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GREEN SYNTHESIS AND BIOLOGICAL EVALUATION OF GOLD NANOPARTICLES USING AQUEOUS EXTRACTS OF WITHANIA COAGULANS

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ABSTRACT
Background: Green synthesis of gold nanoparticles (AuNPs) using medicinal plants offers an eco-friendly and sustainable alternative to conventional nanoparticle production. Withania coagulans contains bioactive phytochemicals with potential reducing, stabilizing, and therapeutic properties. However, the biological activities and safety profile of W. coagulans-mediated AuNPs require further evaluation. This study investigated the green synthesis, characterization, and biological activities of AuNPs synthesized using aqueous extracts of the leaf, stem, and fruit of W. coagulans.
Objective: To synthesize and characterize gold nanoparticles (AuNPs) using aqueous extracts of Withania coagulans.
Methodology: The method used was to use plant extracts (leaf, stem and fruit) as a natural reducing and stabilizing agent to chloroauric acid. UV–Vis spectroscopy, SEM, TEM, FTIR, XRD and zeta potential analysis were used to assess the AuNPs. Antibacterial, antifungal, antioxidant, anti-inflammatory, antipyretic, cytotoxic and phytotoxic properties were evaluated using in-vitro and in vivo assays.
Results: Ruby red AuNPs were obtained, and their surface plasmon peak was found around 550nm. The spherical particles (20-100 nm) were confirmed by SEM and TEM. The antibacterial and antifungal activity of the AuNPs was more potent than the crude extracts; the antioxidant activity was significant; the toxicity to the cells was low and was dose-dependent; antifungal activity was exhibited; and the phytotoxicity to Lemna minor was measurable. AuNPs prepared with W. coagulans showed a promising bioactivity profile along with an acceptable safety profile in line with the trends of sustainable nanotechnology and indicate potential biomedical relevance.
Conclusion: W. coagulans-mediated AuNPs demonstrated promising bioactivity with a favourable safety profile, aligning with sustainable nanotechnology trends and suggesting biomedical relevance.

KEYWORDS: Withania coagulans, gold nanoparticles, green synthesis, bio-nanotechnology, antimicrobial activity, anti-inflammatory activity

INTRODUCTION
Nanotechnology has revolutionized modern biology and medicine by providing an opportunity to create nanoscale systems that exhibit different physicochemical properties.[1] The chemical stability, tunable surface chemistry, and living systems compatibility of gold nanoparticles (AuNPs) make them particularly attractive. The conventional chemical synthesis, however, uses toxic agents which lead to safety and environmental concerns.[2,3] However, green synthesis is a potential alternative, reducing and stabilizing metal ions with biological extracts. Plant-based synthesis is particularly attractive because phytochemicals are reducing and capping agents that can be used to produce nanoparticles without the production of hazardous waste.[4,5]
A summary of the existing literature reveals that Withania coagulans is extensively employed in the traditional medicine of South Asia in inflammatory diseases, diabetes, liver ailments, and gastrointestinal ailments.[6] Reports point to good antioxidant, antimicrobial, and anti-inflammatory effects.[7] However, the production of nanoparticles from this species has yet to be explored. In this study, the aim was to synthesize AuNPs with aqueous extracts of W. coagulans and to see their biological properties, which will support the use of environmentally friendly nanomedicine.
This study aims to synthesize and characterize gold nanoparticles (AuNPs) using aqueous extracts of Withania coagulans.

METHODOLOGY

2.1 Plant extraction and nanoparticle synthesis

Aerial parts of W. coagulans were shade dried, powdered and extracted with distilled water. 1 mM $HAuCl_4$ was reacted with extracts (1% w/v). AuNP formation was seen by ruby-red colour.

2.2 Characterization

The AuNPs were characterized by using UV-Vis spectroscopy [8], SEM and TEM [9], EDX [10], FTIR [11], XRD [12] and zeta potential measurements [13].

