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Optimization of nanosilver purification process with *Camellia sinensis* L. extract as bioreductor

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ABSTRACT: Nanosilver can be described as a nanoparticle synthesized from silver metal with a size range of 1–100 nm. Nanosilver synthesis can be performed using green tea leaf extract (*Camellia sinensis* L.) as a bioreductor due to its flavonoid content. The content of flavonoid compounds is able to reduce silver metal ions (Ag^+) derived from $AgNO_3$ as metal precursors so that silver nanoparticles can be produced. This study aimed to obtain the optimum time and speed of centrifugation in nanosilver purification with green tea leaf bioreductors using the Central Composite Design (CCD) method. This study used two independent variables namely the duration and speed of centrifugation. The effect of centrifugation duration and speed on wavelength, transmittance percentage, and particle size of purified were analyzed by response surface methodology using the R software. The duration and speed of the optimum formula for the nanosilver purification process were obtained using the CCD method. The optimum conditions obtained were the centrifugation duration of 22 minutes and the centrifugation speed of 3500 rpm. Furthermore, the content of tannin compounds in green tea leaves was determined using thin layer chromatography.

KEYWORDS: Nanosilver; green tea leaves; CCD; sonication; purification; tannin.

1. INTRODUCTION

Nanoparticle technology is currently being developed in Indonesia for various applications. Nanoparticles are a part of nanotechnology that develops particles of a pharmaceutical preparation in the size range of 1–1000 nm in a delivery system that can be transferred through diffusion, while increasing the affinity of the system by increasing the contact area. One type of nanoparticle that can be used in pharmaceutical preparations is nanosilver. Nanosilver preparations are nanoparticles produced from silver metal, which have a size of 1–100 nm [1, 2]. Pure particles can be synthesized using physical and chemical methods. However, these methods are expensive and not environmentally friendly. A new method that can be used is biosynthesis using plants as bioreductors, which is more environmentally friendly, simpler, and inexpensive because it uses natural materials (Green Synthesis) and is widespread in Indonesia [3, 4].

Plant organic compounds used as bioreducing agent can help reduce silver ions in metal precursor compounds, such as $AgNO_3$, to form AgNPs in the synthesis process. Green tea leaves (*Camellia sinensis* L.) can be used as bioreductors because it contains flavonoid compounds. Compounds containing flavonoids have hydroxyl groups (–OH) and the ability to bind metals that are oxidized to carbonyl groups (C=O). The hydroxyl functional group can act as a reductant by donating electrons to Ag^+ ions to produce Ag nanoparticles [5]. A plant extract was used as the reducing agent. Green-tea-leaf extract contains secondary metabolites that act as reductants by reducing Ag metal ions. The secondary metabolites found in green tea leaf extracts are terpenoids, alkaloids, phenols, and flavonoids. Flavonoids that are abundant in green tea leaf extract can be used as antioxidants because they are easily oxidized to facilitate the formation of AgNPs by reducing silver metal ions Ag^+ to Ag [6].

If the synthesis is successful, a purification process is required to separate the impurities from the solution by centrifugation. Based on the research conducted by Basule (2021), purification of nanosilver

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synthesis was performed by centrifugation at 2000 rpm for 10 min [7]. The wavelength of the nanosilver after purification was greater than that before purification. This increase in wavelength occurred because the stability of the nanosilver particles formed is lower after the purification process [8]. This low stability is caused by the high ion content in the nanosilver solution, which allows it to form a new nanosilver colloidal system [9]. Hence, it is necessary to optimize the duration and speed of centrifugation to obtain nanosilver particles of suitable particle sizes. The optimization design used in this study was a central composite design (CCD). This design was used to evaluate the effects of a combination of two or more treatments. CCD was chosen in this study because it does not require much time or energy to conduct experiments, even though it has a larger number of levels to maximize the expected response and can provide a relationship between the independent and dependent variables [10].

