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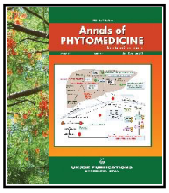

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Biofabrication of silver nanoparticles from the root extract of *Withania somnifera* (L.) Dunal and an investigation of their anti-inflammatory and antibacterial activity

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Abstract

Silver nanoparticles (AgNPs) have been crucial for the advancements in biology and medicine, as well as for environmentally sustainable approaches in the past decade. The aim of the current investigation was to green synthesize AgNPs using *Withania somnifera* (L.) Dunal (Ashwagandha), assessed the characteristics of the synthesized AgNPs using various techniques in the implementation when performing, such as particle size, zeta potential, X-ray diffraction (XRD), ultraviolet (UV-VIS) spectroscopy, Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). The SEM analysis revealed a spherical structure and the XRD imaging for the formulation shows minimized peak intensities. The reduction of Ag^+ ions to Ag^o ions in the synthesized AgNPs was confirmed by FTIR analysis. The antibacterial activity of AgNPs was evaluated using Gram-positive and Gram-negative microorganisms. Bactericidal activity was determined by the maximum zone of inhibition against the bacterial strains, and the better activity of the standard drug. The root extract of *W. somnifera* rapidly reduced Ag^+ to Ag^o and enhanced the synthesis of the AgNPs developed which showed good anti-inflammatory activity.

1. Introduction

Nanotechnology is a rapidly growing science concerned with the production and use of nanosized particles measured in nanometers. Nanotechnology is a prominent area of current research that emphasizes the designing, development, and manipulation of particle structures ranging in size from 1 to 100 nm. AgNPs are among the important and intriguing nanomaterials employed by the various metallic nanoparticles used in the biomedical field. AgNPs are of great importance in the field of nanotechnology and nanoscience, especially in nanomedicine. AgNPs are still being researched extensively, even though there are different noble metals that have been used for different applications.

AgNPs are a topic of great interest for research because of their distinctive qualities (*e.g.*, size and shape as a function of optical, antimicrobial, and electrical properties). *W. somnifera* has been cultivated in several arid regions of India. The term "Ashwagandha" is derived from Sanskrit where 'ashva' means horse and 'gandha' means odour or smell. This name is due to the strong, distinctive odour of the ashwagandha plant, which belongs to the Solanaceae or nightshade family. The herb has been used to cure burns, wounds, and dermatological disorders (Grierson and Afolayan, 1999).

W. somnifera (Solanaceae) is an ancient medicinal plant found in tropical regions of the world, including India. It is a source of stabilizers and bioreductants. The pharmacological effect of *W. somnifera* is considered to be the presence of bioactive compounds, particularly a group of steroidal lactones called withanolides. Plant-based medicines are readily available, less priced, safe, and effective, with little side effects (Vijayalakshmi *et al*., 2021; Sri Bhuvaneswari *et al*., 2021). The traditional use of ashwagandha in Ayurvedic and Unani systems for treating tumors and tubercular glands (Bhattacharya *et al*., 2000). There are several studies revealing the physicochemical and pharmacological properties of *W. somnifera* are anxiolytic, antidepressive, antifungal, antimalarial, and antibacterial (Girish *et al.,* 2006).

The aim of the research was to synthesize AgNPs and examine their antibacterial properties utilizing an aqueous root extract of *W. somnifera* as a reducing agent against silver nitrate solution. The primary goal of this research was to develop AgNPs from *W. somnifera* root extract using green synthesis due to their being environmentally friendly and cost-effective, as well as to investigate their anti-inflammatory and antibacterial activity (Anbalagan *et al*., 2016).

2. Materials and Methods

2.1 Sample collection

The *W. somnifera* plant roots were harvested in Palakkad District and its surroundings. The best quality roots were identified and processed for root extract extraction. Himedia laboratories in India supplied all of the reagents and solvents. Rankem Pvt. Ltd., Hyderabad, provided silver nitrate ($AgNO_3$).

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2.2 Preparation of extract from plant material

The root of *W. somnifera* is chosen and sliced into small pieces. The selected roots were washed with running water and then thoroughly rinsed with water from distillation to remove the foreign particles on the root portion. The covering layer of the root is cleaned and de-skinned roots are allowed to shadow dry for 15 days (Duraisami *et al*., 2021). After completely drying, the root was cut into tiny portions and pulverized to a powder in a suitable apparatus. Approximately 80 g of crushed powder was heated in distilled water (800 ml) for around 1 h. After allowing the mixture was cool to an ambient temperature, the resultant product was filtered through Whatman filter paper Grade 1 in a conical flask, and *W. somnifera* root extract was collected. The above process was repeated periodically to obtain a sufficient quantity of root extract. The formulated root extract was kept at (4ºC) for further study for the synthesis of AgNPs.

2.3 Green synthesis of AgNPs

1 mM $AgNO_3$ was prepared by mixing 0.1698 g of $AgNO_3$ with 1000 ml of de-ionized water in a beaker. The silver nitrate solution was completely mixed by placing the beaker on a magnetic stirrer. The 100 ml of the aqueous root extract of *W. somnifera* was poured gradually into a different proportion of the $AgNO_3$ solution using a pipette: (F1) 100 ml, (F2) 300 ml, (F3) 600 ml, and (F4) 900 ml. The process was performed with constant stirring and maintained at a temperature of 60ºC for 8 h. The $AgNO_3$ was added to the deionized water and stirred using a magnetic stirrer to ensure complete dissolution.

