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OPTIMIZATION OF THE ULTRASOUND ASSISTED EXTRACTION OF TURI LEAF FLAVONOIDS USING RESPONSE SURFACE METHODOLOGY

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Abstract

This research focuses on optimizing the extraction of flavonoid compounds from turi (*Sesbania grandiflora*) leaves using ultrasonic-assisted extraction (UAE) and response surface methodology (RSM). Turi leaves were selected due to their abundance, ease of availability, and potential as a natural antibacterial source, making them a cost-effective material. The aim was to determine the optimal extraction conditions for flavonoids. RSM was applied using a design of experiment (DOE) to set the parameter limits: temperature (40°C–60°C), time (10–30 minutes), and solvent volume (100–200 mL of 95% ethanol). Flavonoid content was analyzed using the $AlCl_3$ colorimetric method. Experimental flavonoid yields ranged from 0.026 to 0.349 mg QE/g sample. The highest yield, 0.349 mg QE/g, was obtained at 60°C, 10 minutes, and 200 mL ethanol. Optimization results using RSM suggested the best extraction conditions were 66.82°C, 3.18 minutes, and 234.09 mL ethanol, with a predicted flavonoid content of 0.445 mg QE/g. These findings confirm UAE as an efficient method for extracting flavonoids from turi leaves under optimized conditions.

Keywords: Optimization, RSM, Ultrasonic wave, Flavonoid

1. INTRODUCTION

Indonesia is a country that is rich in natural resources, including the turi tree (*Sesbania grandiflora*). Turi leaves are planted in many yards and known by the public as turi plants, function as ornamental plants, and are generally used as medicinal plants and vegetables (Joshi et al., 2016). Turi is spread across Indonesia, Malaysia, the Philippines and India. Turi is widely planted in gardens or on the side of roads and rice fields and moors as a plant that divides land ownership (Bhoumik et al., 2016). In previous research (Arimaswati et al., 2019) only analysis of the activity of flavonoid bioactive compounds in turi leaves, but no product has been obtained that can be used by the public.

The active compounds in turi leaves contain the bioactive components arginine, cystine, histidine, isolucine, phenylalanine, tryptophan, valine, threonine, alanine, aspargine, aspartic acid, saponin, oleic acid, galactose, rhamnose, glucuronic acid, flavonoids, and kaempfrol (Bhoumik et al., 2016). One of the high antioxidant contents of the turi plant is flavonoids (Panda et al., 2013), the flavonoid content reaches 12.5%. Flavonoids have antibacterial properties with the following mechanisms, inhibiting nucleic acid synthesis, inhibiting cytoplasmic membrane function and inhibiting energy metabolism in bacteria. Most cell wall structures and cytoplasmic membranes contain proteins and fats (Manner et al., 2013). Turi leaves have a taxonomic classification as in Table 1.

Table 1. Taxonomy of Turi Leaves

Taxonomy	Classification
Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Super division	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Ordo	*Fabales*
Family	*Leguminosae*
Genus	*Sesbania*
Species	*Sesbania grandiflora*

Flavonoids are one of the largest natural phenolic compounds found in all green plants (Markham, K.R. 1988). According to (Pourmorad, F 2006, p 1143) suggests that one of the classes of polyphenol compounds is known to have properties as a free radical scavenger, inhibitor of hydrolysis and oxidative enzymes, and also works as an anti-inflammatory. Based on this description, it is necessary to carry out more intensive optimization research regarding testing the total flavonoid content of the ethanol extract of turi leaves, so that the potential of this plant as an anti-bacterial raw material in various antibacterial products.

