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RESEARCH ARTICLE

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *MORINGA OLEIFERA*, EMPLOYING MULTIVARIATE OPTIMIZATION METHODOLOGIES.

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Abstract

Multivariate optimization was employed in the synthesis of silver nanoparticles (AgNPs) using a greener approach. The flowers, leaves and stem bark water extracts of *Moringa oleifera* were reacted with silver nitrate solution to form the silver nanoparticles. Four factors namely; extraction time, extraction volume, reaction time and reaction temperature were optimized simultaneously with absorption via ultraviolet – visible (UV-VIS) spectrometry used to follow the optimization, i.e., the response. The results showed that there were some interaction factors and as well as curvature that contributed significantly to the response. The silver nanoparticles (AgNPs) showed ultraviolet visible (UV-Vis) absorption peaks at 415, 426 and 420 nm for AgNPs synthesized from the flowers, leaves and stem bark water extracts, respectively. The three different plant parts of *Moringa oleifera* also produced AgNPs of a spherical shape as observed through a scanning electron microscope (SEM). The average sizes of AgNPs obtained when using the flowers, leaves and stem bark extracts were 273.98 nm, 96.72 nm and 95.12 nm, respectively. The stem bark extract produced better NPs in terms of uniform dispersity (mono-dispersed), while the flowers and leaves produced poly-dispersed NPs.

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Introduction:-

Silver (Ag) is commonly referred to as a noble metal due to its inertness. Its use in nanotechnology has received a substantial amount of focus; such as producing silver nanoparticles (AgNPs) for use in various fields such as textile, catalysis, sensing, optics, antibacterial activity and data storage [1]. The AgNPs do not naturally occur hence various techniques have been developed to synthesise them. These include chemical reduction, photochemical reduction, electrochemical reduction, heat evaporation and biological techniques [2]. The latter technique involves the use of plant extracts and micro-organisms for synthesis and as such has been deemed a greener approach towards synthesis of AgNPs. Greener synthesis of NPs provides advancement over other methods as it is simple, cost effective and relatively reproducible and often results in more stable materials [3].

The use of plants for synthesis of AgNPs, however, is advantageous because it does not require elaborate processes such as intracellular synthesis and multiple purification steps or maintenance of microbial cell cultures [4], as is the case with micro-organism oriented NPs production. A number of plants have been used for synthesis of NPs. *Moringa oleifera* (MO) is one such plant. The plant is a member of the *Moringaceae* family, native to India and Pakistan, but widely cultivated in the Middle East, Africa and Southern Asia as a multipurpose crop [5]. The bark

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extract has been shown to possess antifungal, anti-tubercular activity; the extracts of the leaves, seeds and roots have been studied for wound healing antihepatotoxic, antifertility, hypotensive and analgesic activity [6]. The seeds have also been used for removal of heavy metals in aqueous solutions [7], as well as in flocculation studies [8].

In the production of AgNPs, however, research that has been put forth has followed the traditional one-variable-at-a-time (OVAT) approach towards optimization of synthesis of the NPs [9 - 14]. This is where only one variable that affects the experimental response is changed, while the others are kept at a constant level [15]. This approach however, has some disadvantages: i) interactions between factors are not taken into account; ii) many experiments are needed when the number of factors increases; iii) only a small part of the experimental domain is examined; iv) the global optimum might not be found; and v) the found optimal conditions might depend on the starting conditions [16]. Another disadvantage is the increase in the number of experiments necessary to conduct the research leads to an increase of time and expenses as well as increase in consumption of reagents and materials [15]. Hence, the shift towards multivariate optimization.

Multivariate optimization occurs in two phases: screening and response optimization. The screening phase is done as a prelude to an optimization to make sure that the variables being investigated do indeed significantly contribute to the response [17]. It can be carried out through the use of a factorial design or a fractional factorial design. This step identifies only those variables that have a major effect on the experimental response, thus eliminating the less significant and selecting the significant few. Screening designs are thereby capable of giving the main effects as well as the interaction effects [17], of the variables under investigation. The relationship between each variable ($X_1, X_2, X_3 \dots X_i$) and the experimental response (y) is investigated by performing a regression. The mathematical model as such will take the form shown by equation 1 [18].

$$y = b_0 + b_1X_1 + b_jX_j + b_{ij}X_iX_j \quad (1)$$

Where y is the response, b is a constant and X_i and X_j are the input variables. The interaction effect is represented by the term X_iX_j

