

UV-Vis spectrophotometry, analytical balance, ultrasonic bath cleaner, rotary evaporator, suction ball, glass tool, moisture analyzer, spatula, horn spoon, blender, 40 mesh sieve, aluminium foil.

2.1.2 Ingredients

Bay leaf, Aquadest, Ethanol 96%, Ethanol p.a, 2,2-Diphenyl-1 Picrylhydrazyl (DPPH), Vitamin C, 2N hydrochloric acid reagent, Mayer reagent, Dragendroff reagent, Bouchart reagent.

2.2 Bay Leaf Extraction Process

The sample was weighed as much as 10 g, then extracted with 100 ml of 96% ethanol using an ultrasonic bath cleaner with a frequency of 40 kHz for 15, 25, and 35 minutes. Extraction was carried out 3 times. The extract results obtained were filtered using Whatman No. 1 paper. The filtrate was then evaporated using a rotary evaporator and followed by further evaporation in a water bath to produce a thick extract.

2.3 Screening Phytochemistry

2.3.1 Alkaloid

A total of 0.5 grams of simplicia powder was weighed, then 1 ml of HCl 2N and 9 ml of aquadest were added, heated over a water bath for 2 minutes, cooled, and then filtered. The obtained filtrate is divided into 3 parts in the test tube. Tube I is added to the Mayer reagent, white or yellow deposits will be formed, tube II is added to the Bouchart reagent, blackish-brown deposits will be formed, and tube III is added to the Dragendroff reagent, red and orange deposits will be formed. If two of the above three reactions are positive, the sample is declared to contain alkaloids (Martiningsih, 2023).

2.3.2 Flavonoid

Mix 0.5 g of simplicia powder with 100 mL of hot water, then boil for 5 minutes and filter. To 5 mL of the filtrate, add 100 mg of magnesium powder, 1 mL of concentrated HCl, and 2 mL of amyl alcohol. Whisk the solution and let it sit until two layers form. A yellow, orange, or red color in the amyl alcohol layer indicates the presence of flavonoid compounds (Werdiningsih, 2022).

2.3.3 Tanin

Mix 0.5 g of simplicia powder with 10 mL of Aquadest and filter it. Dilute the filtered liquid until it's clear. Take 2 mL of this diluted liquid and add 2 drops of $FeCl_3$ solution. If the mixture turns blackish-green or blue-black, it means tannin compounds are present (Raihan, 2022).

2.3.4 Saponin

A total of 0.5 g of simplicia powder is put into the test tube, then 10 mL of hot aquadest is added and cooled. The solution is beaten vigorously for 10 seconds. The formation of foam at least 1 cm high that is stable for approximately 10 minutes indicates a positive result in the presence of saponin compounds (Raihan, 2022).

2.3.5 Steroid/Triterpenoid

Weighing 1 g of simplicia powder, macerated with 20 ml of ether for 2 hours, and then strained. Evaporate the filtrate in a vaporizer cup and then add 5 drops of the Liberman-Burchard reagent. A bluish-green color indicates the presence of steroid compounds (Raihan, 2022).

2.4 Simplicia Characterization Checks

2.4.1 Determination of Water Rate

Weighted 5 gr of simplicia powder. Leveled simplicia on the moisture analyzer. Close the appliance and wait for the appliance to sound. Amount of standard moisture content <10% (Safrina, 2023).

2.4.2 Determination of water-soluble pollen levels

Weighing 5 grams of simplicia powder, then adding 100 ml of chloroform-saturated water and stirring several times for the first 6 hours, let it sit for 18 hours. Filter the solution after 18 hours. A total of 20 ml of filtrate is steamed to dry in a vaporizer cup that has been previously heated to 105°C and has been tapped out. The filtrate is heated to a fixed weight. The quality requirements for the content of water-soluble juice from bay leaf simplisia, namely >14,8% (Veninda, 2023).

2.4.3 Detemination of Soluble Essence Levels in Ethanol

A total of 5 g of simplicia powder was macerated with 100 mL of 96% ethanol for 24 hours in a sealed container, with occasional shaking for the first 6 hours, followed by 18 hours without stirring. The mixture is then filtered quickly, and as much as 25 mL of filtrate is taken and evaporated to dry in a porcelain cup that has been heated and pre-tapped at 105°C. Evaporation is carried out until a fixed weight is obtained. Based on quality standards, the ethanol-soluble juice content of bay leaf simplicia must meet the minimum requirements of more than 19.9% (Veninda, 2023).

