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Optimization of Nanosilver Synthesis Formula Using Bioreductor from Cassava Leaf Water Extract (*Manihot esculenta* Crantz): Application of Central Composite Design (CCD)

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Abstract: Nanosilver can be described as nano-sized silver particle that is widely used as an antimicrobial. Synthesis of nanosilver can be done by chemical reduction method. The use of bioreductors is considered because it is cheap, environmentally friendly and non-toxic. Precursor concentration and reducing agent concentration need to be optimized to control the nanosilver particle size. This study aims to obtain the formula and the optimum area for nanosilver synthesis from factors optimized using CCD. This research is a quasi-experimental design using a CCD with 2 factors as independent variables, thait is concentration of AgNO3, concentration of cassava leaf aqueous extract. Parameters used as dependent variables are wavelength and %T value. Optimum area and data analysis with ANOVA using Minitab17. The optimum formula results obtained are AgNO3 concentration 1.64 mM and cassava leaf aqueous extract concentration 17.61% which will produce a wavelength of 424.75 nm and %T 95.2%. Two optimum formulas were also selected from the experimental design that had been carried out. It is necessary to do further research related to the relationship between absorbance and the amount of nanosilver formed, validation of the optimum formula solution obtained and paying attention to the critical steps in the synthesis of nanosilver.

Keywords: nanosilver; experimental design; cassava; bioreductor

1. INTRODUCTION

Nanosilver is a nano-sized silver particle that is 1-100 nm [1]. Nanosilver has been widely studied because of its wide application as an antimicrobial [2]. Nanosilver has a broad antibacterial effect on various gram-negative and gram-positive bacteria and antibiotic-resistant bacterial strains [1]. Nanosilver can be applied in wound dressings, cotton fibers, antiseptic sprays and antimicrobial coatings for medical devices that sterilize air and surfaces [3]. The size of silver in the nanoscale was considered to be important due to its reactivity [4] and antibacterial activity [5].

Nanosilver can be synthesized using chemical, physical, and biological methods [6]. The chemical reduction method was chosen because it is an easy, fast and inexpensive method [7]. In the synthesis of nanosilver using chemical reduction methods, chemical reducing agents tend to be toxic [4] and are not environmentally friendly. Hence, the reducing agent obtained from plants can be used as alternative because its minimum and environmentally friendly [6] and can act as a capping agent [8]. Bioreductants can be obtained from plants whose secondary metabolites contain antioxidant compounds [2] such as flavonoids, saponins, tannins [9] and terpenoids, because they have functional groups capable of donating electrons [10].

The use of bioreductants from plants in the synthesis of nanosilver has been widely carried out, including extracts of bitter leaves [9], starfruit leaves [2], and pelawan leaves [3]. Cassava leaves have antioxidant activity because they contain polyphenolic compounds such as flavonoids and tannins. Therefore, cassava leaves can be used as a bioreductant in the synthesis of nanosilver [11]. The flavonoids contained in cassava leaves are clovin, rutin, narcissin and nicotiflorin. Rutin can be a marker because its content is highest in cassava leaves [12].

Polyphenol compounds have different reduction potentials. The best flavonoids in reducing silver were rutin (+0.26 V) and quercetin (+0.23 V) because they had a reduction potential below silver (+0.80 V) and the lowest compared to other flavonoids [13]. In addition, tannins have a reduction potential of +0.605 [14]. The greater the reduction potential value of a compound, the easier it is to undergo reduction and vice versa [15]. Hence, that flavonoids and tannins can reduce Ag+ ions [16].

In the synthesis of nanosilver, silver nitrate ($AgNO_3$) is employed as a precursor due to its high solubility in water [3], the cost is lower as well as the stability is the most stable compared to other silver salts [1]. $AgNO_3$ concentration can affect the size of nanosilver formed [2]. If the concentration of $AgNO_3$ was too high, the reducing agent cannot successfully reduce Ag+ completely. As a result, the size of the nanosilver particles formed is large [17].