Following the screening phase is the response optimization phase. This stage is commonly referred to as response surface methodology (RSM). At this stage all the significant variables are evaluated for their optimum set-points. This step is achieved through the use of either a central composite design (CCD) or a Box-Behnken design (BBD). A polynomial model describing the relation between the response and considered variables is built, with the model usually adopting a second-order polynomial, which caters also for curvature (equation 2) [16].

$$y = b_0 + b_1X_1 + b_jX_j + b_{ij}X_iX_j + b_{ii}X_i^2 + b_{jj}X_j^2 \quad (2)$$

Curvature is accounted for by the terms X_i^2 and X_j^2 .

Herein we report for the first time, to the best of our knowledge, the use of multivariate optimization methodologies, towards synthesis of AgNPs. The multivariate approach offers a fairly simpler and cost-effective route towards synthesis because all experimental variables are varied at the same time, spanning over the whole experimental domain, resulting in fewer performed experiments [19]. The optimum conditions found with this approach are also independent of the starting conditions, as such a more global optimum is found [16].

Experimental:-

Materials and methods:-

Silver nitrate ($AgNO_3$; 99.8%) was purchased from Glassworld, South Africa. Methanol (99.5 % assay) was obtained from Rochelle chemicals, South Africa. *Moringa oleifera* flowers, leaves and stem bark were collected from the National Health Laboratory in Gaborone, Botswana. All solutions were prepared using deionized water produced from a Millipore water purification system obtained from Germany. Multivariate optimization was carried out using Minitab release 14 statistical software.

Plant extracts preparation and AgNPs synthesis:-

After collection, the plant materials were washed 3 times with deionized water then allowed to air dry. To make the plant part extract: 1 g of each plant part was extracted with 100 mL deionized water for the stipulated time (see Table 1).

Factors/variables that were envisaged to affect AgNPs synthesis were identified as plant extraction time, extract volume, reaction time and reaction temperature low levels and high levels (i.e., the ranges) are as shown in Table 1. To produce AgNPs, the plant extract was reacted with 1 mM AgNO₃ by varying the factors shown in Table 1, following the experimental matrix shown in Table 2.

Table 1:-Two-level ½ fraction factorial design data showing the experimental factors and levels used for the screening of AgNPs synthesis

Variable	Factor	Low level	High level
A	Extraction time (minutes)	5	30
B	Extract volume (mL)	2	10
C	Reaction time (minutes)	10	60
D	Reaction Temperature (°C)	25	85

Optimization of AgNPs synthesis:-

A 2-level ½ fraction factorial design was set up using the data shown in Table 1 for screening of significant variables. Table 2 shows an experimental matrix with the response (in this case, absorbance) for each run for stem bark. Similar matrices were produced for the other plant parts. The experimental matrix in each case, was used for screening purposes. Two replicates were carried out for each of the plant parts used for synthesis of AgNPs. A similar set up was used to create a central composite design (CCD) for optimization of the variables. The experimental matrix in this case had a total of 62 runs to be performed (inclusive of the two replicates), of which upon analysis produced an optimized figure for each parameter.

Table 2:-The two-level ½ fraction factorial experimental design and yields (in terms of absorbance) for screening of factors when using the stem bark extract for AgNPs synthesis

Run Order	Extraction time (minutes)	Extract volume (mL)	Reaction time (minutes)	Reaction temperature (°C)	Absorbance
1	30	2	10	85	0.040
2	5	10	60	25	0.021
3	5	2	10	25	0.007
4	5	2	10	25	0.005
5	5	2	60	85	0.045
6	30	10	10	25	0.029
7	5	10	60	25	0.017
8	30	2	60	25	0.031
9	30	10	60	85	0.137
10	5	2	60	85	0.031
11	30	2	10	85	0.030
12	30	10	60	85	0.122
13	30	2	60	25	0.015
14	30	10	10	25	0.031
15	5	10	10	85	0.078
16	5	10	10	85	0.052

UV-Vis analysis:-

UV-Vis absorption measurements of the synthesized NPs were carried out using a double beamed spectrophotometer (Evolution 201; Thermo Scientific, USA). The analysis range was from 250 – 700 nm, with ultrapure water used as the blank for each analysis. Absorbance measurements were the primary means used to follow the optimization processes.

