Research Article

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Phyto-fabrication and characterization of silver nanoparticles using *Withania somnifera*: Investigating antioxidant potential

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Abstract: This study aimed to develop a green and safe method for producing silver nanoparticles (AgNPs) using the root extract of *Withania somnifera* (WS) and evaluate their antioxidant properties. UV-visible spectroscopy revealed a maximum absorption peak at 430 nm. Fourier-transformed infrared spectroscopy confirmed the presence of phenolic coatings on Ws-AgNPs, indicating their role in stabilizing and reducing Ag ions into Ws-AgNPs. Scanning electron microscopy analysis demonstrated that Ws-AgNPs had a spherical shape and a size range of 74–88 nm. Energy dispersive X-ray spectroscopy analysis confirmed silver as the primary element in Ws-AgNPs. X-ray powder diffraction analysis indicated a face-centered cubic crystalline structure for Ws-AgNPs. The potential antioxidant activities of Ws-AgNPs were evaluated using various scavenging assays. At the highest concentration tested (500 µg/mL), 95 ± 1.3%, 98 ± 1.6%, 76.9 ± 1.44%, and 89.6 ± 1.6% scavenging activities were observed with 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, phosphomolybdate, and H_2O_2, respectively. Moreover, the reducing power of Ws-AgNPs was higher than that of the methanolic WS root extract and showed a concentration-dependent trend. In conclusion, the green-synthesized Ws-AgNPs from *W. somnifera* showed remarkable antioxidant activity, as evidenced by their low IC_{50} values. Due to these findings, it is suggested that Ws-AgNPs have the potential to be used as potent antioxidant agents in the cosmetic, food, and pharmaceutical industries.

Keywords: *Withania somnifera*, AgNPs, green synthesis, antioxidant potential, nanotechnology

1 Introduction

The field of nanotechnology is rapidly expanding and is concerned with the development and use of nanoscale materials that exhibit unique physical and chemical properties [1]. In any spatial dimension, nanoparticles typically range in size from 0.1 to 100 nm [2]. Nanoparticles have the advantage over conventional materials in that they have higher surface energy, a higher proportion of surface atoms, and fewer defects [3].

Metallic nanoparticles have gained interest due to their biological properties, such as enzyme inhibition, antimicrobial, anticancer, anti-leishmaniosis, and antioxidant potential [4]. In addition, they have diverse applications in various sectors, such as food, agriculture, catalysis, imaging, health, drug delivery, and cosmetics [5]. Among metallic nanoparticles, silver nanoparticles (AgNPs) are gaining popularity in research and industry, i.e., nano-biotechnology [6,7]. AgNPs exhibit antibacterial, antifungal, anti-inflammatory, anticancer, antibiofilm, and antioxidant properties [8]. In addition, AgNPs are efficient photocatalysts as they remain chemically stable upon exposure to acids and bases. AgNPs offer a range of photocatalytic applications due to their affordability and high oxidizing capacity [9]. AgNPs are formulated by employing chemical and physical routes. Chemicals are toxic and produce

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environmentally harmful products when utilized to synthesize and stabilize nanoparticles [10]. By contrast, physical approaches are expensive and incompatible with the mass production of nanoparticles [11].

In the search for a safer and more sustainable formulation of metallic nanoparticles, green approaches involving the use of plant and other natural substances have recently gained substantial popularity [12]. Moreover, green synthesis employs phytochemicals instead of potentially hazardous ones to reduce and stabilize metal ions during nanoparticle synthesis, resulting in biocompatible, eco-friendly, and cost-effective nanomaterials [13]. Green nanotechnology has been developed to synthesize different metallic nanoparticles using a variety of biocompatible materials such as bacteria, fungi, algae, and plant extracts. Among the aforementioned biocompatible materials, the utilization of plant extracts has drawn significant attention for the biogenesis of AgNPs. This green approach provides a relatively simple, accessible, and quick way to synthesize nanoparticles on a large scale compared to microorganism-mediated synthesis of nanoparticles [14]. Green synthesis of nanoparticles differs from other biological methods because it avoids the tedious process of maintaining cell culture and enables the large-scale production of nanoparticles [15]. Table 1 presents the effectiveness of green-synthesized AgNPs in various applications as compared to other metallic nanoparticles.

Plants have long been thought to be a source of medicinal benefits. Synthetic chemists were motivated to synthesize the corresponding natural compounds after bioactive compounds known as secondary metabolites were identified and isolated from plants, thanks to advancements in chromatography and spectroscopy [16]. A variety of secondary metabolites, such as alkaloids, enzymes, polysaccharides, phenols, tannins, terpenoids, vitamins, and flavonoids, are found in almost all the parts of a selective plant, including the leaves, flowers, fruits, peels, roots, latex, seeds, and stems. Due to their complex structures, these constituents have a strong medicinal value and are also better for the environment [17]. Therefore, plant extracts provide phytochemicals necessary for reduction processes and confer important biological and therapeutic properties to nanoparticles [18]. Reaction conditions are crucial to achieve significant uniformity of the nanoparticle size distribution in the nano-suspension and to improve the overall process. The most important chemical and physical factors influencing the final product's morphological characteristics are the plant extract's component composition and silver ion concentration, the pH value of the reaction, temperature, mixing intensity, external physical influence, and reaction time. Plant extracts with pharmaceutical values should be used to synthesize NPs for use in biomedical applications [19].

Green-synthesized AgNPs exhibit antibacterial, anti-inflammatory, antimicrobial, antibiofilm, and antioxidant properties, can combat multiple drug resistance, and can also be used in cancer theranostics [20]. Moreover, green-synthesized AgNPs, due to their unique properties, possess major therapeutic uses such as anti-diabetic, antioxidant, antibacterial, and antifungal with no human toxicity, and their other biological uses include bio-molecular detection, drug delivery, biomedical, water treatment, enzymes, food production, and agriculture [21].

Withania somnifera (L.) Dunal (WS) is the most significant medicinal plant of solanaceae family. Winter cherry and Indian ginseng are popular names for *W. somnifera* in English and Asghand in Urdu. The Latin term somnifera, which means "sleep-inducer," refers to the herb's widespread use as a stress reliever, while the Sanskrit term ashwagandha means "odor of the horse," referring to the scent of the roots, which is reminiscent of horse sweat. The plant can be found in the American Herbal Pharmacopoeia monograph as well as WHO monographs on Selected Medicinal Plants. *W. somnifera* is one of the key ingredients in a number of Ayurvedic formulas that are currently marketed in India and other countries around the world [22]. The majority of *W. somnifera* products are used and marketed as dietary supplements and come in powder, syrup, ointments, tablets, and capsule form. All parts of the *W. somnifera* (leaves, flowers, fruits, seeds, and roots) are utilized for various medicinal purposes in Ayurvedic medical systems. The roots of *W. somnifera* are the most widely used plant part among those claimed to have numerous health-promoting properties. They are used to make a tonic that slows down the ageing process, increases longevity, strengthens the body's resistance to infectious diseases, and revitalizes the body [23]. Additionally, *W. somnifera* also has other medical use, such as anti-inflammatory, antidiabetic, neuroprotective, immunomodulatory, and anti-cancer [22]. The steroidal lactones, mainly present in the root of the plant, are responsible for therapeutic activities such as anticancer, antioxidants, immunomodulatory, antibacterial, antiageing, hypoglycemic, hypocholesterolemic, memory enhancement, and also have adaptogenic properties [24].

