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Green Synthesis of Silver Nanoparticles with *Dalbergialatifolia* Plant Leaves as Reductant and Its Antimicrobial Properties

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Abstract: ***Green synthesis of nanoparticles has evolved as a low-cost environment friendly, non-toxic and large scale up process. This investigation was carried out on the synthesis of AgNPs using Dalbergialatifolia (DL) aqueous plant leaves extract for the bioreduction of silver ions to nanoparticles, with $AgNO_3$ as the precursor. The bioreduction process was carried out varying certain operational parameters; pH, reaction time, $AgNO_3$ concentration and ratio of extract to silver nitrate solution. UV-Vis spectrophotometer was used to monitor the reaction. Characterization of the synthesized AgNPs was done using Ultravisible/Visible Spectrophotometer (Uv/Vis), Fourier Transform Infra-Red (FTIR), X-Ray Diffraction (XRD) and Scanning Electron Microscope (SEM) analyses. The surface plasmon resonance (SPR) band for the AgNPs was observed to be at 450nm. The optimum conditions for the synthesis of AgNPs with the plant extract were obtained to be; 45 minutes synthesis time, acidic pH, 1mM $AgNO_3$ concentration and ratio 1:1 of plant extract to $AgNO_3$ solution. FTIR analysis for the plant extract revealed the presence of O-H, C=O, C-H, C-N, N-H functional groups as well as the benzyl ring which are found in compounds like flavonoids, phenols and terpenoids. The characteristic peaks for these functional groups were also observed in the synthesized AgNPs, but had almost disappeared, which may indicate that these compounds, present in the plant extract were responsible for efficient reduction, capping and stabilization of the obtained AgNPs. SEM analysis revealed that the synthesized AgNPs were quassi spherical in shape and uniformly distributed. The XRD patterns of the AgNPs revealed Braggs reflections representing b111N, b200N, b220N and b311N planes for fc crystal structure for metallic silver with reference to the JCPDS data. Antimicrobial activity of the nanoparticles was studied against; Staphylococcusaureus, Esterichiacoli, Bacillussubtilus, Pseudomonasaeruginose, Klebsiella, Salmonellaetyphi, Candidaalbican, Aspergillusniger, Rhizophusstolomite and Penicilliumnotatum which in turn showed good inhibition against the microbes at both high and low concentrations. Dalbergialatifolia plant leaves extract proves to be a good option as reductant for AgNPs synthesis, and the synthesized AgNPs were found to be very effective antimicrobial agent.***

Keywords: Dalbergialatifolia, Bioreduction, Silver nanoparticles, Surface Plasmon Band, Microorgarnisms.

I. INTRODUCTION

Manipulations and creations of materials at nanometre sizes (1 – 100nm) is scientifically regarded as nano-technology. At this scale, there are significant differences in material properties such as physicochemical, magnetic and optoelectronic properties that are governed by their size and shape distribution (Daniel *et al*., 2004). It is predominantly, the extremely small size of the nanoparticles and their large surface area to volume ratio that leads to the significant differences in their biological and catalytic activities, their mechanical properties, as well as their thermal and electrical conductivity, which are not observed in the same bulk materials (Philip 2010). Traditionally, nanoparticles have been synthesized and stabilized using physical and chemical techniques. The physical approach includes techniques like laser ablation (Zamiri *et a*l., 2011), lithography (Zhang *et al*., 2008), and high energy irradiation (Chem *et al* 2001). The chemical approach on the other hand uses techniques such as chemical reduction, electrochemistry and photochemical reduction (Chen *et al*., 2006). Green synthesis of nanoparticles involves the techniques for obtaining nanoparticles using naturally occurring reagents such as vitamins, sugars, plant extracts, biodegradable polymers, and microorganisms as reductants and capping agents as against the inorganic chemical approach. Environmental degradation and pollution abatement has led researchers to focus on 'green chemistry'. Utilization of nontoxic chemicals, of environmentally benign solvents and of renewable materials are some of the key issues that merit important consideration in a green synthesis strategy.

Furthermore, green synthesis represents an economic alternative for chemical and physical methods of synthesizing nanoparticles. It employs unicellular and multicellular biological entities such as plants (Philip, 2010), bacteria (Moulton 2010), viruses (Moneteiro *et al*., 2009), and yeast (Vidhu and Philip 2014). Studies have shown that size, shape, stability and physicochemical properties of the nanoparticles are strongly influenced by a variety of process parameters (temperature, concentration, pH and time), and process kinetics involving the interplay between the metal ion precursors and the reducing agents, adsorption kinetics involving the stabilizing agent and the nanoparticles.

Biosynthesis is a process that effectively controls the size, shape, stability and physicochemical properties of materials. It is fast, cost effective and is relatively easy to scale up for the production of large quantities of nanoparticles (Thakkar, 2010). It is also paramount because they contain reducing agents such as citric acid, ascorbic acid, flavonoids, reductases and dehydrogenases and extra-cellular electron shuttlers that play an important role in biosynthesis of metal nanoparticles (Iravani, 2011). Gold and silver nanoparticles (AgNPs) are two prominent inorganic nanoparticles synthesized through plants. Gold nanoparticles have attracted significant interest due to their size, shape and surface properties. As a result of these properties, gold nanoparticles have been investigated for potential application in fields such as biosensors (Chen et al., 2006), in delivery platforms for therapeutic drugs and genetic properties (Ghosh *et al.,* (2002) and as antibacterial drugs (Paciotti *et al*., 2006). Gold nanoparticles are of a considerable interest, but to a much lesser degree than AgNPs (Gurunathan 2010). AgNPs is effective against microbes, acting on a broad range of target sites both extracellularly and intracellularly. Silver, both as a metal and in ionic form, exhibits strong cytotoxicity towards a broad range of microorganisms, and its use as an antibacterial agent is well known (Krutyakov al., 2008; Quang*et al*., 2013). It mode of antibacterial action is similar to that of silver ion. However, the effective biocidal concentration of AgNPs is at a nanomolar level in contrast to a micromolar level of silver ions. Silver has an oligodynamic effect, it is capable of causing a bacteriostatic (growth inhibition) or a bactericidal (antibacterial) impact (Sharma*et a*l., 2005). Research has indicated that silver is also effective in purification systems for disinfecting water or air (Zhang *et al*., 2004). AgNPs are found to possess both anti-bacterial and anti-inflammatory properties that can promote faster wound healing, and because of this, they have been integrated into commercially available wound dressings, pharmaceutical preparations and medical implant coatings (Schluesener 2008).