Scanning electron microscopy-energy dispersive X-ray (SEM-EDX) analysis:-

A Philips XL 30 ESEM coupled with EDX was used to deduce the size, morphology and elemental composition of the nanoparticles (NPs) synthesised from optimized conditions of each factor. Prior to SEM-EDX analysis, the synthesised NPs were centrifuged at 4000 rpm for 15 minutes. The supernatant was decanted off then the NPs were washed three times with ultrapure water. This was achieved by addition of the ultrapure water to the 10 mL mark of the centrifuge tube then vortexing for 2 minutes to re-disperse the NPs. This was followed by centrifuging again at

4000 rpm for 15 minutes. After washing with ultrapure water, the NPs were washed twice with methanol. Washing with methanol was done in order to remove the water molecules thereby speed up the process of drying of the NPs on the SEM grid. A drop of the NP solution was then placed on an SEM grid, and then allowed to air dry. An E6700 Polaron range high vacuum evaporator sputter coater (Quorum Technologies, UK) was used to coat the NPs with carbon. These were then taken for SEM-EDX analysis, which operated under high vacuum and beam acceleration voltage of 20 kV (the recommended operating voltage for material samples).

Results and Discussion:-

Multivariate optimization:-

Fractional factorial design:-

Analysis of the outcome (yield) of the experiments performed when the stem bark extract was used for synthesis are shown by Figure 1. A normal probability plot of standardized effects shows the magnitude of the main effects of each factor as well as the effects brought about by the interaction of factors, towards the obtained yields. The magnitude of each type of effect is represented by its distance from the solid line, as well as the side on which the effect lies with respect to the solid line. Negative effects lie to the left while positive effects lie to the right of the solid line. The solid line indicates where the points would fall if the effects were zero, while the percentage in the y-axis signifies the weightage of each factor's contribution towards the obtained yield. All variables investigated had a significant contribution towards the yields obtained after performance of each experiment. Reaction time had the greatest contribution as it lay furthest to the right, with a weightage of ~90%; while extraction time contributed the least, with a weightage of ~10%. The design also revealed contributory effects of interaction between extraction time-extract volume (AB), extraction time-reaction time (AC), as well as extraction time-reaction temperature (AD). As such all four variables were used to create a CCD to obtain optimum set points of each variable for each plant part. Similar outputs were obtained when the leaves and stem bark extracts were used for synthesis.

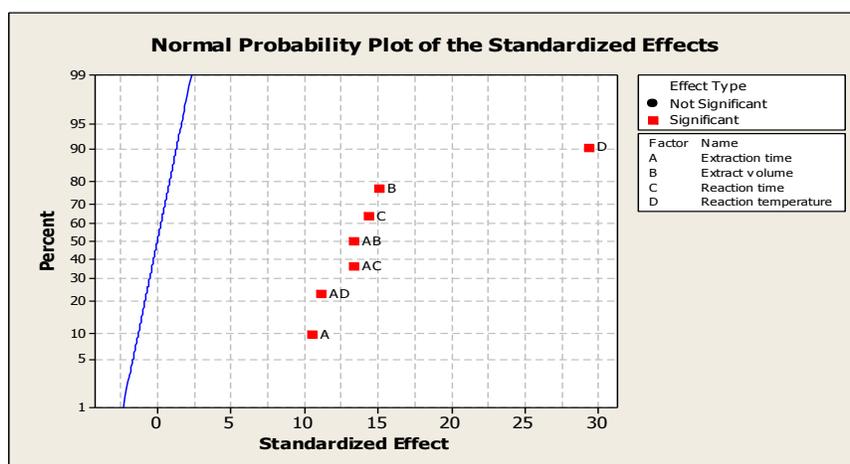


Figure 1:-Normal probability plot of the standardized effects when using stem bark extract for AgNPs synthesis

The statistical significance of the model applied was evaluated through use of Fisher distribution (F-test) for analysis of variance (ANOVA) to validate the linear model at 95% confidence level. Table 3, shows the ANOVA output obtained when using the stem bark extract for AgNPs synthesis; both the main effects and interaction effects were significant (p -value < 0.05). The regression coefficient, R^2 , was also used to assess the fit of the model to the experimental data. The values were found to be 99.58%, 91.74% and 96.62% when using the flower, leaf and stem bark extracts respectively. This suggested that the model fit adequately to the experimental data. For a good fit of a model, the regression coefficient is suggested to be at least 80% [20].