To obtain flavonoid compounds in turi leaves, an extraction method is required. The extraction methods that are often used are maceration, percolation, soxhletation, reflux, and others. Among these methods, there is an extraction method assisted by ultrasonic waves, extraction using ultrasonic waves (UAE) can be the best alternative. This underlies the renewal of the extraction method using ultrasonic waves/Ultrasonic Assisted Extraction (Ramli, et al., 2014). The Ultrasonic Assisted Extraction (UAE) method is an

extraction technique by applying ultrasonic waves to the material to be extracted. Ultrasonic-assisted extraction has been widely used to obtain phenolic compounds from plants using ethanol, water, ethanol/water mixtures, and acetone (Zlabur et al., 2015). Based on the experiments, it was observed that the TPC or FC obtained was quite much higher for each extraction method and time. Therefore, ethanol may be the most suitable solvent system for the extraction of turi leaves due to the different polarity of the constituent active substances (Do Q et al., 2014; Iloki-Assanga et al., 2015).

Seeing the problems above, it is necessary to conduct further research through optimization using the Response Surface Methodology method. This research uses a Response Surface Methodology (RSM) design to obtain an optimal Flavonoid extraction response.

Ultrasonic-assisted extraction is a modification of the maceration method. The extract is processed using high-frequency ultrasound (ultrasonic waves), with high vibrations, namely 20kHz. This working principle is to observe the acoustic properties of ultrasonic waves which are propagated through a medium through which cavitation passes by breaking up microjetting bubbles. When a wave propagates, the medium it passes through will experience vibrations. The liquid propagation medium is known as ultrasonic bath extraction. Vibration will provide intensive agitation for the extraction process. Stirring will increase osmosis between the material and the solvent, thereby increasing the extraction process. Antibacterial extraction of turi leaves using variations in pH, solvent volume, extraction time, and type of solvent have been studied. Several previous studies using UAE obtained quality results but were only limited to the process of taking antimicrobials, not yet reaching the stage of their usefulness as an appropriate product.

Response Surface Methodology (RSM) using Central Composite Design was applied in this research to optimize extraction conditions for turi leaves to produce a response, namely total flavonoid levels (Y, %w/w ER) in optimal amounts. Factor variables and factor level ranges were determined by conducting a preliminary single factor experiment test on the factors (initial variables were temperature 50 Celsius, time 20 minutes, and solvent volume 150 ml ethanol). Then look for a surface plot so that you get 20 variables.

Based on the CCD Central Composite Design, an equation is obtained which is described by the Regression Model Formula. Using the response surface method, 20 extraction design variables were obtained. This design was used to determine the response of flavonoid levels in turi leaf extract with temperature ratio variables, time variables, and solvent ratio variables. This design shows that the flavonoid content of turi leaf extract is

directly proportional to the temperature variable, the interaction of temperature and time, the interaction of time and ratio, and the interaction between ratios. The flavonoid content of meniran leaf extract is inversely proportional to the variables of time, solvent ratio, interaction of temperature and ratio, interaction between temperature, and interaction between time. If the extraction temperature is increased, the flavonoid content of meniran leaf extract will increase. One way to optimize is by using (RSM) Response Surface Methodology.

2. METHODS

Materials

Turi leaves obtained from Sampangan Village, Semarang City, Central Java Province. Solvent for extraction were 96% ethanol, and distilled water. Another materials used were Aluminum Chloride ($AlCl_3$), Quersetin, Aquadest, and Potassium Acetate (CH3COOK).

RSM Work Procedures and Analysis

The raw material used in this research was turi leaves. Turi leaves were sorted based on physical condition and quality, then washed until clean and dried at room temperature 500C. Sampling of turi leaves used the manual method by picking directly from the turi tree.