Based on the above description, the optimal duration and speed of centrifugation for nanosilver purification must be determined to obtain an acceptable response, including satisfactory wavelength and transmittance percentage [11]. This study aimed to optimize the nanosilver purification process, including the duration and speed of centrifugation, using the CCD approach.

2. RESULTS

Extraction of green tea leaves powder was performed using solid-liquid extraction at 60°C. The green tea infusion resulted in a clear brown infusion, and flavonoid compounds that can be used as bioreductors were successfully obtained [12]. A qualitative assay of flavonoids was performed using thin layer chromatography (TLC) to ensure the presence of the flavonoids rutin and tannin in the green tea leaf infusions. Rutin standard displayed an Rf value of 0.51 and tannin standard displayed an RF value of 0.76 while the *C. sinensis* extract presented an Rf value of 0.36 in the first spot and 0.73 in the second spot. The TLC are shown in Figure 1.

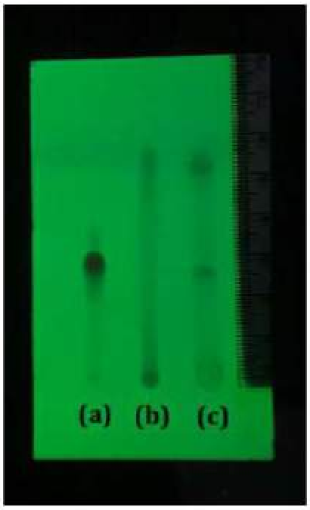

Figure 1. KLT results of rutin standard (a), tannin standard (b), and *Camellia sinensis* extract at a detected wavelength. Mobile phase: butanol acetic acid in a mixture 4:1 (v/v). Stationary phase: silica gel GF_{254}. The elution distance was 7.5 cm.

In the early stages, the mixture of *C. sinensis* extract and $AgNO_3$ appeared light yellow. Changes in color from bright yellow to yellowish-brown indicate the presence of nanosilver [13]. In accordance with previous research, it was reported that a brownish color change occurred because of surface plasmon resonance (SPR), which indicated the presence of a silver ion-reducing process [14]. SPR is an optical sensor that uses surface plasmon waves to observe the interaction between a metal (silver) surface and a dielectric material [15]. Table 1 presents the experimental design using the CCD approximation and the obtained responses, such as the wavelength and percentage transmittance. The higher transmittance percentage indicates the higher quality of nanoproducts [16]. The formation of silver (Ag^0) nanoparticles was indicated by the absorption peak at a wavelength of 434–444 nm [17]. The maximum absorbance wavelength in this study was in the range of 434–444 nm, which proves that the mixing ratio of the two materials between a solution of silver nitrate and green tea extract was successfully synthesized (Figure 2). The obtained percentage also met the target of 91–99%.

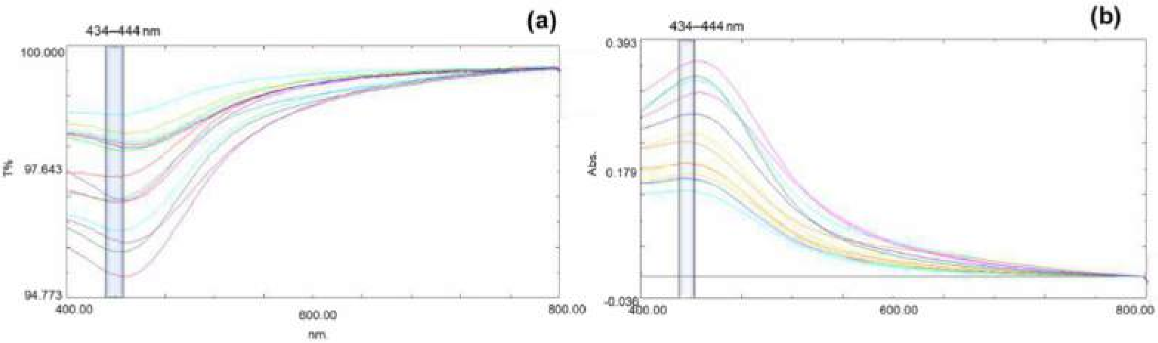

Figure 2. UV-visible spectra of the synthesized product containing silver nitrate and green tea extract in transmittance mode (a) and absorbance mode (b). The blue area indicates the maximum absorbance wavelength of 434–444 nm.