Table 3:-ANOVA table for the yields obtained from the $\frac{1}{2}$ fractional factorial design for AgNPs synthesis using stem bark extract

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	4	2.859	2.859	0.7147	353.9	0.000
2-Way Interactions	3	0.9777	0.9777	0.3259	161.4	0.000
Residual Error	8	0.01615	0.01615	0.002019		

Pure Error	8	0.01616	0.01616	0.002019		
Total	15	3.853				

Central composite design (CCD):-

For each plant part, the factors that were found to significantly affect the response from the screening phase, were used to create a response surface design in order to determine the optimum conditions of each factor. This was achieved through the use of a CCD. Figure 2 shows a three dimensional (3D) surface graphs visualizing the interaction of the variables and the response given. These types of plots show the response in three dimensions, thus, if there are three or more variables, the plot visualization is possible only if one or more variables are set to a constant value [15]. The plots show a high response value (absorbance in this case) towards the extremities of each factor, adopting a curvilinear shape in accordance with the quadratic model fitted.

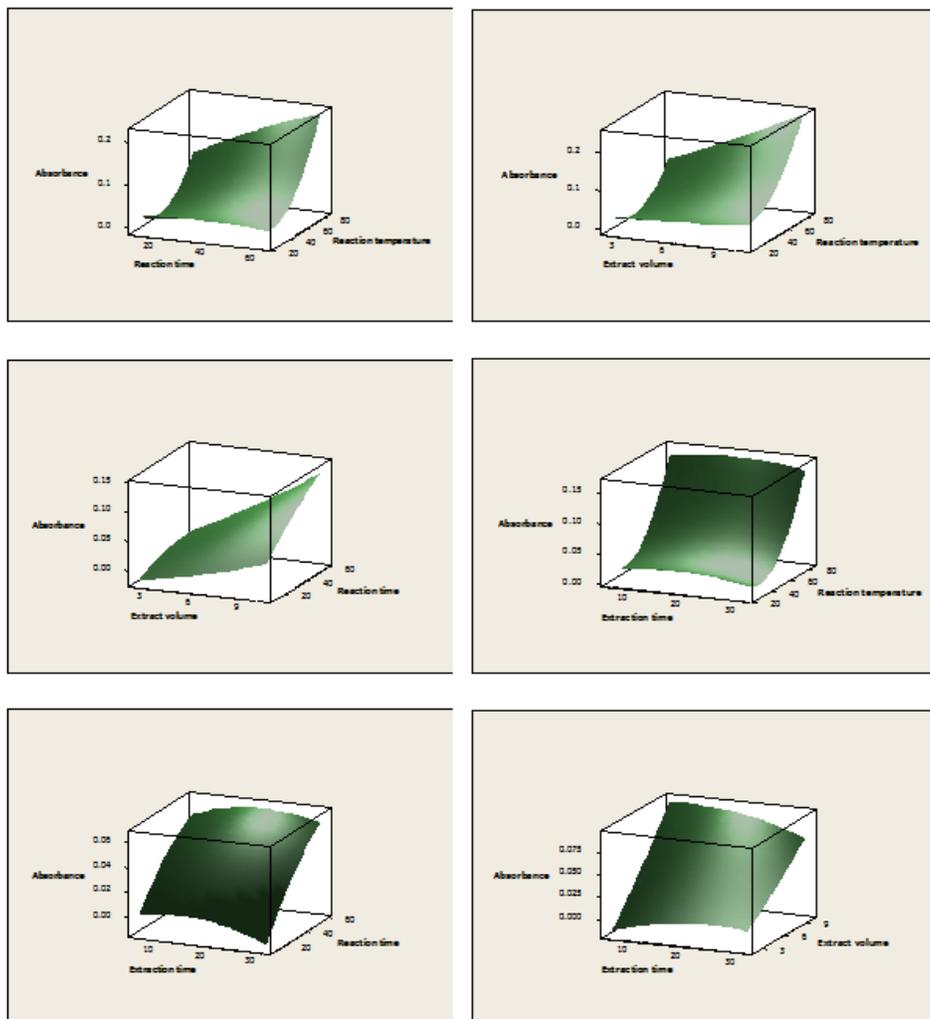


Figure 2:-Response surface plots of the yield (absorbance) obtained due to interaction of factors when stem bark extract was used for AgNPs synthesis.

