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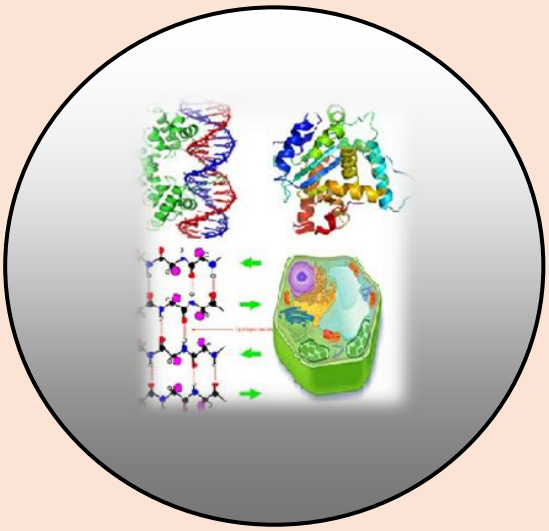

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RESEARCH PAPER

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Green Synthesis of Silver Nanoparticles using *Ocimum sanctum* (Tulsi) leaf extract: Characterization Antibacterial and Catalytic Properties

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ABSTRACT

A recent advance in nanoscience has radically changed the way we diagnose, treat, and prevent various diseases in all aspects of human life. Silver nanoparticles (AgNPs) are one of the most vital nanomaterials among several metallic nanoparticles that are involved in biomedical applications. A conventional method for the synthesis of metal nanoparticles involves toxic reagents, which produce harmful by-products and are harmful for the environment. To overcome these limitations, green synthesis of nanoparticles was established. Eco-friendly methods using plant extracts are gaining popularity due to the abundance of raw materials and the production of non-toxic by-products threatening to the environment. Moreover, the nanoparticles synthesized from the plant extract are cost-effective. In the present study, we report the synthesis of silver nanoparticles using leaf extract of Ocimum sanctum (Tulsi). The resulting AgNPs are characterized using double beam UV-Vis spectrophotometer, FT-IR, XRD. Antibacterial Activity shown by synthesized AgNPs against was tested by Agar well diffusion Method. This route of synthesis is simple, non- hazardous and thus proves to be a novel tool in the study of Nanotechnology.
Key words: Ocimum sanctum, Green Synthesis, Silver Nanoparticles and Antibacterial.

INTRODUCTION

In recent years, metal nanoparticles have been the subject of immense study because of their unique optical, electronic, mechanical, magnetic, and chemical properties that are significantly different from those of bulk materials [Gavhane et al., 2012]. Relative to other metallic nanoparticles, silver nanoparticles (AgNPs) offer various applications in biomedical field. Although they are non-toxic to animal cells, their toxicity to bacterial cells makes them a safe and effective antibacterial agent [Aziz et al., 2014]. Apart from the biomedical applications, they are also used in household products like toothpaste, shampoo, washing machines, water purifiers, cloth, paints, electronics etc [Kim et al., 2010]. Considering their wide applications, synthesis of silver nanoparticles of unique size and composition is a prominent area of research [Iravani et al., 2014]. The "synthesis" of nanoparticles is a milestone of nanotechnology. A number of physical and chemical approaches are explored for their synthesis and stabilization [Klaus-Joerger., 2001]. Physical method includes laser ablation and evaporation condensation methods whereas chemical method utilizes chemical reductants ($NaBH_4$, ethanol, ethylene glycol etc.), aerosol technique, electrochemical or sonochemical deposition, photochemical reduction and laser irradiation technique [Jung et al., 2006].

These approaches are inevitably associated with the involvement of hazardous chemicals such as reductants, stabilizers and organic solvents, or have special requirements for the mentioned techniques such as high energy radiation, microwave irradiation and inert gas condensation [Kim et al., 2004]. Therefore, there is an increasing need to develop the eco-friendly, nontoxic and cost-effective approaches for the preparation of silver nano particles [Mittal & Sharma., 2014].
In recent years, the biological approaches with the use of plant extracts have become valuable alternatives to chemical and Physical Synthesis [Rao & Tang., 2017]. *Ocimum sanctum L.* (Tulsi) is a significant medicinal plant, belonging to family: Lamiaceae, a well-known plant of Indian medicinal system, gaining more attention for potraying a wide spectrum of pharmacological activities [Gupta., 1994]. The major advantage of using Tulsi extracts for biosynthesis of AgNPs is that it is easily available, safe, nontoxic and have plenty of metabolites (Table 1) that can contribute to the reduction of silver ions, and are quicker than microbes in the synthesis [Reddy et al., 2014].