The particle size of nanosilver is very important, because the smaller the particle size, the greater the antibacterial activity [18]. The particle size of nanosilver can be estimated by measuring the wavelength and can indicate the formation of nanosilver [2]. In addition, the %T value which is close to 100% also indicates that the nanosilver formed is already nanometer in size [19]. The nanosilver particle size can be controlled by optimizing the influencing factors, namely silver salt concentration and reducing agent concentration [2]. Notably, the concentration of $AgNO_3$ and extract must be balanced in order to maintain the stoichiometry of the reaction [17].

In this study, optimization was carried out using the Central Composite Design (CCD). The CCD, one of the response surface methodology, was applied to this study in order to evaluate factors and their responses (wavelength and % T value) for obtaining the optimal nanosilver synthesis formula. CCD has fewer trials with more levels compared to the full factorial design [20]. This study aims to obtain the formula and the optimum area for nanosilver synthesis from factors optimized using CCD. These factors are the concentration of $AgNO_3$ and water extract of cassava leaves. Until now there has been no report on the effect of variations in the concentration of cassava leaf aqueous extract as a bioreductant and $AgNO_3$ on the synthesis of nanosilver. Therefore, optimization of nanosilver synthesis formula with cassava leaf extract bioreductant needs to be performed.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in the research were cassava leaves, silver nitrate ($AgNO_3$) from Merck (pro analyst grade), aqubidest, rutin standard, butanol, acetic acid, aquadest, silica gel 60 GF254.

2.2 Method

2.2.1 Obtaining cassava leaves

The cassava leaf samples used were leaves located at positions 4-7 of the 6-month-old plant shoots. 5 kilograms of cassava leaves were taken from the plantation of the Farmer and Fisherman Labor Assistance Institute (LPUBTN).

2.2.2 Plant determination

The determination of cassava leaves was carried out by the Laboratory of Pharmacy Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta.

2.2.3 Cassava leaf water extract

One kilogram of cassava leaves from the Farmers and Fishermen Labor Assistance Institute (LPUBTN) were washed with running water. Only the leaves are taken from the fresh leaves. Cassava leaves were stored in the refrigerator at 6°C for 2 weeks. After 2 weeks the cassava leaves were removed from the refrigerator in a wilted state and had an unpleasant odor.

Cassava leaves that have been stored for 2 weeks are cut into pieces of about 2 cm. Furthermore, the leaves were weighed and then added 100 mL of aquabidest, and heated with a water bath at a temperature of 90° C for 15 minutes (starting from the temperature reached 90° C) while stirring with a stirring rod. The extraction results obtained were then filtered with filter paper [11].

Table 1. Concentration and Weighing Amount of Cassava Leaf Water Extract

Concentration (%w/v) Amount (g) Volume (mL)

Concentration (%w/v)	**Amount (g)**	**Volume (mL)**
9.17	9.17	100
11.57	11.57	100
17.36	17.36	100
23.15	23.15	100
25.55	25.55	100

2.2.4 Routine flavonoid qualitative test by TLC method

The mobile phase used was butanol: acetic acid: water (4:1:5) and the stationary phase used was silica gel 60 GF254 measuring 6x10 cm which was marked with a distance of 2 cm at the lower limit and an elution distance of 8 cm from the lower limit. Then the sample and routine comparison were spotted with a capillary tube at the lower limit on the GF254 TLC plate. Furthermore, it is inserted into the chamber and eluted with the mobile phase to the limit mark. Then lift the TLC plate and observe it visually under UV light at 254 nm and 366 nm and then determine the Rf value [38].

2.2.5 Optimization design of nanosilver synthesis formula

Experimental levels for AgNO3 and cassava leaf extract were presented in Table 2. Experimental design using CCD for nanosilver synthesis was presented in Table 3.