The phytochemical studies of *W. somnifera* revealed that the root contains alkaloids, phenols, flavonoids, glycosides, saponins, tannins, steroids, reducing sugars, and triterpenoids [25]. Until now, more than 12 alkaloids, approximately 40 withanolides, and several sitoindosides have been reported from its roots and aerial parts. Among these metabolites, withanolides promote immune system cell activation, and phenolic compounds are strongly linked to the plant's antioxidant capacity [26]. A limited number of studies have been conducted on the phenolic

Table 1: Effectiveness of green-synthesized AgNPs in various applications compared to other metallic nanoparticles

Plant name	Nanoparticle type	Size and shape	Activity	Ref.
Crinum latifolium	Silver and gold	20.5 nm (AgNPs) and 17.6 nm (AuNPs) Spherical	AgNPs exhibited significant antimicrobial activity than AuNPs	[27]
Aspergillus terreus	Silver and gold	6–20 nm (AgNPs) and 10–50 nm (AuNPs) Spherical	AgNPs showed significant antifungal and cytotoxicity activities than AuNPs However, both these NPs exhibited good antioxidant potential	[28]
Plinia cauliflora, *Punica granatum*	Silver and gold	8–20 nm (AgNPs) and 2–12 nm (AuNPs) Spherical	AgNPs displayed better antibacterial and antifungal activities than AuNPs	[29]
Cymbopogon citratus, *Tridax procumbens*	Silver and copper	70 nm (AgNPs) and 57 nm (CuNPs) Spherical and irregular	AgNPs exhibited high insecticidal activity against fruit flies, while CuNPs caused no mortality	[30]
Ocimum bacilicum	Silver and copper	15 nm (AgNPs) and (CuNPs) Spherical	The larvicidal activity of AgNPs was reported to be more significant against *Epilachna vigintioctopunctata* than CuNPs	[31]
Amomum subulatum	Silver and copper oxide	20.6 nm (AgNPs) and 24.7 nm (CuONPs) Spherical	AgNPs efficacy was reported to be superior to CuONPs against human flora, i.e., *E. coli*, *S. aureus*, and *B. subtilis*, and more cytotoxicity against human cervical cells (HeLa) and human breast cells (MCF-7)	[32]
Allium tuncelianum	Silver, copper, and nickel	(AgNPs) and (CuNPs) Spherical	AgNPs proved more effective than CuNPs and NiNPs when used to treat infection caused by *A. castellanii*	[33]
Mentha spicata	Silver and gold	24 nm (AgNPs) and 19.61 nm (AuNPs) Spherical	AgNPs exhibited better antimicrobial properties and antioxidant potential than AuNPs	[34]
Fusarium pseudonygamai	Silver and gold	5–20 nm (AgNPs) and 6–60 nm (AuNPs) Spherical	AgNPs showed better antibacterial efficacy, antioxidant potential, and anti-cancer activity than AuNPs	[35]

composition and antioxidant activity of *W. somnifera*, despite the abundance of reports on withanolides and alkaloids.

Various studies reported that a number of diseases are caused by excess free radicals produced by stress, and environmental factors that cause oxidative damage to lipids, proteins, and nucleic acids. The primary cause of oxidative stress and cell damage in the body is the emergence of reactive oxygen species (ROS). By triggering chain reactions, ROS damage cells by generating free radicals. Antioxidant molecules are commonly employed to neutralize free radicals and halt the oxidation process. Thus, a variety of proteins, lipids, and organelles within cells can be shielded from oxidation. The human body makes up for any deficiencies in antioxidant enzymes through food. In the food industry, antioxidants are frequently used to prevent food from spoilage. For food preservation, synthetic antioxidants like butylated hydroxytoluene and butylated hydroxyanisole have been utilized, but it has been reported that they cause toxicity. Therefore, the demand for natural antioxidants in food, medicine, and cosmetics has recently increased significantly due to synthetic antioxidants' toxicity and potential health risks.

Various previously reported studies that phytomolecules from medicinal plant extracts can stabilize free radical ions and help lower oxidative stress in the body [36]. Antioxidant capacity is a common metric for measuring medicinal plants' bioactive and active components. The mechanisms by which phenols and flavonoids exert their antioxidative actions are a topic of much debate in the literature. Flavonoids, being polyphenolic compounds, can function as antioxidants through a free radical-scavenging mechanism by generating less reactive flavonoid phenoxyl radicals. The capacity of flavonoid compounds to donate a hydrogen atom from their hydroxyl group may account for their high potential as scavengers of free radicals. Flavonoid phenoxyl radicals are produced when the flavonoid compounds interact with the free radicals. Flavonoid phenoxyl radicals exhibit lower reactivity than that of other phenols. The phenoxyl radicals are prevented from reacting further and form unreactive compounds due to their reduced reactivity, possibly through a process known as radical–radical termination [37]. In recent years, use of natural antioxidants as compared to synthetic antioxidants has increased in cosmetics, food, and medicine [16].

Considering the significance of this plant and its potential uses in nanotechnology, the present work was planned to synthesize silver nanoparticles (Ws-AgNPs) using roots of *W. somnifera* followed by evaluating their antioxidant potential. To the best of our knowledge, this is the first study to report the antioxidant activity of Ws-AgNPs derived from the *W. somnifera* root extract. The antioxidant activities of Ws-AgNPs were evaluated using DPPH, ABTS free radical scavenging assays, phosphomolybdate, and H_2O_2 and reducing power assay.

2 Materials and methods

2.1 Collection and identification of plant specimens

The plant material of *Withania somnifera* (WS) was collected from Jinnah Garden, Islamabad, Pakistan. The roots were cleaned with tap water and then distilled water before being air-dried in the shade for 2.5 weeks. Taxonomist Prof. Rahamatullah Qureshi identified the plant, and a voucher specimen numbering AU-137 was deposited in the herbarium of PMAS-Arid Agriculture University, Rawalpindi, for future reference and record.

2.2 Preparation of the crude extract

For extraction, the dried roots of *Withania somnifera* (WS) were ground to powder using an electric grinder. The cold maceration technique was used for extraction [35]. About 25 g of root powder was added to 300 mL of methanol. After 7 days, this crude extract was filtered off twice and the solvent was evaporated at room temperature. The dried extract was then scraped out using sterilized blades. The crude extract was then collected in Eppendorf tubes and stored at 4°C for subsequent analysis.

2.3 Green synthesis of Ws-AgNPs

2.3.1 Preparation of the plant extract

About 20 g of the root powder was placed in a beaker containing 200 mL of distilled water. The solution was then boiled for 20 min. The root extract of WS was filtered three times using filter paper (Whatman no. 1) to remove the particulate matter. The solution was refrigerated at 4°C in a 500 mL flask for additional use [38].

2.3.2 Plant-based synthesis of Ws-AgNPs

For the plant-based formulation of AgNPs, the method described in the study of Hussain et al. [39] was used

with slight modifications. About 0.17 g of ($AgNO_3$) salt was added in 1 L of distilled water to prepare 1 mM solution of silver nitrate. The $AgNO_3$ solution (900 mL) was boiled and gradually reduced, while the 100 mL plant extract was progressively added until the color of the solution changed to dark brown. The pellet was then collected after centrifuging the reduced solution for 15 min at 10,000 rpm. The centrifugation process was repeated at least three times to eliminate all the impurities The precipitated nanoparticles were dried by lyophilization. The phyto-fabricated Ws-AgNPs were further characterized, and their antioxidant potential was also investigated.