Table 1. List of biomolecules obtained from the Tulsi leaf responsible for the green synthesis of AgNPs.

Plant Specie	Part	Shape and Size	Active Component
Ocimumsanctum	Leaf	18 nm; spherical	Proteins ascorbic acid [Ramteke et al., 2013]
Ocimumsanctum	Leaf	3–20 nm; spherical	Proteins [Mallikarjuna et al., 2011]
Ocimumsanctum	Leaf	42 nm; crystalline	Flavonoids and terpenoids [Rao, et al., 2013]
Ocimumsanctum	Leaf	7–45 nm; spherical	Proteins [Aynul, et al., 2014]

In this paper, we present a rapid, green method for production of Silver nanoparticles using Tulsi leaves extracts, their characterization, their inhibitory effect against Gram-positive bacteria *Staphylococcus aureus*and their catalytic activity in the reduction reaction.

MATERIALS AND METHODS

The present study entitled "Green Synthesis of Silver Nano particles using *Ocimum sanctum* (Tulsi) leaf extract: Characterization and Antibacterial Properties" was performed in the post graduate laboratory of department of Chemistry, Bio chemistry and Microbiology, St. Aloysius College (Autonomous) Jabalpur, Madhya Pradesh. The details of materials, experiments, methodologies employed and techniques adopted are elaborated as follows:

I. Plant collection

Fresh Leaves of Tulsi plant (*Ocimum sanctum*) were collected from the residential areas of Jabalpur.

II. Preparation of the Leaf Extract

10g of fresh Tulsi leaves were weighed and taken in a 250ml beaker. They were washed thoroughly with distilled water. The wet leaves were chopped manually using clean hands and again washed with distilled water. Decontamination of leaves was ensured. These chopped leaves were then transferred to a mortar and crushed thoroughly (adding small amount of distilled water occasionally) using a pestle to obtain a paste of Tulsi leaves. Crushing should be done for at least 10 minutes. The contents of the mortar were then filtered using a clean cotton cloth. The obtained filtrate is what is called as 'Leaf Extract'.

III. Synthesis of Silver Nanoparticles

Leaf extract was taken in a 250ml beaker. To this 0.25g of Silver Nitrate ($AgNO_3$) was added and the whole solution was stirred for approximately 3 hours using a 'Magnetic Stirrer'. Solution was checked till a solid material settled in the base of the beaker, which indicated the formation of Silver Nanoparticles (AgNPs).

IV. Separation of Silver Nano Particles

The solution was then filtered using a Whatman filter paper. The Residue obtained was allowed to dry. When the residue was dried up to 90%, it was collected in a Petri dish containing some Acetone, using a spatula (Acetone was used to remove the excess moisture from the particles) (Figure 1). Acetone was allowed to evaporate so as to obtain completely dried, moisture free Silver Nanoparticles.

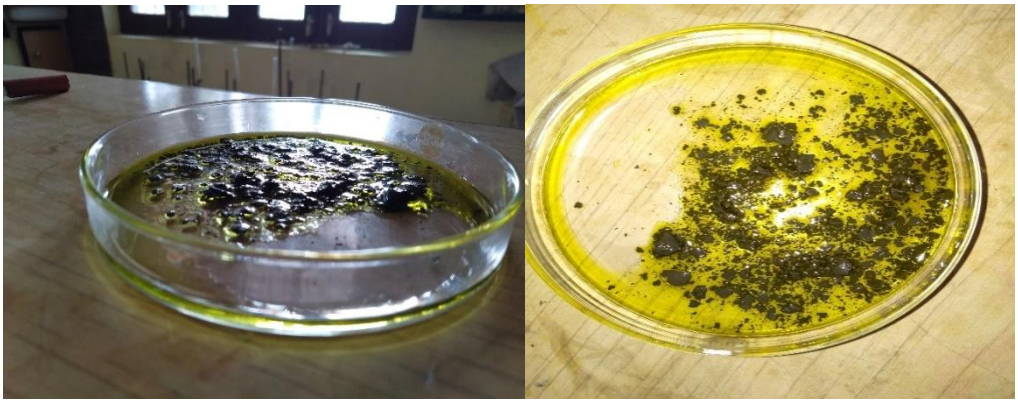

Figure 1. Silver Nano particles dipped in acetone in a Petri dish.

V. Collection of Silver Nanoparticles

The synthesized AgNPs were taken in clean, dry mortar and crushed to maximum possible extent using a pestle. The obtained product (AgNPs) was collected in a clean, dry, air tight packet, and used for its Characterization.