2.3 Biological Assays

The antibacterial and antifungal activities were evaluated using disc diffusion tests [14,15]. The brine shrimp lethality method was used to determine cytotoxicity [16]. The antioxidant activity was determined by the DPPH assay [17]. Lemna minor [18] was used as the model species to assess phytotoxicity. The hemagglutination was studied in the form of RBC agglutination [19]. The anti-inflammatory activity, as well as the antipyretic activity, was examined in classical in vivo models [20,21].

RESULTS

Physical Characterization

First, the synthesizing ability of gold nanoparticles by the aqueous extract of Withania was confirmed by the addition of $AuCl_3$ solution (final concentration 200mg/L) to a freshly prepared 1.0% (m/v) extract and incubated in the dark at 20 °C for 24 hours. The reaction mixture turned ruby-red to bluish colour, indicating the reduction of Au^{3+} to Au^0 and formation of AuNPs as reported earlier. This was also confirmed by UV–Visible spectrophotometry, where the SPF of the biosynthesised nanoparticle was observed at 550 nm (Figure 1a) and the crude extract did not show any absorbance band (Figure 1b). This unique peak further validates the successful bio-reduction and AuNP formation by W. coagulans, thus establishing that the synthesis process was responsible for AuNP formation, and not an inherent property of the plant extract.