Table 2. Nanosilver Synthesis Formula

Formula	High Level	Low Level
$AgNO_3$ (mM)	1	2
Cassava leaf water extract (%w/v)	11.57	23.15

Table 3. Research Design for Optimization of 2 Factor 5 Level Nanosilver Synthesis Using CCD

StdOrder	RunOrder	PtType	Blocks	AgNO3 concentration	Bioreductant concentration
1	1	1	1	1	11.57
2	2	1	1	2	11.57
3	3	1	1	1	23.15
4	4	1	1	2	23.15
5	5	0	1	1.5	17.36
6	6	0	1	1.5	17.36
7	7	0	1	1.5	17.36
8	8	0	1	1.5	17.36
9	9	-1	2	0.79	17.36
10	10	-1	2	2.21	17.36
11	11	-1	2	1.5	9.17
12	12	-1	2	1.5	25.55
13	13	0	2	1.5	17.36
14	14	0	2	1.5	17.36
15	15	0	2	1.5	17.36
16	16	0	2	1.5	17.36

2.2.6 Synthesis and purification of nanosilver

The nanosilver synthesis was carried out according to the modified [3], [39]. The ratio between AgNO3 and cassava leaf extract is 25:1. A total of 2 mL of cassava leaf extract was reacted with 50 mL of AgNO3 solution. The solution was stirred for 5 minutes at a speed of 300 rpm at 75°C using a hotplate. The color change of the solution was observed which indicated the formation of nanosilver. After that, nanosilver purification was carried out by centrifugation at 2000 rpm for 15 minutes to remove impurities, then the supernatant was taken [40]

2.2.7 Determination of the wavelength and transmittance value of nanosilver

The redistilled water was used as blank. Scanning the purified nanosilver sample solution [10] in the range of 400-450 nm using a UV-Vis spectrophotometer [16]. Sample solution was added with 5 mL of redistilled water and then vortexed for 1 minute. The absorbance of the solution was measured at the maximum wavelength with redistilled water [19].

2.2.8 Optimization and data analysis

The optimization process with CCD design (2 factors and 5 levels) and data analysis with ANOVA with 95% confidence level were carried out using the Minitab 17.

3. RESULTS AND DISCUSSION

3.1. Determination results of cassava plants

Determination was carried out on cassava leaves obtained from the Institute for Farmer and Fisherman Labor Assistance (LPUBTN), Pandowoharjo Village, Sleman DIY. The plant was determined at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta. The determination results obtained indicate that the cassava plant used in the study is in accordance with the intended plant, namely cassava (*Manihot esculenta* Crantz).

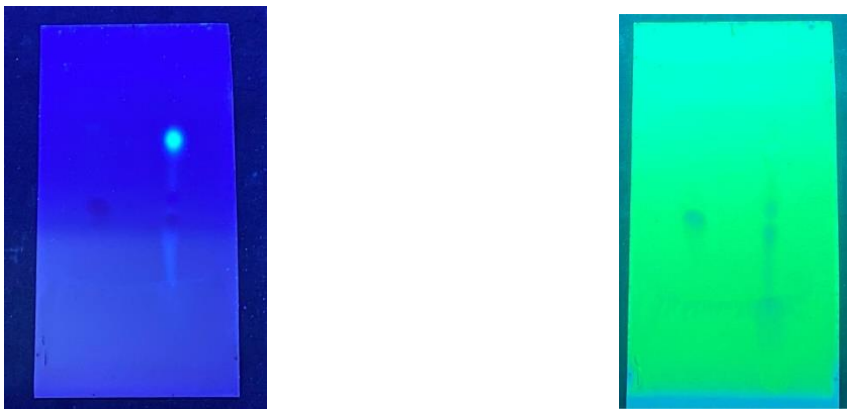
3.2 Cassava leaf water extract

The extract was made by the infundation method. Infundation is a filtration process that is generally used to extract water-soluble active ingredients. Infundament was chosen because it is frequently used and simple [41]. Water is a polar solvent. Consideration of water used as a solvent because water is easy to obtain, cheap and stable [42]. In addition, cassava leaves contain flavonoid rutin [11], saponins and tannins [29] which are soluble in water. The infundation method is suitable for obtaining these compounds. The largest compound content in the aqueous extract of cassava leaves is routine flavonoid [12], hence routine flavonoids are used as a representative of compounds in this extract as bioreductors for nanosilver synthesis.