This research focused on the synthesis of AgNPs using aqueous extracts of *Dalbergialatifolia* leaves as bioreductant; determination of the optimum operational variables such as concentration, pH, ratio of plant extract volume to precursor and time on AgNPs synthesis from the plant extract; characterization of the synthesized AgNPs using UV-Vis and FTIR, SEM and XRD and the evaluation of the antibacterial and antimicrobial properties of the synthesized AgNPs.

II. MATERIALS AND METHOD

$AgNO_3$ of analytical grade was utilized. Leaves of the plant sample were collected from the Botanical garden of the University of Ibadan and were thoroughly washed under running tap water and in distilled water for further purification. 50 g of the cleaned fresh leaves of the plant sample were weighed out and cut in to smaller pieces, 500mL of distilled water ratio 1:10 (w/v) of plant extracted to distilled water was added. The beaker was heated in a temperature regulated steam bath of 70 ^{0}C for 20 minutes to obtain aqueous extracts of the plant sample. The extract was filtered using Whatman No.1 filter paper and kept in a refrigerator at a temperature of 4 ^{0}C for the nanoparticle synthesis. Bulk plant extract was prepared by boiling thoroughly cleaned plant leaves in distilled water in the ratio 1:4 (w/v) in a Clifton temperature regulated water bath at 70 ^{0}C for 20 minutes. The aqueous extract obtained was concentrated using BUCHI Rotavapour 120 rotary evaporator and air dried for 3 days to obtain dry samples of the extracts for FTIR analysis.

A. Synthesis of Silver nanoparticles

150 mL of the plant extract was put into a big conical flask (350 mL) and 150 mL of 1mM $AgN0_3$ was added, (ratio 1:1 v/v). The mixture was placed on a Stuart heat stir magnetic heater and heated to boiling with continual stirring for 1 hour. Reduction of $AgN0_3$ to silver ion was confirmed by change of colour in the reaction medium. Furthermore, confirmation of AgNPs synthesis was carried out by spectrophotometric determination using Perkin Elymer lambda 25 spectrophotometer.

B. Influence of operational variables

1) Effect of Time

Time intervals of 0 min, 5min, 15 min, 30 min, 45 min, and 60 min were employed to measure the effect of time on the AgNPs synthesis by the plant extract. At these time intervals the absorption was observed using the Uv/Vis spectrophotometer. A plot of the absorbance versus the wavelength was made from the generated data.

2) Effect of Concentration

The effect of concentration was observed by using three different concentrations (1mM, 2mM and 3mM) of $AgN0_3$ for the AgNPs synthesis. Each concentration of $AgN0_3$ was added to the plant extract in the ratio 1:1 (v/v), in a conical flask and placed on the Stuart heat stir magnetic heater and brought to boil for 1hour. The formation of AgNPs was confirmed by a change in colour and the absorbance was taken using the Uv/Vis spectrophotometer. A plot of the absorbance versus the wavelength was made from the generated data.

3) Effect of ratio of extract to Silver nitrate solution

The volume of the plant extract was kept constant while that of the $AgN0_3$ solution was varied. 1mM $AgN0_3$ was used. Three ratios were observed, namely; ratio 1:1, 1:2 and 1:3. The plant extract and 1mM $AgN0_3$ were mixed in these three ratios and the adopted process in this study was used to synthesise the AgNPs. The various absorbance values were measured at the end of 1 hour using the UV/Vis spectrophotometer. A plot of the absorbance versus wavelength was made from the generated data.

4) Effect of pH

The effect of pH on the synthesis of AgNPs from the plant sample was studied usingthree pH values of 4,7 and 12. All the pH values used fall within the range of acidic, neutral and alkaline. The usual process adopted in this study for the synthesis of AgNPs was used using the different pH media for the plant extract and 1mM $AgN0_3$ in the ratio 1:1. The absorption was scanned at the end of 1 hour using the Uv/Vis spectrophotometer.

C. Characterization Techniques

1) Uv-Vis spectra analysis

The silver nanoparticles were confirmed andmonitored by measuring the absorbance of the reaction mixture within the wavelength range of 190 – 900 nm in a 2mL quartz cuvette with 1cm path length. The data obtained were plotted on a graph with absorbance values on the y-axis and wavelength on the x-axis.

2) FTIR Spectroscopy (Fourier TransformInfra red Spectroscopy)

The characterization of functional groups on the surface of AgNPs by the plant extracts was investigated using the FT-IR analysis (Perkin Elmer Spectrum BX) and the spectra was scanned in the range of 4000 – 400cm^{-1} at a resolution of 40cm^{-1}. The samples were prepared by dispersing the AgNPs uniformly on a matrix of dry KBr and compressed to form an almost transparent disc.