Table 1. An experimental design utilizing the CCD model and responses acquired after purification

Run Order	Independent variables		Dependent variables	
	Duration (minutes)	Speed (rpm)	Wavelength (nm)	Transmittance (%)
1	15	3500	444	95.798
2	15	3500	440	96.945
3	10	5000	438	97.534
4	20	5000	434	97.002
5	20	2000	446	95.251
6	15	3500	442	96.288
7	10	2000	448	96.040
8	15	5600	270	98.935
9	15	1500	444	96.977
10	15	3500	438	98.103
11	22	3500	436	98.509
12	8	3500	438	98.187
13	15	3500	436	98.318
14	15	3500	438	98.257

The synthesis and purification processes can produce nanoparticles. This can be proven by conducting particle size tests using a particle size analyzer (PSA). This test proved that the nanosilver preparation successfully achieved a size range of 1–100 nm. Figure 3 shows the particle size and size distribution after synthesis for 15 min (before purification) and after purification for 10 min at 2000 rpm. The particle sizes were a size of 82.3 nm (before purification) and 63,5 nm (after purification). The results indicated purification process caused the smaller size of nanosilver. This phenomenon may occur due to the impurities removal in the purification process.

A CCD was used to optimize the duration and speed of centrifugation during the nanosilver purification process. This CCD design had 14 experimental runs and 6 replications in the design recommended by the model. The regression analysis of the response surface for both the wavelength and percentage transmittance is presented in Table 2. The response surface model showed significant results ($p < 0.05$). The design model had no real or significant effect on the wavelength response and percentage transmittance of the nanosilver, where both responses had P-values of 0.11 and 0.6669 respectively. The R^2 and R^2 (adj) values obtained were greater than 5%, and the variables did not significantly affect the wavelength response and percentage transmittance. Figure 4 shows a contour plot of the wavelength and transmittance as functions of the duration and speed of centrifugation. The Lack of Fit of the wavelength response was significant, indicating that the wavelength response could not be predicted using the model. However, the percentage transmittance response was not significant, indicating that the response can be predicted using this model and that it is suitable as an optimization model for the nanosilver purification process [18]. The appearance of the wavelength surface plot and the percentage transmittance between the duration and centrifugation speed are depicted in Figure 5.

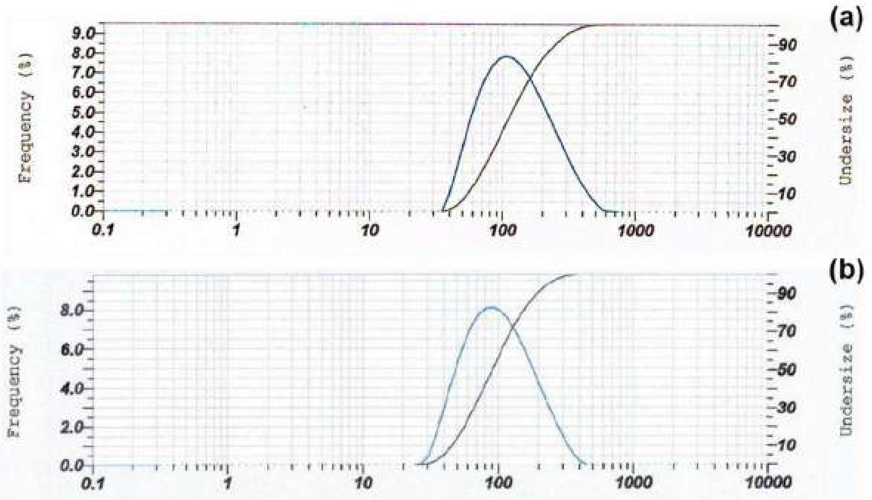

Figure 3. Representative results of particle size analysis of nanosilver before (a) and after (b) purification process