3.3 TLC test results of cassava leaf water extract

TLC is a method of separating chemical components with the principle of adsorption and partition determined by the stationary phase and the mobile phase [43]. This TLC test is to ensure the presence of routine flavonoids in the water extract of cassava leaves. In this test, the stationary phase used was silica 60 GF254 and the mobile phase used butanol: acetic acid: water (4:1:5) and the standard for comparison used was 0.1% routine standard. The results of the TLC plate observed under a 254 nm UV lamp in Figure 1 (b) show that the cassava leaf extract has 2 spots. The standard routine migration distance is 4 cm, whereas the migration distance of the first spot sample is 3.5 cm and the second spot is 4.15 cm. The standard Rf value of the routine is 0.5 and the Rf value of the first spot sample is 0.43 and the second spot is 0.52. Meanwhile, when viewed on a 366 nm UV lamp in Figure 1(a), there are spots that glow in the extract but do not fluoresce on a routine with a migration distance of 6.6 cm and an Rf value of 0.83.

Seen from the elution distance and the Rf value of the standard rutin and extract, it can be concluded that rutin could not be detected in the aqueous extract of cassava leaves. This is because the distance between the routine elution and the extract is quite far. It is possible that this happened because of the small rutin content in the water extract of cassava leaves, making it difficult to detect using TLC. Hence it is recommended to use the HPLC (high performance liquid chromatography) method in detecting routine compounds in the aqueous extract of cassava leaves.



(a) (b)

Figure 1. (a) TLC results at 366 nm; (b) TLC results at 254 nm

3.4 Nanosilver synthesis and purification process

In the nanosilver synthesis, $AgNO_3$ solution was mixed with water extract of cassava leaves in a ratio of 25:1 at 75 °C for 5 minutes. The comparison, temperature and time are obtained from the orientation results. Nanosilver is formed when the compounds in the water extract of cassava leaves such as rutin, saponins and tannins reduce Ag+ ions to Ag0. Then there is nucleation of Ag0 which is followed by a spontaneous coalition of a number of adjacent nanoparticles to form particles with a larger size which can be seen in Figure 2 [40]

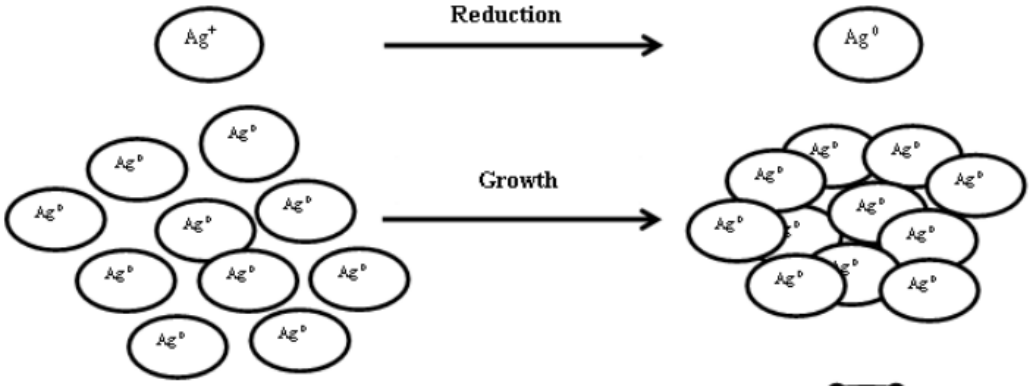


Figure 2. Formation of Nanosilver (Mohammadlou et al., 2016)

The color change to brownish yellow can be a sign of the formation of nanosilver because the color change is a phenomenon of surface plasmon resonance and reduction of Ag+ ions (40). Surface plasmon resonance (SPR) is a phenomenon of electron cloud movement that is influenced by the presence of irradiation on colloidal nanocomposites, which is also known as oscillatory resonance phenomenon [45]. The nanosilver solution formed using a cassava leaf water extract bioreductor is brownish yellow as shown in Figure 3 (a). The solution has a wavelength between 400-450 nm. This is because the number of available bioreductors is sufficient to reduce Ag ions [4].