D. Antimicrobial analysis

100mg of the sample was weighed and dissolved into 1mL of the solvent of extraction for proper dissolution, from which half of it was taken into another 1mL of the solvent, and this was taken to the 5^{th} test tube. The 6^{th} and 7^{th} test tubes were negative control (solvent) and positive control (gentamycin 10mg/ml for bacteria and Toconazole 70% for fungi) for the experiment. The pour plate method was used for the antibacterial analysis. An overnight culture of each organism was prepared by taking a loop full of the organism from stock and inoculated each into the sterile nutrient broth of 5mL, each incubated for 18-24 hours at 37^0C. From overnight culture, 0.1mL of each organism was taken and put into 9.9mL of sterile distilled water to get 1:100 (10^{-2}) of the dilution of the organism. From the diluted organism, (10^{-2}) 0.2mL was taken into the prepared sterile nutrient agar which was at 45^0C, aseptically poured into sterile petri dishes, allowed to solidify for about 45-60 minutes. Using a sterile cork borer of 8mm diameter, the wells were made according to the number of graded concentrations of the sample. In each well, the different graded concentrations of the sample were produced, this was done in duplicates. The plates were allowed to stay on the bench for 2 hours to allow pre-diffusion. The plates were incubated uprightly in the incubator for 18-24 hours at 37^0C. The surface plate method was used for the antifungal analysis. A sterile sabouraud Dextrose Agar (62g/l) was prepared accordingly and aseptically poured into sterile plates in duplicates and allowed to set properly. 0.2mL of the (10^{-2}) was taken, using sterile spreader to cover all the surface of the agar. The wells were made using a sterile borer of 8mm diameter. In each of well, the graded concentrations of the extract were introduced including the controls. The plates were left on the bench for 120 minutes so as to allow proper pre-diffusion of the extract into the agar. The plates were incubated uprightly in the incubator for 48 hours at 26-28^{o}C.The bacteria plates were observed after 24 hours of introduction. It was observed that there were clear zones of inhibition on some plates of higher concentrations and no zones of inhibition at lower concentrations. The fungi plates were also observed after 48 hours of incubation.

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III. RESULTS AND DISCUSSION

Bioreduction of silver occurred due to the presence of reducing agents present in the plant leaf extracts, which reduced Ag^+ in $AgNO_3$ solution into nano size Ag^0 as shown in Equation 1, (Chan and Mashitah, 2012).

$$Ag^+ + e^- \rightarrow Ag^0 \quad 1$$

A. Visual Observation in Colour

Formation of silver nanoparticles is observed by a change in colour from lighter shades to very deep or dark shades when the silver ion solution is added to the plant extract due to the excitation of free electrons in the nanoparticles. The change in colour indicates the presence of AgNPs in solution due to the excitation of surface plasmon vibration (Ahmed *et al*; 2010).The colour formation could occur within a few minutes up to over an hour depending on the plant extract in use. Metal nanoparticles exhibit different colours in solution due to their optical properties. Further confirmation was conducted using UV-vis spectral analysis to determine the surface absorption band of the AgNPs. The colour change was observed within 10 minutes of the biosynthesis, the colour grew gradually deeper until about 45minutes in to the synthesis when there was no further observable colour change. The colour changed gradually from golden yellow to a dark reddish-brown colour (Fig.1).

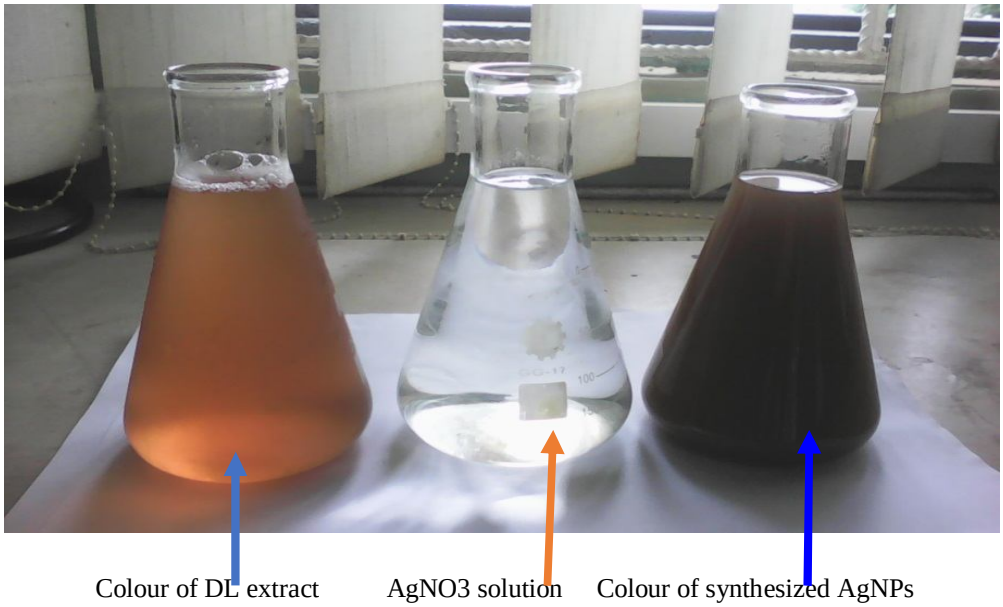

Fig 1: Colour change observed during AgNPs synthesis with *D.latifolia*leaf extract asreductant

B. UV-Vis Spectrophotometer Analyses

The UV absorption peak for silver nanoparticles was reported by Krutyakov*et al.*, 2008, to be in the range of 400-450nm. Fig 2 shows the surface plasmon resonance (SPR) absorption band for the synthesized AgNPSat about 450nm confirming the formation of the AgNPs. The peak has a high intensity which indicates a fairly high concentration of AgNPs formation- (Fig.2).