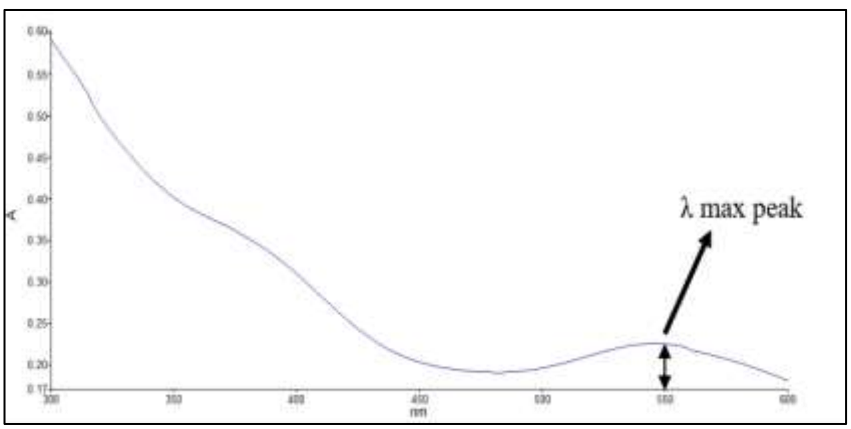


Figure 1(a) : UV-Vis spectrum of the biosynthesized AuNPs

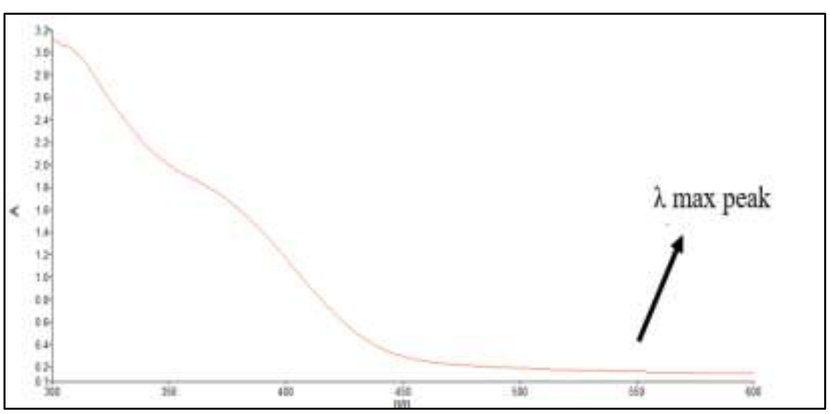
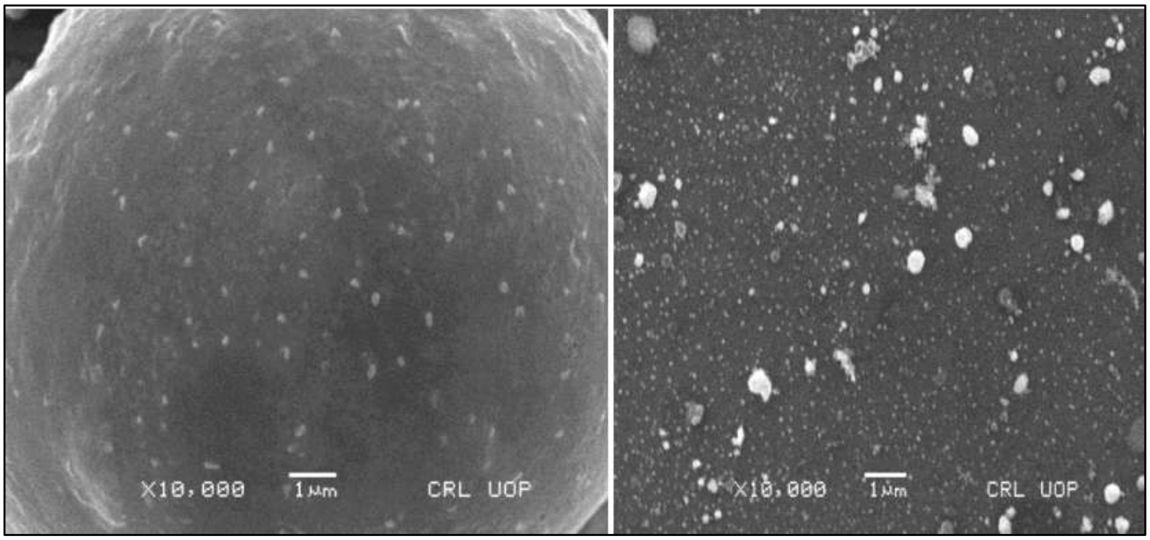


Figure 1 (b): UV-Vis spectrum of the aqueous plant extract

For the analysis of the structural features of the Withania coagulans aqueous extract and biosynthesized AuNPs, scanning electron microscopy (SEM) was used (Figure 2a–b). The plant extract exhibited irregular, heterogeneous and polydispersed surface profile, typical of the plant organic constituents. By contrast, the synthesized AuNPs had a mostly monodispersed spherical shape with a few triangles and irregular shapes. These observations show a successful and stable nanoparticle formation process and uniform particle distribution. The comparative SEM images (Figure 3a–b) clearly showed that the extract of W. coagulans can aid in controlling the formation of nanoparticles in the extract matrix, which is naturally disordered while the structures of the nanoparticles are well defined.



(a) (b)

Figure 3: (a) (b) SEM image of biosynthesized gold nanoparticles (AuNPs) using Withania coagulans leaf extract

Transmission electron microscopy (TEM) analysis

The synthesized AuNPs using the leaf extract of Withania coagulans were analysed by transmission electron microscopy (TEM), which confirmed that the prepared AuNPs were mostly spherical in shape with a few irregular shapes, which may be AuNP aggregation of nano-spheroids. The size of the particles varied from 20 to 100 nm in diameter, which is in the range of stable metal nanoparticles and is essential for their optical and physicochemical characteristics. Figures 3.3(a) and 3.3(b) are representative TEM images, with Figure 3.3(a) displaying the uniform spherical morphology and Figure 3.3(b) accentuating the sometimes-non-spherical morphology. The observations confirm the successful synthesis of AuNPs with well-defined morphology, which is well-suited for biomedical and catalytic applications.