Response Surface The response surface method is a combination of mathematical and statistical techniques that are useful for modeling and analyzing problems whose response is influenced by several variables and the aim is to optimize the response. In many problems, the form of the relationship between the response and the independent variable is unknown. The first step of the response surface method is to find an approach that matches the actual relationship function between y and the independent variables (Montgomerry, 1997). This method use Equation (1):

$$y = f(x_1, x_2, \ldots, x_k) + \varepsilon \qquad (1)$$

With :

y : variabel respon
x_i : factor, i = 1, 2,..., k
ε : Residual with assumtion IIDN (0, σ^2)

Most problems occur when the form of the relationship between the response variable and the predictor variable is unknown. Therefore, in the response surface method, the first step is to find a suitable approach for the correct functional relationship between the

response variable and the predictor variables using a linear equation model as in equation (2).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_k x_k + \varepsilon \quad (2)$$

If there is curvature in the system then an order polynomial with a higher degree must be used such as the second-order model which is stated in Equation (3).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i,j} \sum \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

Mixture Design is a Design of Experiment (DOE) method where the response is assumed to depend only on the relative proportions of the ingredients included in the mixture (Cornell, 1990). The general definition of mixture is the proportion of ingredients in the mixture. So in mixture design, the constituent factors are the existing materials. The total mixture proportion is one as in Equation (4).

$$\sum_{(i=1)}^{p} X_i = X_1 + X_2 + \ldots + X_p = 1 \quad (4)$$

Optimization is needed because the extraction process can involve various variables and influences. Analysis of the simultaneous influence of multiple variables can be carried out using Response Surface Methodology (RSM), a mathematical and statistical tool that uses a series of experiments designed to optimize the experimental conditions of a process (Chen et al., 2015). Optimization of extraction conditions is carried out by varying one parameter and improving others, using RSM based on Centered Composite Design (CCD). The influence of the three independent variables (X1: ethanol; The level of the independent variable is selected based on the results achieved in single factor testing. Twenty independent variable associations were selected per experimental design for three parameters, with a centered test (0, 0, 0) repeated six times to verify standard errors and reproducibility of the extraction process (Brahmi et al., 2022)

Fixed Variables and Changing Variables

The fixed variable consists of 2 liters of distilled water as an intermediary for ultrasound waves in the extraction media. while the independent variables consist of Temperature (0C), Time (M), Solvent Volume (ml). Then a surface plot was carried out so that 20 experimental variables were found. Experimental variable statistics with minimum, median, and maximum limits are in Table 2.

Table 2. Statistics of Turi Leaf Extract Variables

Variable	-1	0	1
Temperature (C)	40	50	60

time (min)	10	20	30
Vol Solvent (ml)	100	150	200

Then is a summary of the results of the design experiment which are presented in the central composite design in Table 3.

Tabel 3 Central Composite Design

Factors:	3	Replicates:	1
Base runs:	20	Total runs:	20
Base blocks:	1	Total blocks:	1
α = 1,68179			

Extraction Stages

Turi leaves are weighed according to requirements and then put into an Erlenmeyer with a solvent that has been determined according to the design on the response surface, then set at a predetermined temperature, with a predetermined time according to the variables. This is done continuously until all variables are completed. Then it is purified using conventional distillation at a temperature of 70-750C to separate the extract from the ethanol solvent.

Determination of Flavonoid Content Calorimetric Method - $AlCl_3$

A stock solution of quercetin (1000 μg/mL) was prepared by dissolving 25 mg of quercetin in 25 mL of 80% ethanol. Standard solutions of 20, 40, 60, 80, and 100 μg/mL were then prepared. From each, 0.5 mL was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixtures were incubated at 25°C for 30 minutes. Absorbance was measured at 434.2 nm using a UV-Vis spectrophotometer, and a calibration curve was constructed by plotting absorbance (Y) against concentration (X). Five grams of ethanol extract of turi leaves were dissolved in 25 mL of ethanol and stirred for 24 hours at 200 rpm. The mixture was then filtered, and the filtrate was adjusted to 25 mL with ethanol.