2.4 Characterization of AgNPs

2.4.1 UV-visible spectrophotometer

UV-visible spectrophotometer is a common and simple method for concluding the formation of nanoparticles. The presence of AgNPs was confirmed using a UV-visible spectrophotometer that detected the surface wavelength of the plasmon resonance area (300-700 nm).

2.4.2 FTIR spectroscopy

FTIR spectroscopy (FTIR-8400S SHIMADZU, Japan) was used to identify the biomolecules responsible for reducing Ag^+ ions and capping the AgNPs. The *W. somnifera* root extract of synthesized nanoparticles contains uncapped biomolecules, which can be removed by dissolving them in de-ionized water and centrifuging at 500 rpm for 10 min. This procedure was continued several times, and the resulting pellet was collected and heated to 60°C in a hot air oven using FTIR measurements ranging from 4500 to 500 cm^{-1} (Amsa *et al.,* 2022).

2.4.3 Particle size analysis

Microtrac blue wave-particle size analyzer can confirm the formation of nano sized particles in *W. somnifera* and synthesized AgNPs. Diluting the sample with de-ionized water prior to measuring is necessary to obtain the appropriate concentration for measurement and the formation of nano sized particles was confirmed using the particle size distribution findings (Zhang *et al.,* 2016).

2.4.4 Zeta potential

AgNPs that had been produced were dispersed in deionized water and then sonicated. After filtering and centrifuging the resulting mixture at 25°C for 15 min at 5000 rpm, the supernatant was collected. Before being tested in an automated particle size analyzer (Malvern instrument nano zeta sizer), the residue was carefully diluted repeatedly (Zaka *et al*., 2016).

2.4.5 SEM analysis

SEM investigation was performed on the *W. somnifera* root powder and the AgNPs were gently scattered on an adhesive tape adhered to the aluminum stub, a thin layer of platinum was applied to the sample stubs, about 10Å. A copper sputter module was utilized in a high vacuum evaporator at a voltage of 1,000 K under an argon atmosphere to accomplish the platinum coating. SEM image was captured at a magnification of 50.00 KX, and a working distance of 8.5 mm. SEM was used to reveal the detailed structure and surface characteristics of the coated sample stubs (Anandalakshmi *et al.,* 2016).

2.4.6 XRD analysis

XRD analysis is a powerful tool used to analyze the crystallographic structure of AgNPs. The detection of AgNPs was confirmed by the XRD spectrum and then compared to the root powder from *W. somnifera*. The phase composition, grain size, and crystallographic structures of prepared nanoparticles were analyzed by X-ray diffraction spectroscopy (Philips PAN analytical). The AgNPs were investigated with Copper (Cu) Kα radiation. Scanning the samples over a 2θ range from 10º to 80º at a rate of -5.0 deg/min (Moodley *et al*., 2018).

2.4.7 Antibacterial activity

The disc diffusion technique was used to evaluate the AgNPs synthesized from the *W. somnifera* root extract and its antibacterial studies. Four different bacterial strains gram positive and gram negative (Sethumathi *et al*., 2021), including *Staphylococcus aureus, Bacillus subsites, Escherichia coli,* and *Klebsiella pneumonia* have been introduced into nutritional broth and incubated for 24 h at 37°C in a rotary incubator shaker. A sterilized glass rod was used to evenly disperse 100 µl of these bacterial cultures that had been incubated overnight using different numbers of dilutions. Then, using sterilized tweezers, the sterile Whatman filter papers with a thickness of 25 mm that were applied to the developed silver nanoparticles of various proportions were placed on the agar plates and incubated at 37°C for 24 h. The zone of inhibition appeared in millimeters and amoxicillin was used as the standard (Lakshmanan *et al.,* 2018).

2.4.8 *In vitro* anti-inflammatory activity

The *in vitro* anti-inflammatory effect of the AgNPs was assessed by protein denaturation technique and aceclofenac was taken as a reference drug. The preparation of varying doses of *W. somnifera* root extract and synthesized AgNPs, ranging from 20 µg/ml to 100 µg/ml, to test their potential anti-inflammatory or other biological activities. In addition, a standard aceclofenac was also prepared at the same dose levels for comparative purposes. The resulting solution consisted of various proportions of AgNPs, and the aceclofenac was added to 2 ml of bovine serum albumin, which was adjusted to pH 6.4 and incubated for 15 min at 27ºC. After incubation, the resultant solution was heated for 10 min at 70°C to produce denaturation. Allow the mixture to cool to room temperature, and measure spectrophotometrically at 660 nm and distilled water can be used as a blank in triplicate. The percentage inhibition for denaturation of

protein was determined by using the formula below (Sriramulu and Sumathi, 2017):

$$\% \text{ Inhibition} = (A_t - A_c/A_t) \times 100$$

where,

A_t = Absorbance of test,

A_c = Absorbance of control

3. Results

3.1 Color change of solution

The successful synthesis of AgNPs prepared by *W. somnifera* root extracts was identified by the colour change and spectral analysis of the reaction medium and synthesized nanoparticles. The colour change of the AgNPs from pale yellow turn to dark brown is one of the visual indications of the formation of AgNPs. In this case, it appears that the inclusion of a *W. somnifera* root extract in an $AgNO_3$ solution suggests the reduction of silver ions (Ag^+) to metal ions (Ag^o) to form AgNPs. The colour change observation method is a simple and practical way to optimize conditions of synthesizing AgNPs by using the *W. somnifera* root extract.