2.4 Characterization of Ws-AgNPs using UV-visible, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), energy dispersive X-ray (EDX) spectroscopy, and X-ray diffraction (XRD) analysis

Ws-AgNPs were first placed in Eppendorf tubes, and distilled water was added immediately. Ws-AgNPs were subjected to sonication for 10–15 min. The bioformulation of Ws-AgNPs was then analyzed by recording UV-Vis spectra from 300 to 800 nm. FTIR analysis was performed using an FTIR spectrophotometer in the 400–4,000 cm^{-1} range to determine the functional groups responsible for the reduction of silver ions originating from the plant extract. Moreover, an SEM study was carried out to determine the surface morphology of the phyto-fabricated Ws-AgNPs. For this purpose, the sample was prepared on a carbon-coated copper grid using the drop-coating method. Samples were dried for 7 min under a mercury lamp and the excess solution was wiped off with blotting paper. The green-synthesized Ws-AgNPs were morphologically analyzed at various magnifications. EDX studies were also carried out to validate the presence of elemental silver in Ws-AgNPs. In addition, XRD analysis of the Ws-AgNP thin film was performed to investigate their crystalline nature. The Bragg angle in the range from 10° to 70° was used to record the XRD pattern.

2.5 *In vitro* antioxidant activities

The plant extract and phyto-fabricated Ws-AgNPs were examined for their antioxidant activities using various assays. Three replicates were prepared for each sample.

2.5.1 DPPH radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical and the most frequently employed assay to measure the ability of antioxidant compounds to scavenge free radicals. Antioxidant activity was determined using DPPH following the protocol of Nagajyothi et al. [40] with slight modifications. To prepare the plant extract aliquots, 5 mg/mL of the methanolic Ws ext solution was taken and dissolved in methanol to prepare solutions at six different concentrations (15, 30, 60, 125, 250, and 500 µg/mL). In this assay, 1 mL of each plant aliquot from the different concentration solutions was mixed with 3 mL of 0.004% DPPH solution. For the standard DPPH solution (Abs = 0.8–0.9), the prepared stock solution's absorbance was assessed using a UV-Vis spectrophotometer at 517 nm.

Similarly, 1 mL of the solution of Ws-AgNPs samples at different concentrations was mixed with 3 mL of 0.004% DPPH solution. To prepare aliquots of NPs, 5 mg of Ws-AgNPs was dissolved in 5 mL of methanol to obtain six different concentration solutions. At room temperature, the solution mixtures were left to stand in the dark for 30 min after vortexing. For the control sample, 3 mL of DPPH solution was added and the sample was replaced with methanol. Ascorbic acid served as the standard for comparison. The experiment was repeated three times. The sample absorbance was recorded using a UV-Vis spectrophotometer at 517 nm and the % radical scavenging activity of each concentration was estimated by the following formula:

$$\text{Inhibition}(\%) = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100$$

where AC is the solution absorbance without the addition of sample, and AS is the solution absorbance when the extract or NPs are added.

The IC_{50} (µg/mL) values, defined as the quantity of antioxidants required to decrease the free radical's concentration by 50%, were calculated from the % inhibition vs concentration graph.

2.5.2 ABTS radical scavenging assay

The 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, also known as the ABTS assay, was performed following the protocol of Re et al. [41] with some modifications. Equal quantities of ABTS (7 mM solution) and potassium persulfate solution (2.45 mM) were mixed to get a working solution. This solution was then left in the dark at room temperature for an entire night to produce the dark

solution containing the ABTS radical cations. Before being used in the assay, the solution was diluted by mixing ABTS radical cations (1 mL) with methanol (60 mL) to attain the solution absorbance of 0.701 ± 0.02 at 745 nm. Different concentrations of methanolic Ws ext and Ws-AgNPs (300 µL) were mixed with 3 mL of ABTS solution. The standard was ascorbic acid. The decrease in the absorbance after 1 min of mixing the solution and then up to 6 min was recorded. The percentage scavenging activity was determined as follows:

$$\text{Scavenging activity of ABTS}(\%) = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100$$

where AC is the solution absorbance without the addition of the sample and AS is the solution absorbance when the extract or NPs are added.

The amount required to reduce ABTS by 50% was used to express the sample's antioxidant activity in terms of IC_{50}.

2.5.3 Phosphomolybdate assay (total antioxidant capacity [TAC])

Using the protocol of Umamaheswari and Chatterjee [42], the phosphomolybdate assay was used to measure the TAC of the plant sample. An aliquot of 0.4 mL of methanolic Ws ext and Ws-AgNPs was mixed with 4 mL of reagent solution (28 mM sodium phosphate, 4 mM ammonium molybdate, and 0.6 M sulfuric acid) and incubated for 90 min at 95°C in a water bath. After the mixture had been cooled to room temperature, the absorbance at 765 nm was recorded against the blank. For the blank, 4 mL of the reagent was combined with a sufficient quantity of methanol and incubated under similar conditions. The standard was ascorbic acid. The formula used to calculate the total antioxidant activity was as follows:

$$\text{Antioxidant Activity}(\%) = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100$$

where AC is the solution absorbance without the addition of the sample and AS is the solution absorbance when extract or NPs are added.

2.5.4 Hydrogen peroxide scavenging assay

The procedure of Aryal et al. [43] was slightly modified to measure the sample's ability to scavenge hydrogen peroxide. A 50 mM phosphate buffer (pH 7.4) was utilized to formulate a 40 mM solution of hydrogen peroxide. For all six concentrations, 0.6 mL of hydrogen peroxide solution was combined with 0.4 mL of the sample extracts and Ws-AgNP solution in test tubes. After the tubes were vortexed for 10 min, hydrogen peroxide absorbance at 230 nm was calculated against the blank. As a blank, phosphate buffer without hydrogen peroxide was employed. The formula used to calculate the percentage scavenging activity was as follows:

$$\text{Hydrogen peroxide scavenging activity}(\%) = \left(\frac{\text{AC} - \text{AS}}{\text{AC}}\right) \times 100$$

where AC is the solution absorbance without the addition of the sample, and AS is the solution absorbance when extract or NPs are added.

2.5.5 Reducing power assay

This assay was carried out following the procedure used by Hue et al. [44] with slight modifications. About 2.5 mL of different concentrations of the plant extract and Ws-AgNPs were mixed with 2.5 mL of potassium ferricyanide (1%) and sodium phosphate buffer (0.2 M, pH 6.6). Then, the mixture was allowed to stand for 20 min at 50°C. After adding 10% of 2.5 mL trichloroacetic acid (TCA), the mixture was centrifuged for 10 min at 3,000 rpm. The pellet was discarded, and 2.5 mL of the upper layer of the supernatant was mixed with 0.5 mL of $FeCl_3$ (0.1%) and 2.5 mL of distilled water. To compare the results, ascorbic acid was employed as a reference, and the absorbance at 700 nm was measured. An increased sample absorbance suggested a strong reducing power.

2.6 Statistical analysis

Replicates of readings were taken, and findings were presented as mean ± standard deviation. IC_{50} values were calculated from the regression line. The significance of the results was analyzed using the analysis of variance (ANOVA). Statistics were considered significant at $P < 0.005$.

