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Multifarious Uses of UV-VIS Spectroscopy for Green Synthesis of Silver Nanoparticles for Antibacterial Textiles

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Article

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ABSTRACT

Green synthesis is an easy and economical method of synthesizing silver nanoparticles (AgNPs). However, many variables, including the ideal temperature, the volume and concentration of silver nitrate, the amount of plant extract, etc., affect the green synthesis. This article aims to determine the ideal temperature and plant extract amount using the UV-VIS spectrum. It was found above 40 °C was required for both Calendula arvensis (field marigold) leave extract and silver nitrate to synthesize AgNPs. The optimum temperatures were 55 °C and 60 °C for $AgNO_3$ and plant extract, respectively and it was determined using Taguchi orthogonal array based on the UV-VIS spectrum. Silver nanoparticles (AgNPs) can be incorporated into textiles by padding and in-situ methods, but they need accurate plant extract amounts for good antibacterial efficiency. Two equations were proposed for determining the optimum plant extract amount for padding and in-situ methods. Several AgNPs loaded samples were prepared following the derived equations. For evaluating the efficiency of the equations C.reticulata (orange) peel extract was used besides C.arvensis plant extract. The samples were also characterized by antibacterial tests, Scanning Electron Microscope (SEM), Energy Dispersive Spectroscopy (EDS), and X-ray diffraction (XRD). It showed when plant extract was taken according to the equations, around 45 nm nanoparticle size and 100% antibacterial activity were obtained. However, taking 1 ml more plant extract than that of the calculated amount of plant extract increased nanoparticle size to 107 nm and reduced antibacterial activity to 90%.

KEYWORDS

UV-VIS spectrum, antibacterial textiles, green synthesis, silver nanoparticles, Taguchi design

INTRODUCTION

Silver nanoparticles (AgNPs) are widely used antibacterial agents for almost all types of bacteria and viruses [1]. The AgNP synthesis methods are broadly categorized into top-down and bottom-up processes. The bottom-up process includes sol-gel, solvothermal, Chemical Vapor Disposition (CVD), polyol, green synthesis, etc., where nanoparticle forms through reduction and nucleation. On the contrary, the top-down process (e.g., mechanical milling, sputtering, laser ablation, etc.) produces nanoparticles through breaking and emulsification.

The bottom-up synthesis of AgNPs comprises three stages: reduction, nucleation, and limited growth or capping, which requires at least two different agents, namely reduction and capping agents. Unlike chemical synthesis, in green synthesis, the used biological substance accomplishes all three tasks simultaneously. The biological substance includes all parts of plants, ranging from sprouts to leaves, and microorganisms such as fungi, yeast, and algae [1].

Green synthesis is most advantageous over other chemical or physical methods regarding cost, eco-friendliness, simplicity, etc. The application of green synthesised nanoparticles in textiles is pretty common. Still, green synthesis depends on numerous factors such as temperature, plant extract, silver nitrate volume, molarity, etc. Numerous previous kinds of research on green synthesis claimed that room temperature is enough for green synthesis [2]. However, the room temperature varies with region and season. The temperature does not affect the particle size unless it decomposes the organic extract [3,4].

On the other hand, the role of plant extract amount in green synthesis is a debated issue. For example, Sangeetha et al. demonstrated that increased Aloe Vera extract would cause larger nanoparticles [5]. Again, Khalil et al. demonstrated small amount (0.5 to 1 ml) of olive leaf extract would cause larger nanoparticles (24-36 nm). In contrast, a higher amount of olive leaf extract (5 ml) would cause smaller nanoparticles (8-15 nm) [6]. Hamelin et al. showed that an extended amount of Thymus Kotschyanus plant extract (10 ml) creates comparatively larger nanoparticles ranging from 50-60 nm [7].

The same controversy was found in the antibacterial activity of silver nanoparticles (AgNPs) since the nanoparticle size and antibacterial activity are closely related. For example, Shaik et al. experimented with 1 to 7.5 ml of Oregano plant extract, found that 7.5 ml of plant extract had the best antibacterial activity [8]. However, a further increase of Pulicaria plant extract from 7.5 ml to 10 ml decreased the antibacterial activity due to nanoparticle agglomeration [9]. On the other hand, the lower silver concentration and higher volume were found better for antibacterial activity. However, the reducing agent (plant extract) played a significant role here [10].

Although plant extract plays a significant role in green synthesis there is no formula or a rule of thumb to calculate the ideal amount of plant extract for green synthesis considering antibacterial activity and nanoparticle sizes. This study aims to formulate a way to determine the ideal amount of plant extract. For this purpose, the UV-VIS spectra were focused to determine the optimum plant extract amount, as the blue shift of UV-VIS causes smaller nanoparticles [11]. An equation for determining an optimum plant extract amount required to produce smaller nanoparticles was derived empirically based on the UV-VIS spectroscopy results. For green synthesis, a widely found Mediterranean weed named *Calendula arvensis* (field marigold) was selected considering its excellence in green synthesis.

The *C.arvensis* provides some exceptional behaviors such as high wash fastness and shape diversity [12]. It also showed some unexpected behaviour such as temporary loss of antibacterial properties and

recovered it while washing [13]. *C.arvensis* grows in empty fields, rocky hillsides, and cultivated fields, and it contains flavonoids, different natural components (l-threonic acid, ß-sitosterol, z-jasmone, etc.), phenolic acid, etc. [14]. It contains different phytochemicals including monoterpene, Sesquiterpene, alcohol, aldehyde, ketones, Esters, phenolic acids, and flavonoids, which act as both reducing agent (converting silver ions to silver atoms) and capping or limiting agent (to limit the silver atom aggregation) [15]. Furthermore, as the AgNPs can also be incorporated into textiles using the *in-situ* method, another equation was derived for the *in-situ* method. The efficacy of the equations was justified by another plant extract (*C.reticulata* peel extract). The AgNPs incorporated samples were characterized by SEM images to evaluate the size and shape of both *C.arvensis* and *C.reticulata* synthesized AgNPs. The EDS images were used to confirm the presence of Ag in the samples. Again, XRD to determine the crystal size and crystallinity for both *C.arvensis* synthesized AgNPs and *C.reticulata* synthesized AgNPs.

EXPERIMENTAL

Required Materials

Analytical grade Silver Nitrate ($AgNO_3$), Ethanol, Muller Hinton agar, Nutrient broth, and Citric acid were used and purchased from Merck, Germany. The plain woven cotton fabrics (GSM 123) were collected from local textile mills. Gram-positive (*S.aureus*) bacteria were used for the antibacterial test. The bacteria were collected from the microbiology laboratory of the Cukurova University. The *C.arvensis* (field marigold) plants and *C.reticulata* (mandarin orange) peels were collected in the month of February from the university garden and university agricultural products sell centre, respectively.

Equipment

FT/IR-6700 (Jasco, Japan) was used for FTIR, and Shimadzu UV- 1800 UV/Visible spectrophotometer was used for the UV spectrum. PANalytical EMPYREAN (UK) XRD was used for taking the XRD data. FEI Quanta 650 (USA) Scanning Electron Microscope (SEM) was used For size and shape.

Plant Extract preparation

The leaves and stalks were separated and washed several times with 95% ethanol. Then it was shade-dried and chopped into small pieces. 5 g of the dried leaves were kept in 200 ml of water and boiled at 90 ^{o}C until the liquor became 100 ml, to acquire maximum phytochemical. After cooling, the extract was filtered with Whatman no.1 filter paper. In this way, a 90 ml plant extract solution was obtained. This solution was stored in a jar labelled as *C.arvensis* and kept at 3 ^{o}C for future use. In the same way,

an extract from mandarin orange (*C.reticulata*) peel was also prepared and labelled as *C.reticulata* and stored at 3 ^{o}C for future use.

Fabric pre-treatment

Pretreatment is a crucial matter in AgNP incorporation. Therefore, the fabrics were pretreated using NaOH. The fabric sample was boiled at 4 g/L NaOH solution for 1 hour at 90 ^{o}C and again boiled for 1 hour in distilled water to reduce alkalinity.

AgNPs synthesis

$AgNO_3$ with various molarities and volumes were used. For 0.001 M, 0.002 M, 0.005 M $AgNO_3$ 100 ml volume, and 0.1 M $AgNO_3$ 20 ml volume were prepared using double distilled water. The used plant extract was ranged from 2 drops to 2 ml. Both the $AgNO_3$ solution and plant extract were heated at different temperatures ranging from 40 ^{o}C to 90 ^{o}C with a magnetic stirrer having 20 rpm. A few drops of plant extract were added to the $AgNO_3$ solution, and the colour of the solution changed within a few seconds which indicates the AgNP formation. The nanoparticle solution was kept at room temperature and checked every day for stability.

Antibacterial tests

The antibacterial tests were done with a small modification of the AATCC-100 test standard. *S. aureus* (a Gram-positive bacteria) and *K. pneumoniae* (a Gram-negative bacteria) were used in the antibacterial tests. Before conducting the test all equipment such as Petri dishes, agar, broth, test tubes, vials etc. were sterilized using an Autoclave machine. Then, using an inoculation loop few bacteria were collected and added to the neutrient broth, and the solution was mixed well using a vortex machine. Next, the broth solution's turbidity was measured using a McFarland Densitometer and necessary adjustment was taken to set McFarland standard 0.5 (2x 10^5 CFU/ml).