2.4.4 Determination of Total Ash Content

A total of 2 g of simplicia powder is weighed and inserted into a silicate cross that has been primed and tapped beforehand. The baking process is carried out in a kiln at 600°C for 3 hours. After the demolition process is completed, the ash formed is cooled in a deciser, then weighed until a fixed weight is obtained. Next, the calculation of the total ash content was carried out. Based on the quality requirements, the total ash content of bay leaf simplicia should be less than 2.5% (Yana, 2022).

2.4.5 Determination of Acid Insoluble Ash Content

The sample was boiled for 5 minutes in 25 ml of diluted hydrochloric acid, and the ash from the total ash content was obtained. The acid-insoluble parts are collected with ash-free filter paper and washed with hot water. Then it was heated again for 15 minutes. Then cooling and weighing are carried out until the weight is constant. The quality requirements for the content of acid-insoluble ash from bay leaf simplicia are less than 1,8% (Yana, 2022).

2.5 Antioxidant Activity Test

2.5.1 DPPH Raw Solution 100 ppm

Weigh 10 mg of DPPH powder, then dissolve with ethanol p.a, and add up to 100 ml (Pzutri & Mahfzur, 2023).

2.5.2 DPPH Raw Solution 40 ppm

A 10 ml (DPPH 100 ppm) is bottled, put into a 25 ml measuring flask, and the volume is filled with ethanol p.a to the line of mark.

2.5.3 Maximum Wavelength

Take 2 ml of DPPH solution (40 ppm) and then add 2 ml of ethanol to the test tube and shake until homogeneous. Taken 3 ml and measured at wavelengths of 400-800 nm (Faidah et al., 2024).

2.5.4 Determining Operating time

A 1 ml solution of Vitamin C with a concentration of 8 ppm is taken into the test tube. Added 2 ml of 40 ppm DPPH solution. Homogenized and measured absorbance every 5 minutes for 60 minutes (Pzutri & Mahfzur, 2023).

2.5.5 Raw Solution of Bay Leaf 100 ppm

Weighing 10 mg of the extract is put into a beaker, and ethanol p.a is added to taste. Stir until dissolved. Then put it in a 100 ml measuring flask and add ethanol p.a to the limit mark.

2.5.6 Vitamin C Raw Solution

Put 10 mg of vitamin C powder in a beaker, add enough ethanol p.a, and stir until dissolved. Then put it in a 100 ml measuring flask and add ethanol p.a to the limit mark.

2.5.7 Blank Absorption Measurement

2 mL of 40 ppm DPPH solution was put in a test tube, and 2 ml of ethanol p.a was added. Then the absorption of the blank solution was measured with a UV-Vis spectrophotometer at a wavelength of 516 nm, carried out 3 times (Faidah et al., 2024).

2.5.8 Measurement of Vitamin C Antioxidant Activity

Pinched as much as 0.4 ml, 0.6 ml, 0.8 ml, 1 ml, and 1.2 ml of vitamin C raw solution. Put each in a 10 ml measuring flask and then add ethanol p.a to the limit mark so that a concentration of 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm. Pinched 1 ml of each concentration series and put it into the test tube. 2 ml of 40 ppm DPPH solution was

added, then incubated for 30 minutes. Measured the absorbance at 516 nm. Repeated 3 times (Pzutri & Mahfzur, 2023).

2.5.9 Measurement of Antioxidant Activity of Bay Leaf Extract

Pinched as much as 0.4 ml, 0,6 ml, 0,8 ml, 1 ml, and 1.2 ml of raw solution of bay leaf ethanol extract. Put each one in a 10 ml measuring flask and add ethanol p.a to the limit mark so that a concentration of 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm. Pinched 1 ml of each concentration series and put it into the test tube. 2 ml of 40 ppm DPPH solution was added, then incubated for 30 minutes. Measured the absorbance at 516 nm. Repeated 3 times (Pzutri & Mahfzur, 2023).