Different proportions of root extract from *W. somnifera* and $AgNO_3$ were prepared for formulation F1, F2, F3, and F4 and identified F4 (1:9 ratio) as the one that exhibited an acceptable color change. This indicates that F4 is the most promising for further studies involving the synthesizing of AgNPs using *W. somnifera* root extract. Figure 1 represents the biofabrication of root extract of *W. Somnifera* silver nanoparticles and their colour changes at different time intervals, *viz*., 30 min, 4 h, 6 h, and 24 h, respectively.

3.2 FTIR spectra analysis

In FTIR analysis results for AgNPs observed the band between in the range of 3500-3200 cm^{-1} corresponding to O-H stretching suggests the presence of H- bonded alcohols and phenols. Peaks in this region between 1500-1550 cm^{-1} indicate C-H bond stretching, while peaks between 1450-1500 cm^{-1} exhibited the bond stretching vibration of N-H bond stretching. This group can help stabilize and disperse the nanoparticles. Table 1 shows the FT-IR spectrum of *W. somnifera* root extract, $AgNO_3$, and synthesized AgNPs (F4).

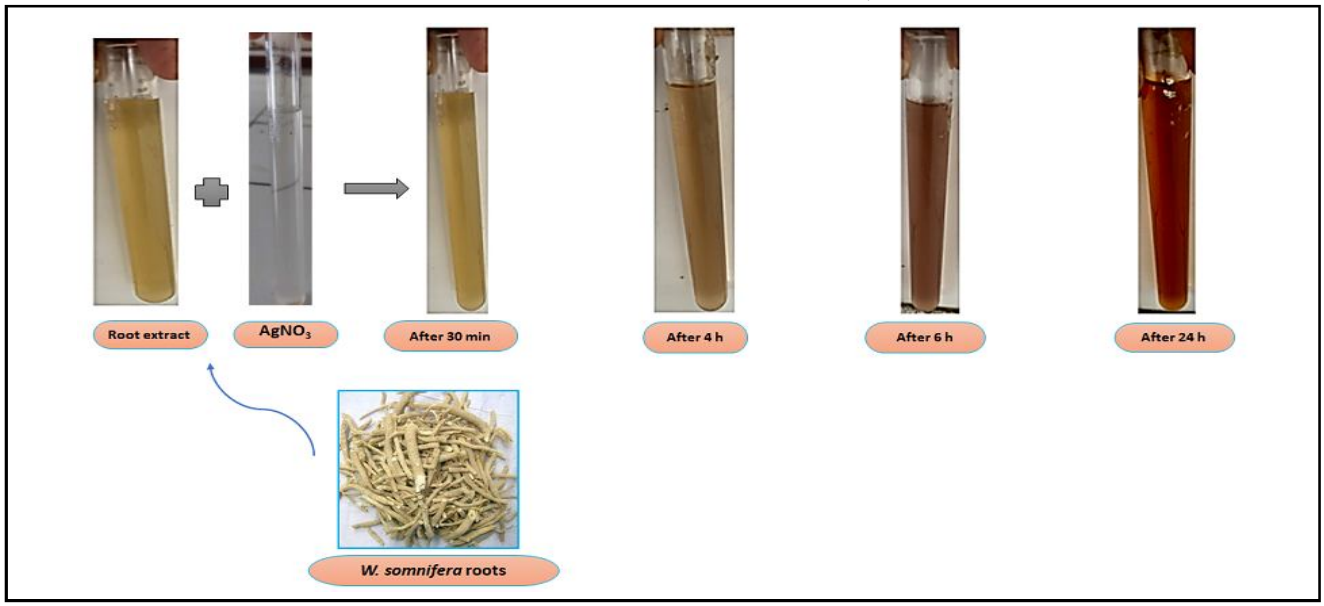

Figure 1: Biofabrication of root extract from *W. Somnifera* silver nanoparticles and their colour changes at different time intervals.

Table 1: Interpretation of IR spectrum of silver nitrate, *W. somnifera* and formulation (F4)

Transition	IR range (cm^{-1})	Absorption wave number (cm^{-1})		
		Silver nitrate	*W. somnifera*	F4
O-H Stretching Alcohols, phenols	3500-3200	3238.26, 3407.02, 3456.20	3423.41, 3422.4	3366.52, 3260.44
O-H Stretching Carboxylic acid	3300-2500	3097.47, 3149.54, 2938.35, 2948.96, 2778.27, 2733.91, 2470.64	2917.13	3260.44, 3081.07, 2886.27, 2835.16
C-H Stretching Alkane	3000-2850	2938.35, 2733.91, 2778.27, 2346.24	2849.63	2886.27
HC≡CH Stretching Alkynes	2260-2100	2064.66, 2108.05	2298.99, 2128.30	2250.77, 2250.77, 2162.05
C=O Stretching Carbonyl	1760-1665	1683.74, 1662.52	1702.06	1677.95, 1649.02