A blank was prepared by replacing the standard with 0.5 mL of ethanol, followed by the same reagents as above. For the test solution, 1.0 mL of extract was diluted to 10 mL with ethanol. Then, 0.5 mL of this solution was treated with 1.5 mL of 95% ethanol, 0.1 mL $AlCl_3$ (10%), 0.1 mL potassium acetate (1 M), and 2.8 mL distilled water. After 30 minutes of incubation at 25°C, absorbance was measured at 434.2 nm. Testing was carried out in triplicate (Azizah et al., 2014). Flavonoid levels can be calculated using Equation (5):

$$F = \frac{c\ x\ V\ x\ f\ x\ 10^{-6}}{m}\ x\ 100\% \qquad (5)$$

Information:
F: flavonoids $AlCl_3$ method (mg QE/g)
c : Quercetin equivalent (μm/ml)
V: total volume of extract
f: dilution factor
m: sample weight (gr)

3. RESULTS and DISCUSSION

Research Design and Extract Measurement Response Results

The results of extraction and testing of turi leaf flavonoids under various conditions of extraction time, temperature, and solvent volume are presented in Table 2. The data in Table 2, shows that the highest extraction process yield value (0.35%) was obtained at 10 minutes, temperature 600C, Solvent Volume 200 ml 95% ethanol, ANOVA test results show that there is no significant difference in the process yield values for all factors with F-value and P-value more than 0.05. The coefficient of determination (R Squer) value in this model is 43.67%, meaning that the influence of time, temperature, and solvent volume is around 43.67% for the extraction process. The remaining 56.33% is influenced by other factors.

S	R-sq	R-sq(adj)	R-sq(pred)
0,103931	43,76%	0,00%	0,00%

The results of the turi leaf flavonoid optimization experiment is presented in Table 4

Table 4. Research Design and Response Results for Measurement of Turi Leaf Extract

Temperature (C0	Time (min)	Vol Solvent (ml)	Flavonoid (mg QE/g)
50	20	150	0,087
50	36,82	150	0,027
50	20	234,1	0,309
50	20	150	0,153
50	20	150	0,287
60	10	100	0,173
60	30	100	0,17
60	10	200	0,35
33,2	20	150	0,167

40	30	200	0,334
50	20	65,91	0,125
40	30	100	0,107
50	20	150	0,171
40	10	200	0,078
66,8	20	150	0,132
50	20	150	0,069
40	10	100	0,064
50	20	150	0,337
50	3,182	150	0,255
60	30	200	0,219

In Table 4, the best flavonoid results were obtained at 0.337 with a solvent volume of 150 ml, temperature of 500 C, and time of 20 minutes.

ANOVA (Analysis of Variance)

The results of testing the first-order model are shown in Table 5.

Tabel 5. *Analysis of Variance*

Source	**DF**	**Adj SS**	**Adj MS**	**F**	**P**
Model	9	0,08406	0,00934	0,86	0,582
Linear	3	0,052766	0,017589	1,63	0,244
Temperature	1	0,005355	0,005355	0,5	0,497
Time	1	0,003475	0,003475	0,32	0,583
Vol Solvent	1	0,043936	0,043936	4,07	0,071
Square	3	0,006986	0,002329	0,22	0,883
Temp*Temp	1	0,000962	0,000962	0,09	0,772
Time*Time	1	0,001861	0,001861	0,17	0,687
Vol Solvent*Vol Solvent	1	0,003489	0,003489	0,32	0,582
2-Way Interaction	3	0,024308	0,008103	0,75	0,547
Temp*Time	1	0,023382	0,023382	2,16	0,172
Temp*Vol Solvent	1	0,000028	0,000028	0	0,96
Waktu*Vol Solvent	1	0,000898	0,000898	0,08	0,779
Error	10	0,108017	0,010802		
Lack-of-Fit	5	0,050216	0,010043	0,87	0,559
Pure Error	5	0,057801	0,01156		
Total	19	0,192077			

Based on Table 5. ANOVA (analyst variation) Test to determine the best model for the Extraction response. The best model testing is carried out through simultaneous parameter tests to determine the influence of time, temperature, solvent volume together, and partial parameter tests to determine the influence of each predictor on the extraction response. C.V.% (coefficient variation), df degrees of freedom (free variation) are significant at p value < 0.05 so there is no significant effect.