Here, a fabric sample having a 4.8 cm x 4.8 cm size was used. First, a 100 ml inoculum was cultured for 24 hours at 37 ^{o}C in nutritional broth. Each fabric sample subsequently absorbed roughly 1 ml of this inoculum. Following that, each sample was incubated for 24 hours at 37 ^{o}C. Following the incubation time, 100 ml of water was added as a buffer solution, and the samples were thoroughly mixed by shaking. To determine the quantity of living bacteria, serial dilution was performed. The process was repeated 3 times. Finally, the following formula (equation 1) calculates the percentage of bacterial reduction after 24 hours incubation period:

$$\text{Reduction rate \%} = \frac{(B-A)}{B} \times 100 \quad (1)$$

where B = bacterial colony for the untreated sample; A = bacterial colony for AgNPs treated sample

Characterizations

The functional groups of the phytochemicals of the plant extract were examined by the FTIR. Simultaneously the synthesized solution was characterized by UV-VIS spectroscopy. For this purpose the reference cuvette was filled with water and referencing was done. Then the synthesized AgNPs were poured into the sample cuvette the absorption spectrum was taken and the maximum absorption peak was recorded. Later for analysing the spectra Python 3.0-based Scikit-Learn machine learning library was used. This maximum absorption peak of the UV-VIS spectrum forecasts the AgNP formation. For determining AgNP size and shape, SEM was used. For this purpose, the synthesized AgNPs were incorporated into cotton fabrics by padding, immersion, and in-situ methods. The AgNPs-loaded fabric samples were used for SEM and EDS analysis. After taking the SEM images the sizes of the appeared AgNPs were measured. The AgNPs-loaded samples were also used for XRD analysis.

RESULTS AND DISCUSSION

The plant extracts may have various properties such as antibacterial, anti-mutagenic, anti-inflammatory, antioxidant, hemolytic, etc. Among various components monoterpene, sesquiterpene, alcohol, aldehyde, ketones, esters, etc. are present in *C. arvensis*. The *C.arvensis* contains different types of phenolic acids and flavonoids [16]. On the other hand, *C.reticulata* peel extracts contain flavonoids, hesperidin, hesperidin, vitamin-C, flavanones, flavones, naringenin, nobiletin, polymethoxyflavones (PMF), etc. [17]. The presence of different types of phytochemicals indicates a very good potential for green synthesis. The *C.arvensis* and *C.reticulata* extracts were characterized by FTIR to determine the contained functional groups.

FTIR

The FTIR spectrum of *C.arvensis* and *C.reticulata* have been depicted in Figure 1. The *C.arvensis* extract showed 4 peaks at 3300 cm^{-1}, 1640 cm^{-1}, near 1340 cm^{-1}, and 1470 cm^{-1} representing –OH, >C=C< groups, trans-wagging band of $–CH_2$ and $–CH_3$ asymmetrical or symmetrical stretching, respectively. On the other hand, the *C.reticulata* showed only 2 major peaks at 3300 cm^{-1}, and 1640 cm^{-1} which represent –OH, >C=C< groups. The hydroxyl groups of different phytochemicals reduce the Ag+ to Ag0 atoms and it continues to aggregate due to the creation of new nanoparticles which increases the free energy. Hence, the aggregation must have to be stopped after a certain limit. In the case of green synthesis with *C.arvensis* this limiting growth is accomplished by the phytochemicals such as flavonoid, monoterpene, etc. [18].

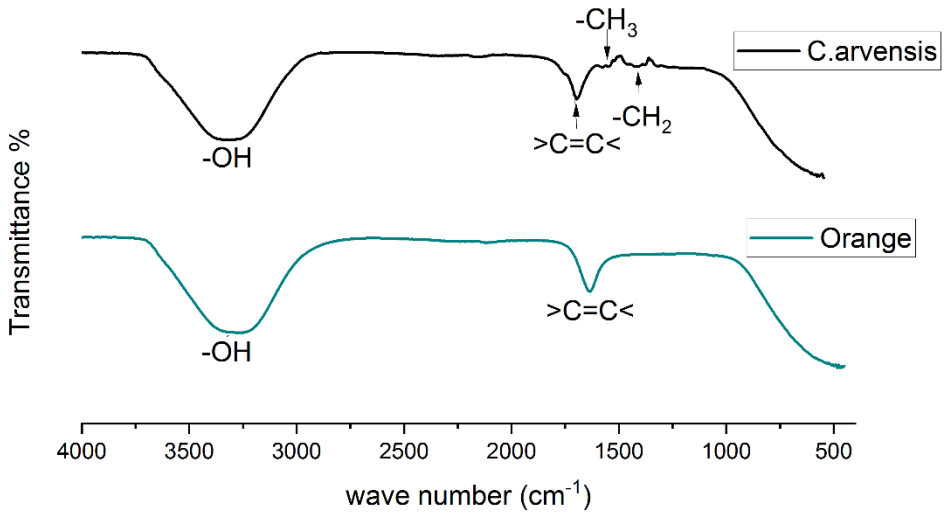


Figure 1. FTIR spectrum of *Calendula arvensis* and *C.reticulata* (orange) extracts

AgNPs preparation

At room temperature, AgNPs were tried to synthesize from 0.1 M $AgNO_3$ with *C.arvensis* plant extract at various ratios. However, every attempt to synthesize AgNPs failed. In most of the cases, silver nitrate ($AgNO_3$) got oxidized and turned to black precipitation, depending on the amount of reducing agent (plant extract). At one stage, a bottle of unsuccessful $AgNO_3$ and plant extract solution along with a thermometer was placed in an oven, surprisingly, when the temperature reached 50 ^oC the solution started to change color. The solution was taken to the UV-VIS spectroscopy and a 431 nm absorption peak was recorded. As the temperature played a crucial role it was further investigated in various ways as described following:

Temperature on AgNP synthesis

At room temperature, $AgNO_3$ (0.1 M) quickly precipitated to the amount equivalent to the plant extract, i.e. the more plant extract the more precipitation. AgNPs were synthesized from $AgNO_3$ (0.1 M) at ice cube immersion, room temperature, 40 ^oC, and 60 ^oC. For this purpose, 5 ml $AgNO_3$ (0.1 M) was taken into 4 laboratory bottles, and 2 drops of plant extract were added to each bottle. The bottles were placed in ice cube boxes, at room temperature (25 ^oC), 40 ^oC, and 60 ^oC, respectively. It was seen only the 60 ^oC sample successfully synthesized AgNPs and the other 3 samples failed to synthesize AgNPs (Figure 2). Besides, to determine the effect of temperature on plant extract, the plant extract was kept at 0 ^oC and 40 ^oC. It was observed that the 0 ^oC samples failed to synthesize AgNPs due to the low plant extract temperature. However, the *C.arvensis* extract worked well at above 40 ^oC temperature in AgNP synthesis (Table 1).

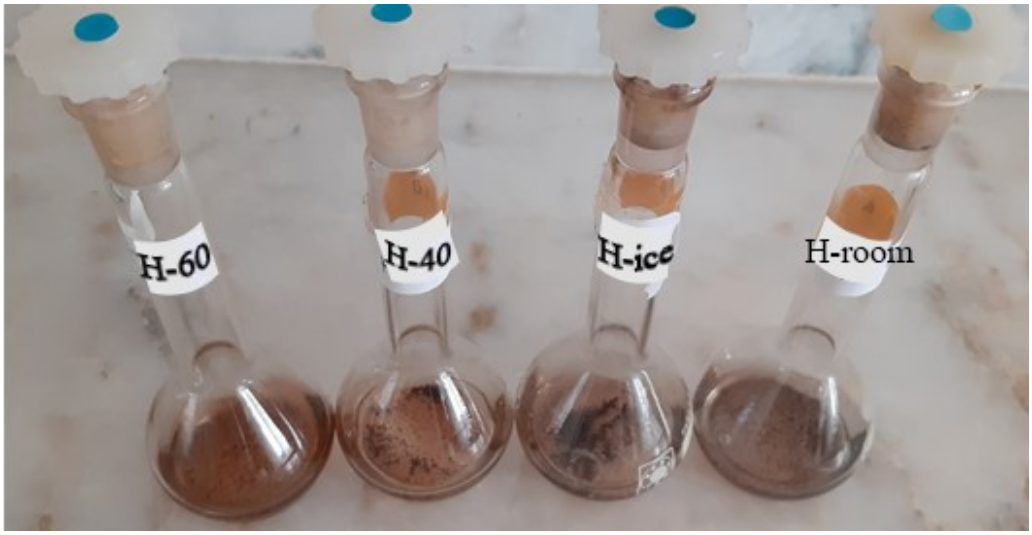


Figure 2. Temperature on AgNP synthesis

Table 1. Different temperatures (°C) on AgNP synthesis

Sample	$AgNO_3$ (0.1M) ml	Leaf extract (drops)	Temp (°C)	Plant extract (°C)	Result
H-ice	5	2	0	25	Instant black precipitation
H-room	5	2	25	25	Black precipitation in 5 min
H-40	5	2	40	25	Black precipitation in 5 min
H- 60	5	2	60	25	synthesized
P-0C	5	2	40	3	Failed to synthesized
P-40 C	5	2	60	40	Well synthesized

However, the synthesized silver nanoparticles were very unstable and aggregated within 3 hours. To improve the stability different molarity was taken such as 0.01 M, 0.002 M, 0.05 M, and 0.1 M. Each synthesized sample showed different colours as shown in Figure 3-b. Here, the best performances were obtained from 0.002 M and 0.001 M. So, the rest experiments were decided to be performed with 0.002 M with 100 ml $AgNO_3$.