A pale brown solution like Figure 3 (b) produces a wavelength below 400 nm. At wavelengths below 400 nm, Ag+ is still formed, which means that the chemical reduction process has not worked perfectly [46]. A dark brown to black solution as shown in Figure 3 (c) produces a wavelength above 450 nm. This is because the number of available bioreductants exceeds the available Ag+ ions so that Ag+ is reduced very quickly and causes agglomeration which causes the resulting nanosilver to have a large particle size [4].

(a) (b) (c)

Figure 3. (a) Nanosilver Yield 400-450 nm (b) Nanosilver Yield <400 nm (c) Nanosilver Yield > 450 nm

Nanosilver purification was carried out by centrifugation at 2000 rpm for 15 minutes. The precipitate formed is an impurity and what is taken is part of the supernatant (40). Furthermore, the supernatant was used for the characterization of nanosilver. After purification, a longer wavelength shift occurred but still met the target. Judging from the PSA test results, the average nanosilver size for the center point (experiment 5) before and after purification had similar sizes. However, the wavelength before and after purification is different [47]. Further, it can be stated that the purification carried out has obtained pure nanosilver.

3.5 Results Wavelength and %T

Nanosilver characterization can be done by measuring the wavelength. Silver nanoparticles can interact strongly with certain wavelengths of light and the unique optical properties of these materials are the basis of their plasmonic properties [34]. The absorbance at a wavelength of 400-450 nm is a nanosilver SPR. If the absorbance read below 400 nm is the silver ion wavelength, it indicates that the silver ion reduction process has not been running perfectly [40]. Nanosilver colloids have different colors based on the absorption of light and emission in the visible light region, the frequency of the conduction vibrations of electrons in response to electric fields resulting from electromagnetic radiation. However, only free electrons that have plasmon resonance in the visible light spectrum can give good color [48]. In this study, the experiment was replicated 2 times. Here's the result of the average wavelength:

Table 4. Observation results of wavelength

Trial	AgNO3 (mM)	Extract (%)	Wavelength (nm)	CV (%)
1	1	11.57	428.67	0.27
2	2	11.57	260.67	0.44
3	1	23.15	422	4.52
4	2	23.15	440	1.98
5	1.5	17.36	442	1.86
6	1.5	17.36	442	1.86
7	1.5	17.36	442	1.86
8	1.5	17.36	448	1.86
9	0.79	17.36	419.33	1.81
10	2.21	17.36	251.67	7.46
11	1.5	9.17	435.33	0.70
12	1.5	25.55	430	0.47

13	1.5	17.36	422	1.86
14	1.5	17.36	444	1.86
15	1.5	17.36	446	1.86
16	1.5	17.36	436	1.86

In this study, 2 replications were carried out in trials 1-4 and 9-4 to reduce the possibility of random errors. From the replication, the CV value was obtained. The CV value is said to be eligible if <10%, which means the data has high precision for each replication [49]. The CV related to the precision of the method obtained in all experiments meets the requirements. However, it can be seen in Table 4, the CV obtained in the various results. In the research conducted, the increase in temperature will affect the particle size which will affect the wavelength [40]. However, the results obtained meet the nanosilver wavelength target of 400-450 nm [40].