-C=C-Stretching Alkenes	1680-1640	1662.52	1655.77	1621.06
C=C Stretching Heterocyclic aromatic	1550-1475	1481.23	1431.08	1541.02,1520.77,1491.84
C-O Stretching Alcohol, Carboxylic acid	1320-1000	1275.82, 1081.99, 1043.42	1316.33, 1239.18, 1077.17	1317.29, 1266.18, 1220.86, 1076.21, 1032.81
=C-H Bending Alkenes	1000-650	863.09, 825.48, 732.90, 684.68	926.73, 880.19, 668.29	895.87, 838.98, 775.33, 653.82

3.3 Particle size and polydispersity index

The data in Table 2 represents the average size and PDI values for *W. somnifera* root powder and synthesized AgNPs formulations. The PDI values for the synthesized AgNPs are given as a range from (F1 to F4) 0.171 to 0.0440, respectively. F4 has the lowest PDI value (0.0440), indicating a relatively narrow and uniform particle size distribution compared to the other formulations.

Table 2: Particle size and polydispersity index

S.No.	Formulations	Average particle size (d. nm)	Polydispersity index
1	*W. somnifera* root powder	218.40	0.1710
2	F1	70.40	0.0410
3	F2	66.85	0.0430
4	F2	65.14	0.0442
5	F4	64.14	0.0440

The *W. somnifera* root powder has a particle size of about 218.4 nm and a relatively wide size distribution (PDI 0.171). The AgNPs synthesized from *W. somnifera* root extract (Formulations F1 to F4) have sizes of 70.40 d.nm, 66.85 d.nm, 65.13 d.nm, and F4 (64.14 d.nm), respectively. The formulation F4 indeed has the smallest particle size among the other formulations with an average particle size of (64.14 nm). This confirms that F4 contains AgNPs in the nanometer size range. The particle size of the F4 formulation 1:9 ratio is promising for its relatively small and uniform AgNPs size distribution.

3.4 Zeta potential

The zeta potential measurement is indeed a crucial aspect of studying colloidal systems, such as nanoparticles, as it provides valuable information about their stability and characteristics. Zeta potential is the assessment of the electrostatic potential at the shear plane (the boundary between the particle's diffuse double layer and the bulk solution), and it can help predict the tendency of particles to aggregate or disperse. The zeta potential of *W. somnifera* root powder is measured at 130.5 mV. The zeta potential values of synthesized AgNPs formulations F1-F4 have that are correspondingly F1 (– 20.3 mV), F2 (– 18.7 mV), F3 (– 16.8 mV), and F4 (– 14.9 mV), while the values are lower in magnitude compared to the root powder. The negative zeta potential values indicate the surface charge of these nanoparticles carries a negative charge. The negative zeta potential of the AgNPs suggests good physical stability. Figures 2 and 3 represent the zeta potential graphs.

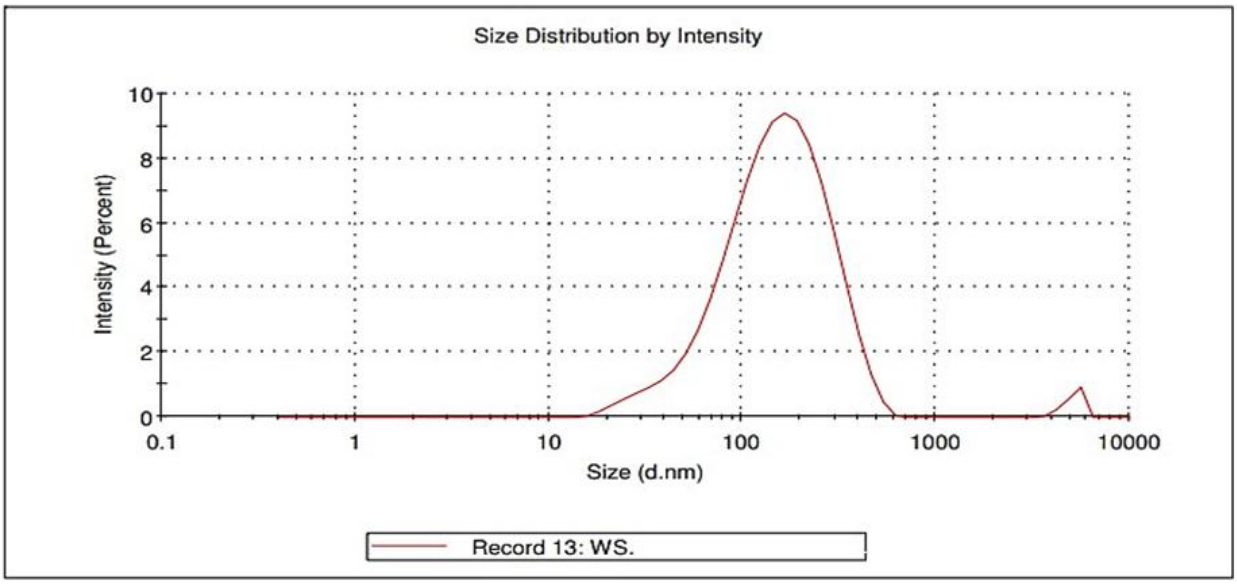

Figure 2: Zeta potential of *W. somnifera* root extract.