Standardization Effects

In this study, simultaneously increasing temperature and time caused an increase in extraction yield. However, it should be noted that extreme increases in temperature also have the potential to damage phenolic compounds (Santos et al., 2009). Increasing the temperature during UAE extraction increases the vapor pressure of the solvent, resulting in a decrease in cavitation forces and a decrease in the resulting phenolic content. Taking into account previous findings, this study considers that higher temperatures may damage the extract yield. Thus, the extraction temperature range was set between 30 to 600C to further optimize extraction.

The extraction results of biomolecules such as polyphenols are very time dependent (Wang et al., 2008; German et al., 2010). Determining the right extraction time will save time and save energy. The time required for extraction is usually determined by the type of material and extraction procedure. Standardization data is presented in Figure 1.

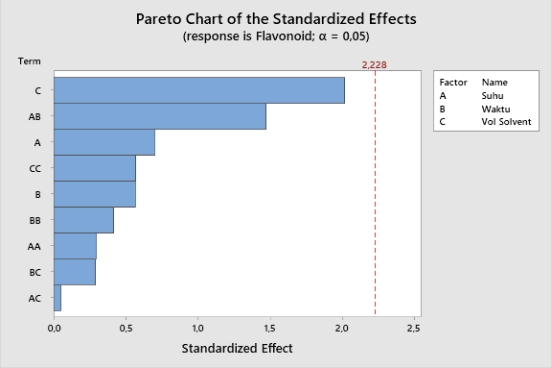

Figure 1. Pareto Chart of the Standardized Effects

In the Pareto chart, the results for flavonoids are α=0.05. The solvent volume and temperature factors together with time have values that are almost close to the standard effect response of flavonoids with each solvent volume having a value of 2 and temperature together with time having a value of 1.5, both of which do not meet the standard figures because they are still < 2.228.

Surface Plot Response Results

Next, the results of the measurement data were subjected to statistical analysis using the Minitab Statistical Software 19 program. The results of the response surface and response count for the total phenol content of turi leaf extract can be seen in Figures 2 to 4

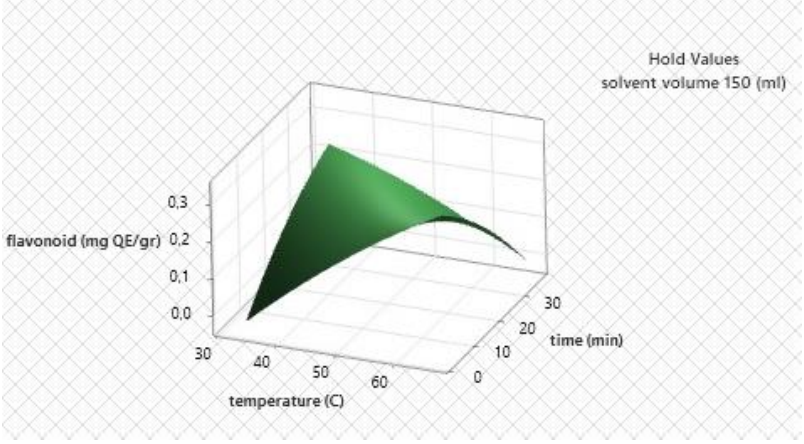

Figure 2. *Surface plot* Flavonoid vs Time and Temperature

In Figure 2, with a solvent volume value of 150 ml with a fixed time variable of 20 minutes, if the temperature is increased from 300C to 600C, the resulting flavonoid extract will also increase with a value from 0 to 0.3. Because temperature is an important indicator in an extraction, extraction results increase with increasing temperature and extraction time (Injilauddin et al., 2015).