VI. Characterization of Silver Nanoparticles

- UV-Vis Absorption Spectroscopy

The optical property of the AgNPs was analysed by UV – visible absorption spectroscopy (using the UV Spectrophotometer- Shimadzu UV-1800). 0.1g of synthesized AgNPs was diluted with 2 ml of distilled water and subjected to spectral analysis in the wavelength range from 200-800 nm.

- X-RAY Diffraction Analysis (XRD)

The XRD pattern was obtained by placing the prepared samples on a glass slide and dried under hot air oven at 50°C.

- Fourier – Transform Infrared (FTIR) Analysis

The infrared spectra for the green synthesized AgNPs were attained for the identification of functional groups using a Fourier Transform Infrared Spectrophotometer (Shimadzu IR Affinity – 1S) by employing KBr pellet technique and registering amplitude waves ranging from 450 to 4000 cm^{-1}.

- Zeta Potential Measurements and Particle size distribution of AgNPs

When nanoparticles are administered intravenously, they are easily recognized by the body immune systems, and are then cleared by phagocytes from the circulation [Muller & Wallis., 1993].

Apart from the size of nanoparticles, their surface hydrophobicity determines the amount of blood components adsorbed, mainly proteins. The zeta potential is a measure of the repulsive forces between particles and since most aqueous colloidal systems are stabilized by electrostatic repulsion, the larger the repulsive forces between particles, the less likely they will come closer to forming an aggregate [Bajpai et al.2014].

The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the nanocapsule or adsorbed onto the surface [Mohanraj & Chen., 2006].

The particle size distribution and zeta potential of the prepared AgNPs colloid were determined by the DLS technique using Zetasizer within the range of 0.1–10 000 nm at a scattering angle of 90° and 25 °C and 80 °C. For the hydrodynamic diameter measurement, 1 ml of the sample was transferred into a disposable plastic cuvette, and automatically equilibrated in the instrument for 2 min. The data were recorded in triplicate. For the zeta potential analysis, 1 ml of the sample was injected into the zeta cell, and the measurements were repeated three times after equilibration.

VII. Antibacterial Activity

The Antibacterial property of synthesized Silver Nanoparticles (AgNPs) was tested against *Staphylococcus aureus* bacterial culture (Figure 2), by Agar well diffusion method. *S. aureus* is a gram positive bacteria. It is found in grape-like (staphylo-) clusters. This is why it is called Staphylococcus. *S. aureus* was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston.

Figure 2. Staphylococcus aureus bacterial culture.

The experiment was performed in the Microbiology Department of St. Aloysius College (Autonomous) Jabalpur. It involved following Procedural steps:

- Nutrient Agar Media Preparation
- Plating
- Inoculation (of Leaf extract and Synthesized AgNPs) using well diffusion method.
- Incubation
-

Figure 3. Nutrient Agar Media (NAM).

Nutrient Agar Media Preparation

Consider Table 2.

Table 2. Reagents required for Media Preparation.

S. No	Reagent	Weight/Volume
1	Peptone	0.5g
2	Sodium Chloride (NaCl)	0.5g
3	Agar	2g
4	Beef Extract	0.3g
5	Distilled water	100ml

After mixing all the ingredients given in Table 2 in a 250ml volumetric flask (Figure 3), heat the contents to boiling to dissolve the media completely. Ensure a clear solution. Sterilize by autoclaving at 15lbs pressure (121°C) for 20 minutes. Then cool to 45-50 °C.

Plating

After the Media preparation, every step was performed using Laminar Air Flow.
Autoclaved Petri plates were first sterilized using the flame of burner in the Laminar Air Flow. NAM was poured in the sterilized petri plates. Culture of the test microorganism (Staphylococcus aureus) was inoculated by spread plate method in the solidified NAM plates.

Inoculation

Well diffusion method: Discovered by Magldi in 1997. The well diffusion method is simple, easy to reproduce, inexpensive, easy both to read and interpret. It is widely used to evaluate the antimicrobial activity of plant or microbial extracts.
Media in the three Petri Plates was cut into one well 5mm in the middle with help of cork borer. Small amount of Tulsi leaf extract was put in well on any one of the plate, while Synthesized Silver Nanoparticles in another plate. One of the Petri plate was left blank, to be taken as a Reference plate.

Incubation

The above three NAM plates were incubated for in 37°C for 24 hours (37°C because this is optimum temperature for the growth of bacteria).