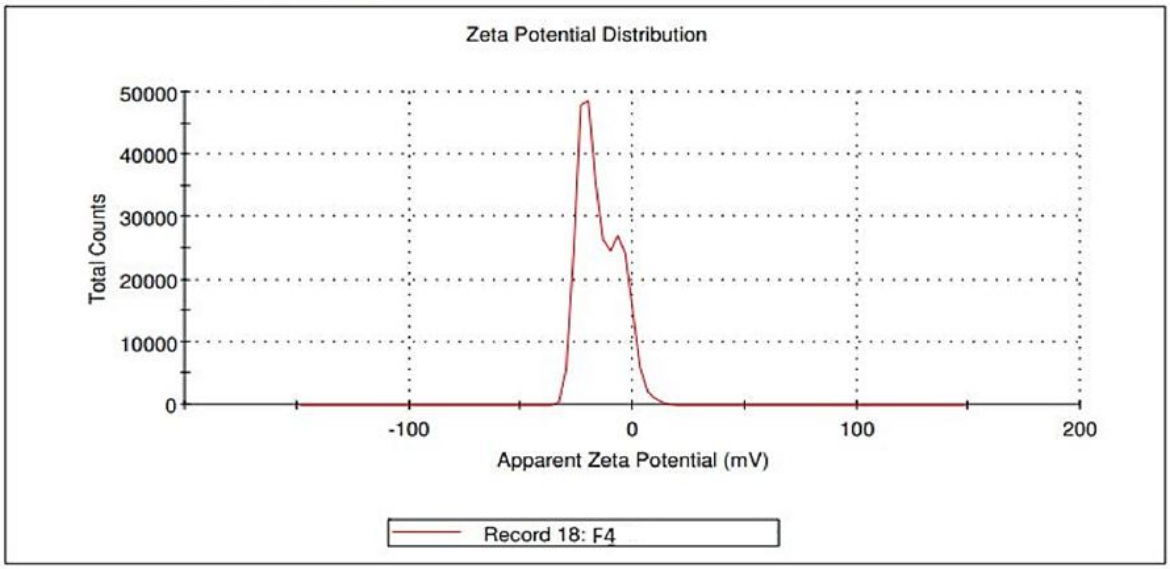

Figure 3: Zeta potential of synthesized AgNPs (F4).

3.5 SEM analysis

The SEM observation of the *W. somnifera* root powder shows an uneven shape when compared to the prepared AgNPs. The appearance of the root powder shows a larger particle size whereas synthesized AgNPs exhibit a small rectangular shape. The specific shape and morphology of AgNPs can be influenced by factors such as temperature and pH during the synthesis process. The SEM micrograph of the prepared nanoparticulate of the *W. somnifera* root extract exhibits that they are indeed in the nanoscale range and have better surface morphology. The SEM images Figure 4 provide valuable insights into the structural differences between root powder and AgNPs. The smaller size and uniform shape of the AgNPs are desirable features for many applications, especially in nanotechnology.

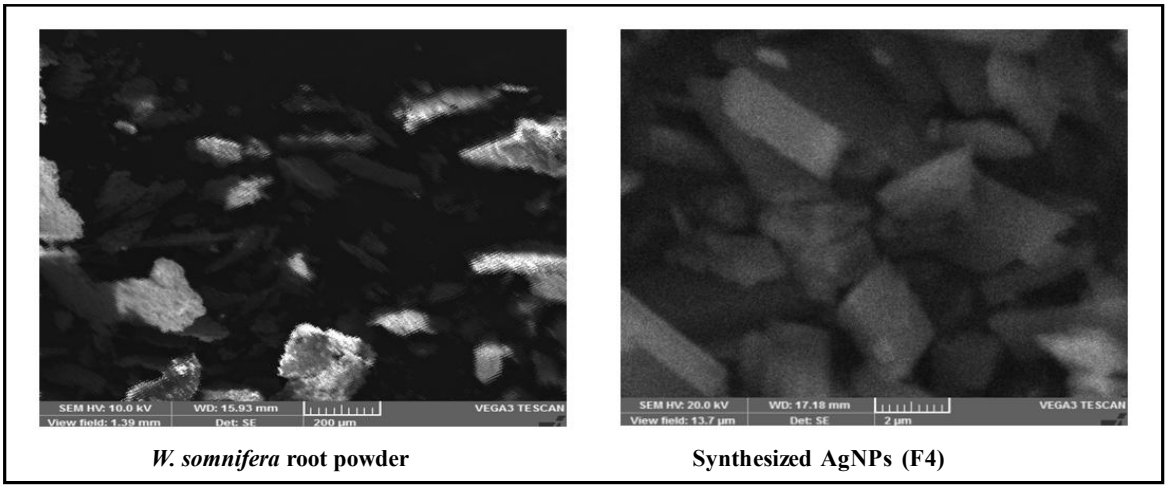

Figure 4: SEM images of *W. somnifera* and AGNPs,

3.6 XRD analysis

Powder XRD is used to characterize the synthesized nanoparticles and it can help to confirm the presence of AgNPs and to identify the detail of the structure. The provided Figures 5 and 6 likely represent the XRD patterns of the *W. somnifera* root powder and the synthesized AgNPs.