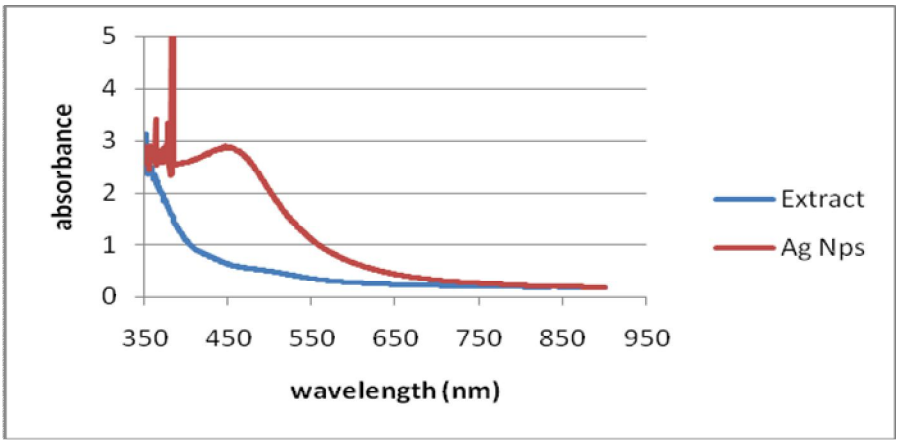

Fig. 2: Graph of wavelength vs absorbance for *Dalbergialatifolia*leaves Extract and AgNPs

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C. Effect of Time

From Fig 3, it was observed that there was no AgNPs formation at the start of the biosynthesis. At 5 min and 10 min, there is an overlap of bands, which indicates AgNPs formation as from 5 minutes into the biosynthesis and no further AgNPs synthesis up to 10 minutes of the biosynthesis. The bands for 15 min and 30 min occured at a higher absorbance and also overlap, indicating further synthesis of the nanoparticles between 10 to 15 minutes, and from 15 minutes to 30minutes, there was no appreciable AgNPs synthesis. The bands for 45 min and 60 min also overlap, this reveals that AgNPs were fully formed as from 45 minutes of the biosynthesis, and there was no further AgNPs synthesis between 45minutes till the end of the one-hour synthesis period. It can therefore be reported from this study, that AgNPs were fully formed by the aqueous extract of *D.latifolia*in 45 minutes.

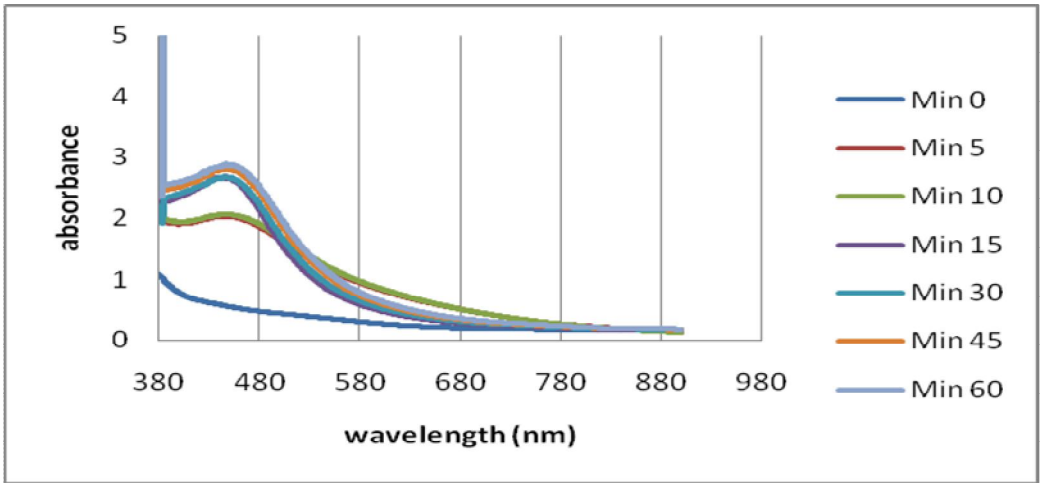

Fig. 3: Graph of absorbance vs wavelength for AgNPs synthesis at different time intervals

D. Effect of Extract to Silver nitrate ratio

The ratio of *D.latifolia*leaves extract to silver nitrate solution was also considered an important factor in the biosynthesis of AgNPs.From Fig 4, the band for ratio 1:3 shows no appreciable absorption at 450nm wavelength indicating that no AgNPs were formed with this ratio. The absorption band for ratio 1:2 at this wavelength shows a fairly high intensity indicating the formation of AgNPs. However, the band for ratio 1:1 gives the highest peak showing quite a high intensity which indicates that the concentration of AgNPs formed using ratio 1:1 were of high concentration. It can therefore be concluded that ratio 1:1 of *D.latifolia* leaves extract to $AgNO_3$ solution gave the optimum condition for synthesis of AgNPs.

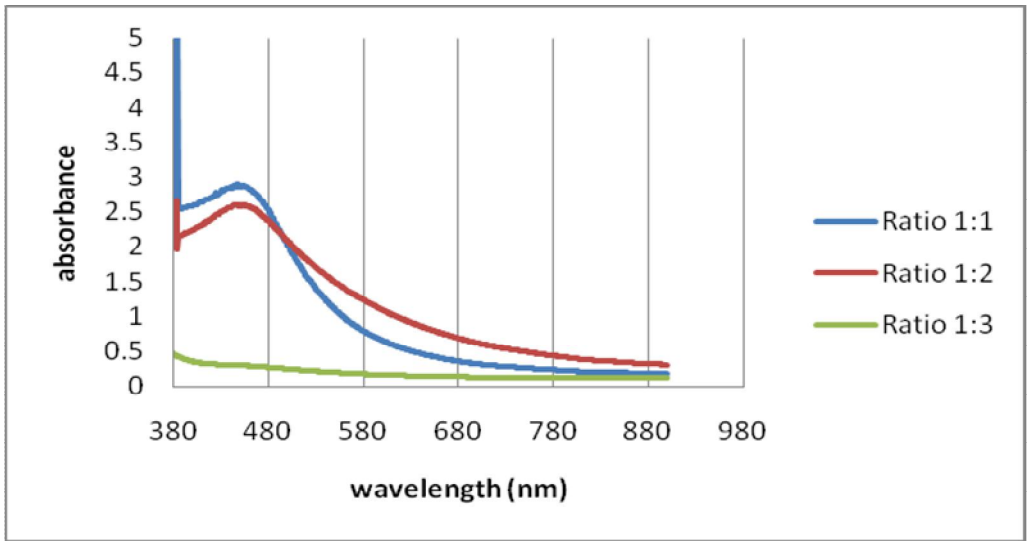

Fig. 4: Graph of absorbance vs wavelength for different ratios of *D.latifolia* extract and $AgNO_3$ solution