VIII. Catalytic Property of AgNPs

AgNPs synthesized using the *Ocimum sanctum* Leaf Extract were found to have the capability as a catalyst for the reduction of para-nitro phenol to para-aminophenol [Khodadadi, Bordbar & Nasrollahzadeh 2017]. Following Method was used to test the catalytic property of Silver Nanoparticles in the reduction of para-nitro phenol to para-aminophenol:

Figure 4. Solution after addition of p-nitro phenol.

Step 4. When bubbling stopped, Hydrochloric acid was added (Slowly) to remove the excess Sodium borohydride and dissolve p-amino phenol. HCl was added till the ph of the solution became approximately equal to 1.
Step 5. Solution was filtered using Whatman filter paper and washing was done using cold water. It was ensured that the filtrate having p-amino phenol is protected from light and so the filtration was done in a dark room.
Step 6. The filtrate was neutralized by adding Sodium Bicarbonate ($NaHCO_3$) and a light brown product precipitates out of the solution (Figure 5). This solution was filtered over a Whatman filter paper (washing was done using cold water). Dry the precipitate by placing it in a dark space.

Table 3. Reagents used for the reduction of para-nitro phenol to para-aminophenol in the presence of Silver Nanoparticles.

Reagents	Volume/Weight
2M Hydrochloric Acid	30ml
Sodium Bicarbonate	Few grams
Sodium Hydroxide	0.75g
p-nitro Phenol	1.82g
Silver Nanoparticles	Few grams
Sodium Borohydride	1.01g

Step 1. Preparation of Sodium Borohydride: In a 250ml Beaker 0.75g Sodium Hydroxide (NaOH) was taken. To this 18ml water and 1.01g Sodium Borohydride ($NaBH_4$) was added. Stir it on a Magnetic Stirrer to get a clear solution.
Step 2. Few milligrams of Silver Nanoparticles were added to the above solution and the resultant mixture was cooled to 13◦.
Step 3. P-nitro phenol was added with continuous stirring. Meanwhile a constant temperature, between 13-17◦ was maintained. After the addition, a small quantity of water was added. Stirring was continued for further 30-45minutes (Figure 4).

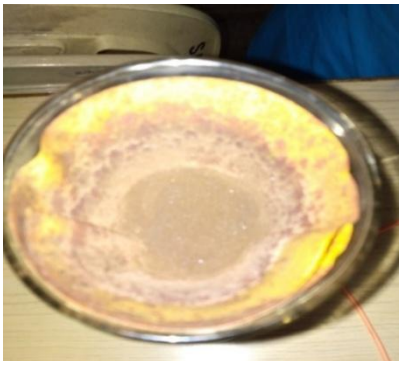

Figure 5. Brown Precipitate of p-amino phenol.

RESULTS AND DISCUSSION

UV-Vis Analysis

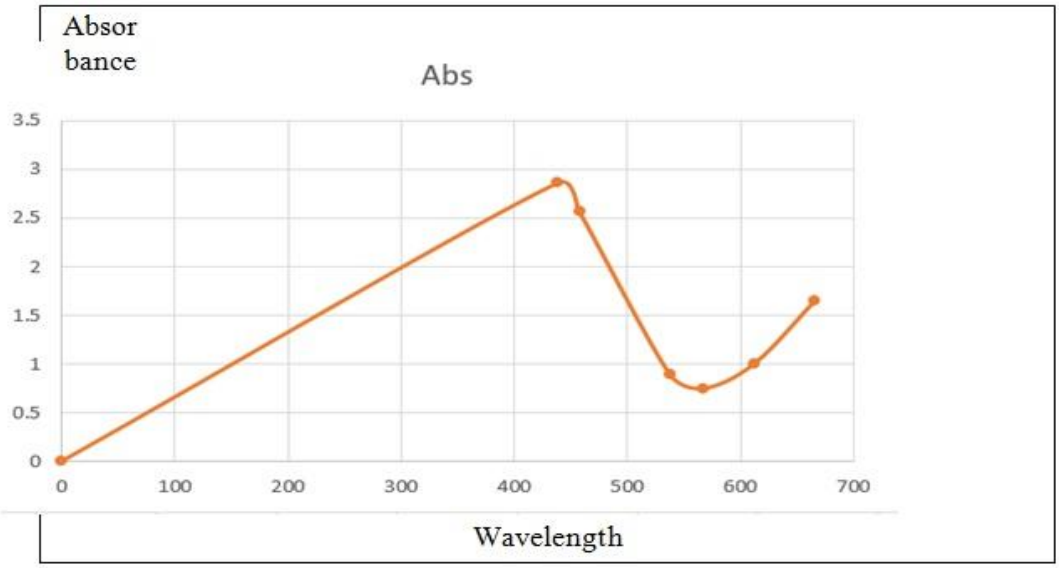

Figure 6. UV-Vis Spectra of Synthesized AgNPs.