Table 4 shows the desirable or D values along with the optimum conditions for the variables investigated following the use of response optimizer. The response optimizer is a tool that enables identification of the optimum conditions of each factor under investigation, to yield a desirable response. This brings in the concept of the desirability function (D). Its aim is to find operating conditions that ensure compliance with the criteria of all the involved responses and, at the same time, to provide the best value of compromise in the desirable joint response [21]. Desirability ranges between 0 (representing an undesirable response) and 1 (a desired or ideal response). For AgNP synthesis using the flowers leaves and stem bark extracts, the set conditions gave D values of 1.00, 0.991 and 0.999.

This signified that at the set conditions of each factor, their combination would result in obtaining the highest response. Hence, these were highly desirable conditions.

Table 4:-Optimum conditions and D value for each variable for each plant part

Plant part	Variable				D value
	Extraction time (min)	Extract volume (mL)	Reaction time (min)	Reaction temperature (°C)	
Flower	22	10	60	85	1.00
Leaf	5	10	40	85	0.991
Stem bark	11	10	60	85	0.999

Table 5 presents the results of the CCD showing the magnitude of each factor, interactions and the quadratic terms, which is presented with equation 3 (derived from equation 2, coefficients of which were shown in Table 5), where x_1 is the extraction time, x_2 is the extract volume, x_3 is the reaction time and x_4 is the reaction temperature; y represents the response, respectively.

Table 5:-Estimated regression coefficients for the yields obtained when using stem bark extract for AgNPs synthesis

Term	Coef	SE Coef	T	P
Constant	0.0412	0.00440	9.37	0.000
Extraction time	-0.00191	0.00350	-0.548	0.586
Extract volume	0.0431	0.00350	12.3	0.000
Reaction time	0.0309	0.00350	8.83	0.000
Reaction temperature	0.0676	0.00350	19.3	0.000
Extraction time*Extraction time	-0.00840	0.00921	-0.912	0.366
Extract volume*Extract volume	0.00410	0.00921	0.445	0.658
Reaction time*Reaction time	-0.00740	0.00921	-0.804	0.426
Reaction temperature*Reaction temperature	0.0543	0.00921	5.90	0.000
Extraction time*Extract volume	-0.0100	0.00371	-2.71	0.009
Extraction time*Reaction time	0.00416	0.00371	1.12	0.268
Extraction time*Reaction temperature	0.000656	0.00371	0.177	0.860
Extract volume*Reaction time	0.0175	0.00371	4.71	0.000
Extract volume*Reaction temperature	0.0350	0.00371	9.43	0.000
Reaction time*Reaction temperature	0.0320	0.00371	8.64	0.000

S = 0.02098 R-Sq = 94.9% R-Sq(adj) = 93.4%

$$y = (41.2 - 1.92x_1 + 43.1x_2 + 30.9x_3 + 67.6x_4 - 8.40x_1^2 + 4.10x_2^2 - 7.40x_3^2 + 54.4x_4^2 - 10.0x_1x_2 + 4.16x_1x_3 + 0.656x_1x_4 + 17.5x_2x_3 + 35.0x_2x_4 + 32.0x_3x_4) \times 10^{-3} \dots \dots \dots (3)$$

However, to assess the significance of the contribution that each effect had on the response, a p-value for each effect was used. For this work a p-value of 0.05 was used and any effect that shows a value less than 0.05 shows that it significantly affected the response and above 0.05 shows it did not affect the response significantly. Therefore, from Table 5, all the main effects except, extraction time (x_1) significantly affected the response; all second order interactions terms except extraction time*reaction time (x_1x_3) and extraction time* reaction temperature (x_1x_4) were significant while for the quadratic term for only the reaction temperature, i.e., reaction temperature*reaction temperature (x_4^2) was significant. Based on this, the full quadratic model showing only the significant variables is shown by in applied to the data obtained from the AgNPs synthesised from the stem bark extract showing only the significant terms is represented by equation 4. Similar equations and tables for flowers and leaf extracts were obtained.

$$y = (41.2 + 43.1x_2 + 30.9x_3 + 67.6x_4 + 54.4x_4^2 - 10.0x_1x_2 + 17.5x_2x_3 + 35.0x_2x_4 + 32.0x_3x_4) \times 10^{-3} \dots \dots \dots (4)$$

Therefore, time taken to extract the plant material that was reacted with the silver salt to form AgNPs, did not a significant effect on the formation of AgNPs. The reaction temperature played a significant role as it contributed to

the curvature (a maxima) in the optimization process and the other four interaction terms – these would not have been picked using OVAT optimization strategy.