2.5.10 Value Analysis of IC_{50}

The determination of the percent of free radical inhibition by bay leaf extract with vitamin C as a positive control, using the DPPH free radical suppression method, can be calculated with the following formula:

$$\% \text{ Inhibition} = \frac{A \text{ kontrol} - A \text{ Sampel}}{A \text{ Kontrol}} \times 100\%$$

2.6 Data Analysis

The data from the research results were analyzed using the SPSS program, or statistical product and service solution, with the One-Way Anova method to determine the significant influence of each extraction time used

2.7 Method Validation

2.7.1 Accuracy

The absorbance of the solution was measured at a concentration of 8 ppm using UV-Vis spectrophotometry. Absorbance is obtained and calculated to obtain a measured level value. Accuracy is shown as % recovery, and an acceptable accuracy range is 80-110% (Melasasi et al., 2021).

2.7.2 Precision

Calculated absorption of the vitamin C solution with a concentration of 8 ppm. Tests were carried out with 6 repetitions. Accuracy is determined from the standard deviation (SD) and % RSD. A method is said to have good precision if the value of RSD is less than 2%.

2.7.3 LOD and LOQ

The detection limit and the quantity limit are calculated from the linear regression equation of the calibration curve obtained using the formula:

$$\text{LOD} = \frac{3 \, x \, SD}{s}$$

$$\text{LOQ} = \frac{10 \, x \, SD}{s}$$

2.7.4 Linearity

Put 10 mg of vitamin C powder in a beaker, add enough ethanol p.a, stir until dissolved. Then put it in a 100 ml measuring flask and add ethanol p.a to the limit mark (100 ppm). Pinched 0.4 ml, 0.6 ml, 0.8 ml, 1 ml, and 1.2 ml of vitamin C parent solution (100 ppm) obtained concentrations of 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm. Calculated the results of absorbance measurements from linear regression equations and calculations of their correlation coefficients. The correlation coefficient is said to be good if $r \geq 0{,}99$.

3. RESULT AND DISCUSSION

3.1 Extraction Results

Time (Minute)	Result (g)	Yield (%)
15	0,827 g	8,26%
25	1,226 g	12,24%
35	1,421 g	14,19%

Table 1. Extraction Results

Based on the results obtained, each time the extraction produces a different yield. This is because the longer the solvent's contact time with the sample, the more time the solvent has to attract secondary metabolite compounds from within the sample. So the longer the extraction time, the more bioactive components are extracted.

3.2 Screening Phytochemistry Results

The results showed that bay leaves contain secondary metabolites, including alkaloids, flavonoids, tannins, saponins, and steroids.

3.3 Result of Characterization Simplicia

Characterization checks	Requirements (FHI, 2017)	Result
Determination of Water Rate	<10%	5,33%
Determination of water-soluble pollen levels	>14,8%	15,24%
Detemination of Soluble Essence Levels in Ethanol	>19,9%	29,62%
Determination of Total Ash Content	<2,5%	2,17%

Determination of Acid Insoluble Ash Content	<1,8%	1,58%

Tabel 2. Result of Characterization Simplicia

The minimum limit or range of water content in simplicia powder is less than 10 % (Depkes RI, 1989). The results of determining the moisture content obtained from bay leaf simplicia powder were 5.33%. This shows that the moisture content of bay leaf simplicia powder is qualified.

According to the Herbal Pharmacopoeia, the quality requirements for the ethanol-soluble juice content from bay leaf simplicia are more than 19,9%. The results of determining the level of ethanol-soluble juice obtained from bay leaf simplicia powder was 29.62%. This shows that the soluble essence of ethanol simplicia bay leaf powder is acceptable. According to the Herbal Pharmacopoeia, the quality requirements of water-soluble juice content from bay leaf simplicia >14,8%. The determination of water-soluble juice obtained from bay leaf simplicia powder was 15,24%. This shows that the water-soluble essence content of bay leaf simplicia powder is qualified. When compared to the value of ethanol-soluble juice content, the value is lower. This indicates that the compounds that are abundant in bay leaf simplicia powder are semipolar or have fewer compounds that are attracted to water solvents.

According to the Herbal Pharmacopoeia, the quality requirements for the total ash content of bay leaf simplicia are less than 2,5%. The results of determining the total ash content obtained from bay leaf simplicia powder are 2,17%. This indicates that the total ash content of bay leaf simplicia powder is qualified.

The determination of the content of insoluble acid ash aims to determine the amount of ash content obtained from external factors. The Herbal Pharmacopoeia requires bay leaf simplicia to have less than 1.8% insoluble ash. Testing showed bay leaf simplicia powder had 1.58% insoluble ash. This meets the required quality standard.