In green synthesis, first, the reducing agent (plant extract) reduced the silver ions to silver atoms (Figure 3c). The newly created silver atoms tend to aggregate due to their free energy, if the aggregation does not get stopped silver particles will be precipitated and no AgNPs will be obtained. Hence a capping agent is required to stop the aggregation after a certain size. In green synthesis, the various phytochemicals of plant extract such as flavonoids, terpenoids, etc. act as capping agents.

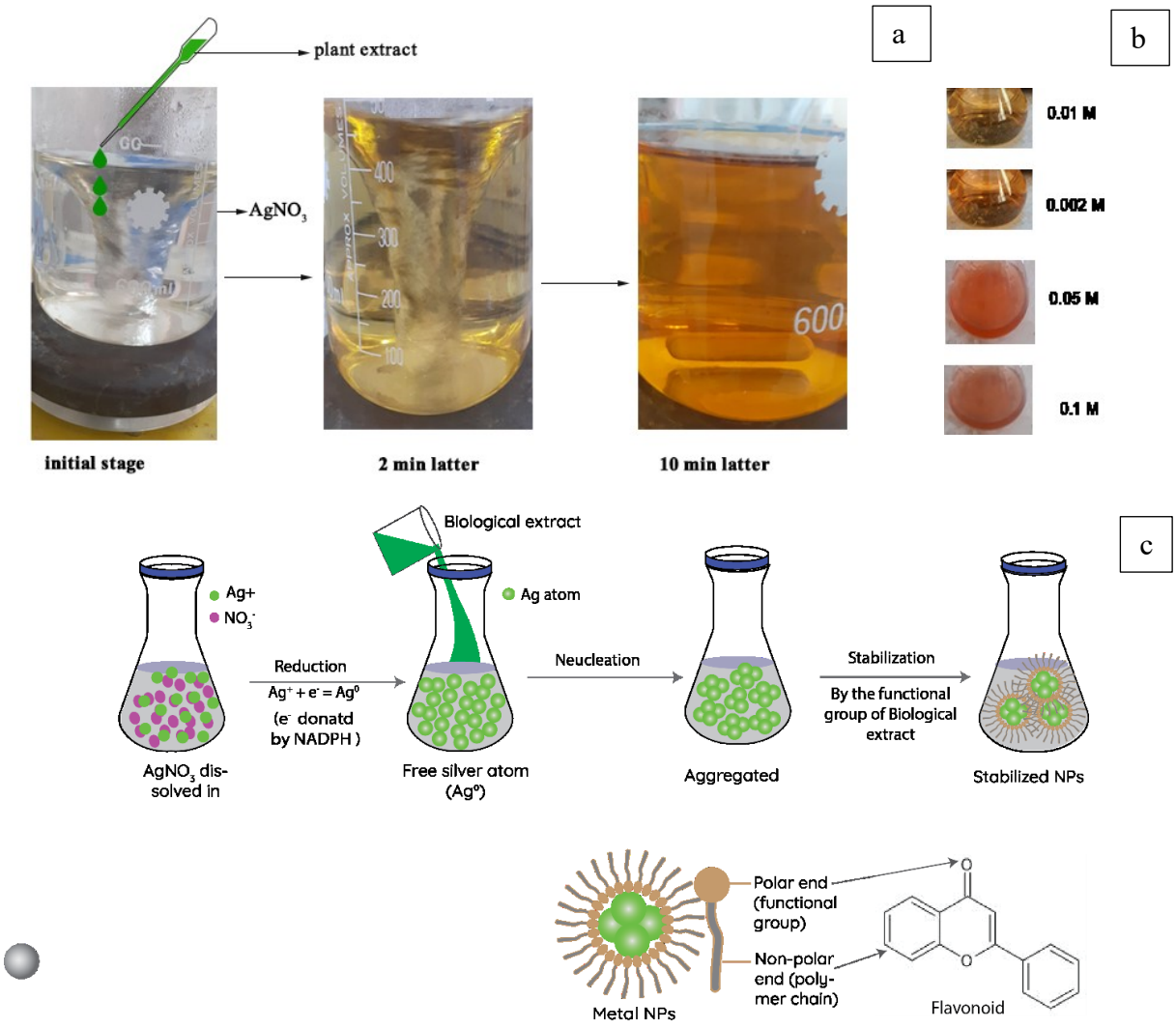


Figure 3. Green synthesis: a) process b) colours of AgNPs solution for various molarity c) green synthesis mechanism [19]

UV-VIS spectroscopy

The colour change of the solution is a primary indicator of AgNPs formation. For confirmation, the synthesized AgNPs suspension was tested in a UV-VIS spectrophotometer. AgNPs produce absorbance peak or Surface Plasmon Resonance (SPR) bands at 350-500 nm [20]. The $AgNO_3$ can not produce an absorbance peak whereas the synthesized AgNPs produced a visible absorbance peak (figure 4-a). In this study, a wide range of absorbance peaks ranging from 416 nm to 441 nm were obtained for different samples depending on $AgNO_3$ concentration, temperature, plant extract amount, etc. (figure 4-b). The maximum SPR and Molarity are very poorly correlated having an r^2 score of 0.06, similarly, the maximum SPR and Plant extract amount are also poorly correlated having an r^2 score of 0.11. So, only $AgNO_3$ concentration or plant extract can not determine maximum SPR.

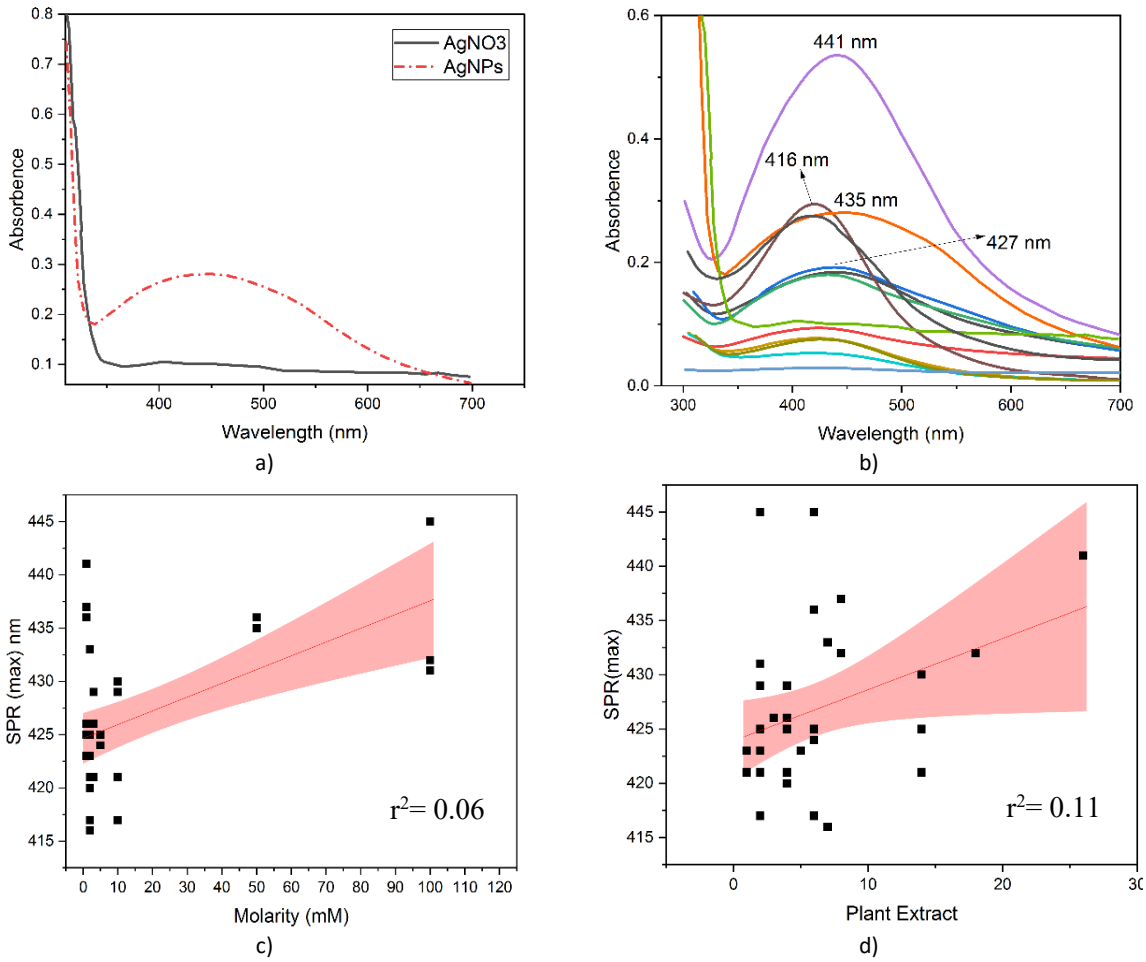


Figure 4. UV-VIS spectrum for various AgNPs: (a) UV-VIS spectrum of only $AgNO_3$ and synthesized AgNPs; (b) various UV spectra for various AgNPs; (c) Surface Plasmon Resonance (SPR) of AgNPs synthesized from various concentrations of $AgNO_3$; (d) SPR of synthesized AgNPs from various plant extract amount

UV-VIS spectra to determine the optimum temperature

As mentioned earlier, temperature was found very crucial in green synthesis with *Calendula arvensis*. Hence, to investigate in detail various temperatures from 40 °C to 70 °C with 100 ml of 0.002M $AgNO_3$ and 4 drops (153 µl) of plant extract were tried. The samples were checked daily for stability. Table 2 shows the first day and 4th day's UV-VIS absorbance peak (maximum SPR) for different sets of temperatures of $AgNO_3$ and Plant extract.