3 Results and discussion

3.1 Green synthesis and characterization of AgNPs

3.1.1 Optical observation

In the current study, the root retract of *W. somnifera* was used to reduce silver nitrate ($AgNO_3$) to Ws-AgNPs. Ws-

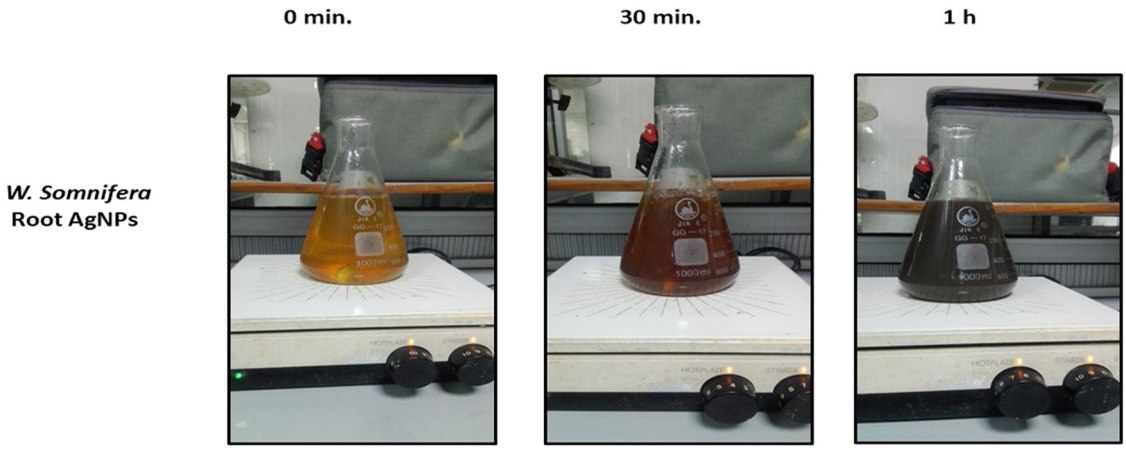

Figure 1: Optical confirmation of the synthesis of Ws-AgNPs by color observation at 0, 30, and 60 min of mixing.

AgNPs were reduced and capped by the root extract. The secondary metabolites present in the plant could be the possible cause for the reduction of the $AgNO_3$ salt in a variety of redox processes, and possible functional groups could confer bioactivity while preventing the nanosilver core from clumping. The initial evidence of phyto-fabrication of Ws-AgNPs was the color change of the reaction mixture, which was later confirmed by recording the reaction mixture's absorbance using a UV-vis spectrophotometer. When the reaction mixture color changed from light yellow to brown and finally to dark brown, it indicated the phyto-fabrication of *W. somnifera* root-mediated AgNPs (Figure 1). This color change is the first indicator of Ws-AgNP synthesis due to the optical properties of AgNPs.

3.1.2 Mechanism of Ws-AgNP synthesis

The mechanism for the green synthesis of Ws-AgNPs is shown in Figure 2. The mechanism of Ws-AgNP synthesis involves the reduction of Ag^+ ions to Ag^0 by the functional groups of secondary metabolites found in the plant extract.

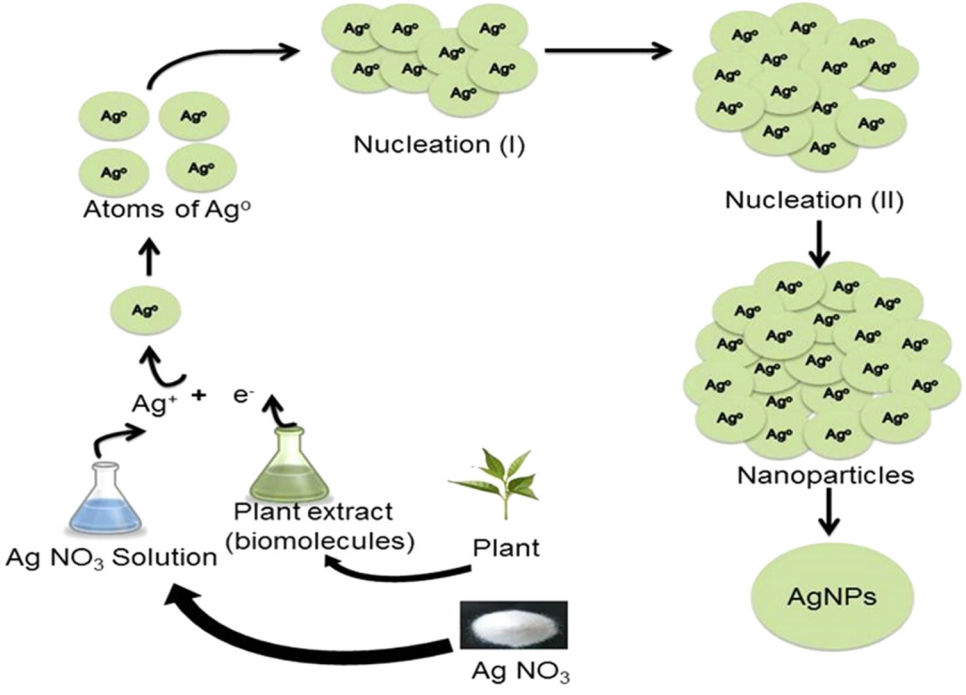

Figure 2: Mechanism of green synthesis of Ws-AgNPs.

After reduction, Ag^0 undergoes a sequence of agglomeration, denoted as nucleation (I) and (II), to form a nanoparticle. Nucleation of silver zero atoms leads to the formation of nanoparticles, i.e. AgNPs, as shown in Figure 2. Thus, the proposed mechanism of synthesis of AgNPs involves the reduction of ions, clustering, and ultimate growth of nanoparticles [45]. The presence of various significant bioactive compounds and the bonding of their functional groups with metallic salt stabilizes nanoparticles. The presence of phenolic compounds on the surface of nanoparticles is attributed to preventing their coalescence [46].

3.1.3 UV-visible spectrophotometer analysis

uv-visible spectrophotometry is one of the key approaches to assess the reduction, stabilization, and formation of AgNPs. This technique uses the principle of surface plasmon resonance (SPR) for characterization. SPR can be explained as a resonant, cumulative oscillation of valence electrons in a solid that is stimulated by light incidence. The resonance occurs when the light's frequency matches the surface electrons' oscillation frequency. SPR establishes the basis for various standard tools for measuring material adsorption on the metal surface (usually silver and gold) or on the surface of metallic nanoparticles [47]. The change in color of the reaction mixture from light yellow to dark brown indicated the reduction of Ag^o. Moreover, UV-Vis analysis also confirmed the formation of Ws-AgNPs. The UV-Vis spectrum of Ws-AgNPs has a strong band in the visible region of 350–550 nm [36]. The AgNPs synthesized using the WS root showed an adsorption peak at 430 nm (Figure 3a), confirming the formation of Ws-AgNPs. Similarly, Chirumamilla et al. [48] observed the peak of AgNPs synthesized from the *Calendula officinalis* flower extract at 430 nm. Moreover, Anbalagan et al. [49] also reported the absorption peak of