The addition of *Ocimum sanctum* leaf extract to silver nitrate solution resulted in colour change of the solution from transparent to dark yellowish brown due to the synthesis of Silver Nanoparticles. These colour changes arises due to the excitation of surface plasmon vibrations with silver nanoparticles [Mulvaney., 1996]. It was observed that the highest absorbance peak of the produced Silver Nanoparticles was centered near 430nm.

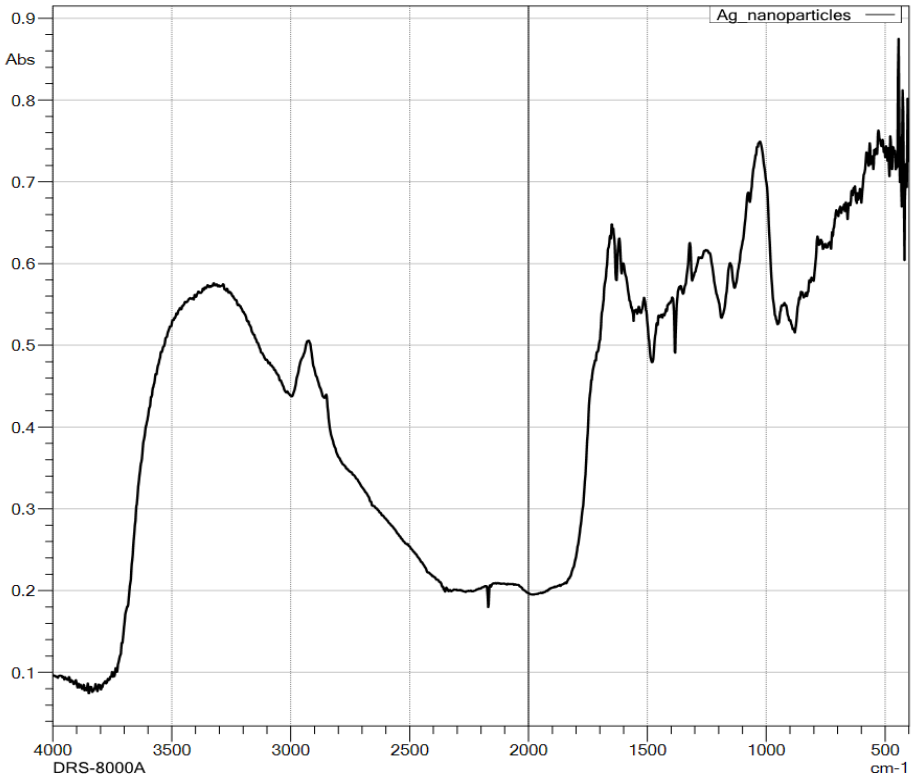

Figure 7. FT-IR Spectral Representation of Synthesized AgNPs.

These Results confirm the presence of possible proteins acting as reducing and stabilizing agents for Silver Nanoparticles.

XRD Analysis

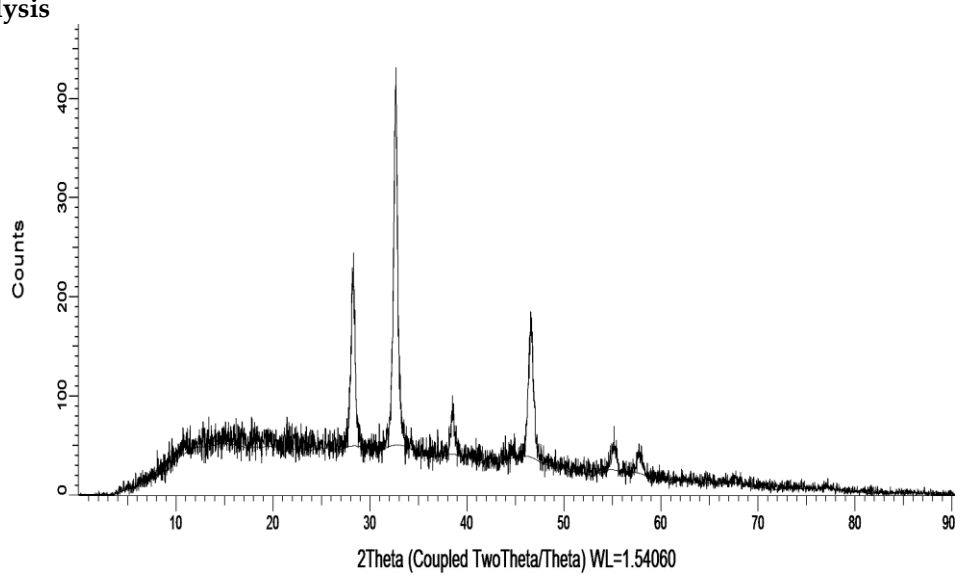

Figure 8. XRD pattern of biosynthesized silver nanoparticles.