Table 2. Effect of temperature on AgNPs UV-spectrum and absorption

Sample	0.002M $AgNO_3$ (ml)	$AgNO_3$ Temp (°C)	PE temp (°C)	reaction time (min)	Max SPR Day-1	Absorption Value Day-1	Max SPR Day-4	Absorption value day-4
X1	100	70	70	3	421	0.1829	423	0.2230
X2	100	70	60	1	422	0.2187	423	0.2773
X3	100	70	50	4	421	0.2647	424	0.3174
X4	100	70	40	4	420	0.2505	421	0.2901

Sample	0.002M $AgNO_3$ (ml)	$AgNO_3$ Temp (°C)	PE temp (°C)	reaction time (min)	Max SPR Day-1	Absorption Value Day-1	Max SPR Day-4	Absorption value day-4
X5	100	60	70	14	425	0.0912	428	0.2428
X6	100	60	60	14	417	0.1205	425	0.2922
X7	100	60	50	12	423	0.1298	424	0.3918
X8	100	60	40	12	423	0.1599	424	0.2379
X9	100	50	70	36	424	0.1034	425	0.1908
X10	100	50	60	26	416	0.0764	426	0.1633
X11	100	50	50	42	424	0.1334	425	0.2322
X12	100	50	40	42	425	0.0884	428	0.1613
X13	100	40	70	180	416	0.0523	429	0.1244
X14	100	40	60	180	424	0.0533	430	0.1342
X15	100	40	50	180	424	0.0683	431	0. 1658
X16	100	40	40	180	424	0.0804	428	0.1686

Table 2 shows the lowest UV-VIS absorbance peak or SPR was achieved on day 1 at X10 (416 nm), X13 (416 nm), and X6 (417 nm). In these cases, the plant extract temperature was between 60-70 °C, and $AgNO_3$ temperature varies between 40 °C to 60 °C, but the UV-VIS absorbance peaks of day 4 were beyond 425 nm, which indicates nanoparticle aggregation. The lowest UV-VIS SPR of day 4 was from X4 (421 nm), having 70 °C and 40 °C for $AgNO_3$ and plant extract temperature, respectively.

The Surface Plasmon Resonance (SPR) of various $AgNO_3$ and Plant extract temperature pairs were plotted in Figure 5. The absorption values were also depicted here. Interestingly, it shows the UV-VIS SPR decreases with increasing the plant extract temperature where $AgNO_3$ temperature was kept at 70 °C (Figure 5). On the contrary, when the $AgNO_3$ temperature is 40 °C and the plant extract's temperature increases, the UV-VIS spectrum for the first day was unchanged. But it increases with time. The overall stability (in terms of UV-VIS spectrum) depends immensely on $AgNO_3$ temperature, as the volume of $AgNO_3$ is much higher than the plant extract. But the temperature is vital in both cases. As mentioned earlier, a temperature below 40 °C for plant extract will not disperse or activate and consequently fail to form AgNPs.

Another interesting phenomenon is when the plant extract temperature is 50 °C the synthesized AgNPs showed a sharp rise in absorption value (X7) which indicates a high concentration of AgNPs. So it can be hypothesized that the optimum temperature can increase the AgNP synthesis. However, more study is needed in support of this hypothesis. It is beyond the scope of the present study.

The low temperature cannot synthesize AgNPs, because the high temperature may disorder $AgNO_3$ structure (Pbca) and exhibit slight thermal expansion along the c-axis. More specifically, the O1-N-O2 angle decreases with temperature whereas the O2-N-O3 angle increases non-linearly with

temperature [21]. The optimum temperature was determined from this dataset using the Taguchi Orthogonal array design.

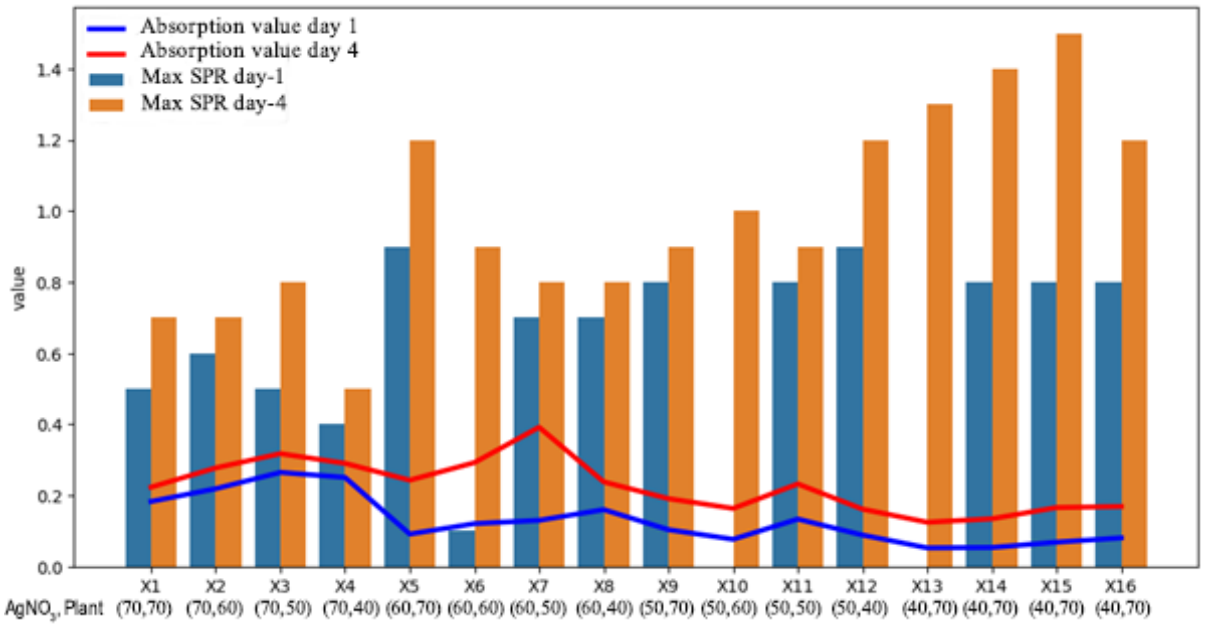


Figure 5. Temperature on AgNPs formation and UV spectrum

Temperature determination by Taguchi design

As mentioned earlier, it needs at least 40 °C temperatures for AgNP synthesis. Hence, to find the optimum temperature, Taguchi orthogonal array experimental design was used. The Taguchi method is based on an orthogonal array and ANOVA [22]. Here, two factors are $AgNO_3$ temperature and plant extract temperature. Each factor has four levels (40 °C, 50 °C, 60 °C, 70 °C). Therefore, the L_{16} (4^2) An orthogonal array was selected to determine the optimum temperature and the minimum number of experiments. The experimental results are shown in Table 3. In the coded matrix each group was numbered as 1 to 4. In the actual matrix columns for each temperature group of the $AgNO_3$ four different temperatures of plant extract were used.

Table 3. L16 orthogonal design, Levels of the factors, and experimental results

Exp. No.	Coded matrix		Actual matrix		Experimental results	
	A	B	$AgNO_3$ Temp (°C)	Plant Extract Temp (°C)	Max SPR (nm)	Time (min)
1.	1	1	70	70	421	3
2.	1	2	70	60	422	1
3.	1	3	70	50	421	4
4.	1	4	70	40	420	4
5.	2	1	60	70	425	14

Exp. No.	Coded matrix		Actual matrix		Experimental results	
	A	B	$AgNO_3$ Temp (°C)	Plant Extract Temp (°C)	Max SPR (nm)	Time (min)
6.	2	2	60	60	417	14
7.	2	3	60	50	423	12
8.	2	4	60	40	423	12
9.	3	1	50	70	424	36
10.	3	2	50	60	416	26
11.	3	3	50	50	424	42
12.	3	4	50	40	425	42
13.	4	1	40	70	416	180
14.	4	2	40	60	424	180
15.	4	3	40	50	424	180
16.	4	4	40	40	424	180

The outcome of the Taguchi orthogonal array is depicted in Figure 6 as contour plots. It shows the optimum $AgNO_3$ and plant extract temperature should be around 55 °C and 60 °C, respectively. The results are slightly different than that of Heydari and Rashidipor, who found that 45 °C is the best among a temperature range from 4 °C to 60 °C for AgNP synthesis from oak fruit hull [23].