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E. Effect of $AgNO_3$ concentration

From Fig 5, the band for 3mM concentration of $AgNO_3$ showed no absorbance at 450 nm, the same goes for the band for 2mM concentration of $AgNO_3$. This indicated that AgNPs were rarely formed using 2mM and 3mM concentrations of $AgNO_3$with *D.latifolia*leaves extract. However, the band for 1mM $AgNO_3$ concentration gave a sharp peak of high intensity at a wavelength of 450nm indicating an intense formation of AgNPs. Therefore, this result indicated that the use of 1mM of $AgNO_3$ is the optimum condition for the synthesis of AgNPswith*D.latifolia*leaves extract.

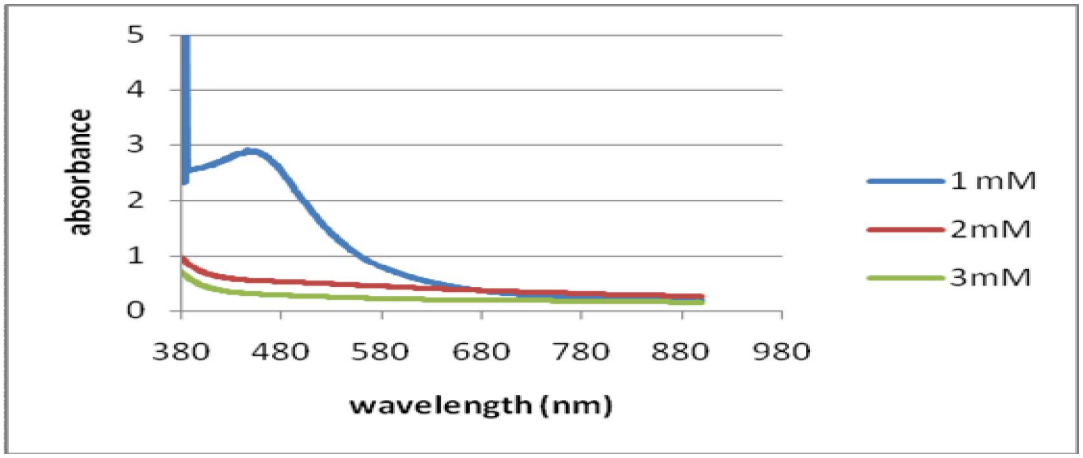

Fig 5: Graph of wavelength vs absorbance for different $AgNO_3$ concentration with *D.latifolia*

F. Effect of pH

Researchers have reported that pH plays an important role in shape and size control in the synthesis of silver nanoparticles. (Quang*et al.*, 2013). From Fig 6, the band for alkaline pH shows a very broad peak of low intensity indicating that the concentration of AgNPs formed using an alkaline extract of *D.latifolia* is low, it also indicated that the AgNPs formed are large-sized. The band for neutral pH shows a higher peak of better intensity which indicates the formation of a higher concentration of AgNPs, while the band for acidic pH shows a band of high absorbance at 450 nm, with a very sharp peak. The very sharp peak indicatedthe formation of a high concentration of AgNPs, and suggests that they could be spherical-shaped AgNPs. (Sharma et al., 2005). Therefore, the present investigation revealed that for optimum AgNPs synthesis with*D.latifolia* leaves extract, the acidic medium is most preferable.

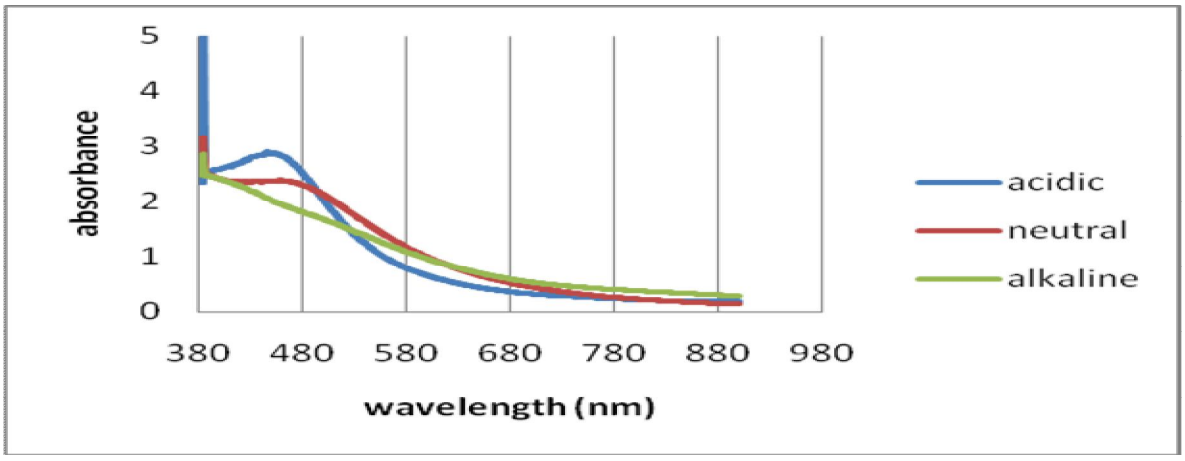

Fig. 6: Graph of wavelength vs absorbance for *D.latifolia* pH variation

G. FTIR Analyses

Fig 7 shows the FTIR graph of both the extract and the synthesized AgNPs. In the extract, prominent peaks were observed at 3474cm^{-1}2998 cm^{-1}, 1638cm^{-1}, 1432cm^{-1},1118cm^{-1} which are associated with O-H str, CH_2, CH_3def, C=O str, aromatic ring and C=N str. In the synthesized AgNPs, peaks which were not so prominent were observed at 3590 cm^{-1}, 3020 cm^{-1}, 2432 cm^{-1} and 1756 cm^{-1} which are associated with N-H str, C=O str and aromatic rings.