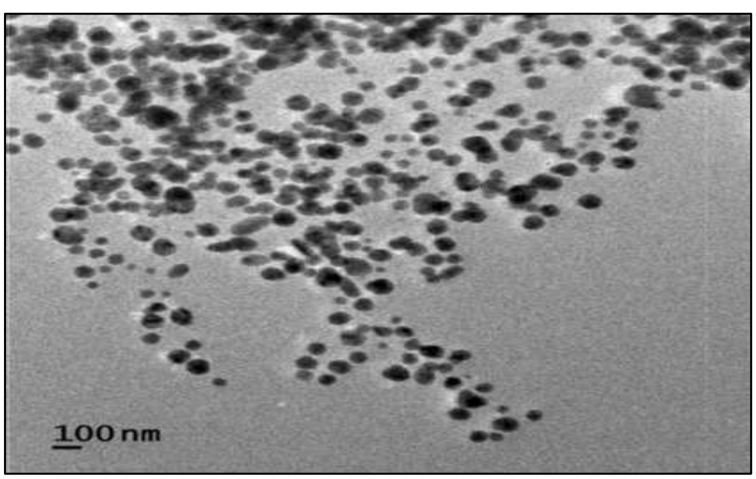


Figure 3(a): TEM image illustrating the generally spherical shape of the AuNPs

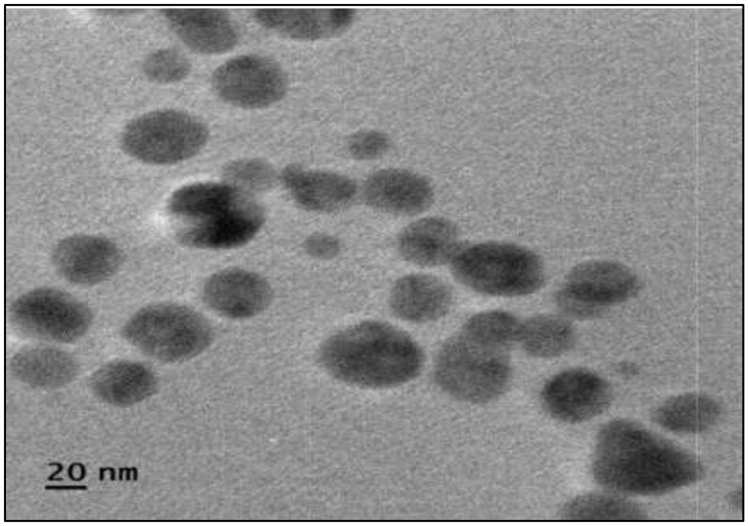


Figure 3(b): TEM showing some of the irregularly shaped nanoparticles

EDX analysis
According to the EDX analysis, the successful biosynthesis of AuNPs was confirmed by the presence of a broad peak of elemental gold (Au°) in the spectrum of the nanoparticles. To confirm AuNPs were only formed after the reduction process, a gold signal did not appear in the crude extract. The additional elements C, N, O, K, Ca, Na, Cl and Mg were indicative of the organic matrix of the plant from which the nanoparticles were produced (Figure 4a,4b).