The XRD analysis of *W. somnifera* root powder revealed several diffraction peaks at 2θ values are 30.16^0, 36.02^0,4 7.02^0, 52.75^0, 61.72^0, 67.97^0, 72.66^0, 78.12^0. These peaks are confirmation of the crystalline nature of the *W. somnifera* root powder. The XRD analysis of the synthesized AgNPs was observed in diffraction peaks at 2θ values of 27.7^0, 32.1^0, 39.80^0, and 43.5^0, which correspond to the reflection of planes of a face-centered cubic in nature. The presence of these peaks indicates the synthesis of AgNPs and exhibits their crystalline nature (Dhand *et al*., 2016). It was noted that the AgNPs showed a significant reduction in the intensity of diffraction peaks at various 2θ angles. The reduction in peak intensity suggests an amorphous or partially crystalline nature for the formulated AgNPs.

Based on the distinctive peaks shown by the F4 formulation (27.7^0, 32.1^0, 39.8^0, 43.5^0), has been identified as the most promising one for the biosynthesis of AgNPs from *W. somnifera* root extract. So, the F4 formulation is selected for further study.

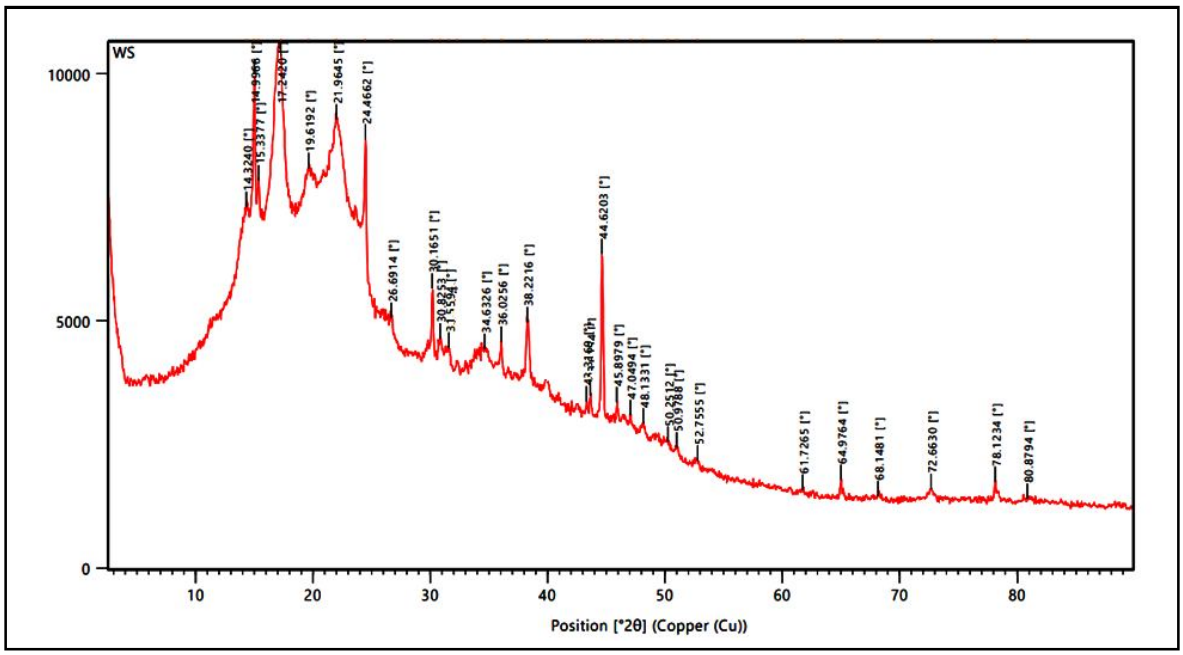

Figure 5: XRD pattern of *W. somnifera* root extract.