The plant extract shows a broad peak at 3484cm^{-1} which indicates the presence of OH group or carboxyl group. There is a shift in the broad peak to the right at 3590 indicating N-H str. These carboxyl and amide groups indicate the presence of secondary amines which is a pointer to proteins, also confirming the biosynthesis of the nanoparticles by the action of the proteins or phytochemicals in the plant extracts. Also, most of the functional group peaks indicated in the extract which are found in molecules like terpenoids, polyphenols and alkaloids were also shown on the AgNPs but have reduced drastically in intensity, and had almost disappeared. This indicates that they had been used up in the AgNPs synthesis for reduction, capping and stabilization of theAgNPs(Kong and Yu 2007). Generally, in the AgNPs spectra, the prominent peaks observed denote C-O-C, ether linkages, C-OH, N-H, C=O bonds, and also aromatic rings. These denote stretching vibrational bonds found in compounds like flavonoids, terpenes and alkaloids, which have been found to be responsible for efficient reduction, capping and stabilization of AgNPs synthesized with plant extracts (Philip 2010).

Flavones, organic acids, and quinones are water-soluble phytochemicals that are responsible for the immediate reduction of the ions. Studies have revealed that plant extracts contain emodin, an anthraquinone that undergoes tautomerization, leading to the formation of the silver nanoparticles. It was suggested that the phytochemicals are involved directly in the reduction of the ions and formation of silver nanoparticles (Suba and Rao 2013).These compounds have been revealed in this investigation to be present in *D.latifolia*leafextract, and may be responsible for the biofabrication of the AgNPs with $AgNO_3$ as the precursor.

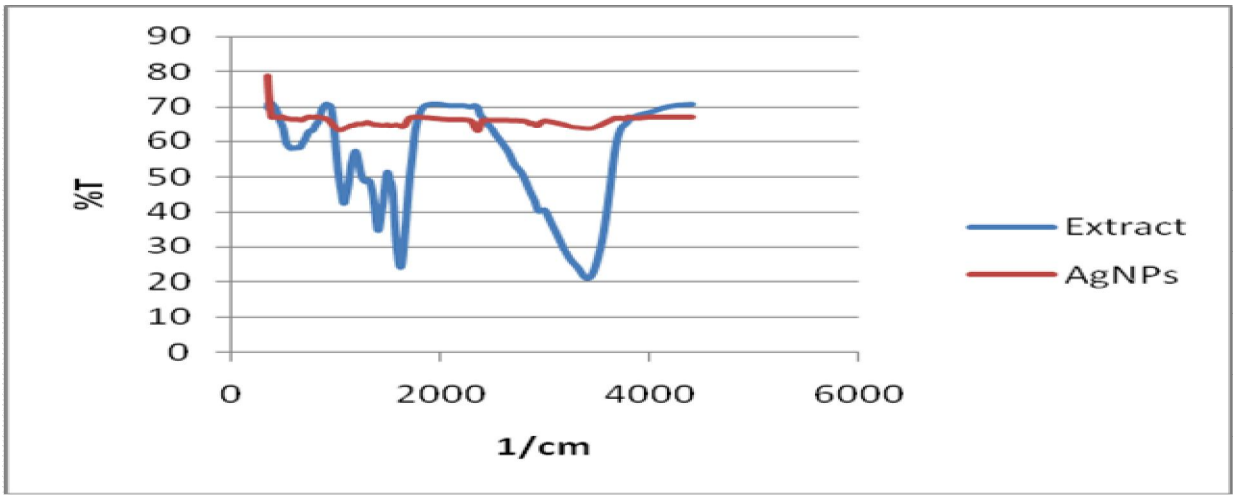

Fig. 7: Graph of FTIR analysis on *D.latifolia*leaves extract and AgNPs

H. SEM Analysis

A scanning electron microscope was employed to analyze the shape of the synthesized AgNPs. SEM analysis showed that the plant leaves extract of *D.latifolia*have tremendous capability to act as reductant in the AgNPs synthesis. The AgNPs werefound to be quassi spherical in shape and were uniformly distributed, as shown in Fig 8.