FT-IR Analysis

The peak in a range of 3700cm^{-1} to 3850cm^{-1} represents the O–H stretching vibration in alcohols, flavonoids and phenolics. Peaks situated at 1635cm^{-1} 1436 cm^{-1}, 1407 cm^{-1} corresponds to the bonding vibrations of the amide I bond of proteins arising due to carbonyl stretch in proteins while Peaks at 2996 cm^{-1} and 2912 cm^{-1} probably attribute to the N–H stretching vibration of the amide II of proteins. Bands at 2095 cm-1, 1312 cm-1 and 1031 cm-1 can be assigned to strong stretching vibrations of C–N aromatic and aliphatic amines. These IR Spectroscopic studies confirmed that the carbonyl group of amino acid residues have strong binding ability with metal suggesting the formation of layer covering metal nanoparticles and acting as capping agent to prevent agglomeration and providing stability to medium [Sathyavathi et al.,2010].

The XRD analysis showed diffraction peaks corresponding to fcc structure of silver. Intense peaks were observed at 32.23°, 38.098°, 47.154° and 64.51° corresponding to 101, 111, 200, 220 Bragg's reflection, respectively. The broadening of the Bragg peaks indicates the formation of nanoparticles. This result confirmed the formation of AgNPs by the reduction of Ag+ ions with leaf extract of Ocimum sanctum which is crystalline in nature. Full width at half maximum (FWHM) data were used with Scherrer's formula to determine the average particle size. The average particle size estimated was approximately 18 nm.

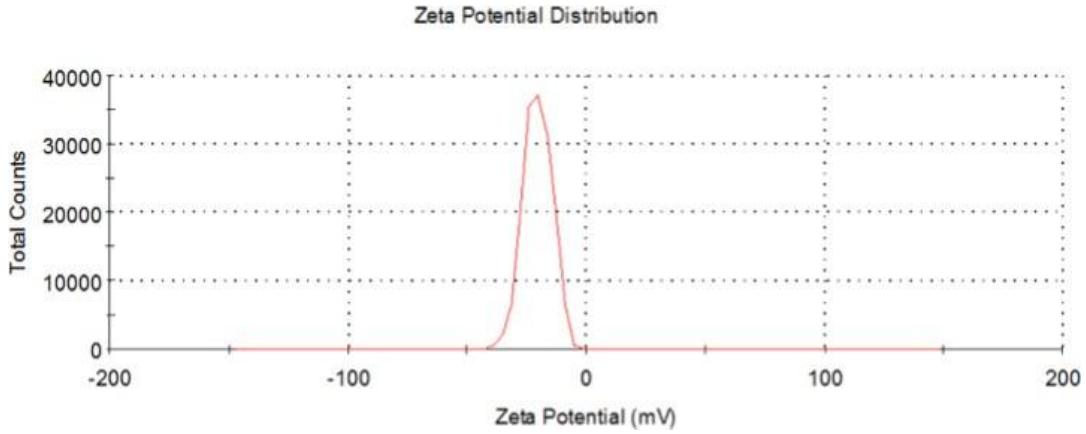

Figure 9. Zeta potential of the AgNPs biosynthesised at the optimum condition.

Zeta Potential Measurements

The zeta potential distribution was measured between zeta potent (mV) versus total counts.
The zeta potential value of *Ocimum sanctum* leaf extract meditated AgNPs was: 20.4 mV (figure 9), proving the interaction of AgNPs with the biomolecules from *Ocimum sanctum* leaf extract. Thus, there exist repulsive forces between negatively charged AgNPs, which are responsible for the moderate stability of nanoparticles in the suspension.

It should be noted that the mean particle size (around 18 nm) determined by the XRD analysis was significantly smaller than that (about 51.883 nm) measured by the DLS method. This discrepancy could be possibly due to the adsorption of organic stabilizers from the extract on the surface of AgNPs, the aggregation of some small particles and the adsorption of water on the stabilized AgNPs, all of which could have a negative effect on the average particle size obtained by the DLS method.