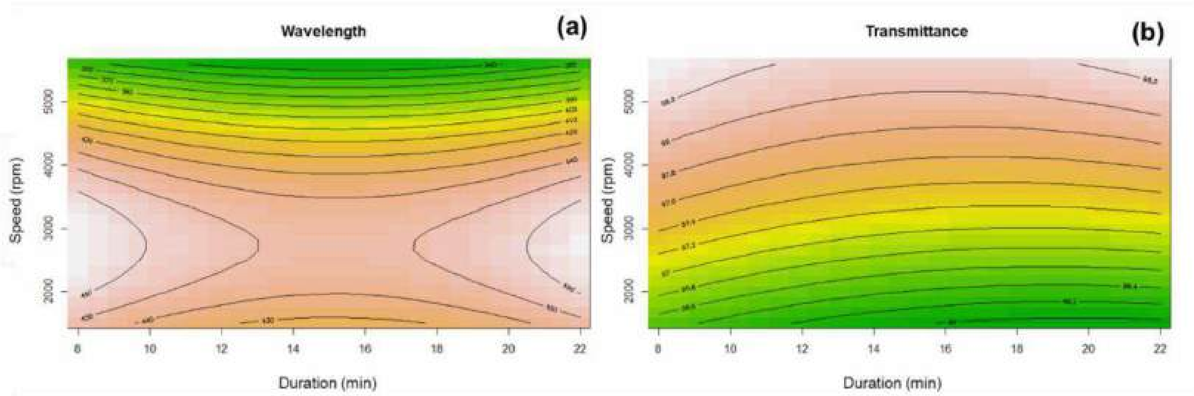

Figure 4. Contour plot of the wavelength (a) and transmittance (b) for duration versus speed of centrifugation.

The optimum formula was optimized using R software, as has never been done in previous studies. This was inferred to result in a desirability value for the composite of 0.643 at a duration of 22 min and a speed of 3500 rpm. A desirability value close to 1 implies a high ability of the model to produce the desired value. Figure 6 presents a plot of the optimization surface for both responses involving wavelength and percentage transmittance. The optimum formula for the production of nano silver was determined using the RSM model. The results of the observations using six replicates for each formula are presented in Table 3. The RSD values, which indicate random errors in measuring the observation results for wavelength and transmittance before purification, were 2.04% and 0.75%, respectively. The RSD values for wavelength and transmittance after purification were 2.94% and 0.217%, respectively. These results revealed that the optimum formula for nanosilver particles was reached.

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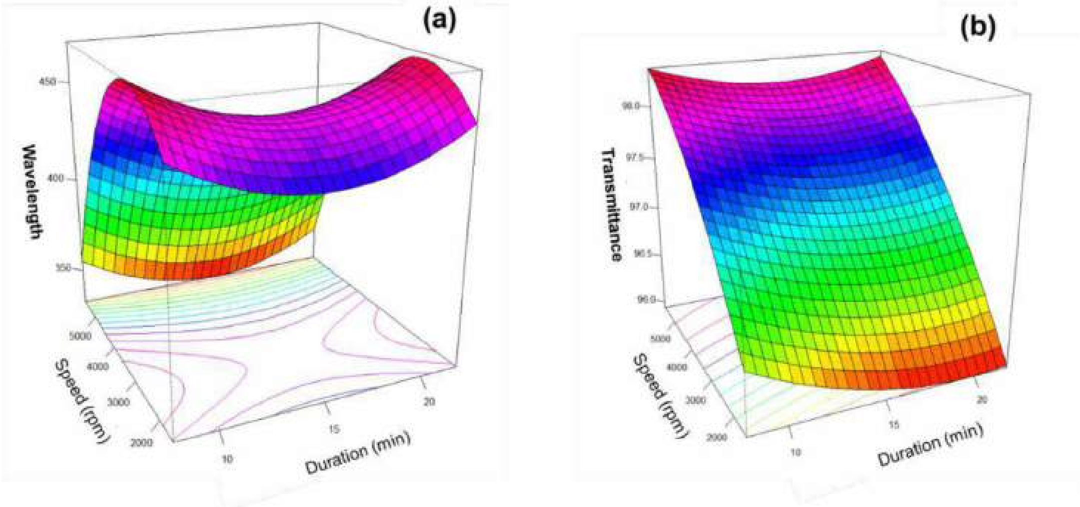