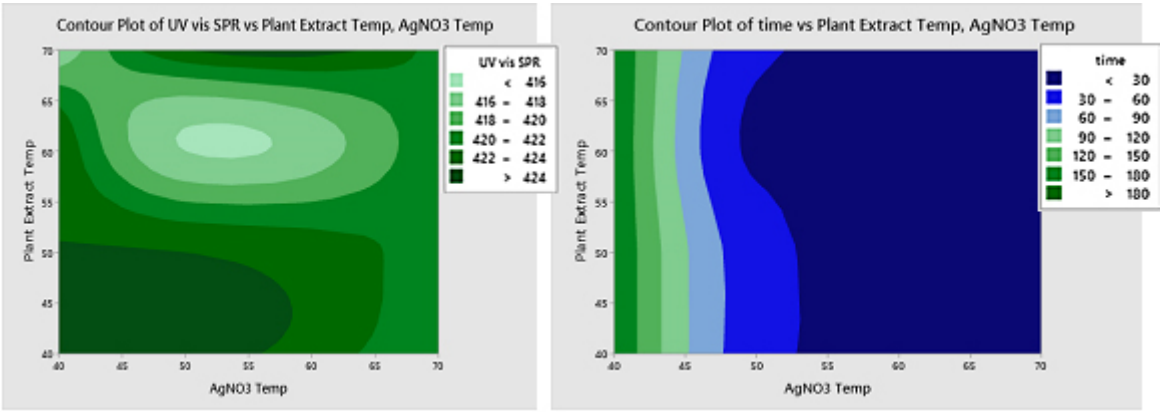


Figure 6. Contour plot of UV- VIS SPR and reaction time

The concentration of $AgNO_3$ on UV-VIS Spectrum and stability

To evaluate the effect of $AgNO_3$ concentration on nanoparticle stability, different concentrations were taken with the same volume (table 4). The plant extract was determined according to the molarity and volume. The first UV-VIS spectra were recorded after 2 hours of solution preparation so that the solution had enough time to get synthesized. Figure 7 depicts the interaction among $AgNO_3$ molarity, UV spectrum, and stability.

Table 4. $AgNO_3$ concentration on UV- VIS spectrum and stability

$AgNO_3$ molarity (M)	$AgNO_3$ amount	Plant extract (drops)	Time	Temp for both Solution (°C)	SPR (day-1) (nm)	Absorption value	UV- VIS day-2 (nm)	Stability
0.001M	100	2	15 min	70	423	0.1699	425	60 days
0.002 M	100	2	10 min	50	417	0.072	424	60 days
0.003 M	100	2	24 hrs	60	421	0.0985	425	50 days
0.005 M	100	6	50 sec	60	424	0.1978	426	25 days
0.01 M	100	14	6 min	50	430	0.618	430	7 days
0.1 M	100	18	instant	65	432	0.8210	Aggr. 20 hours	20 hours
0.1 M	20	2	instant	60	445	0.272	no	3 hours

* 1 drop=38 µl

From Table 4 the $AgNO_3$ volume was 100 ml in all the cases but the $AgNO_3$ concentration varied from 0.001 M to 0.1 M as well as plant extract. The stability, UV-VIS and $AgNO_3$ molarity are depicted in Figure 7. It shows that high molarity causes high UV-VIS SPR and low stability, and vice versa. It shows that with the increase of molarity, the UV-VIS APR increases while the stability decreases.

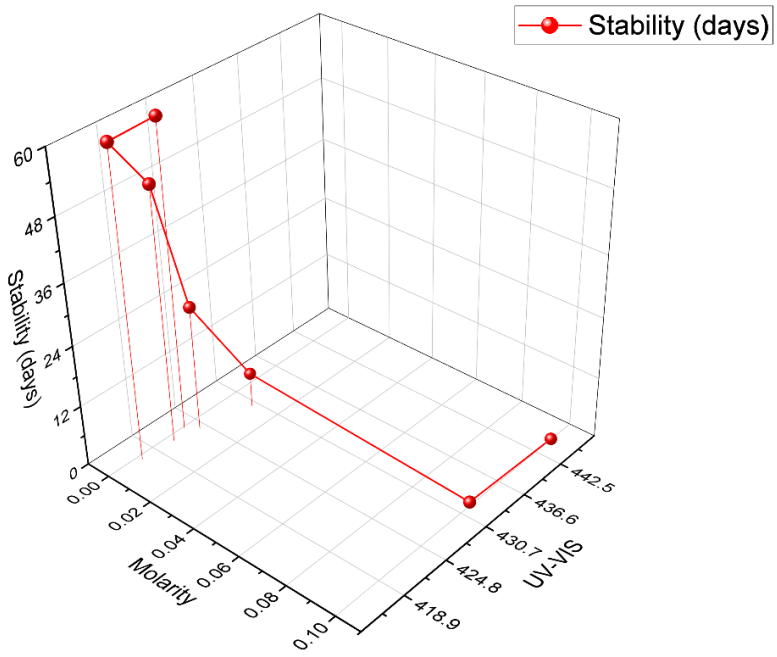


Figure 7. UV spectrum of $AgNO_3$ having various molarity

The stability of high morality silver nitrate is very short whereas for low molarity it is high. The high concentration of $AgNO_3$ produced too many silver nanoparticles which tended to attach due to their

newly created surface free energy. The capping action of the plant extract is not enough to stop the aggregation. Hence, the stability of the AgNPs from high concentric $AgNO_3$ was very low as illustrated in Figure 8.

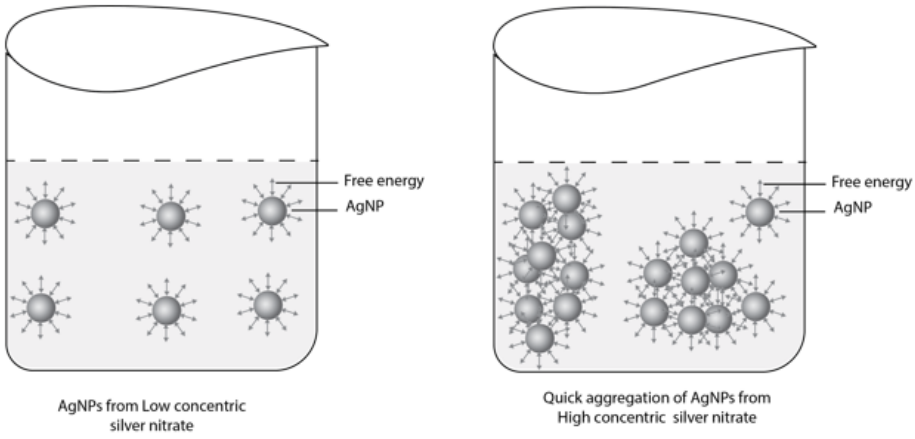


Figure 8. Silver nanoparticle stability with concentration

Different volumes of $AgNO_3$ on UV-VIS SPR

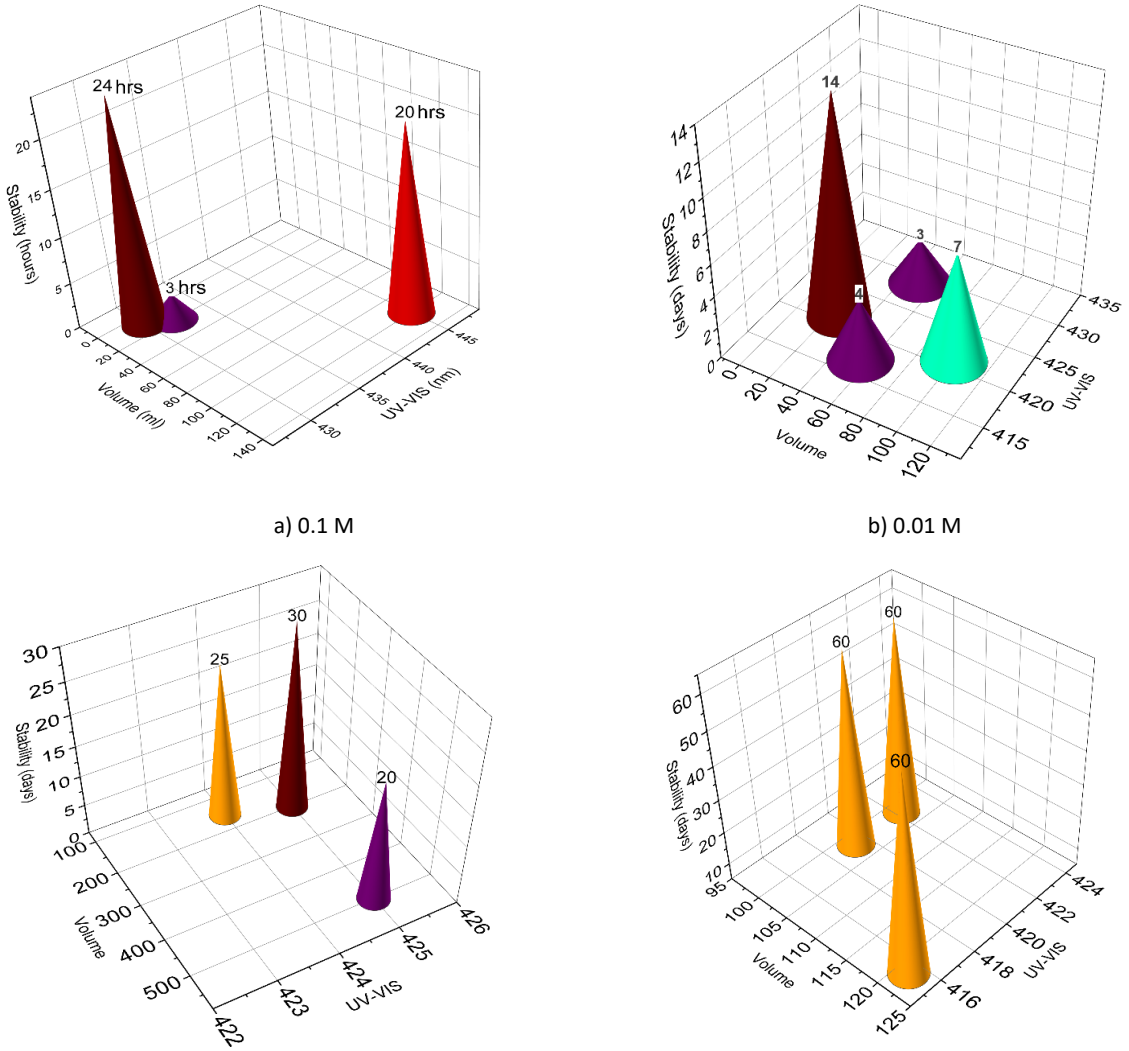
To determine the influence of $AgNO_3$ volume different volumes were tried keeping the silver nitrate molarity the same. Plant extract cannot be kept constant as high molarity and high volume need more plant extract. Table 5 depicts 4 categories of $AgNO_3$ molarity (0.01 M, 0.1 M, 0.005 M, and 0.002 M) with different volumes in each category. It is clear from Figure 8 that there is no noticeable relationship between $AgNO_3$ volume and UV-VIS spectrum stability. Rather $AgNO_3$ concentration plays the most crucial role in this aspect, as shown in Figure 9.