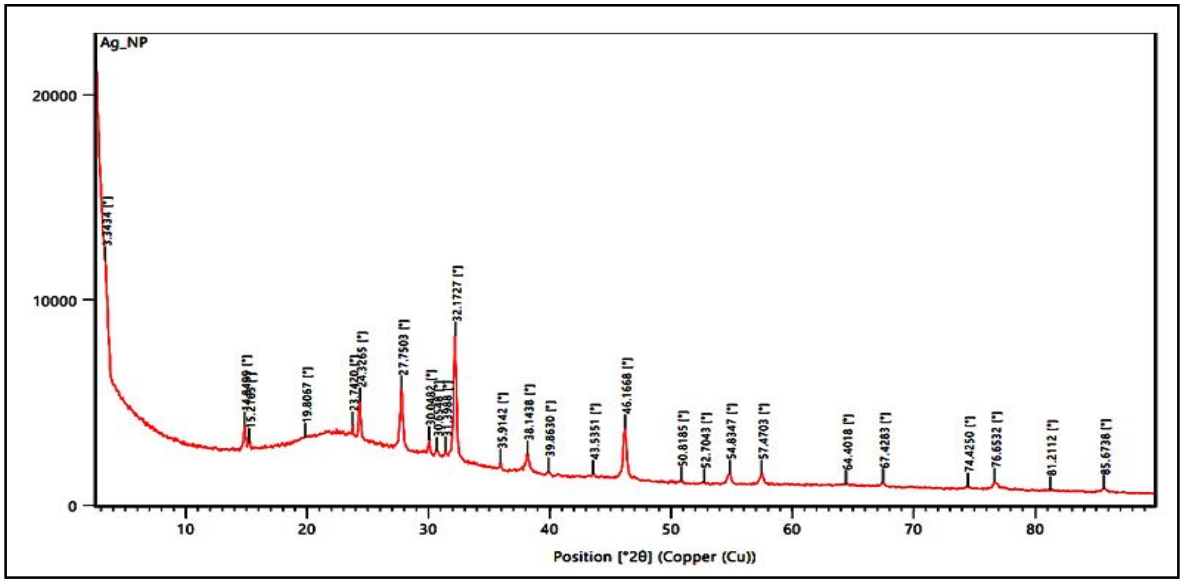

Figure 6: XRD pattern of synthesized AgNPs (F4).

3.7 Antibacterial activity

W. somnifera root extract biosynthesized AgNPs (F4) were tested for their antibacterial properties. The four distinct bacterial strains *S. aureus, B. subtilis, E. coli*, and *K. pneumonia* were subjected to the antibacterial activity of *W. somnifera* root extract and its synthesized AgNPs. Table 3 represent the diameter of the zone of inhibition around each disc with the *W. somnifera* root extract and synthesized AgNPs.

Table 3: The zone of inhibition of *W. somnifera* extract and synthesized AgNPs (F4)

S.No.	Treatments	Zone of inhibition (mm) (Gram-positive)		Zone of inhibition (mm) (Gram-negative)	
		S. aureus	*B. substilis*	*E. coli*	*K. pneumonia*
1	Root extract	21	20	17	18
2	Formulation (F4)	24	21	20	19
3	Standard (Amoxicillin)	25	23	21	20

The inhibition zone results clearly indicate the concentration of the formulated AgNPs has enhanced antibacterial activity compared to the *W. somnifera* root extract. According to the study, synthesized AgNPs revealed the highest antibacterial activity when compared to the extract. The effective antibacterial effect of the AgNPs may be attributed to the released silver ions, which may interact with microorganisms by adhering to the membranes of the bacterial cell, penetrating bacterial cells, and altering membrane permeability and respiration. AgNPs can interact with DNA, which contains sulfur and phosphorus. This interaction can lead to DNA damage, impairing the genetic material and disrupting bacterial replication and function.

3.8 *In vitro* anti-inflammatory study

The anti-inflammatory study of *W. somnifera* root extract, prepared AgNPs (F4), and aceclofenac, a standard drug. The anti-inflammatory activity of the *W. somnifera* root extract was also approximately 25%, 37%, 50%, 59%, and 68%. It proved that the *W. somnifera* root extract had anti-inflammatory properties and that the anti-inflammatory activity increased by increasing the amounts of the *W. somnifera* root extract.

The synthesized AgNPs (F4) formulations revealed that 30%, 44%, 53%, 63%, and 71% of the proteins had been denatured. It proved that when AgNPs concentrations increase, consequently increases anti-inflammatory activity. Figure 7 illustrates the *in vitro* comparative anti-inflammatory activity of root extract, *W. somnifera* root extract of AgNPs, and a standard drug.