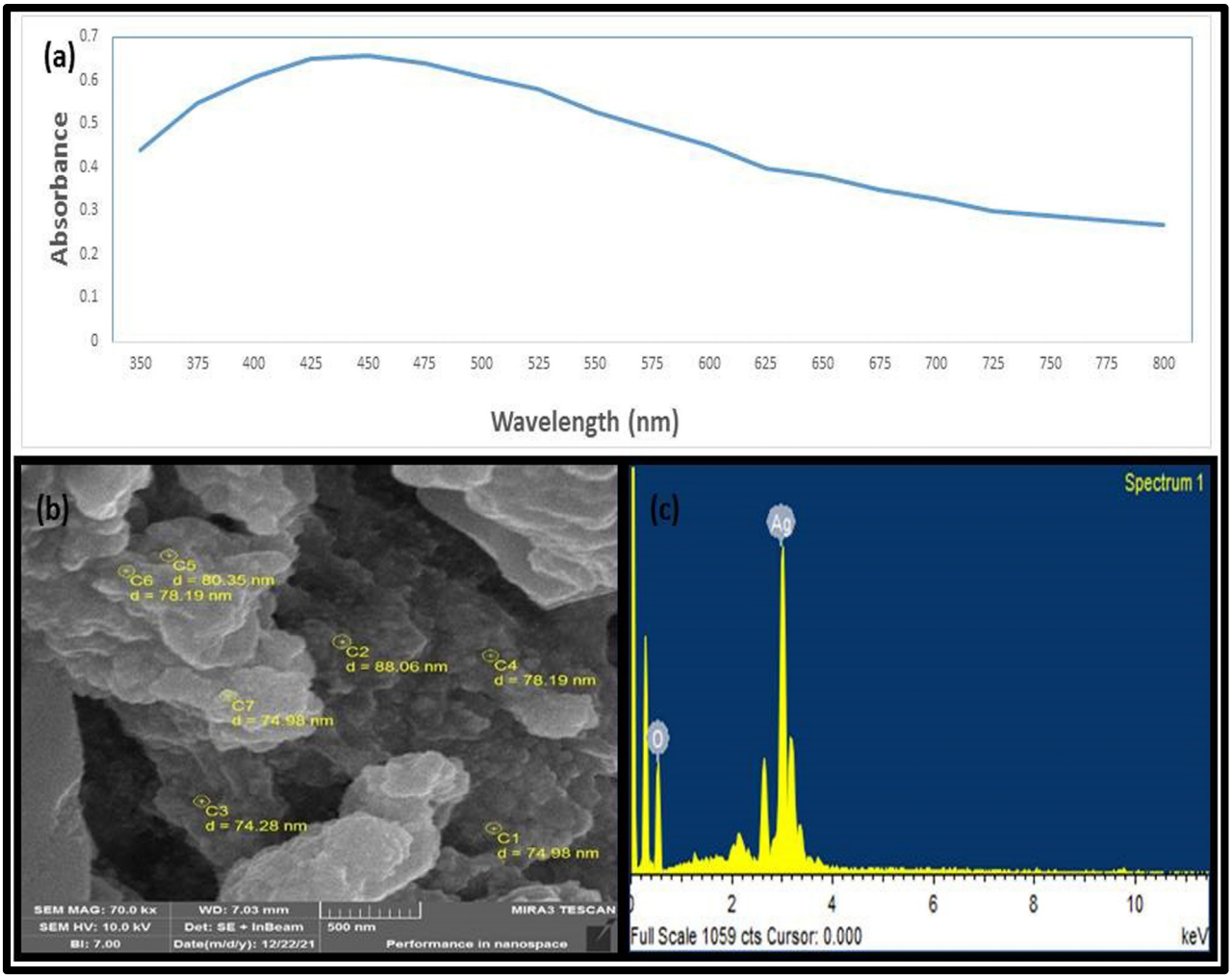

Figure 3: UV-visible spectra (a), SEM micrograph (b), and EDX spectra (c) of Ws-AgNPs.

green-synthesized AgNPs in the 430–440 nm range. The peak broadening indicated the polydispersed nature of the nanoparticles. In addition, the synthesis of AgNPs using the WS extract with a maximum absorption peak at 420 nm was also reported by Javed et al. [50].

3.1.4 SEM analysis

Structural investigation of Ws-AgNPs was conducted using SEM. The SEM image indicated that agglomerated clusters of Ws-AgNPs are dispersed over the surface in a fairly large random pattern with empty space [36] and that Ws-AgNPs are variable in shape, although most of them are spherical. These findings ensure that AgNPs mediated by *W. somnifera* are in the nano range. The average size of nanoparticles was found in the range between 74 and 88 nm (Figure 3b). Ws-AgNPs had a spherical morphology with a high density and homogeneous dispersion. Likewise, Adelere et al. [51] documented similar results. The morphology of the nanoparticles is affected by numerous variables, such as the extract concentration, contact time, pH, and quantity of the silver salt [52].

3.1.5 EDX analysis

EDX analysis was used to examine the elemental composition of the phyto-fabricated Ws-AgNPs. The purity and presence of elemental silver (Ag) were verified using the EDX analysis. The maximum characterization peak of Ag was recorded at 3 keV and a silver signal with high intensity was also observed (Figure 3c). The peak around 3 keV is the characteristic peak indicating AgNP synthesis. The EDX spectrum also showed a signal for oxygen, which may indicate the proteins and enzymes found in the plant extract that are important for the capping of the AgNPs [53].

3.1.6 FTIR analysis

FTIR analysis was performed to reveal the potential biomolecules involved in the surface coating and as capping agents to stabilize *W. somnifera* root-mediated Ws-AgNPs. Additionally, this analysis helped identify the functional groups present in the suspension of AgNPs and may be responsible for the reduction of metal ions. The sample was screened with an FTIR spectrophotometer in the 400–4,000 cm^{-1} range to determine the chemical interaction of NPs with phytochemicals from WS.

The FTIR spectra of Ws-AgNPs synthesized using the WS root (Figure 4) produced peaks at 3,419, 2,962, 2,922, 1,618, 1,383, 1,317, 1,076, 1,024 and 800 cm^{-1}. The peak at 3,419 cm^{-1} is due to stretching vibrations of O–H, attributing the presence of phenolic and alcoholic groups on the surface of Ws-AgNPs. Other peaks at 2,962 and 2,922 cm^{-1} are attributed to the C–H stretching vibration of alkanes. The peak at 1,618 cm^{-1} is attributed to C–O bond vibrations and C–C stretching vibrations. The peaks at 1,383 and 1,317 cm^{-1}

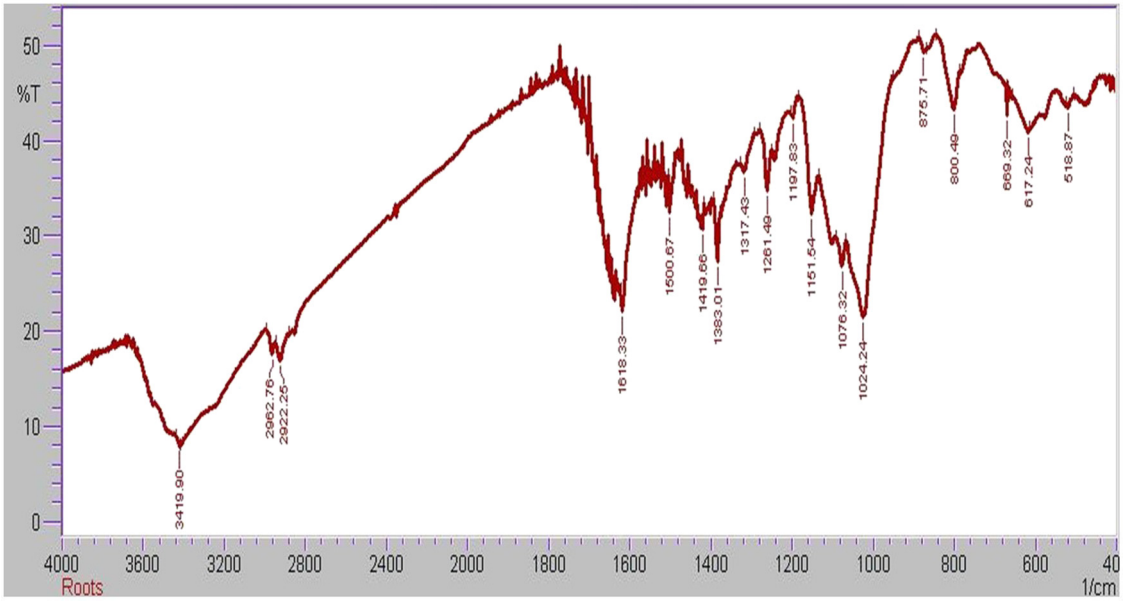

Figure 4: FTIR spectra of Ws-AgNPs.