Table 5. $AgNO_3$ volume on UV spectrum and stability

$AgNO_3$ Molarity	$AgNO_3$ amount	PE amount (drop)	Time	Temp (°C)	UV-VIS SPR (nm)_2 hrs	Absorption value	UV-VIS SPR (nm)_24hrs	Stability (at least)
0.01 M	25	1	5 min	60	421	0.2715	429	14 days
0.01 M	50	2	1800 min	60	429	0.1349	430	3 days
0.01 M	55	6	instant	60	417	0.5059	430	4 days
0.01 M	100	14	6 min	50	421	0.618	429	7 days
0.1 M	20	6	instant	60	445	0.272	0	3 hours
0.1 M	10	2	instant	70	431	0.6076	440	1 day
0.1 M	116	8	instant	65	432	0.7604	445	20 hours
0.005 M	155	6	50 sec	60	424	0.5321	429	25 days
0.005 M	200	4	2 min	60	425	0.4509	433	30 days
0.005 M	500	14	5 min	60	425	0.4106	425	20 days

$AgNO_3$ Molarity	$AgNO_3$ amount	PE amount (drop)	Time	Temp (°C)	UV-VIS SPR (nm)_2 hrs	Absorption value	UV-VIS SPR (nm)_24hrs	Stability (at least)
0.002 M	120	7	3 min	50	416	0.2298	421	60 days
0.002 M	100	2	15 min	70	423	0.1699	425	60 days
0.002 M	100	4	10 min	70	420	0.2505	421	60 days

In Table 5 the $AgNO_3$ concentration is kept the same and the $AgNO_3$ volume varies, the plant extract amount is also varied according to the $AgNO_3$ molarity and plant extract amount. The stability and UV-VIS spectrum change accordingly. Here, the influential roles are played by both $AgNO_3$ molarity and plant extract amount. The high molarity tends to aggregate more quickly. Figure 9 shows that each molarity of silver nitrate the volume affects the UV-VIS spectrum differently. For example, 0.1 M, 0.01 M $AgNO_3$, and 0.005 M synthesized AgNPs showed the highest stability for low $AgNO_3$ volume. But, it depends on the plant extract amount which has to be determined by $AgNO_3$ volume and molarity.



a) 0.1 M

b) 0.01 M

c) 0.005 M

d) 0.002 M

Figure 9. $AgNO_3$ volume on UV-VIS and Stability of AgNPs for different concentrations

On the other hand, 0.002 M silver nitrate showed a high volume for longer stability.

Because 0.002 M is very dilute and the other parameters do not influence the AgNP aggregation. However, in general, molarity is strongly correlated with stability. The average stability of different concentrations of $AgNO_3$ is shown in Figure 10. The highest stability was reported for 0.002 M and the lowest was 0.1 M.

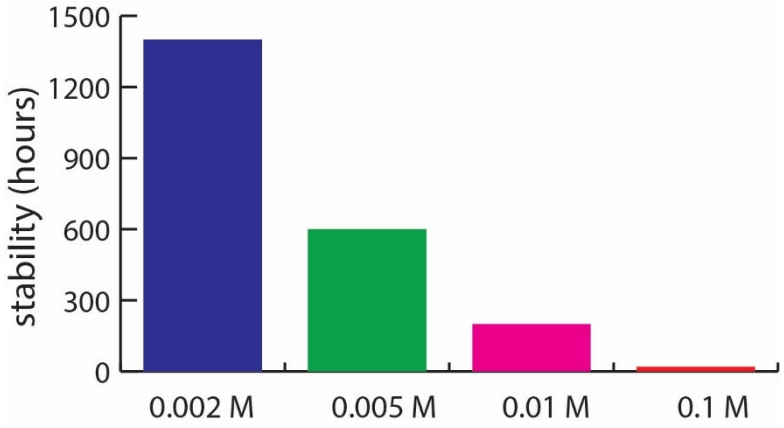


Figure 10. $AgNO_3$ molarity on AgNps stability

From the above discussion, it is obvious that the maximum UV-VIS Surface Plasmon Resonance (SPR) does not depend solely on the molarity, plant extract, or volume. But, all these 3 factors combine to determine the UV-VIS SPR.

Amount of Plant Extracts on UV-VIS of AgNps

To examine the effect of plant extract on the UV-VIS spectrum, different amounts of plant extract ranged from 2 drops to 26 drops with constant molarities were tried. (Table 6).

Table 6. Effect of different plant extracts on AgNPs

$AgNO_3$ (100 ml) (M)	Temp (both)	Plant Extract (drops)	Reaction time	UV-VIS SPR (nm)	Absorption value
0.001	70	2	48 hours	425	0.01699
0.001	70	4	24 hours	426	0.0528
0.001	70	6	24 hours	436	0.1867
0.001	70	8	24 hours	437	0.1687
0.001	70	26	5 min	441	0.5437
0.002	60	2	10 min	417	0.072
0.002	60	4	7 min	421	0.1538
0.002	60	5	6 min	423	0.1092
0.002	60	6	5 min	425	0.194

$AgNO_3$ (100 ml) (M)	Temp (both)	Plant Extract (drops)	Reaction time	UV-VIS SPR (nm)	Absorption value
0.002	60	7	4 min	433	0.2464
0.002	60	1	24 hrs	423	0.1178
0.003	60	3	5 min	426	0.1928
0.003	60	4	2 min	429	0.2343
0.003	60	2	24 hrs	421	0.0985
0.003	60	2	4 min	429	0.1779

Table 6 depicts the influence of various plant extract amounts on the same $AgNO_3$ concentration and volume. The UV-VIS, molarity and plant extract amount are shown in Figure 11. It is seen in almost all cases, the amount of plant extract is proportional to the UV- VIS spectrum. The higher the plant extract, the higher the UV-VIS spectrum as well as absorbance. Plant extract plays a crucial role in AgNP synthesis depending on $AgNO_3$ molarity and volume [10]. Plant extract in combination with $AgNO_3$ volume and molarity play a significant role in AgNP synthesis. $AgNO_3$ volume and molarity were known, but the plant extract amount was unknown. Therefore, to formulate an equation for determining the plant extract amount is described in the following section.

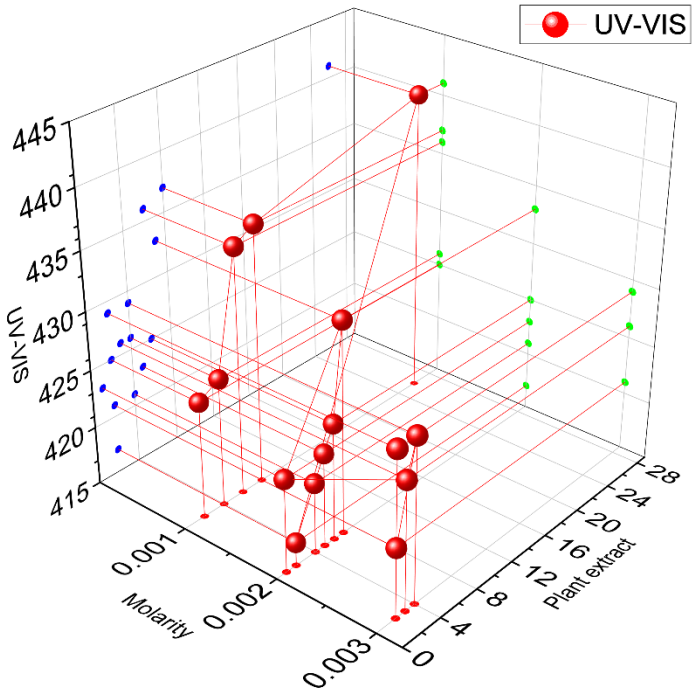


Figure 11. Plant Extract on the UV-VIS spectrum

Determining the amount of plant extract

To derive an equation for the optimum plant extract amount, the above-mentioned influence of $AgNO_3$ molarity, volume, *C.arvensis* extract amount, and UV-VIS spectra of *C.arvensis* synthesized AgNPs were considered. 1 mole of $AgNO_3$ contains 6.02 x 10^{23} molecules. But there is no such number for the plant extract solution. Hence, we determined an empirical constant based on hundreds of trials. To ease our work we used Python 3.0 and the Python-based Numpy library (supplement 1). The main objective of this equation is to determine the plant extract quantity for the lower UV-VIS spectrum (which will ultimately give a smaller nanoparticle size).