The R^2 values were found to be 94.9%, 95.4%, 84.9% and for when using the stem bark, flowers, and leaf extracts for the synthesis of AgNPs, respectively. This indicated the adequacy of the quadratic model used. The lack-of-fit test was also used to further examine the adequacy of the model. The p-value of the LOF test must be less than the significance level (α) for there to be an insignificant LOF (Noordin *et al.*, 2004). An insignificant LOF implies that the model accounts for the errors brought about by the regressor-response relationship. In this work, α was 0.05. The p-value for the LOF was found to be 0.000 for the stem bark extract, 0.001 for the leaves extract and 0.000 for the flower extract.

UV-Vis analysis:-

Particle shape, size and aggregation affect the absorption spectra of NPs [22]. The region of between 400 – 450 nm has been shown to be the characteristic region at which silver plasmons resonate [23 - 26], giving strong absorbance peaks in that region. Figure 3 shows the absorption spectra of the synthesized AgNPs using the optimized conditions from the three plant parts. AgNPs obtained from the flower extract showed the characteristic peak at 415 nm [Figure 3(a)]; while from the leaves extract the peak was located at 426 nm [Figure 3(b)]; and lastly from the stem bark extract the peak was located at 420 nm [Figure 3(c)]. Occurrence of the peaks centered at around 420 nm also suggested that the general shape of the NPs obtained was spherical. Small spherical nanoparticles exhibit a single surface plasmon band at small wavelengths, whereas large anisotropic particles reveal two or three bands at longer wavelengths [27]. A similar observation was made by Sathyavathi *et al.* [28], Prasad and Elumalai [29], whereby AgNPs was synthesized using *Moringa oleifera* leaf extract using the OVAT approach. Both works [28, 29] showed AgNPs that were spherical in shape.

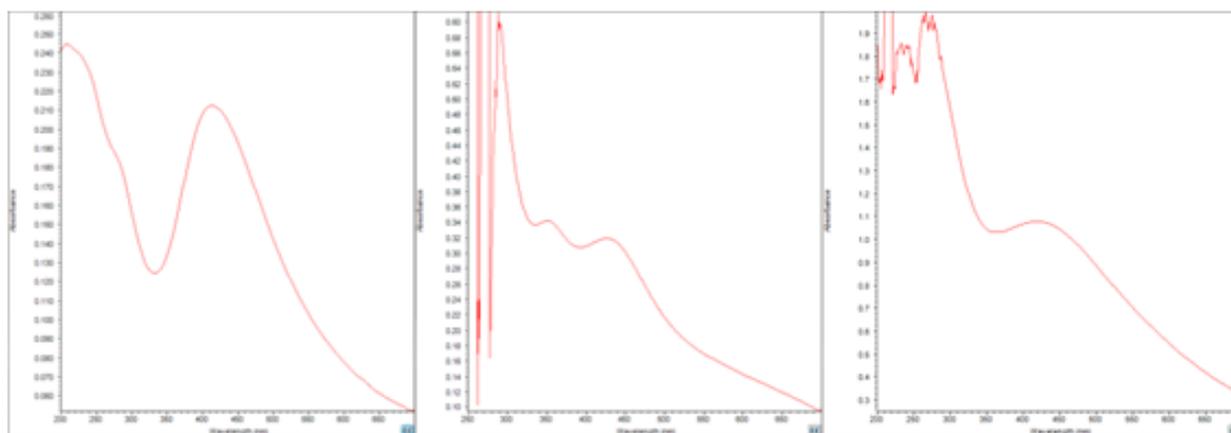


Figure 3:-UV-Vis absorption spectra of AgNPs synthesized from (a) flowers (b) leaves and (c) stem bark extracts

Scanning Electron Microscopy (SEM) analysis:-

The morphological characteristics of the synthesized AgNPs were observed using a SEM. The images obtained revealed that the NPs were spherical in shape, as shown by Figure 4. Well defined images, in terms of resolution and shape elucidation were obtained at a magnification of 15000x. The average sizes of the AgNPs were found to be 273.98 nm, 96.72 nm and 95.12 nm when using the flowers, leaves and stem bark extracts for AgNPs synthesis, respectively. Figure 4 also revealed that amongst the three, the stem bark mediated AgNPs were of uniform dispersity. The dispersity of the flowers and leaves AgNPs was not uniform, showing varying sizes of the NPs obtained.