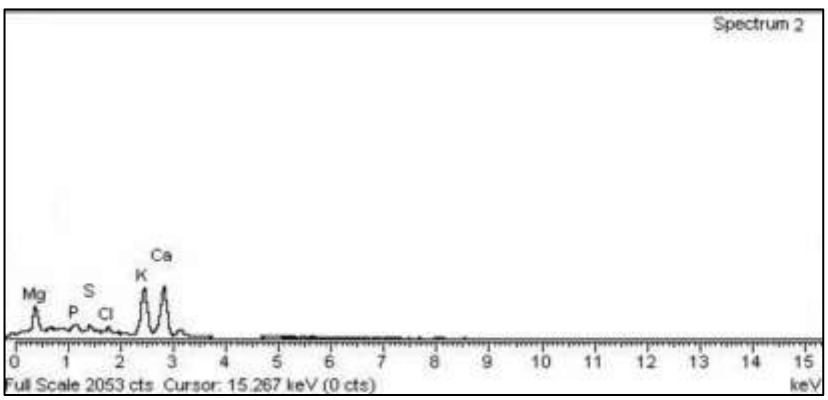


Figure 4(a): EDX micrograph of aqueous extract of Withania coagulans

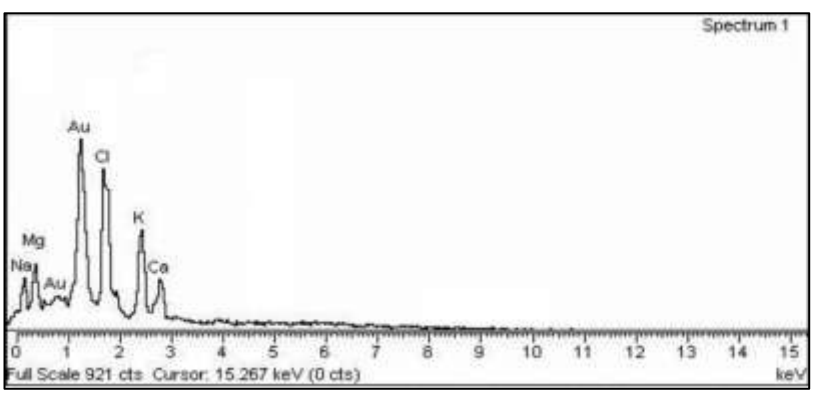


Figure 4(b) EDX analysis of biosynthesized AuNPs from aqueous extract of Withania coagulans

XDR Analysis
The results of X-ray diffraction indicated good evidence of crystalline gold. The observed peaks at specific 2θ angles—38.10°, 44.13°, 64.43°, and 77.32°—were consistent with the (111), (200), (220), and (311)

planes of an FCC-type gold structure (fig 3.5). The high intensity of the planes (111) indicated that the crystals aligned favourably, and the peaks were broadened, confirming the nanoscale dimensions of the particles. Size estimation was done by applying the Scherrer equation and it is found that the average crystallite size is about 28 ± 1.5 nm, which is a successful result of the formation of the nanoparticles by the plant-mediated synthesis.
The X-ray diffraction (XRD) pattern of biosynthesis of AuNPs using an aqueous extract of Withania coagulans is presented in figure 5. X-ray diffraction (XRD) pattern of biosynthesis of AuNPs using an aqueous extract of Withania coagulans is shown in figure 3.5.

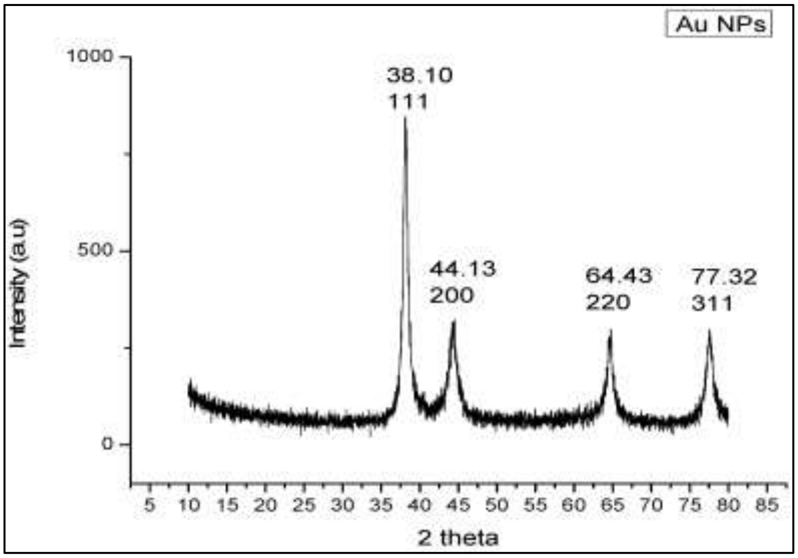


Figure 5: X-ray diffraction (XRD) patterns of biosynthesis of AuNPs using an aqueous extract of Withania coagulans