3.4 Maximum Wavelength

Maximum wave measurements were carried out at wavelengths in the range of 400-800, and the measurement results showed that a 40 ppm DPPH solution produced maximum absorption at a wavelength of 516 nm with an absorbance value of 0.940.

3.5 Operating Time

Minute	Absorbance
0	0,540
5	0,538
10	0,537
15	0,533
20	**0,531**
25	**0,531**
30	**0,531**
35	**0,531**
40	0,534
45	0,536
50	0,537
55	0,535
60	0,537

Table 3. Operating Time Result

The purpose of the operating time is to show the most accurate time at which the test solution has reacted perfectly in inhibiting free radicals (DPPH). DPPH absorbance was stable from 20 to 35 minutes, so it can be concluded that the operating time used in this study was 30 minutes. Molyneux (2004) stated that the operating time in the ideal DPPH test is 30 minutes, which is used as the sample incubation time.

3.6 Analysis of Vitamin C Antioxidant Activity

Vitamin C is used as a benchmark to prove that the method used in determining antioxidant activity in the test solution is correct. In this study, a vitamin C value of 9.28 ppm was obtained, which shows that vitamin C has very strong antioxidant activity. The mechanism of action of vitamin C as an antioxidant is based on the mechanism of Hydrogen Atom Transfer (HAT), which is the process of neutralizing free radicals through the donation of hydrogen atoms. This antioxidant activity can also be visually observed through the discoloration of DPPH compounds that were originally purple to yellow after reacting with vitamin C (Nurkhasanah et al., 2023).

3.7 Antioxidant Activity Analysis of Bay Leaf Ethanol Extract

Time (Minute)	Yield (%)	IC_{50} (ppm)
15	8,26%	66,97
25	12,24%	58,53
35	14,19%	42,25

Table 4. Yield Value and IC_{50}

As shown in Table 3, the highest value of bay leaf ethanol extract was obtained at an extraction time of 35 minutes. Based on the analysis of the One Way ANOVA test, it is shown that the

extraction time by the ultrasonic method has a significant effect on antioxidant activity (sig < 0,05).

In Kristina's (2022) study, the highest antioxidant level was obtained at the 25 minute with a value of 99.74 ppm and a yield of 30,24%. Meanwhile, in the study of Ulfa (2024), the highest flavonoid levels were obtained at 30 minutes with a yield value of 21.4%, where flavonoids are secondary metabolites that have the potential to be antioxidants.

This is due to the longer contact time between the material and the solvent, which allows the chemical components to move with the help of ultrasonic waves. Ultrasonic waves are able to break down the cell walls of plants and make the compounds contained in them easily attracted by solvents. However, if the extraction time is too short, then the yield value will be low because not all materials are successfully extracted (Susanti, 2021).

The high yield produced is due to an increase in the level of antioxidant chemical components such as phenols and flavonoids at 35 minutes. Some studies have also shown that antioxidant activity is influenced by the content of phenols and flavonoids, i.e. by separating hydrogen atoms from phenol and flavonoid donors so as to produce the non-radical compound diphenylpycrylhydrazine, which is indicated by a change in color. However, once optimal conditions are reached, antioxidant activity will decrease as the number of chemical components produced decreases (Kristina, 2022).

3.8 Value Analysis of IC_{50}

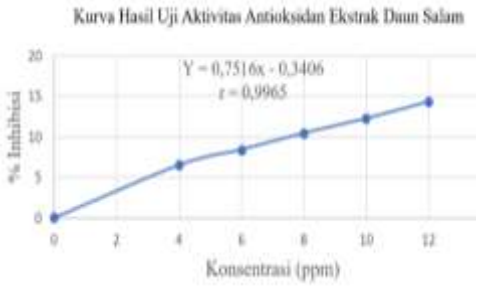
IC_{50} (*Inhibition concentration*) is the concentration needed to inhibit 50% of free radicals (DPPH) (Manurung, 2021).