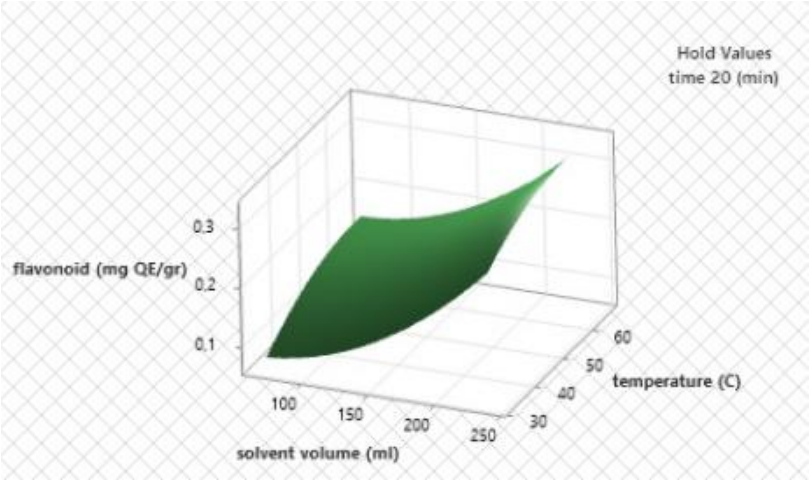

Figure 3. *Surface plot* Flavonoid vs Temperature and Solvent volume

Furthermore, in Figure 3, with a solvent volume value of 50 ml, the time variable remains 20 minutes. If the solvent volume is increased from 100 ml to 250 ml, the resulting flavonoid extract will also increase with a value from 0.1 to 0.3. Likewise, increasing

temperature and solvent volume can be factors in the extraction rate to stimulate more flavonoid compounds to be extracted (Sulihono et al., 2012).

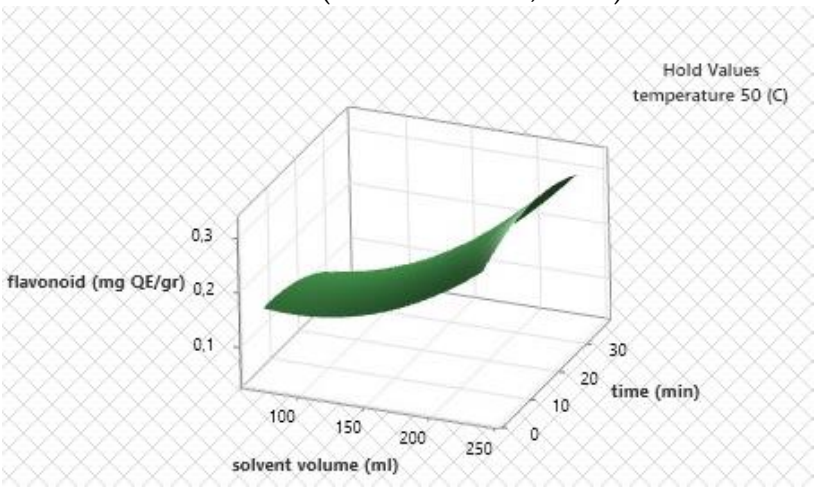

Figure 4. *Surface* and *Contour plot* Flavonoid vs Time and Vol Solven

Meanwhile, in Figure 4, with a time value of 20 minutes and a fixed variable, the solvent volume is 150 ml. If the temperature is increased from 300C to 600C, the resulting flavonoid extract will also increase with a value from 0.1 to 0.3. The combination of a large volume of solvent and an increase in temperature can be a factor in increasing the mass of flavonoids extracted as expected (Sulihono et al., 2012).

Normal Probability Test

After the surface analysis is carried out, the next step is the Normality test. In the normality test, we refer to the Normal Probability Plot which refers to the points following the diagonal line in Figure 5.