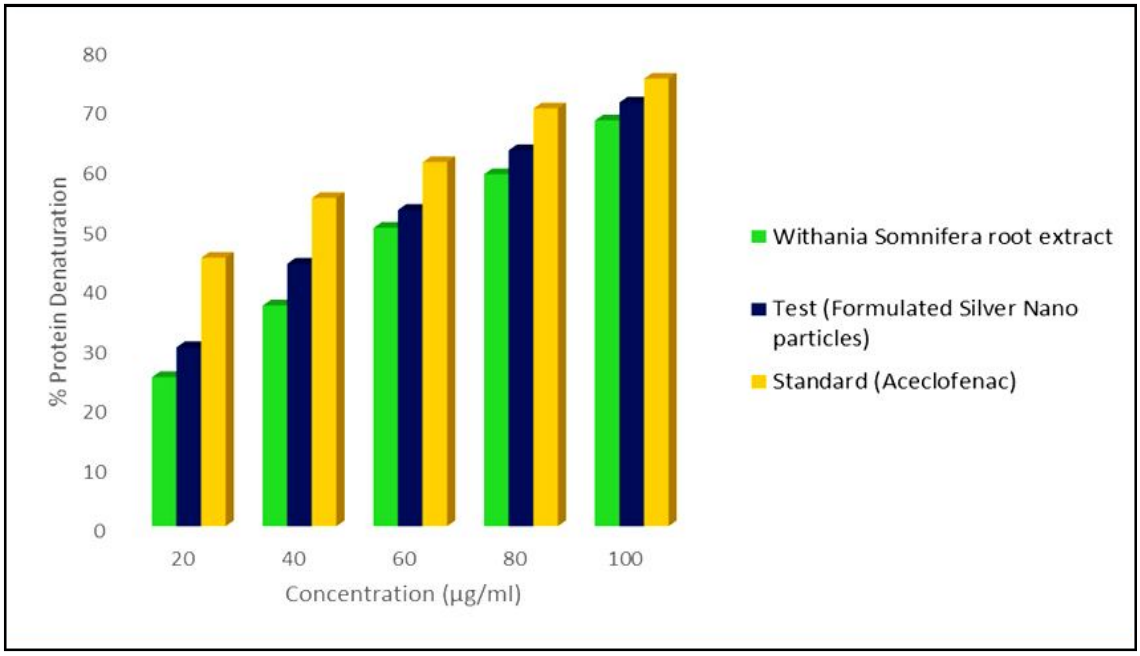

Figure 7: Anti-inflammatory activity of AgNPs, *W. somnifera* root extract, and a standard drug.

4. Discussions

The synthesized AgNPs from *W. somnifera* were successfully prepared by isolation technique and various characterization methods were applied to confirm their formation of AgNPs. It was observed that the first sign was the visible change in colour from light yellow to dark brown. This colour change was due to the excitation of surface plasmon resonance in the synthesized AgNPs. Further analysis of the UV spectrum revealed a peak at 431 nm for the F4 formulation, confirming the formation of AgNPs. FTIR analysis of the synthesized AgNPs shows that there is no interaction between the $AgNO_3$ and the extract, indicating that there was no significant chemical interaction with the constituents of *W. somnifera* root extract during the synthesis of AgNPs from $AgNO_3$. A uniform size and narrow particle size distribution in synthesized AgNPs suggest a controlled and precise synthesis process. The AgNPs in the different formulations, with formulation F4 having the smallest particle size and a low polydispersity index to provide a certain degree of particle size distribution, indicate that the AgNPs are physically stable.

The negative charge of the AgNPs is a sign of their stability and prevents them from aggregating. A negative zeta potential indicates that the interface was negatively charged as a result of bilayer repulsion between the droplets associated with the AgNPs. The capping of the AgNPs on the *W. somnifera* root extract's surface is a result of its negative charge. A zeta potential value of ± 20 mV is often considered a threshold for stability of the nanoparticles. It suggests a higher degree of electrostatic repulsion between particles, making the nanoparticle dispersion more stable. This electrostatic repulsion helps prevent particles from aggregating or flocculating. The high negative zeta potential therefore indicates strong repulsion between the particles, confirming their stability in the formulation.

The surface morphology of the nanoparticles was found to be in good shape when viewed with SEM. Microscopic imaging SEM proved a valuable tool for both the structure and size range of the AgNPs, root extract as a reducing agent, facilitates the conversion of silver ions into the synthesis of AgNPs, and as an overlaying agent; may help stabilize the nanoparticles and prevent them from agglomerating or clumping.

XRD analysis indicates that the synthesized AgNPs have a distinct crystalline structure compared to the *W. somnifera* root powder. Formulation F4 appears to have the strongest effect on reducing diffraction peaks at a 2θ angle. XRD analysis confirms that the AgNPs synthesized from *W. somnifera* root powder have an amorphous nature.

The presence of unassigned peaks at certain 2θ angles (31.390 and 46.160) in the diffraction pattern of the synthesized AgNPs indicates the presence of unpredicted crystalline structures. These peaks, although weaker than the main peaks corresponding to silver, indicate the presence of additional crystallographic phases or compounds in the sample.

Based on these results, the biosynthesized AgNPs showed much greater antibacterial activity than the *W. somnifera* root extract, supporting the previously reported data (Ronavari *et al*., 2017). Moreover, AgNPs synthesized with *W. somnifera* root extract were shown to be more active against Gram-positive bacteria (such as *S. aureus,* and *B. substilis*) than against the negative bacteria (*E. coli* and *K. pneumonia*). The synthesized AgNPs formulation F4 also has an anti-inflammatory effect, which was evaluated by an *in vitro* approach in which the protein albumin was denatured. The F4 formulation, which contained AgNPs, demonstrated a more effective anti-inflammatory effect than the *W. somnifera* root extract. The study successfully synthesized AgNPs using *W. Somnifera* root extract as a reducing and stabilizing agent. This is a significant achievement in the field of nanotechnology and materials science.

5. Conclusion

The study successfully synthesized AgNPs using *W. somnifera* root extract is a significant achievement in the field of nanotechnology and materials science. The results of the FTIR analysis indicate that there was no discernible interaction between the *W. somnifera* root extract and $AgNO_3$ during the synthesis process. This suggests that the extract effectively reduced the silver ions without chemical interaction, and the AgNPs were stabilized without significant alteration of the extract's composition. The particle size of the AgNPs had a uniform size distribution. The zeta potential of the F4 formulation indicates good quality and it was suggested that the F4 formulation had the most stable and desirable characteristics in terms of surface charge. The SEM image of the AgNPs showed a good surface morphology in a small rectangular needle-shaped structure, as contrasted to that of the *W. somnifera* root extract. The X-ray diffraction of the F4 formulation shows diminished diffraction peaks compared to the extract. Additionally, the AgNPs were found to have a cubic nature and a face-centered crystal structure. The study concluded that the AgNPs fabricated through green synthesis exhibited good anti-inflammatory action.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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