correspond to the C–H bending of alkanes and C–O stretching in the carbonyl group of protein residues. Two other peaks at 1,076 and 1,024 cm^{-1} may indicate the C-stretching of ether groups, the C–N stretching vibration of aliphatic amines, and the C–O vibration [36]. Stretching vibrations of C–X of alkyl halides were observed at 800 cm^{-1}. Similar characteristic peaks of AgNPs were reported in other studies [54,55]. Finally, we can point out that any biomolecule with these bonds can function as a capping or reducing agent.

3.1.7 XRD analysis

XRD analysis verified the crystalline nature of the phyto-fabricated Ws-AgNPs. The XRD diffraction pattern demonstrated the diffraction peaks at 32.1°, 38°, 64.5°, and 76.5°. The crystalline structure of NPs is represented by high intensity of peaks (Figure 5). The XRD spectra show absorption peaks at 32.1°, 38°, 64.5°, and 76.5° and could be assigned to the (111) (200) (220) and (311) planes of the face-centered cubic crystalline structure of Ag. The findings are in line with Devanesan and AlSalhi [56], who described the biosynthesis of AgNPs and recorded the XRD peaks at 38.1°, 44.2°, 64.5°, and 76.5°. The phytochemicals from plant extracts were deposited on the surface of AgNPs, which was also proved by FTIR studies and several related research corroborated these results [57]. The average crystalline particle size was calculated by using the Debye–Scherrer equation $D = k\lambda/\beta\cos\theta$, where D is the particle size, k = 0.94 is a constant, λ is the X-ray wavelength (0.1541 nm), and β and θ represent the full width at half-maximum. The average crystalline size was found to be 63.8 nm.

3.2 *In vitro* antioxidant activities

3.2.1 DPPH radical scavenging assay

An antioxidant inhibits the oxidation by neutralizing free radicals. The antioxidant itself is oxidized to counteract free radicals [58]. The phenolic compounds and flavonoids in medicinal plants are well acknowledged for sequential reduction and capping of plant-based nanoparticles and these metabolites are attributed to a range of useful activities by NPs. The capping biomolecules found in the extract of plants and their adsorption on the surface of NPs may be responsible for the antioxidant activity [59]. Free radicals are normally produced during cellular metabolism and act as regulatory and signaling molecules. However, excessive production of free radicals can damage cellular components and hamper their ability to function normally [60]. DPPH is a stable molecule that can be reduced to hydrogen by the electron reduction process. DPPH produces a purple-colored solution after being dissolved in methanol, which then becomes a colorless solution upon reduction by an antioxidant. This process involves an antioxidant donating hydrogen, which creates the non-radical DPPHH [61].

The current study compared the antioxidant potential of phyto-fabricated Ws-AgNPs with that of the plant root extract (Ws ext) using the DPPH free radical scavenging assay. Antioxidant potential was determined in the form of inhibition percentage and IC_{50} value. The highest radical % scavenging potential observed for ascorbic acid was 99 ± 1.58 at 500 µg/mL. The maximum percentage scavenging activities of Ws-AgNPs were found to be 95 ± 1.3 and 40.2 ± 1.5 at 500 and 15 µg/mL, respectively, while the %

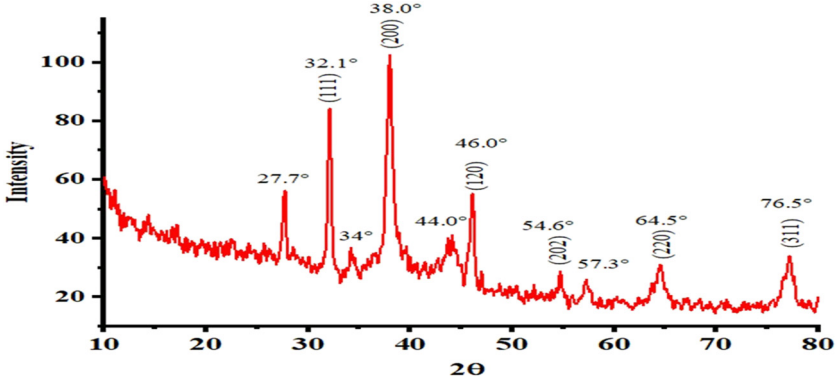

Figure 5: XRD pattern of Ws-AgNPs.

scavenging potentials for methanolic Ws ext at the highest and lowest concentrations of 500 and 15 µg/mL were 81.2 ± 1.44 and 33.57 ± 1.6, respectively. Similarly, *Atrocarpus altilis* leaf extract-mediated AgNPs demonstrated significant DPPH free radicals scavenging action [62]. Another study also reported excellent antioxidant activity for *Elephantopus scaber*-mediated AgNPs [63].

The IC_{50} value of ascorbic acid was 21.6 µg/mL, while those of Ws-AgNPs and Ws ext were 74.2 and 144.2 µg/mL, respectively. A lower IC_{50} value indicates high antioxidant activity while a higher IC_{50} value indicates poorer antioxidant activity. Figure 6a graphically represents the IC_{50} values and the scavenged DPPH percentage. The results indicated that phyto-fabricated AgNPs have higher scavenging potential than the plant root extract alone. Due to this potent antioxidant activity, Ws-AgNPs have the potential to be used in the cosmetic, food, and pharmaceutical industries [64]. With increased interest in using natural antioxidants as food additives, green-synthesized Ws-AgNPs can act as potent agents, ensuring food safety.

3.2.2 ABTS radical scavenging activity

WSR AgNP and Ws ext samples scavenged the ABTS radicals in a dose-dependent manner. Among these concentrations, 500 µg/mL of both the plant extract and Ws-AgNPs exhibited maximum inhibition activity. In the case of Ws-AgNPs, the maximum percentage scavenging activities of 98 ± 1.6 were observed at 500 µg/mL and 14.4 ± 1.4 at 15 µg/mL. Meanwhile, for Ws ext, they were 91 ± 1.49 and 11.3 ± 1.7 at 500 µg/mL and 15 µg/mL, respectively (Figure 6b).

The IC_{50} value of Ws-AgNPs was minimal (156.9 µg/mL), followed by Ws ext (195.5 µg/mL). Ascorbic acid had a lower IC_{50} value (57.52 µg/mL) than those of both the Ws-AgNPs and the root extract of the plant sample. The IC_{50} value indicated that Ws-AgNPs have the highest antioxidant potential. The IC_{50} value of the root is consistent with the results reported by Udayakumar et al. [65]. Similarly, Niraimathi et al. [66] reported the antioxidant potential of leaf and root extract from *W. somnifera* and their IC_{50} values. These findings suggest that *W. somnifera* possesses many metabolites, which can donate hydrogen from their hydroxyl group (–OH) to free radicals, creating stable and extremely reactive hydroxyl radicals. Another study using *Alternathera sessilis*-mediated AgNPs also reported their excellent antimicrobial and antioxidant potential [67]. Moreover, AgNPs synthesized from *Echium vulgare* also demonstrated significant antioxidant activity and catalytic degradation [68]. Our results are also in accordance with Mittal et al. [69], who reported excellent antioxidant and antimicrobial activities of *Eucalyptus globulus*- and *Salvia officinalis*-mediated AgNPs.