Moles = $M \times V$

Where M = molarity; V = volume

Empirical constant = $\left(10 + \frac{log\, V}{log\, M}\right) = 10 + log_M V$ *[since,* $log_v M = \frac{log_e M}{log_e v}$ *]*

Plant extract quantity = Mole × empirical constants+$log10$ (drops)

$$= M \times V \times (10 + \log_M V) + \log_{10} V \text{ (drops)} \quad (2)$$

1 ml = 25 drops (for the used dropper)

1 ml = 1000 μl

Hence for convenience, the plant extract quantity in μl, needs to be divided by 0.025

Hence concisely, it can be written:

$$\text{Plant extract amount} = \frac{M \times V \times (10 + log_M V) + log_{10} V}{0.025}\ \mu l \quad (3)$$

For example, 0.002 M of 100 ml $AgNO_3$ will require $\frac{0.002x100(10+log_{0.002}100)+log_{10}100}{0.025}$ = 153 μl

Formulating the amount of plant extract for the in-situ method

For in-situ synthesis, the formula is the same as above. But there, the plant extract should be prepared in a different vessel. According to our study, the minimum amount of liquid to wet the fabric is 2.5 times of fabric weight.

The required volume of liquid to wet a fabric sample = $fabric\ sample\ weight \times 2.5$ ml

Sometimes multiple samples are needed to prepare. Hence a high amount of plant extract solution may be needed. For this reason, the dilution volume term was used here. If the dilution volume is 100 ml, then the calculated plant extract (according to equation 4) will be needed to add in 100 ml of water.

Required volume for fabrics, $v = \frac{GSM}{10000} \times L \times W \times 2.5 \times dilution\ volume$

Here, GSM = gram per square meter; L = sample length; W = sample width

$$\text{The Required Plant extract} = \frac{0.002 \times 100(10 + log_{0.002}100) + log_{10}100}{0.025 \times 1000}\ \text{ml} \quad (4)$$

If the dilution volume is one, the calculated plant extract will be added to the 1 ml of water.

For example, to find out the required plant extract for in-situ synthesis of 230 GSM fabric having a 10 cm x 10 cm sample size, if we're going to prepare 100 ml plant extract solution for the in-situ method, then,

Required volume $v = \frac{230}{10000} \times 10 \times 10 \times 2.5 \times 100 = 575$

Plant extract amount $= \frac{0.001 \times 575 \times (10 + log_{0.001}\, 575) + log_{10}\, 575)}{0.025x1000} = 0.5\ ml$ in 100 ml water

Several samples were prepared using the abovementioned equations (equations 1 and 2). In addition to it, SEM, EDS, XRD, and finally, antibacterial tests were done to characterize the nanoparticle-loaded fabrics. To evaluate the efficacy of the equations, *C.reticulata* (orange) peel extract was used.

Evaluating the equations

Several fabric samples were prepared according to the proposed formulae in Equation 3 and Equation 4. To determine the efficiency of the equations, a few samples with a deviation from the calculated plant extract amount were also prepared. The antibacterial activity of the samples was determined by the antibacterial tests. The antibacterial test results were identical for both *S.aureus* and *K.pneumonia*, henceforth the test results were not mentioned separately. Besides, the nanoparticle size was determined by the SEM image. The applied plant extract, calculated plant extract amount, application method, antibacterial activity, and AgNP size for two different plant sources are tabulated in Table 7. It reveals that the calculated plant extract amount seems enough to have 100% bacterial reduction in all three methods: immersion, in-situ, and padding.

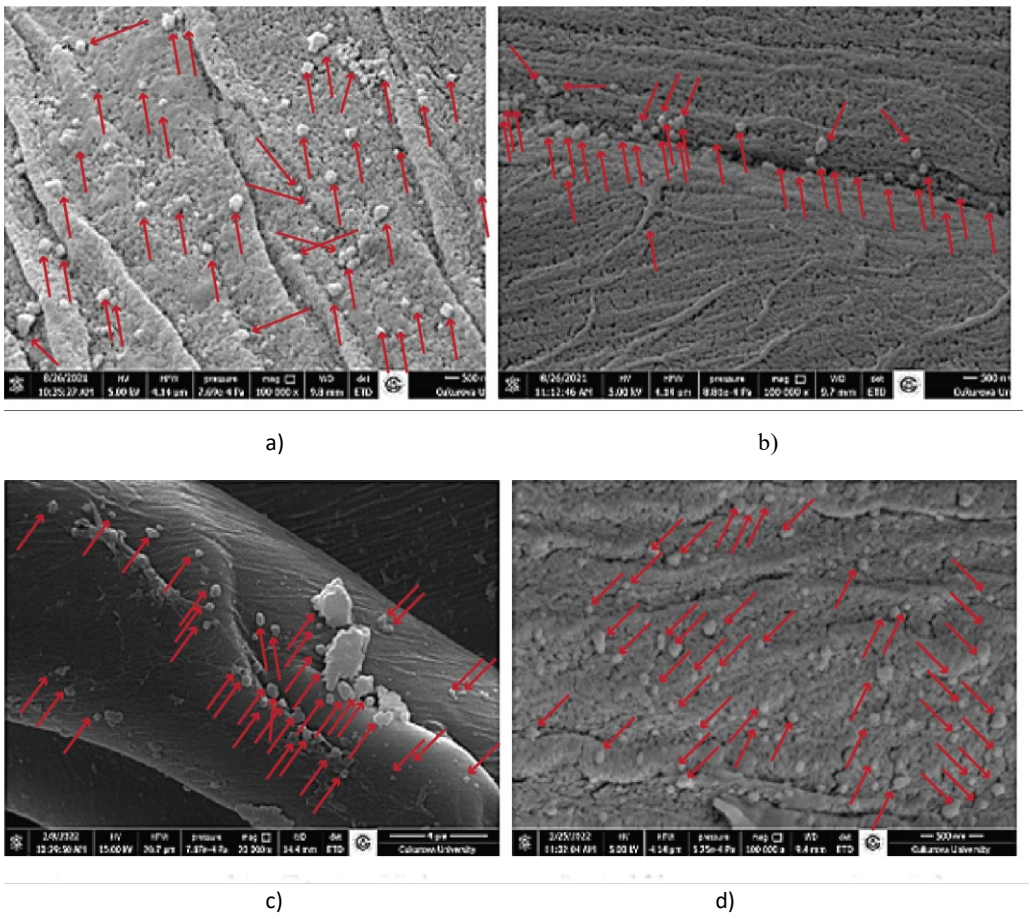
Table 7. Evaluation of proposed equations in antibacterial activity

Sample	Plant source	Method	$AgNO_3$ (M)	$AgNO_3$ Volm	Calculated Plant Ext.	Applied PE	Deviation (μl)	Mean Size (nm)	Antibacterial Activity (%)
CA-1	*C.arvensis*	Padding	0.002	100	153 μl	153 μl	0	45	100
CA-2	*C.arvensis*	immersion	0.002	200	238 μl	230 μl	8	58	100
CA-3	*C.arvensis*	In-situ*	0.001	100	0.5 ml	0.5 ml	0	-	100
OE-1	Orange peel	In-situ*	0.001	100	0.5 ml	2 ml	1500	107	90
OE-2	Orange peel	immersion	0.002	50	105 μl	105 μl	0	53	100
OE-3	Orange peel	immersion	0.002	100	150 μl	115 μl	35	-	99.90

* CA= *Calendula arvensis* , OE= Orange peel Extract, In-situ (GSM= 250, length = 10, width= 10, dilution volume =100)

Table 7 shows that CA-1 and CA-3 have the same plant extract amount according to equation 3 (padding method) and equation 4 (in-situ method), respectively. In both cases, 100% antibacterial activity was found. CA-2 and OE-2 have very low deviation (about 10 µl) but exhibit 100% antibacterial activity. OE-3 also has a slight deviation (35 µl) from the calculated plant extract amount, showing 99.9% antibacterial activity. However, the OE-1 sample has a very high deviation (1500 µl) from the calculated plant extract amount. It creates large nanoparticles (107 nm), which cause low antibacterial activity of 90% since the toxicity of nanoparticles is directly related to the nanoparticle size due to the large surface area [24].