Antibacterial Activity
The antibacterial activity of the synthesized gold nanoparticles (AuNPs) was investigated by the well-diffusion method against gram-positive bacteria (S. aureus and S. pyogenes) and gram-negative bacteria (E. coli, P. aeruginosa and S. Typhi). The metabolic extract and N-hexane fraction exhibited moderate inhibition with zones of 14–16 mm and 12–15 mm, respectively, while the AuNPs exhibited the maximum inhibition with zones of 18–24 mm. The aqueous extract showed very little inhibition (10-12 mm). As a positive control, ciprofloxacin always yielded zones of 26 mm.
The enhanced activity of AuNPs has been explained by the high surface area and the ability of drug molecules to interact with the bacterial cell wall due to their affinity for the drug molecules. The literature indicates that AuNPs are able to disrupt the peptidoglycan layer and penetrate the cells, leading to cell damage and destruction. The results presented here show that the prepared AuNPs are indeed highly effective in combating bacteria and their activity is better than all the plant extracts tested (Table 1).

Table 1: Antibacterial activity of plant extract and synthesized gold nanoparticles (AuNPs) measured by the disc diffusion method. Values represent the zone of inhibition (mm) ± standard deviation (n = 3).

S. No	Tested solution	Zone of inhibition in (mm) against Pathogenic bacteria					Statistical Significance (p-value)
		S. aureus	E. coli	S. pyogenes	S. Typhi	P. aeruginosa	
1	AuNPs	24 ± 0.74	22 ± 0.97	23 ± 0.73	22 ± 0.97	18 ± 0.97	0.001
2	Metabolic extract	15± 0.67	16± 0.56	16± 0.78	14±0.98	14±1.02	0.001
3	N-Hexane	14±0.69	14± 0.78	15±0.95	12±0.45	13±0.66	0.001
4	Aqueous extract	10±0.45	12± 0.56	12±0.76	11± 0.78	12 ± 0.87	0.001
5	Positive control	26 ±1.02	25±0.92	27± 0.78	26±0.87	24±0.69	0.017
	ANOVA p-value	7.46×10^{-9}	1.01×10^{-10}	7.24×10^{-9}	2.21×10^{-9}	$7.48x\ 10^{-9}$	<0.05: Significant

The antifungal Activity

Gold nanoparticles (AuNPs) were the most effective at inhibiting all fungal species, with the highest response being seen with A. flavus, with an inhibitory value of 34.7% and 23.2% for the other species. The methanolic and n-hexane extracts gave moderate suppression, especially against A. niger and A. flavus, which was less active than that of AuNPs. The aqueous extract exhibited the poorest inhibition, probably because the water is not the most appropriate solvent for extracting the antifungal compounds. The differences between treatments were significant ($p < 0.05$) as confirmed by the statistical analysis. The results point to the broad-spectrum antifungal activity of AuNPs, which can now be formulated more optimally or increased in concentration to enhance their activity, while the activity of the crude plant extracts can be improved with better formulation or higher concentrations. The overall antifungal results are presented in Table 2.

The details of various extracts and biosynthesised AuNPs evaluation against pathogenic Fungi are presented in Table 2.

Table 2: Results of various extracts and biosynthesized AuNPs against pathogenic Fungi

S. No	Tested compound	% inhibition of various fungal species				Statistical Significance (p-value)
		A. niger	F. solani	A. flavus	A. fumigatus	
1	AuNPs	32.2 ± 1.36	29.4 ± 2.35	34.7 ± 2.50	23.2 ± 1.00	< 0.05: Significant
2	Methanolic Extract	21.7 ± 2.00	10.6 ± 1.43	20.8 ± 1.62	20.4 ± 1.37	< 0.05: Significant
3	H-Hexan	21.5 ± 2.66	14.6 ± 1.98	24.9 ± 2.18	19.1 ± 1.88	< 0.05: Significant
4	Aqueous Extract	11.4 ± 1.08	10.5 ± 1.02	17.6 ± 1.39	12.0 ± 1.72	< 0.05: Significant
5	Positive control	43.3 ± 2.39	34.6 ± 1.74	53.2 ± 1.42	44.6 ± 1.43	< 0.05: Significant
	ANOVA p-value	**7.46×10^{-9}**	**1.01×10^{-10}**	**7.24×10^{-9}**	**2.21×10^{-9}**	**Significant**