Test Solution	Regression equations	IC_{50} (ppm)
Vitamin C	Y = 5,712x – 3,04092	9,28
15th Minute Bay Leaf Ethanol Extract	Y = 0,7516x – 0,3406	66,97
25th Minute Bay Leaf Ethanol Extract	Y = 0,8431x + 0,6449	58,53
35th Minute Bay Leaf Ethanol Extract	Y = 1,1611x + 0,9370	42,25

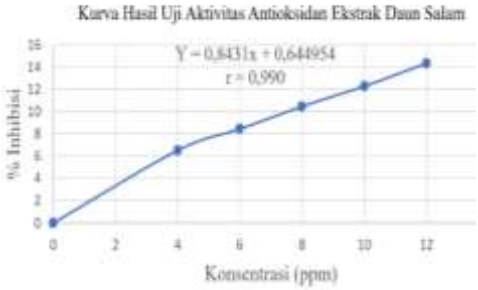
Table 5. Calculation Result of IC_{50}

The IC_{50} was obtained from the calculation of the linear regression equation, which shows the relationship between the concentration of the test solution and the percent of inhibition.

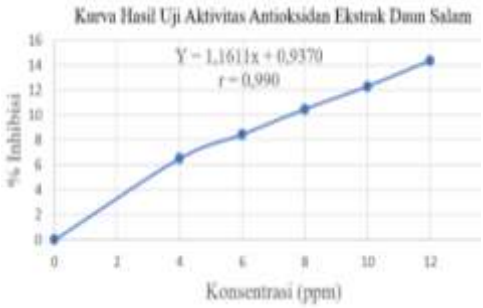
Picture 1. Antioxidant Activity Curve Of Vitamin C



Picture 2. Antioxidant Activity Curve Bay Leaf Ethanol Extract 15th minute



Picture 3. Antioxidant Activity Curve Bay Leaf Ethanol Extract 25th minute



Picture 4. Antioxidant Activity Curve Bay Leaf Ethanol Extract 35th minute

3.9 Method Validation Test Result

3.9.1 Accuracy

Consentration (ppm)	Absorbance	Measured Concentration	% Recovery
8	0,454	8,550	106,875
	0,456	8,589	107,362
	0,456	8,589	107,362
Σ	**1,366**	**25,728**	**321,599**
Mean	**0,455**	**8,576**	**107,199**

Table 6. Accuracy Test Results

The accuracy objective is to show the proximity between the measured rate result and the actual rate expressed by the % recovery. The % recovery value obtained is in the range of 107%. The results are acceptable because the % recovery is in the permissible range of 80-110%. This shows that the method used is accurate.

3.9.2 Precision

SD	%RSD
0,0169	0,19

Table 7. Precision Test Results

The precision test aims to see the proximity of a series of repeated measurements on the sample. Precision is expressed in percent of the relative standard deviation (% RSD). The results of the precision test showed that % RSD is 0,19%. The results show that the precision test is good because the value of % RSD < 2%.

3.9.3 LOD and LOQ

LOD	LOQ
1,0123	3,3744

Table 8. Test Results of LOD and LOQ

XThe LXOD test shows the lowest concentration that can still be detected, while the LOQ test shows the smallest amount of analyte in the sample that can still be measured and meets the careful and through criteria. The results showed that LOD value is 1,0123 ppm, while LXOQ value is 3,3744 ppm.

3.9.4 Linearity

No	Consentration	Absorbance
1	0	0
2	4	0,219
3	6	0,323
4	8	0,456
5	10	0,531
6	12	0,611
Slope (a)		0,05183
Intercept (b)		0,0108122
Correlation coefficient (r)		0,9970

Table 9. Linearity Test Result

Picture 5. Vitamin C Calibration Curve

The standard curve give an overview of the value of the correlation coefficient (r) in the linear regression equation, namely y = ax+b. From the standard curve, a linear regression equation was obtained, namely y = 0,05183x + 0,0108122 that y is the absorbance and x is the concentration with correlation coefficient value (r) namely 0,9970. The value of the correlation coefficient (r) is 0,9970 which indicates a strong relationship between sample concentration and absorbance. A

value (r) that is close to 1 has a very close relationship between the two variables by striking a linear curve.

4. CONCLUSION

Based on the research that has been carried out, it can be concluded that:

1. The extraction time has a significant effect (sig<0,05) on antioxidant activity. Antioxidant activity at extraction times of 15 minute, 25 minute dan 35 minute was obtained values of 66,97 ppm, 58,53 ppm and 42,25 ppm, respectively.
2. Based on the results of the research, the optimal time in the extraction of bay leaves by the ultrasonic method was obtained at 35 minutes with a value of 42.25 ppm.

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