The SEM images were taken to determine the mean nanoparticle size and shape. Figures 12a and 12b show the AgNPs formed from *C.arvensis* plant extracts. The used amount is specified in Table 7. Figures 12c and 12d show the AgNPs synthesized by *C.reticulata* fruit peel extract. The shape of nanoparticles is irregular in all the samples. The mean nanoparticle size ranges from 45 nm to 107 nm. However, when the calculated plant extract deviates conspicuously from the applied plant extract, the nanoparticle size gets larger because a large amount of plant extract will aggregate the nanoparticles and make them bigger.



a) b)

c) d)

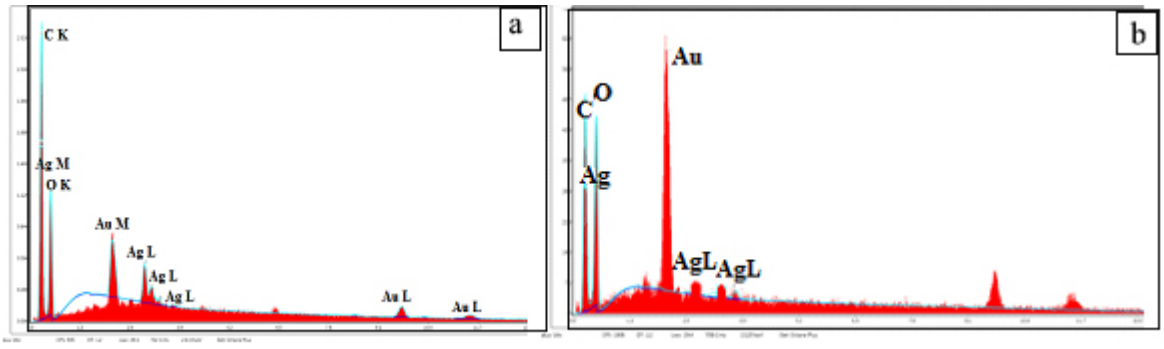
Figure 12. SEM images of different samples, red marks indicate some nanoparticles: (a) SEM image of AgNPs using CA-1; (b) SEM image of AgNPs using CA-2; (c) SEM image of AgNPs using OE-1; (d) SEM image of AgNPs using OE-2

The derived equations worked for both *Calendula arvensis* and *Citrus reticulata* plant extract.

It shows a high deviation of calculated plant extract will increase the AgNP size and decrease the antibacterial activity to a greater extent. In Table 7, the sample OE-1 has the lowest antibacterial activity (90%). Here the applied plant extract (2 ml) is much more than the calculated plant extract (0.5 ml). For the OE-3 sample, the deviation between the applied amount and the calculated amount is about 35 µl. Here the antibacterial activity is a little bit less than 100%. In the case of other samples, the applied plant extract is the same or very close to the calculated plant extract; hence, the antibacterial activity is also higher. The deviation is inversely proportional to the antibacterial activity; in other words, a high deviation is associated with low antibacterial activity. On the contrary, low or no deviation shows 100% antibacterial activity.

EDS

The EDS spectrum (Figure 13) has different peaks for Ag, C, and O; the EDS profile shows Ag peaks, confirming that the AgNPs were formed in the reaction medium for both *C.arvensis* and *C.reticulata* extracts. Further, at around 3 keV, some peaks are typically caused by the absorption of silver nanocrystals due to the Surface Plasmon Resonance (SPR) of AgNPs [25]. The C and O elements indicate the presence of carbon and Oxygen in plant extract and cotton fibre. Other strong peaks are from Au due to the gold plating of the sample before SEM sample preparation. The K and L beside the metal elements represent the shell of the metal.



a) b)

Figure 13. EDS images: (a) EDS of *C.arvensis* synthesized AgNPs (b) EDS of *C.reticulata* peel synthesized AgNPs

X-ray Detraction (XRD) Measurements

XRD is a widely used nondestructive analytical technique to determine the crystalline properties of film and powdered samples. First, the AgNPs-loaded fabric was analyzed using X-ray diffraction. The minimum step size is 2theta 0.0001, and the range of 2θ is 10 to 90. The operation voltage was 40 kV,

and the current was 40 mA with Cu anode. Finally, HighScore plus was used for matching the peaks with reference silver materials. Figure 14 shows an XRD image of green synthesized AgNPs incorporated fabrics. It shows several peaks with a close match to the Silver Fulminate (ICSD:30516).

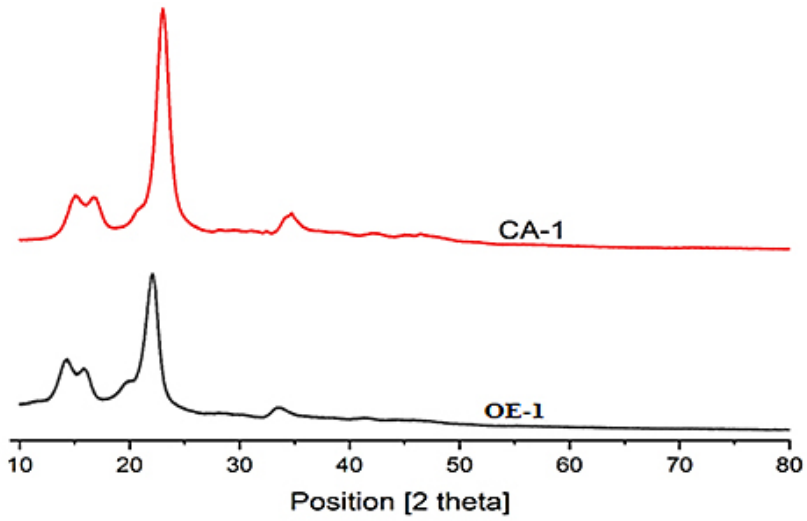


Figure 14. XRD image of green synthesized AgNPs incorporated fabric

The XRD pattern has two regions: from 2θ=10° - 36° and 2θ=36° -80°. The first region represents the cellulosic crystals, and the second represents AgNP crystals [26]. The C.arvensis and C.retulata plants synthesized, having no deviation and high deviation from the calculated plant extract amount, show similar major peaks for cellulose crystalline form. It shows the primary diffraction angle at 2θ = 15.03 and 16.94 for the (010) plane, 2θ = 22.7 for the (002) plane, and 34.5 for the (110) plane. The crystal sizes (D) were calculated by the Scherrer equation where K= 0.90 and λ = 1.540598 (CuKα).

$$D = \frac{K\lambda}{\beta cos\theta}$$

OE-1 (peel extracts amount is 1500 µl more than the calculated amount) has a 3.73 nm crystal size with 31% crystallinity, whereas the CA-1 (plants extract amount is exactly same as the calculation) has a 5.10 nm crystal size and 69% crystallinity. Although the crystal size of OE-1 is small, the SEM image shows that the average size of AgNPs for OE-1 is 107 nm since the high amount of plant extract (1500 µl excess) aggregated the crystals and caused a larger particle size.

CONCLUSION

Synthesizing AgNPs from plant extract needs some special considerations regarding temperature, plant extract amount, silver nitrate concentration, and volume. *Calendula arvensis* extract needs at least 40 °C for synthesizing AgNPs. 55 °C and 60 °C were determined as the optimum temperatures for $AgNO_3$ and plant extract, respectively using Taguchi orthogonal array. For greater stability, low

concentrations (such as 0.002 M or 0.001 M) are preferable with accurate plant extract amount. To determine the appropriate plant extract amount an equation was proposed that utilized Silver nitrate molarity and volume to ensure a lower UV-VIS absorption peak or Surface Plasmon Resonance (SPR). The efficiency of the derived equation was examined by *C.reticulata* (orange) peel extract. The equation was found very efficient in calculating plant extract amount to achieve smaller nanoparticle size and high antibacterial properties for both extracts. The more deviation from the equation causes poor antibacterial performance and larger nanoparticle size due to nanoparticle aggregation.

The equation was tested for 2 plant extracts, more biological extracts are needed to be tried for the derived equation. Besides depth of scholarly peruse is needed to investigate the failure of AgNPs at low temperature.

Author Contributions

Conceptualization – Ahmed T; Methodology – Ahmed T; Formal analysis – Ahmed T; Investigation – Ahmed T; Resources – Gülnaz O; writing-original draft preparation – Ahmed T; writing-review and editing – Ahmed T; Visualization – Ahmed T; Supervision – Ogulata R. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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SUPPLEMENT

Determination of plant extract

```
[ ]: import numpy as np

[ ]: def insitu(m, gsm, l, w):
       v = (gsm/1000)*l*w*5
       return m*v*

[ ]: def insitu(m, gsm, l, w, V):
       v = (gsm/(10**4))*l*w*2.5*V
       return ((m*v*(10 + (np.log(m)/ np.log(v)))*np.log10(v)))/2 # in drop
     insitu(0.001, 230, 10,10, 100)

[ ]: 7.071545053482689

[ ]: def insitu(m, gsm, l, w, V):
       v = gsm/(10**4)*l*w*3*V
       return ((m*v*(10+ ((np.log10(m))/(np.log10(v))))+np.log10(v)))/(0.025*1000)
      #in ml
     insitu(0.002, 254, 10,10, 50)

[ ]: 0.3761629203507831

[ ]: def pe_amt(m,v):
       return  (m*v*(10+ ((np.log10(v))/(np.log10(m))))+np.log10(v))/(0.025) #micro
      liter

[ ]: pe_amt(0.002, 575)

[ ]: 154.07181258987862
```