Table 5. Observation results of %T

Trial	AgNO3 (mM)	Extract (%)	%T	CV (%)
1	1	11.57	95.80	0.55
2	2	11.57	96.37	5.01
3	1	23.15	92.03	4.84
4	2	23.15	93.90	3.07
5	1.5	17.36	94.30	2.31
6	1.5	17.36	98.30	2.31
7	1.5	17.36	96.70	2.31
8	1.5	17.36	97.80	2.31
9	0.79	17.36	89.50	10.67
10	2.21	17.36	94.13	4.26
11	1.5	9.17	97.00	2.22
12	1.5	25.55	92.63	4.64
13	1.5	17.36	93.10	2.31
14	1.5	17.36	92.90	2.31
15	1.5	17.36	93.50	2.31
16	1.5	17.36	93.80	2.31

Based on the table of results of %T nanosilver above, it can be seen that almost all experiments met the target of %T nanosilver, namely 91-99% [19], [50]. On the 9th try, %T did not meet the target. This happened because in the first replication in the experiment, the %T value was far below the target of 78.5% while other replications met the target. So when the average results obtained are below the target. The difference in %T obtained in each replication is because it is difficult to obtain a constant temperature. Temperature can affect the amount of nanosilver formed, where the higher the temperature, the faster the nanosilver formation reaction will be and more will be formed [51]. The absorbance value indicates the amount of nanosilver formed (50), where the more nanosilver formed, the higher the absorbance value, while the %T value is inversely proportional to the absorbance [2], [35]. The CV value is said to be eligible if <10%, which means the data has high precision for each replication [49]. The CV obtained in all experiments meets the requirements so that it can be said that the data obtained is precise. However, it can be seen in table IV, the CV obtained varies. However, the results obtained meet the target of %T nanosilver, which is 91-99% [19,49]

3.6 Optimization results using CCD design

The CCD design in this study was used to optimize two factors, namely the concentration of AgNO3 and water extract of cassava leaves. The response seen is the wavelength and %T. This CCD design has 16 trial runs. The experimental data results will be analyzed using Minitab 17 software (Minitab, Inc, State College, PA, USA). Responses will be evaluated using ANOVA statistical analysis.

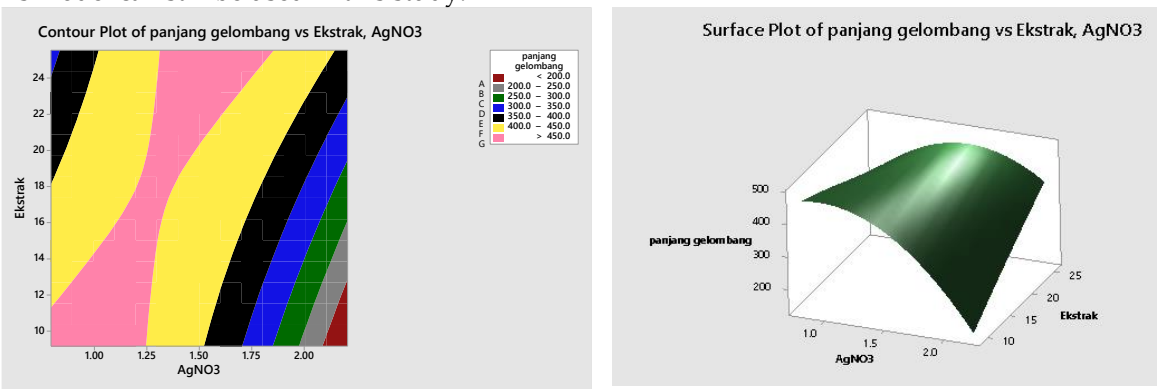
3.6.1 Response to wavelength

The model has a real or significant effect if it has an F-value>P-value and a P-value <0.005 [52]. From Figure 4 (a) it can be seen that the model has a real or significant effect on the response statistically which has a P-value of less than 0.05, which is 0.000 and an F-value that is greater than the P-value of 14.48. So this model can be used as an optimization model. The regression equation for the wavelength model is as follows:

$$453 + 242\ AgNO_3 - 17{,}6\ Extract - 205{,}7\ AgNO_3^2 - 0{,}085\ Extract^2 + 16.06\ AgNO_3.Extract \quad (1)$$

The value of R2 which is close to 80% indicates a significant effect of independent variables on the response [53]. The R2 value obtained is 90.62% which indicates that the $AgNO_3$ concentration factor and the water extract concentration of cassava leaves have an effect of 90.62% on the wavelength response value while 9.38% is influenced by other factors that are not used in this model. Hence, it can be concluded that the factors used have a significant effect on the response.