Cytotoxic Activity

The cytotoxic activity of AuNPs and plant extracts was evaluated through the brine shrimp lethality assay at three concentrations, 10,000, 100,000 and 1,000,000 µg/L. Increased mortality was recorded at the highest concentration (1,000,000 µg/l) of AuNPs, with a mild concentration-dependent response of 20% mortality in the two lower concentrations. The methanolic extract was non-dose-responsive, with constant 30% cytotoxicity at all concentrations. The n-hexane extract, on the other hand, showed a definite increase in toxicity with concentration, with the highest level at 1,000,000 µg/L. In general, the aqueous extract was not lethal at all, with slightly more lethality at the highest dose. The results suggest that the aqueous extract is relatively non-toxic, while AuNPs and n-hexane extracts are toxic. The complete results are given in Table 3.

The synthesised nanoparticles (AuNPs) were found to be cytotoxic to the brine shrimp (Artemia salina) at different concentrations (Table 3) with the percentage mortality presented ($n = 3$).

Table 3: Cytotoxic effect of synthesized gold nanoparticles on brine shrimp (Artemia salina) at different concentrations, showing percentage mortality (n = 3).

S. No	Tested compound	% Cytotoxic effect of various concentration			p-Value (Significance)
		10000 µg/L	100000 µg/L	1000000 µg/L	
1	AuNPs	20.0 ± 2.00	20.0 ± 2.00	30.0 ± 10.0	< 0.05: Significant

2	Methanolic Extract	30.0 ± 10.0	30.0 ± 10.0	30.0 ± 10.0	< 0.05: Significant
3	H-Hexan	20.0 ± 2.00	30.0 ± 10.0	40.0 ± 10.0	< 0.05: Significant
4	Aqueous Extract	20.0 ± 2.00	20.0 ± 2.00	30.0 ± 10.0	< 0.05: Significant
5	Positive control	50.0 ± 10.0	50.0 ± 10.0	60.0 ± 10.0	< 0.05: Significant
	ANOVA p-value	0.0017	0.0044	0.0003	Significant

The findings suggest that the cytotoxicity levels of all the tested compounds are characteristic with a concentration-dependent rise in the case of AuNPs and n-hexane extract while methanolic and aqueous extract remain consistent.

Antioxidant Assay

The antioxidant activity of AuNPs was evaluated by the DPPH radical scavenging assay with comparison to crude methanolic, n-hexane and aqueous extracts at concentration of 1mg/mL. AuNPs showed high scavenging activity (64.56%) close to the activity of ascorbic acid (72.00%) in the positive control. The methanolic extract exhibited moderate activity (23.77%) and the n-hexane (17.05%) and aqueous extracts (7.50%) exhibited much lower effects. The negative control DMSO (without test compound) did not inhibit, indicating that the scavenging activity was due to the test compounds. The statistical analysis showed that the activity of AuNPs was not significantly different from the standard at highly stringent levels, and that all crude extracts had significantly reduced activity ($p < 0.001$). It was found that AuNPs have significant antioxidant activity similar to ascorbic acid while plant extracts, especially aqueous extract, showed weak antioxidant activity. Complete results are given in Table 4.

The antioxidant activity of plant extract and synthesised gold nanoparticles (AuNPs) as determined in DPPH radical scavenging assay is presented in Table 4.

Table 4: Antioxidant activity of plant extract and synthesized gold nanoparticles (AuNPs) measured by DPPH radical scavenging assay.