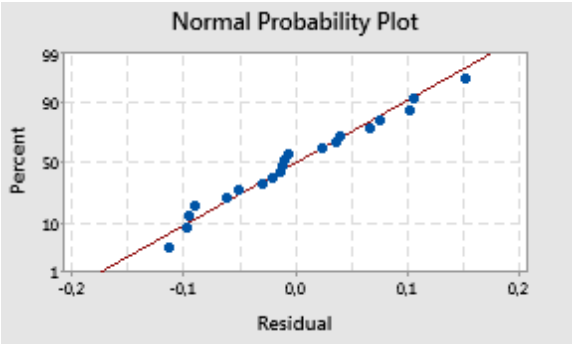

Figure 5. Normal Probability Plot

In pictures like the one above, plots like this often give rise to a lot of doubt or multiple interpretations, therefore they must be strengthened with Basic Statistics through the Basic Statistical Normality Test (Select Receipt and Kolmogorov-Smirnov) to bring up the

P value or P-Value. If the P value > 0.05 then the data has a normal contribution. The following plot is shown in Figure 6

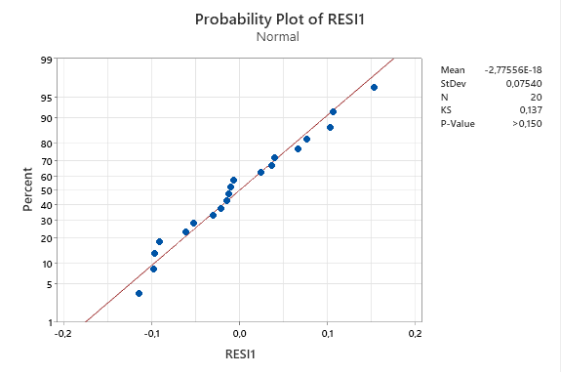

Figure 6. Probability Plot RESI

After the normality test is carried out and the data is declared a correct response, the next test is the Heteroscedasticity test, which is one of the factors that cause the simple linear regression model to be inefficient and inaccurate, also resulting in the use of the maximum likelihood method in estimating regression parameters (coefficients) being disrupted. The analysis involves the Versus Fits plot presented in Figure 7:

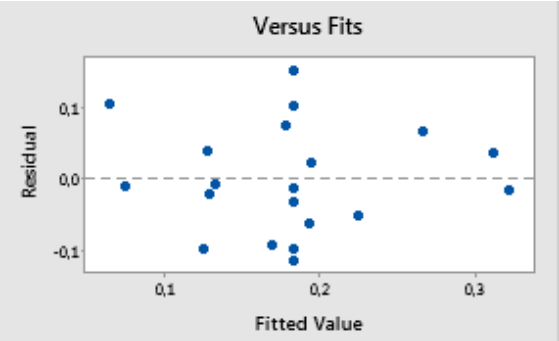

Figure 7. Plot Versus Fits

In the heteroscedasticity test, if the blue dots spread across the (x, y) axis, then heteroscedasticity does not occur.

Regression Equations

Flavonoids = -0.67 + 0.0215 Temperature + 0.0268 Time – 0.00097 Vol Solvent – 0.000082 Temperature*Temperature – 0.000114 Time*Time + 0.00000 Vol Solvent*Volsolvent – 0.000541 Temperature *Time – 0.000004 Temperature*Vol Solvent + 0.000021 Time*Vol Solvent

4. **CONCLUSION**

This research aims to determine the optimal variables for obtaining natural flavonoid compounds from Turi Leaves (Sesbania grandiflora). This opportunity I used Minitab Statistical Software 19 to simulate the variables that will be used to run the Mixture Design method, which is a Design of Experiment (DOE) where the response is assumed to depend only on the relative proportions of the ingredients included in the mixture (Cornell, 1990). After the design has been obtained as many as 20 experimental variables consisting of time, temperature, solvent volume, after that it is run using an extraction tool in the form of Ultrasonic Steam Ultraclean to obtain heat waves for the extraction process according to the variables that have been obtained. After that, concentration was carried out using a reflux heater and then analyzed using UV-Vis Spectrophotometry consisting of a blank, standard solution using quercetin, and sample. After obtaining the results, they were entered into Minitab Statistical Software 19 using the Analyze Response Surface Design method to obtain the results of this research with the best optimization at a variable temperature of 500C, time 20 minutes, solvent volume 150 ml ethanol, with a flavonoid yield of 0.35.

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