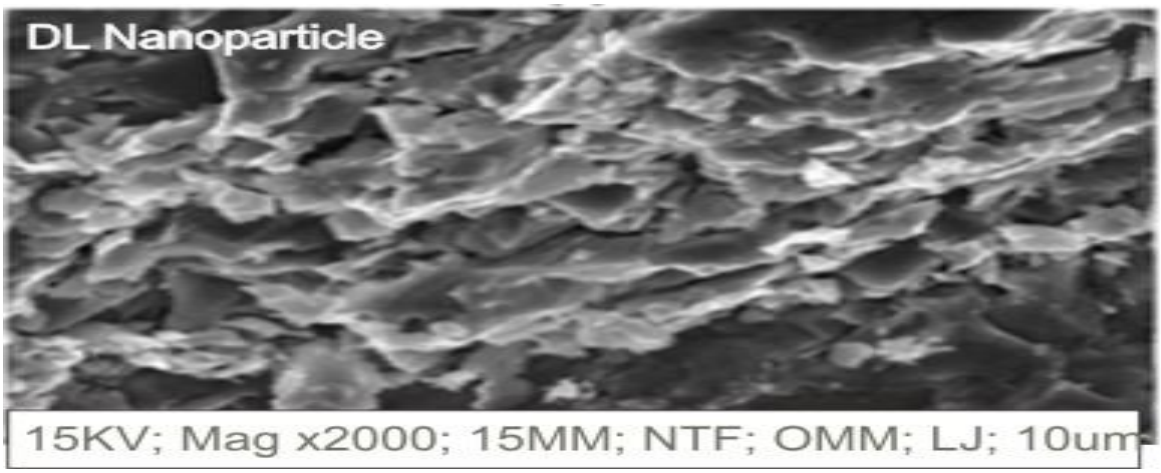

Fig 8: SEM analysis of synthesized AgNPs

I. XRD Analysis

XRD analysis was used to determine the phase distribution, crystallinity and purity of the synthesised AgNPs with *D.latifolia*leaves extract. Fig 9 shows the XRD pattern, and with reference to the JCPDS data file No. 04-0783, it was concluded that the nanoparticles were crystalline in nature having a quassi spherical shape.Braggs reflections representing b111N, b200N, b220N and b311N planes for crystal structure for metallic silver was observed. With reference to the JCPDS data file No 04-0783, it was concluded that the AgNPs are crystalline in nature, with no such impurities.

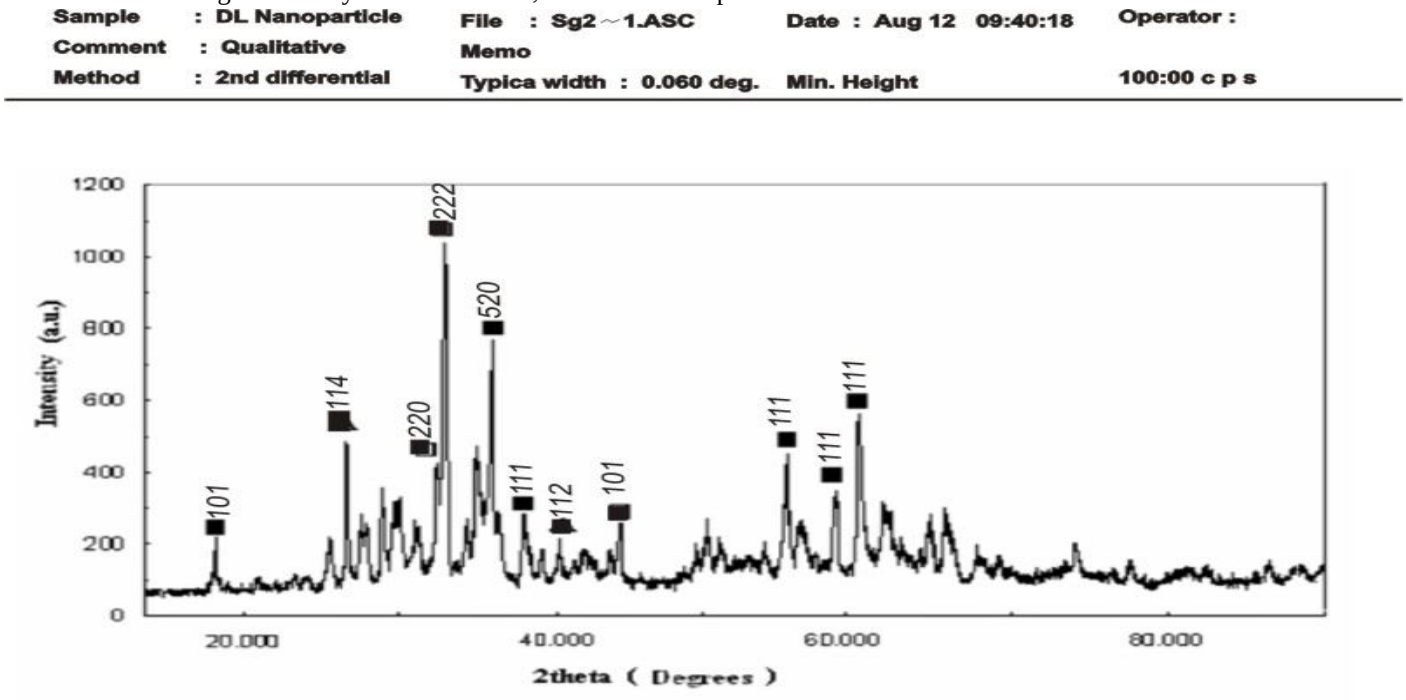

Fig 9: XRD analysis of AgNPs synthesized from *D.latifolia*leavesextract

J. Antimicrobial Analysis

The antimicrobial analysis was investigated using pathogenic strains of some bacteria and fungi. The result of the antimicrobial analysis revealed that the synthesized AgNPsshowed good inhibition at bothhigh and low concentrations against most of the microbes, except at very low concentrations where it showed no inhibition against *Bacillus subtilus, Salmonellae typhi, Candida albican, Rhizophusstolomite*and *Penicilliumnotatum*..

Table 1: Antimicrobial activity of the synthesized*AgNPs*

	Zones of inhibition (mm)										
	S.a	E.c	B.sub	Ps.a	Kleb	Sal	C.a	A.n	Rhiz	Pen	Conc of AgNPs (mg/mL)
1	20	22	18	22	18	16	18	18	16	16	100
2	18	18	16	18	16	14	16	16	14	14	50
3	16	16	14	16	14	12	14	14	12	12	25
4	14	14	10	14	12	10	10	12	10	10	12.5
5	10	10	-	10	10	-	-	10	-	-	6.25
-ve	-	-	-	-	-	-	-	-	-	-	
+ve	36	38	38	36	38	38	28	26	26	26	

Integers 1-5 represent D.latifoliaAgNPs at various concentrations, -ve = negative control (methanol); +ve = positive control(gentamycin at 10ug/ml)/tioconazole (70%));- = no inhibition; S.a = Staphylococcus aureus ; E.c = Esterichia coli; B.sub = Bacillus subtilus; Ps.a = Pseudomonas aeruginose; Kleb = Klebsiella Sal = Salmonellae typhi; C.a = Candida albican; A.n = Aspergillusniger; Rhiz =Rhizophusstolomite; Pen = Penicilliumnotatum; 1-9=Low inhibition; 10-19= Moderate inhibition; 20-29= High inhibition; 30-39= very high inhibition; - = No inhibition.