The lack of fit test aims to show the suitability of the data obtained from the experimental with the results of the model data. This test is seen from the results of center point replication. In this study the center point was replicated 8 times, this center point replication aims to investigate experimental errors [54]. However, in the Lack of fit test, a significant P-value was obtained, namely 0.001 which indicated that the response data obtained did not match the response predicted by the model [55]. However, the experimental results showed as many as 14 experiments had wavelengths that met the nanosilver wavelength target of 400-450 nm [40], so this model can still be used in this study.



(a) (b)

Figure 4. (a) Wavelength Response Contour Plot vs Extract, AgNO3. (b) Surface Plot of Wavelength Response vs Extract, $AgNO_3$

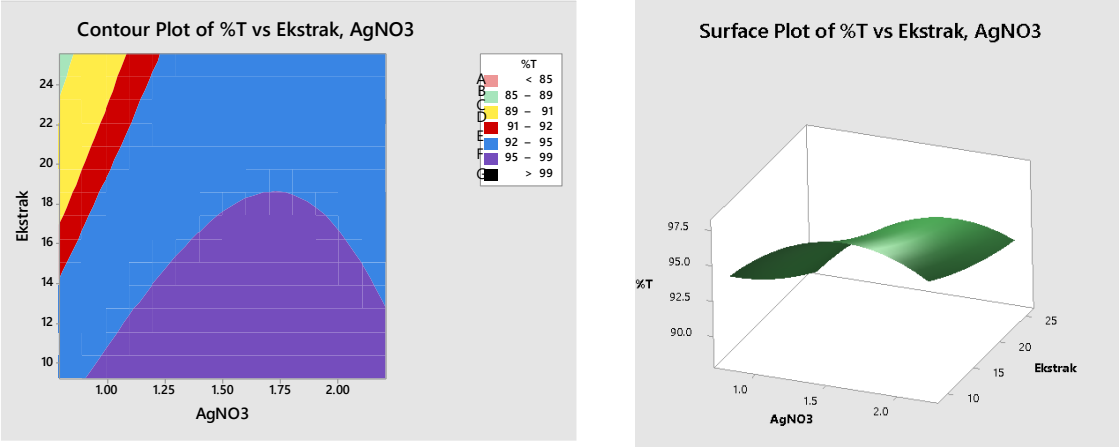
The RSM method provides a more detailed explanation of the effect of the two independent variables used, namely and their interaction effect on the response. In RSM there are Contour plots (2D) and Surface Plots (3D). Figure 4(b) shows a 3D illustration of the interaction between the independent variables and the response. Figure 4(a) presented a contour plot to illustrate the interaction of the independent variables and the response in 2D [56]. It can be seen that that the

optimum area of $AgNO_3$ concentration to the wavelength response is located in the yellow area. This is because in that area, the nanosilver wavelength that meets the target is 400-450 nm (40).

3.6.2 Response to %T

Figure 5 (a) shows that the model has a statistically significant or significant effect on %T which has a P-value of less than 0.05, which is 0.010 and an F-value that is greater than the P-value of 5.75. The R2 value obtained is 79.31% indicating that the $AgNO_3$ concentration factor and the concentration of cassava leaf aqueous extract have an effect of 79.31% on the wavelength response value and 20.69% is influenced by other factors that are not used in this model. So that it can be said that the factors used are quite influential on the %T response. In the Lack of fit test, an insignificant P-value of 0.325 indicates that the response data obtained is in accordance with the model [55] (Appendix 12). So this model can be used as an optimization model. The regression equation for the %T model obtained is as follows:

$$89{,}09 + 16{,}08\ AgNO_3 - 0{,}628\ Extract - 5{,}26\ AgNO_3^2 + 0{,}0055\ Extract2 + 0{,}112\ AgNO_3.Extract \quad (2)$$