Figure 5. Surface plot of wavelength and transmittance percentage between duration versus speed of centrifugation.

Table 2. Regression analysis results of response surface for wavelength and percentage transmittance.

Response	P-*value*	R^2	R^2 (Adj)	*Lack of Fit*
Wavelength	0.11	0.6195	0.3817	$2.328.10^{-6}$
Transmittance	0.6669	0.2906	-0.1528	0.2864

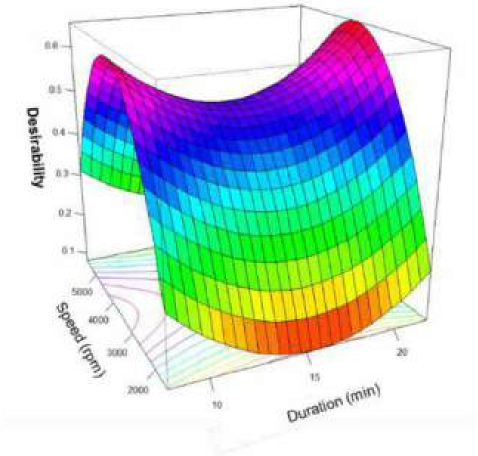

Figure 6. Surface plot of both responses versus desirability

3. DISCUSSION

The solvent used in the extraction process was selected based on having the same polarity as the flavonoid compounds, according to the principle of dissolution. In accordance with this principle, the solvent used was redistilled water because it has similar polarity properties to those of most flavonoid compounds that can dissolve in water. Redistilled water was also chosen because it is readily available, inexpensive, and nonvolatile. The green tea leaf extract contained tannins, as indicated by the closely related Rf values for the reference standard and *C. sinensis* extract. Rf values between samples and rutin standards were considerably different because of the small content of rutin contained in green tea leaves compared with the other compounds. Nanosilver synthesis by the sonication method can form nano-sized particles in

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Table 3. Optimal formula test results on response wavelength and percentage transmittance.

No	Before Purification		After Purification	
	Wavelength (nm)	transmittance (%)	Wavelength (nm)	transmittance (%)
1	432	94.754	438	98.409
2	430	94.467	440	98.134
3	435	96.117	436	98.624
4	434	96.242	434	98.065
5	430	95.726	432	98.344
6	432	95.985	434	98.112
Mean	432.167	95.549	435.667	98.281
SD	2.040	0.750	2.940	0.217
RSD	0.47%	0.78%	0.68%	0.22%

the wavelength range of 400–450 nm, which indicates the presence of nanosilver. The sonication method converts electrical signals into physical vibrations in the presence of ultrasonic electricity such that the particles break into smaller particles. The formation of nanosilver with smaller size led to shorter wavelengths with increased monodispersity [19].