Table 1 revealed that treatment of the microbes with a fairly high concentration of the AgNPs (100mg/mL), resulted to high inhibition of the microbes. At lower concentrationsof 50 mg/mL and 25mg/mL, the inhibition was appreciable. At concentrations of 12.5mg/mL, the inhibition had reduced, while at 6.25mg/mL, five of the microbes (*Bacillus subtilus, Salmonellae typhi, Candida albican, Rhizophusstolomite*and *Penicilliumnotatum.*) showed no inhibition at all to the AgNPs, even though they showed a fairly high inhibition at higher AgNPs concentrations. This indicated that level of inhibition of the microbes is dependent on the concentration of the AgNPs. The higher the concentration of the AgNPs, the higher their inhibition of the microbes studied.

IV. CONCLUSION

This present investigation reports the green synthesis of AgNPs using *D. latifolia*plant leaves extract. Certain operational parameters were varied to determine the optimum conditions for AgNPs synthesis. This plant-based nanotechnology is free from poisonous and dangerous solvents or wastes. Rapid synthesis of AgNPs was attained when some parameters, namely; time, pH, $AgNO_3$ concentration and ratio of extract to $AgNO_3$ were varied. The extracts served as reducing, capping and stabilizing agents in the synthesis. The method is a single step process with economic viability. This study revealed that *D. latifolia* leaves extract results to a high concentration of AgNPs and that the AgNPs were fully formed with the aqueous extract of *D.latifolia*in 45 minutes. Also, using ratio 1:1 of plant extract to $AgNO_3$ solution gave the optimum ratio for the synthesis of AgNPs. Furthermore, this investigation revealed thata high concentration of AgNPs was formed with 1mM $AgNO_3$ concentration. The present investigation also revealed that for optimum AgNPs synthesis with *D.latifolia* leaves extracts, the acidic medium is most preferable.

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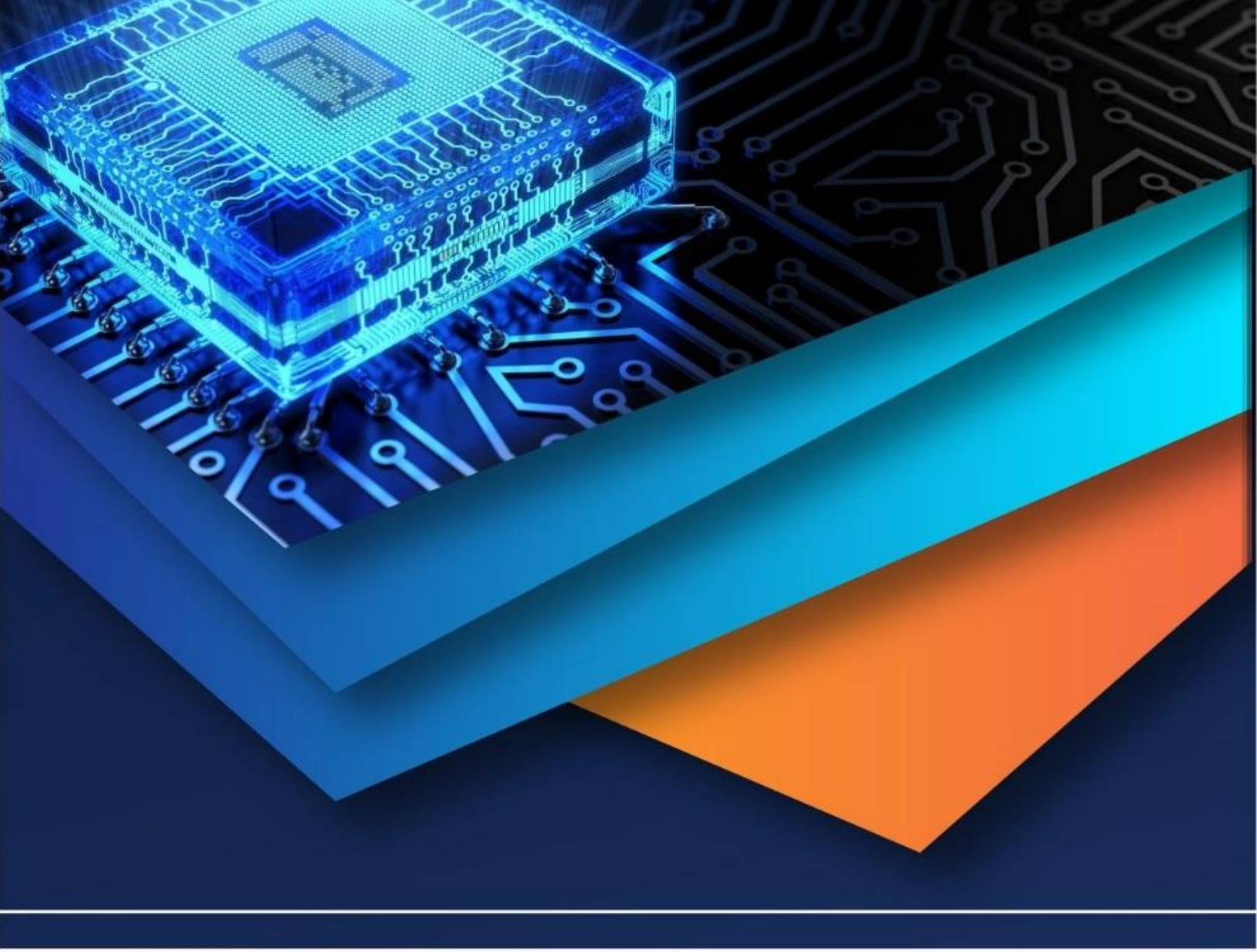

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