3.2.3 Phosphomolybdate assay

TAC is used to estimate the ability of plant samples to arrest oxidative stress. This procedure relies on using plant extracts to reduce molybdate ions and their conversion into phophomolybenum complex. In the current study, the phosphomolybdate assay was used to determine the antioxidant potential of the methanolic root extract and phyto-fabricated Ws-AgNPs.

The TAC% scavenging activities of Ws-AgNPs at the highest (500 µg/mL) and lowest (15 µg/mL) concentrations were 76.96 ± 1.57 and 40.61 ± 1.75, while the TAC% scavenging potentials for methanolic Ws ext at the highest and lowest concentrations were 64.02 ± 1.46 and 33.67 ± 1.44, respectively. The TAC% scavenging activity of ascorbic acid was 88.41 ± 1.49 at 500 µg/mL. The increase in the TAC was reported with increasing sample concentration. Similarly, the antioxidant activity of *Pteris tripartita* leaf extract-mediated AgNPs was reported by Alzubaidi et al. [70] and described significant radical scavenging potential using the phosphomolybdenum assay.

The IC_{50} value of Ws-AgNPs was the lowest (73.94 µg/mL) followed by Ws ext (175.23 µg/mL). The IC_{50} value indicated that Ws-AgNPs have the highest antioxidant potential. Ws-AgNPs had a higher antioxidant capacity than the extract; this discrepancy could be attributed to the different chemical compositions of the samples utilized in the study. Previous studies have demonstrated the significant antioxidant activity of the *W. somnifera* extract due to the presence of flavonoids, withanolides including withaferin A, withanolide B, withanoside V, and withanone [26]. Therefore, Ws-AgNPs' potential as antioxidants may be related to the complex of these antioxidant metabolites found in plants, which shield the biological components from oxidation and damage [71]. In addition, Ws-AgNPs possessed significant antioxidant activity due to stability gained by coating and capping of phenolic compounds [72]. Similar studies were carried out by Sathiyaraj et al. [73], who reported the antioxidant potential of green-synthesized AgNPs. Many studies exhibited similar results of antioxidant potential of phyto-fabricated AgNPs [74–76].

3.2.4 Hydrogen peroxide scavenging assay

The free radical scavenging activity of both the plant extract and Ws-AgNPs was also determined using the

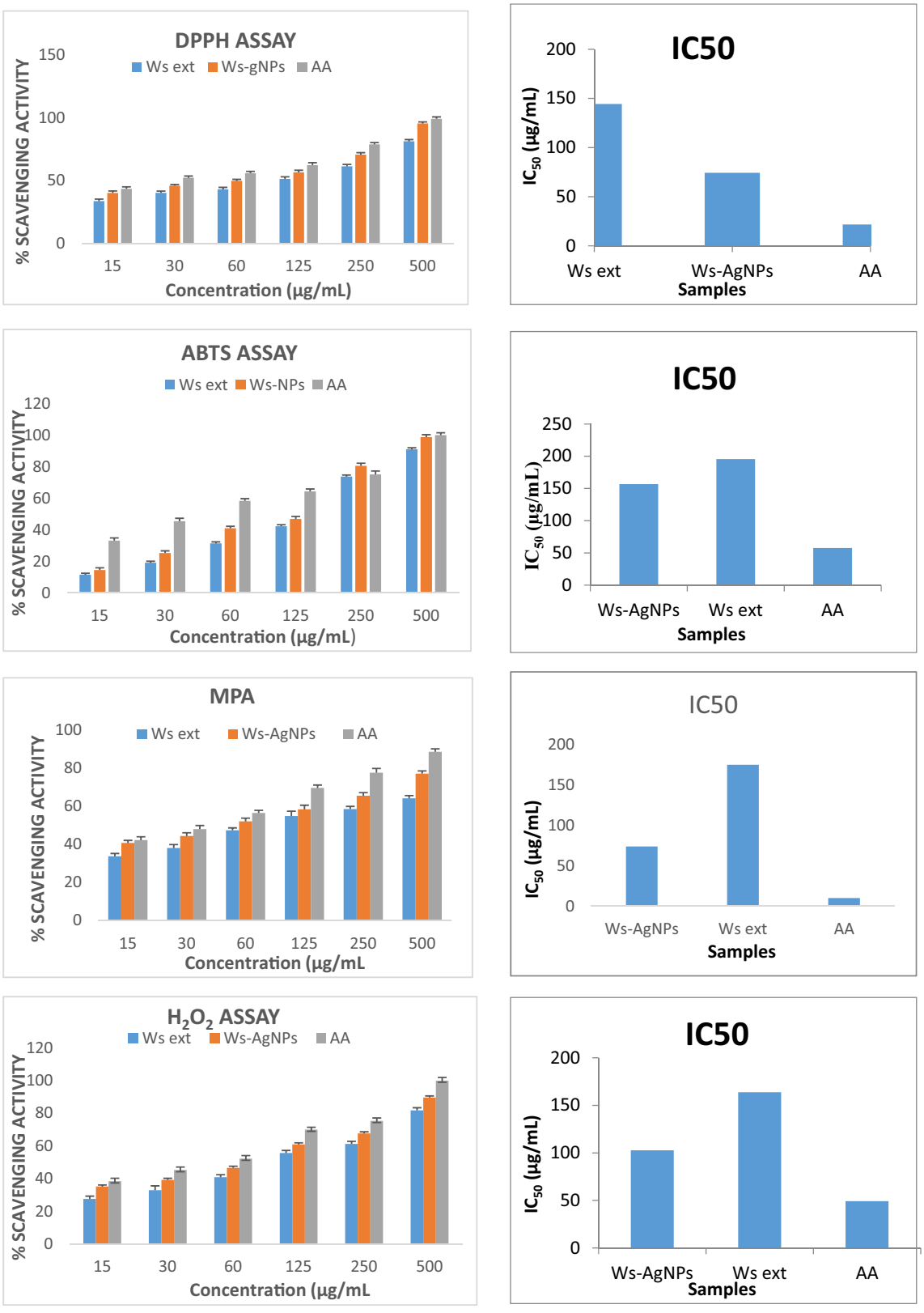

Figure 6: Graphical presentation of *in vitro* antioxidant studies using DPPH, ABTS, MPA, and H_2O_2 scavenging assays.