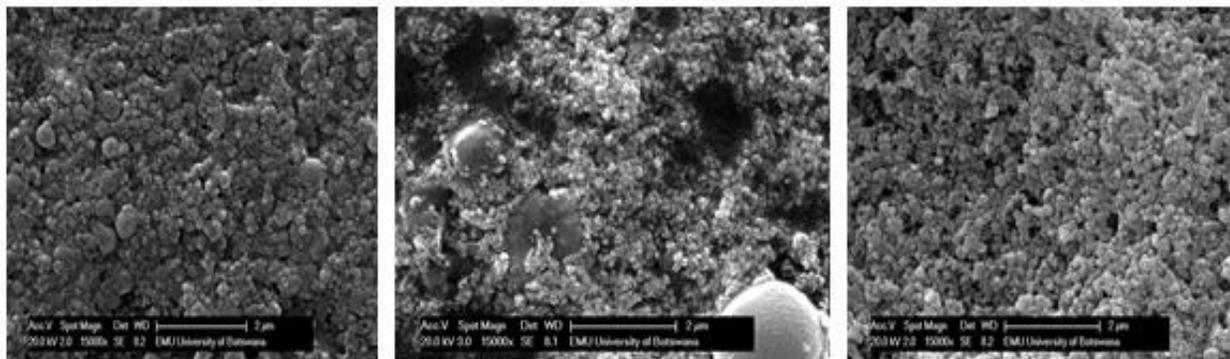


Figure 4:-SEM micrographs of AgNPs obtained when using (a) flowers, (b) leaves and (c) stem bark extracts for synthesis

Energy-dispersive X-ray spectroscopy (EDX) analysis:-

Results obtained from EDX analysis are shown by Figure 5. The spectra show the most prominent peak as the silver peak, and thus confirmed the formation of AgNPs. The second prominent peak was that of carbon, which came about as a result of the carbon coating that was done to prevent charging of the sample prior to SEM-EDX analysis. Figure 5(c) shows another significant peak due to chlorine. This may have been brought about by the plant material, eventually ending up being adsorbed onto the surface of the NPs.

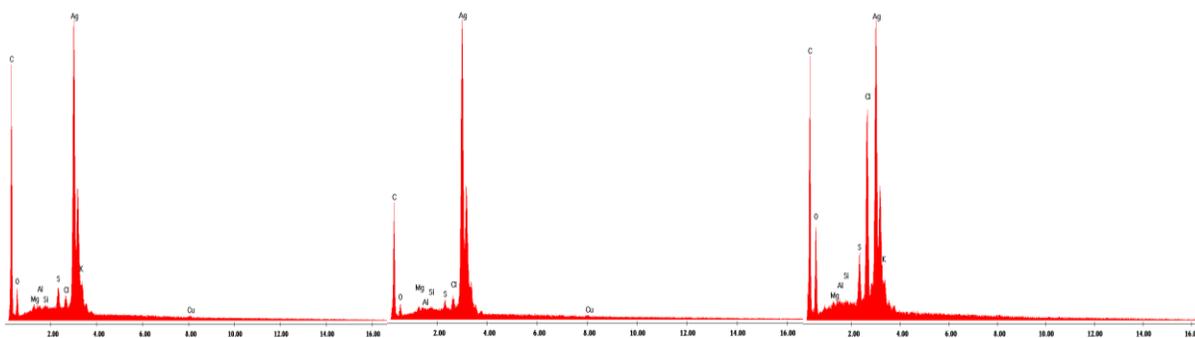


Figure 5:-EDX spectra of the AgNPs obtained when using (a) flowers, (b) leaves and (c) stem bark extracts for AgNPs synthesis

Conclusion:-

The present work demonstrated the capability of employing multivariate optimization for a greener approach of AgNPs synthesis. There were interaction terms and quadratic terms that significantly affected the response – these would not have been observed when using one-variable at a time (OVAT) optimization approach. As such a more global optimum was obtained and the optimized values synthesized relatively good AgNPs of spherical shape, with average sizes of 273.98 nm, 96.72 nm and 95.12 nm for AgNPs obtained when using the flowers, leaves and stem bark extracts, respectively. The stem bark extract produced better NPs in terms of uniform dispersity (mono-dispersed), while the flowers and leaves produced poly-dispersed NPs.

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