Antibacterial Activity

The presence of zones of inhibition was regarded as the evidence of antimicrobial action. From the inhibition zones, antimicrobial activity was expressed in terms of average diameter of the zones of inhibition. Zone of inhibition was seen when the extract of both *Ocimumsanctum* leaves as well as synthesized Silver Nanoparticles was inoculated in NAM plates containing the test microorganism: Staphylococcus aureus (Figure 11).

Particle Size Distribution

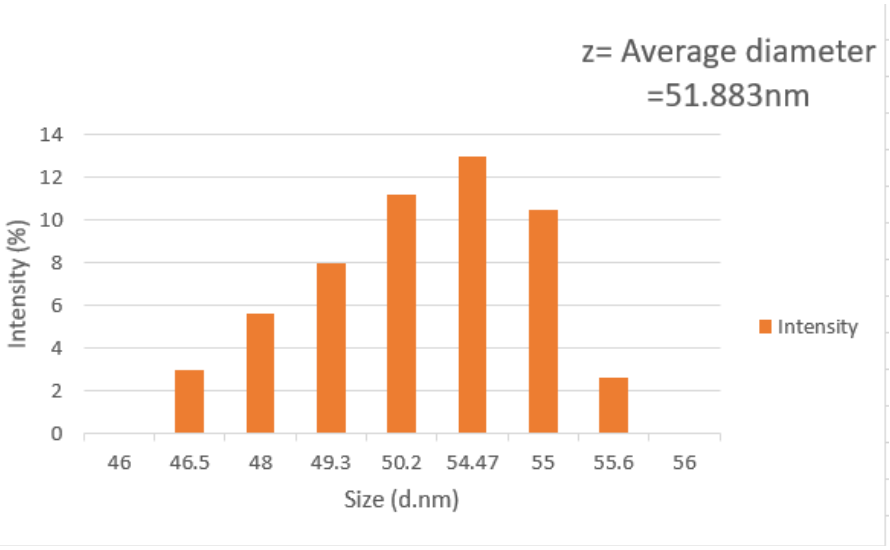

Figure 10. Particle size distribution of the biosynthesised AgNPs at 25 °C (a) and 80 °C (b).

Figure 11. Anti-bacterial activity of synthesized AgNPs (a) Blank or Reference plate, (b) Extract of Tulsi Leaves, (c) AgNPs.

Catalytic Activity of AgNPs

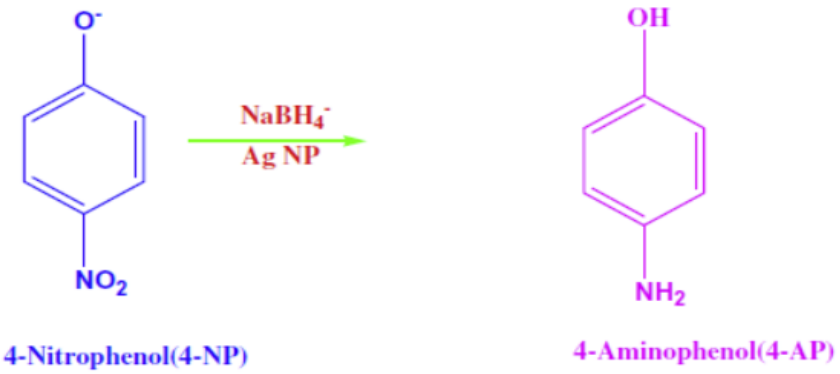

Figure 12. Schematic representation of the performance of Synthesized Silver Nanoparticles (AgNPs) as catalysts in the reduction of 4-nitrophenol to 4-aminophenol by $NaBH_4$.

Table 4. Zones of inhibition by leaf extract and Synthesized AgNPs in mm.

Bacteria	Tulsi leaf Extract	AgNPs
Staphylococcus aureus	0.5mm	1.25mm

Maximum antimicrobial activity was exhibited by Silver Nano Particles, as the zone of inhibition against Staphylococcus auerus had the largest diameter in comparison with the Tulsi leaf extract.
But it has been observed that, after immediate addition of freshly prepared aqueous solution of $NaBH_4$, the peak due to 4-nitrophenol was red shifted from 319 to 400 nm (Figure 14). This peak was due to the formation of 4-nitrophenolate ions in alkaline condition caused by the addition of $NaBH_4$.