S. No	Tested compound	Concentration	DPPH (inhibition %)	p-value (vs. Ascorbic acid)
1	AuNPs	1mg/ mL	64.56	**< 0.005** (highly significant)
2	Methanolic Extract	1mg/ mL	23.77	**< 0.001** (highly significant)
3	H-Hexan	1mg/ mL	17.05	**< 0.001** (highly significant)
4	Aqueous Extract	1mg/ mL	07.50	**< 0.001** (highly significant)
5	Positive control (Ascorbic acid)	1mg/ mL	72.0	Reference
6	Negative Control (DMSO)	N/A	0.00	**< 0.001** (highly significant)

Phytotoxic Activity

The potential for phytotoxicity of AuNPs and plant extracts was assessed using the Lemna minor lethality assay with concentrations of 10, 100 and 1000 µg/mL. AuNPs demonstrated strong, dose-dependent inhibition, reaching 95.56% at 1000 µg/mL, 71.10% at 100 µg/mL, and 27.76% at 10 µg/mL. The methanolic extract had moderate phytotoxicity (55.55%, 37.77% and 18.88% at the respective concentrations) while the n-hexane extract had high inhibition at the highest concentration (93.33%) but declined in a steep manner at lower concentrations. The aqueous extract had the least inhibitory activity (32.99 % at 1000 µg/mL); it was very low at the lower concentrations. The positive control paraquat was the most toxic in all the concentrations tested, thus validating the assay. No significant difference was found between AuNPs and the standard at 1000 µg/mL ($p > 0.05$), with significant differences observed at the lower concentrations ($p < 0.01$). The results show that the aqueous extracts are not as effective as herbicidal agents as AuNPs and n-hexane extracts. Table 3.5, on pages 68–69, contains complete data.

Table 4.5: Concentration dependent phytotoxic potential of AuNPs in comparison with plant extracts

S. No	Tested compound	Concentration wise % inhibition		
		1000 µg/ml	100 µg/ml	10 µg/ml
1	AuNPs	95.56 ± 1.10%	71.10 ± 1.10%	27.76 ± 1.90%
2	Methanolic Extract	55.55 ± 2.223%	37.77 ± 1.113%	18.88 ± 1.113%
3	H-Hexan	93.33 ± 1.90%	56.66 ± 1.90%	42.23 ± 2.90%
4	Aqueous Extract	32.99 ± 2.502%	19.99 ± 1.925%	7.77 ± 1.113%
5	Positive control (Paraquat at 0.015 µg/ml)	96.67 ± 1.90%	82.23 ± 2.90%	51.10 ± 1.10%

DISCUSSION

The study shows the successful synthesis of AuNPs by using aqueous extract of Withania coagulans as green reducing agents. The formation of the nanoparticles was confirmed by ruby-red colour and SPR of ~550 nm, which is consistent with the earlier reported results that Au^{3+} ions were reduced to metallic gold using metabolites from plant [22,23]. SEM and TEM images showed predominantly spherical AuNPs ranging in size from 20 to 100 nm, which was similar to the results obtained from other medicinal plants [24]. The XRD results confirmed the crystalline nature of the gold with FCC structure, and is in good agreement with JCPDS data and previous studies of biologically reduced AuNPs [25].

In terms of biological activity, AuNPs exhibited potent antibacterial activity, with inhibition zones ranging from 18 to 24 mm, which is similar to the inhibition zones of AuNPs prepared from Withania somnifera and Azadirachta indica [26,27]. The antimicrobial activity is due to the interaction between the nanoparticles and the membrane and to the oxidative stress. Anti-fungal results also revealed good inhibition (23-35%), in line with the previous reports of damage to the fungal cell membranes caused by metal nanoparticles [28]. The cytotoxicity assay revealed slight toxicity of the biosynthesized AuNP (20-30 %), which is comparable to previous studies on biosynthesized AuNP [29]. Antioxidant evaluation revealed high radical scavenging activity (64%), which is consistent with reports indicating that the ability of these nanoparticle-phytochemical conjugates to donate electrons is enhanced [30]. The phytotoxicity results indicated significant and dose dependent inhibition as found in studies related to the oxidative stress induced after the application of nanoparticles in plants [31]. Umbilical cord stem cells from W. coagulans showed excellent antibacterial, antioxidant, antifungal, phytotoxic and anti-hemagglutination properties in general. The properties of these materials underpin their potential application in biomedical and agricultural research as environmentally friendly alternatives to nanoparticles.

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