Purification was performed after nanosilver synthesis to remove impurities. Nanosilver purification was conducted by centrifugation at a specific speed and duration based on the optimal design. Centrifugation was used to remove impurities and large particles in the nanosilver preparation. An increase in the wavelength was observed before and after purification. This increase in wavelength suggests that the nanosilver particle size increased because of delocalized and exchanged electron conduction on the particle surface, which caused a redshift [17]. Nanosilver was characterized using a UV-Vis double-beam spectrophotometer to determine the optimum wavelength and percentage transmittance for nanosilver formation. These two responses were analyzed before and after purification. This indicated that silver ions (Ag^+) were completely reduced to Ag or nanosilver. The percentage transmittance was observed to quantitatively measure the clarity of the solution. Both showed that the nanosilver solution was successfully synthesized and had a nano size.

The transmittance percentage, which was close to 100%, indicates that the particle size of the nanosilver formed decreased. If the particle size is smaller, the subsequent Brownian motion is faster, which prevents the sedimentation process and causes the solution to become clearer [20]. The absorbance was inversely proportional to the transmittance percentage. The higher the percentage transmittance, the lower the absorbance. The absorbance value indicates the amount of nanosilver formed: the lower the absorbance value, the less nanosilver is formed. Wavelengths that did not reach the target occurred because of the high centrifugation speed during the purification process, causing agglomeration and low stability [3]. In addition, the purification process removes the capping agent from the surface of the nanosilver so that clumping or aggregation occurs, causing nonuniform nanoparticle sizes because the SPR energy shifts to a lower energy level.

The experimental data and responses were analyzed and evaluated using R software. The software analyzed the model that best suited the response conditions so that it will determine the optimal point of the given response. After obtaining the optimization results, validation was performed to determine the level of accuracy of the optimization model. A p-value that does not coincide or is above 0.05 indicates that the difference produced out of the model is not significant. This could have occurred because the wavelength response and percentage transmittance in all experiments were still in the optimum wavelength range of the nanosilver, and there was no significant difference such that neither response could be predicted. The adjusted R^2 value could decrease if the variables added to the model had no effect.

A model suitability test was conducted to determine whether the model was in accordance with the predicted model by examining the lack of fit (inaccuracy) in a response. The experimental results of 13 experiments have wavelengths that meet the target wavelength of nanosilver, and only one experiment does not meet the target wavelength; therefore, this model can still be used. The resulting plot shows the response area of the centrifugation duration and speed in the nanosilver purification process using a green tea leaf extract bioreductor. The optimized areas have various colors that show the area of the wavelength response and percentage transmittance. The three-dimensional surface wavelength response was curved downward, indicating that the area had the maximum response resulting from the independent variables.

Optimization provides the best results by finding variable values that are considered optimal, effective, and efficient for achieving the desired results [21]. The test results of centrifugation duration and speed that have been performed are then entered into the software and then the optimum point of the response given in the most suitable model will be recommended. The optimum point was obtained from the highest combined desirability value, which was considered the optimal condition. The highest desirability value from the predicted optimization process was 0.643 for a duration of 22 min and a speed of 3500 rpm. This indicated that 64.3% of the software's ability to produce the best condition combines all the objective functions.

Data validation was the final stage of the optimization process and is required to test the accuracy of the model in describing certain conditions. In this study, the validation was performed by comparing the prediction results obtained from the software with the research results after six replicates. All replications obtained RSD at the wavelength and percentage transmittance that met the requirements, so that it can be said that the replicated data had good precision.

4. CONCLUSIONS

The optimum centrifugation duration and speed in nanosilver purification was achieved by the CCD approach. The optimal conditions attained in this research were a duration of 22 min and a speed of 3500 rpm. This condition has a composite desirability result which is 0.643 in the process of computational optimization. Observation of the formulation under optimum conditions resulted in favorable repeatability with prediction errors that were <2% for the response of wavelength and percentage of transmittance. Further, the purification process in nanosilver formulation enable the removal of unwanted substances or contaminants that may have formed during synthesis stages, such as residual reactant materials or binding agents.