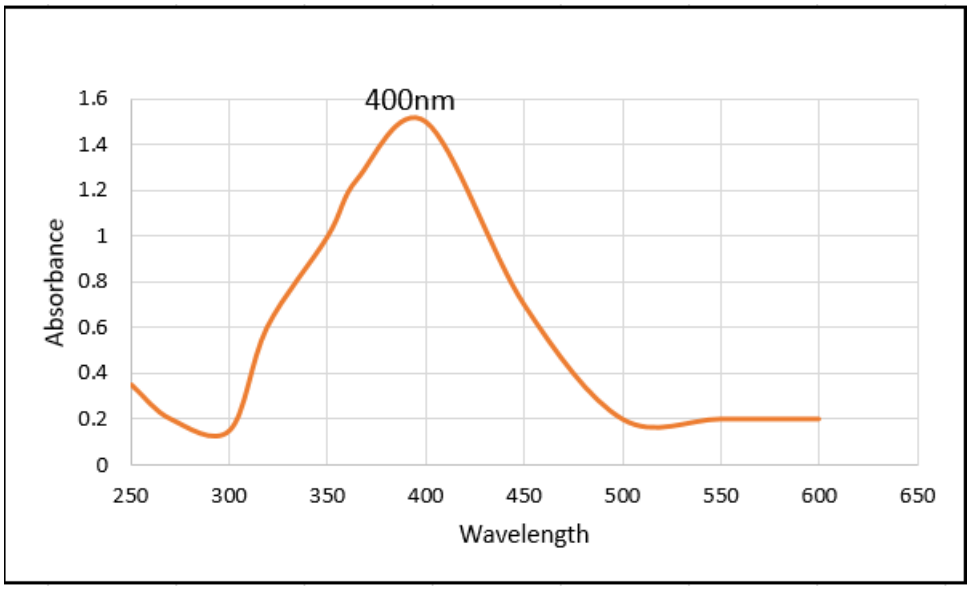

Figure 14. UV-Vis absorption spectra of 4-nitrophenol taken after addition of NaBH4.

In the absence of proper catalyst, the thermodynamically favourable reduction of 4-nitrophenol (4-NP) was not observed. Under alkaline conditions, the decomposition of borohydride is much slower. Borohydride is relatively environmentally friendly because of the low toxicity of borates. The advantage of the catalytic reduction of 4-NP is the easy monitoring of the reactant 4-nitrophenolate anion (λmax=400nm) through spectrophotometry. The 4-nitrophenolate anion formation from 4-NP in the initial step upon addition of borohydride is indicated when the peak at 319 nm (due to 4-NP) is shifted to 400 nm. The sole product 4-AP (λmax=300 nm) can also be monitored easily, and when required to know whether the reduction is actually taking place. Under certain situations, a significant decrease in absorbance at 400 nm may not be associated with the concomitant evolution of a peak at 300 nm indicating that the process does not involve any reduction, rather it is a mere adsorption of the nitro phenolate ion. The addition of small amount of AgNPs, however, causes fading and ultimate bleaching of the yellow colour of the reaction mixture., time dependent UV–vis spectra of this catalytic reaction mixture display the disappearance of 400 nm peak and the gradual development of the new peak at 300 nm which substantiates the formation of the 4-aminophenol.
These results indicate that AgNPs indeed catalyse the reduction process. In this method, the concentration of the borohydride ion, used as reductant, largely exceeds (50 times) that of 4nitrophenol. As soon as we added the $NaBH_4$, the AgNPs started the catalytic reduction by relaying electrons from the donor BH_4^- to the acceptor 4-nitrophenol right after the adsorption of both onto the particle surfaces [Manisha et. Al 2003]. As the initial concentration of Sodium borohydride was very high, it remained essentially constant throughout the reaction. The AgNPs are very effective for the catalytic reduction of 4-nitrophenol. At the end of the reaction, the catalyst particles remained active and were easily separated from the product, 4-aminophenol, using an external magnet.

CONCLUSION

The present investigation concluded that the green synthesis of AgNPs, using Tulsi leaf extract as reducing and capping agent, having advantages such as, ease in availability, eco-friendly with which the process can scale up economic viability. The synthesized silver nanoparticles were crystalline in nature possessed antimicrobial property and were seen to be stable due to the presence of proteins which may act as a capping agent, yet further research is needed in this area to explore the possible biomolecule responsible for bio reduction process.

A thorough understanding of biochemical mechanism involved in the plant mediated NPs synthesis is a prerequisite in order to make the approach economically more competitive and sustainable. Further betterment in green synthesis, purification and sterilization methods will take nanotechnology to new eco-friendly approach in near future.

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