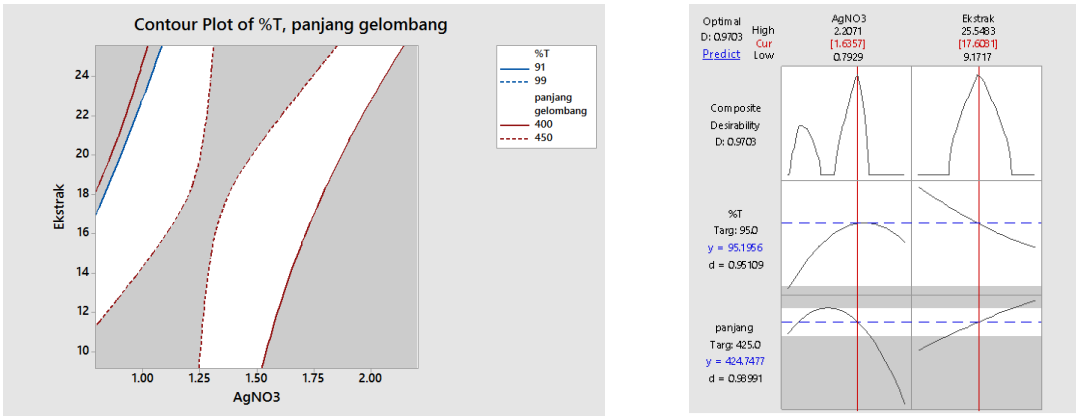
(a) (b)

Figure 5. (a) Contour Plot Response %T vs Extract, AgNO3. (b) Surface Plot Response %T vs Extract, AgNO3.

Based on Figures 5 (a) and (b) above, it can be concluded that the optimum area for the %T response lies in the red, blue and purple areas. This is because in that area a response that meets the target is obtained, namely 91-99% [19,4]. The concentration of $AgNO_3$ and water extract of cassava leaves in this area is a concentration pair that meets the target. While the concentration outside the area is a concentration pair that does not find the target**.**

3.6.3 Determination of optimum area and composition

In Figure 6 (a) the white area shows the superimposed contour plot area which is the intersection area of the wavelength response contour plot with %T. This area is the optimum area for AgNO3 concentration and water extract of cassava leaves with wavelength response and %T that meet the target. Figure 6 (b) shows that the optimum formula solution for nanosilver synthesis given by the RSM model is $AgNO_3$ concentration of 1.64 mM and cassava leaf aqueous extract concentration of 17.61%. In the prediction of the optimal formula, it will produce a wavelength of 424.75 nm and %T 95.20%. The desirability value of the optimum formula solution is 0.9703. A good desirability value is close to 1 which indicates the higher the precision value of the formula solution with the desired target response [57]. Hence, it can be concluded that the optimum formula solution has a high accuracy to produce the target response.



(a) (b)

Figure 6. (a) Superimposed contour plot area (b) Optimum Formula Solution

Table 6. Optimum formula and response prediction

Formula	$AgNO_3$	Extract (%)	Wavelength (nm)	%T
Solution	1.64 mM	17.61	424.75	95.20
1	1.5	17.36	440.22	95.05
2	1.5	9.17	405.54	97.62

From the optimum formula area, 2 optimum formulas were selected in the design. Based on the regression equation of the optimization model, the two formulas have a wavelength and %T that meet the target. The two formulas were chosen because they had the lowest concentrations of $AgNO_3$ and water extract of cassava leaves in the design and entered the optimum area. Hence it is more efficient in the materials used and the less for unwanted interactions to be occured. The two optimum formulas and the predicted response results can be seen in Table 6.

4. CONCLUSION

The result of this research is the optimum area of nanosilver synthesis formula using the Central Composite Design (CCD) method. Three optimum formulas for the synthesis of nanosilver were obtained, consisting of the optimum formula solution obtained using the RSM model and 2 formulas from the experimental design.

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