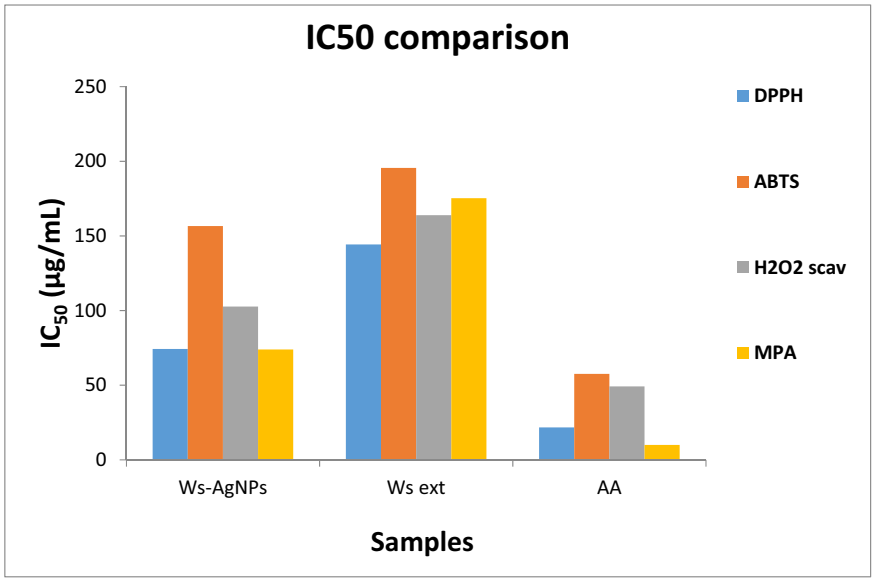

Figure 7: Comparison of the antioxidant activity (IC_{50}) of Ws-AgNPs, Ws ext, and ascorbic acid.

hydrogen peroxide scavenging assay at different concentrations. The maximum percentage scavenging activity observed for Ws-AgNPs was 89.65 ± 1.66 at 500 µg/mL while those for methanolic Ws ext at the highest and lowest concentrations of 500 and 15 µg/mL were 81.76 ± 1.64 and 27.51 ± 1.72, respectively.

Ws-AgNPs have shown significant antioxidant potential as they have a lower IC_{50} of 102.6 µg/mL compared to that of the Ws ext (163.8 µg/mL). Figure 5d presents the H_2O_2 scavenging percent inhibition vs the concentration. Antioxidant activity was found to be directly related to the concentrations, as shown in Figure 6d. The hydrogen peroxide assay is a frequently used method for assessing natural antioxidants' capacity to donate electrons. Numerous studies have shown the direct correlation between the reducing power of some plant extracts, which are further enhanced by nanoparticles, and their antioxidant activities. The IC_{50} value of the root extract is consistent with the results reported by Saleh and Mahdi [75]. Similarly, AgNPs synthesized from the hydroalcoholic extract of Triphala also exhibited significant antioxidant activity using the H_2O_2 scavenging assay [77]. The comparison of IC_{50} values of the root extract, Ws-AgNPs, and ascorbic acid for all the performed scavenging activities is graphically presented in Figure 7.

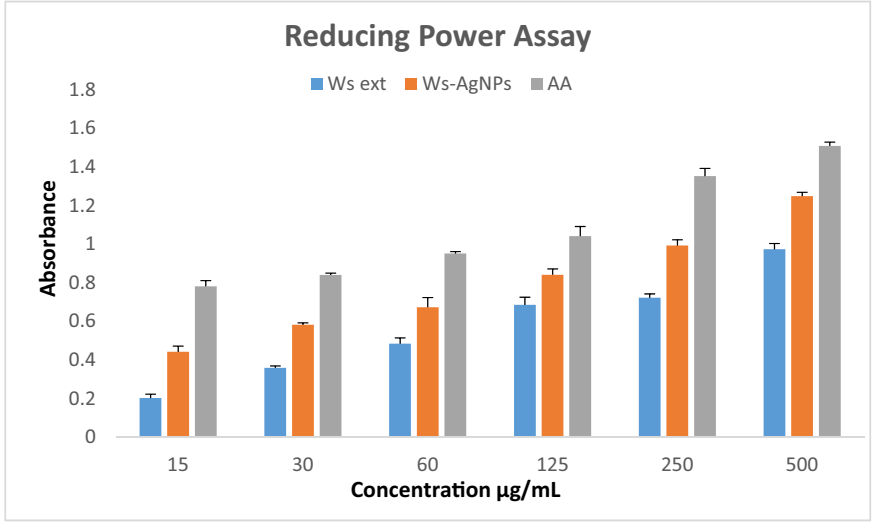

Figure 8: Ferric reducing power determination of phyto-fabricated Ws-AgNPs, root extract, and ascorbic acid.

3.2.5 Reducing power assay

This technique is employed to assess the reducing capacity of plant samples by reducing Fe^{3+} to Fe^{2+} through electron donation from antioxidants present in the extract. The concentration of reduced Fe^{2+} produced upon the addition of the plant extract was determined using a UV-Vis spectrophotometer at 700 nm. In the present study, the reducing potential of the root extract and phyto-fabricated AgNPs was assessed at different concentrations, i.e., 15, 30, 60, 125, 250, and 500 µg/mL. A direct relationship was found between the concentrations of Ws ext and Ws-AgNPs and their reducing power as the absorbance of samples increased with increasing concentration.

The maximum absorbance was observed for ascorbic acid, which was 1.508 ± 0.006 at 500 µg/mL. In the case of AgNPs, the maximum absorbances were found to be 1.248 ± 0.008 and 0.442 ± 0.01 at 500 and 15 µg/mL, respectively, while the absorbances of the methanolic Ws ext at highest (500 µg/mL) and lowest (15 µg/mL) concentrations were 0.973 ± 0.01 and 0.203 ± 0.01, respectively [60]. The greater antioxidant capacity was observed for green-synthesized AgNPs compared to ascorbic acid and the antioxidant potential of AgNPs increased with the concentration. Our results are also consistent with the findings of Balčiūnaitienė et al. [68], who reported strong antioxidant activity of AgNPs synthesized from *Eucalyptus globulus* and *Salvia officinalis* leaf extracts. The results indicated that phyto-fabricated AgNPs are significantly efficient candidates for free radical inhibition as compared to the plant extract. This antioxidant activity can be attributed to polyphenolic metabolites such as flavonoids, withanolides including withaferin A, withanolide B, withanoside V, and withanone, which are reported for their free radical scavenging potential [26] (Figure 8).

4 Conclusion

Extracts from medicinal plants are used for the phyto-fabrication of AgNPs, enabling biocompatible, user-friendly, cost-effective, and environmentally sustainable green chemical processes. AgNPs were successfully phyto-fabricated, characterized, and evaluated for their antioxidant potential. AgNPs were tested for their antioxidant activity using DPPH, ABTS, H_2O_2, phosphomolybdate, and reducing assays. The results indicated that AgNPs have significant antioxidant potential compared to the root extract. This study proved that *W. somnifera* contains numerous bioactive substances including polyphenols and flavonoids, which act as reducing and capping agents to stabilize AgNPs and enhance their antioxidant activity. Functional groups, especially the hydroxyl groups (−OH) of bioactive substances, may confer stability to the phenoxyl radicals by donating their −OH groups. Therefore, the pharmaceutical industry has a lot of potential for using surface coating of plant-bioactive compounds. Moreover, these results support the considerable antioxidant efficacy of Ws-AgNPs, which can be employed in the treatment of various illnesses due to the overproduction of free radicals in the body. Utilization of phyto-fabricated AgNPs offers a benefit over the extract. Phyto-fabricated AgNPs are easier to penetrate the cells because they are more stable and nanoscale. Thus, these results suggest that Ws-AgNPs could be employed in food, cosmetics, and pharmaceutical industries with various biomedical applications. However, there is a need for prior clinical testing and to check their toxicity. To the best of our knowledge, this is the first study to report the antioxidant activity of Ws-AgNPs derived from the *W. somnifera* root extract.

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Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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