5. MATERIALS AND METHODS

5.1. Experimental Section

Some of the materials used in this study were green tea leaves (*C. sinensis L.*) from Kepala Djenggot (production code 09022211HC3), filter paper; $AgNO_3$ pro analysis; distilled water; and pharmaceutical grade butanol, acetic acid, tannin, and rutin (Merck Millipore). The equipments required for this study were glassware (PT. Iwaki Glass), thermometer, analytical balance (Ohaus), UV-Vis spectrophotometer (Shimadzu UV-Vis 1800), hot plate (Thermo Scientific), GF254 TLC plate (Merck), vortex (Thermo Scientific), a centrifuge (Thermo Scientific), microtube, pipette pump, micropipettes, and R software with the package of 'rsm.'

5. 2. Methods

5.2.1. Extraction of green tea leaves

A total of 100 mL of redistilled water was heated to 60°C. Thereafter, the green tea leaf (*C. sinensis* L.) powder of 0.2 g was put into the aqueous and heated for 20 minutes at 60°C followed by stirring. The resulting solution was filtered [22].

5.2.2. Qualitative test by thin layer chromatography

The mobile phase butanol-acetic acid at a ratio of 4:1 (v/v) was prepared by blending both materials and then shaking [17]. The mobile phase was placed in a chromatography chamber and saturated for 1 h. The stationary phase used in this study was silica gel GF254 thin-layer chromatography, with a measurement of 6 × 10 cm and an elution separation of 7.5 cm. The plate was then heated at 110°C for 30 min. The samples, tannin standard comparator, and rutin reference standard were dotted 2 cm from the bottom edge of the plate. After saturation with the eluent, the chromatography plate was placed in the chamber and eluted with the mobile phase to a certain volume. After elution, plates were examined under UV light at 254 nm. The RF value was then measured [23].

5.2.3. Preparation of $AgNO_3$ solution

The solid $AgNO_3$ (0.169 g) was dissolved in distilled water. The solution was poured into a 100 mL volumetric flask, and distilled water was added to the volume, which was then shaken until completely dissolved. The solution was diluted by transferring 20 mL of the solution to a 200 mL volumetric flask and adding distilled water, which was then shaken until completely solvated [22].

5.2.4. *Nanosilver purification optimization design*

The variation of centrifugation duration and speed according to Basule (2021) which have been modified [7]. The duration and speed of centrifugation for nanosilver purification were determined using a two-factor, five-level CCD. Table 4 shows the duration and speed of centrifugation at each experimental level. The optimization in this study utilized R software, which has an experimental design that conducts 14 experiments to generate an optimization in this model.

Table 4. Optimized factors utilizing the method of CCD

Factors	Experimental stage of CCD				
	(-α)	(-1)	(0)	(+1)	(+α)
Duration (minute)	8	10	15	20	22
Speed (rpm)	1500	2000	3500	5000	5600

5.2.5. *Synthesis and purification of nanosilver*

Three milliliters of green tea leaves solution was mixed with 27 mL of $AgNO_3$ solution followed by heating process in a sonicator bath at 80°C for 15 minutes. This method was developed by modifying the approach from an earlier study [7]. Nanosilver was purified by centrifuging the colloid for a specific duration and speed. The supernatants from the centrifugation were examined for wavelength, percent transmittance, and particle size [8].

5.2.6. *Characterization of nanosilver*

The nanosilver formed was characterized using a UV-Vis spectrophotometer in the 400–450 nm wavelength range [6]. Maximum absorbance wavelength and transmittance measurements were conducted using a UV-Vis spectrophotometer. Total sample of nano silver (100 µL) was solvated in 5 mL distilled water on the reaction tube and swirled by vortexing for 1 min. The absorbance of the samples was measured at the optimal wavelength. Redistilled water was used as the blank [20].

5.2.7. *Statistical analysis*

The optimization process was implemented using the CCD method (two factors and five levels). The results of this study were optimized with RSM using R software.

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