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Article

ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF SILVER NANOPARTICLES SYNTHESIZED USING *RHEUM EMODI* ROOTS EXTRACT

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ABSTRACT

Antimicrobial and cytotoxic potential of silver nano particles (AgNPs) synthesized through greener route using *R. emodi* root extract was investigated. The synthesis of AgNps was confirmed by visualizing the colour change which shows surface plasmon resonance (SPR) peak at 425 nm. FTIR analysis indicates the involvement of anthraquinone pigments, in capping and stabilization of AgNps. FESEM, XRD, TEM, with EDX display the formation of crystalline AgNps with face centered cubic (FCC) symmetry with average size 27.5nm. AgNps were found to exhibit significant antimicrobial activity. Similarly, synthesized AgNps show substantial change in cytotoxicity against human breast lines (MCF-7 cell lines).

Keywords: *Rheum emodi*, Green synthesis, Silver nanoparticles, Antimicrobial, Cytotoxic potential.

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1. INTRODUCTION

In the present era of nanotechnology, nanoparticles have found tremendous applications in various fields such as optical polarizability, catalysis, electronics and medicine [1-3]. Copper, silver, gold have unique properties that offer numerous application in audiophile, photonics, photography, biological labelling and also have scientific application [4-5]. Literatures have reported the use of diverse biological regime such as plants extract, algae, diatom, fungi and bacteria for the synthesis of nanoparticles [6-10]. But, the biogenic synthesis of these nanoparticles using plant biomass has been found to be more viable owing to its simplistic methodology and easy to scale up processes. This increases the scope of investigation and regulation of nanoparticles at cellular stage, drug delivery, hepatitis B, cancer detection and treatment, environmental pollution control, ceramic coatings, HIV/AIDS treatment, textile industries etc. [11-16].

Among diverse nanoparticles, silver nanoparticle has been found to be more promising due to its anti-inflammatory, anti-angiogenesis, antifungal, antiviral, anti-inflammatory, antibacterial, anti-platelet activity, diagnosis and against cancer cells which makes them vital [17-21]. It has gained attention over the years because of distinctive properties, such as good conductivity, low sintering temperature, chemical stability, optical properties and antimicrobial coating [22-25].

Rheum emodi is one of the natural sources which belong to polygonaceae family. It is an important medicinal plant and the main part used as drug is root. The plant contains several biomolecules such as flavonoids, saponins especially anthraquinones. The anthraquinone derivatives such as Aloe emodin, Emodin, Physcion, Rhein and Chrysophanol etc. show remarkable antimicrobial, antiviral, antifungal, antioxidant, anti-Parkinson's and anticancer activities [26-27].

Only few studies have been reported on root mediated synthesis of biogenic metal nanoparticles using various plant resources such as *Rumex hymenosepalus* [27], *Erythrina indica* [28], *Delphinium denudatum* [29], *Nelumbo nucifera* [30], *Streptomyces hygroscopicus* [31], *Momordica charantia* [32], *Acacia nilotica*[33]. The present study focusses on biogenic synthesis of silver nanoparticles using methanol root extract of *R. emodi* evaluated for its antimicrobial, antifungal and anticancer applications.

Figure 1: (a) UV-vis spectra of *R.emodi* root crude extract and Synthesized Ag NPs. (b) XRD pattern of Synthesized Ag NPs. FTIR transmittance of (c) *R.emodi* root crude extract and (d) Synthesized Ag NPs.

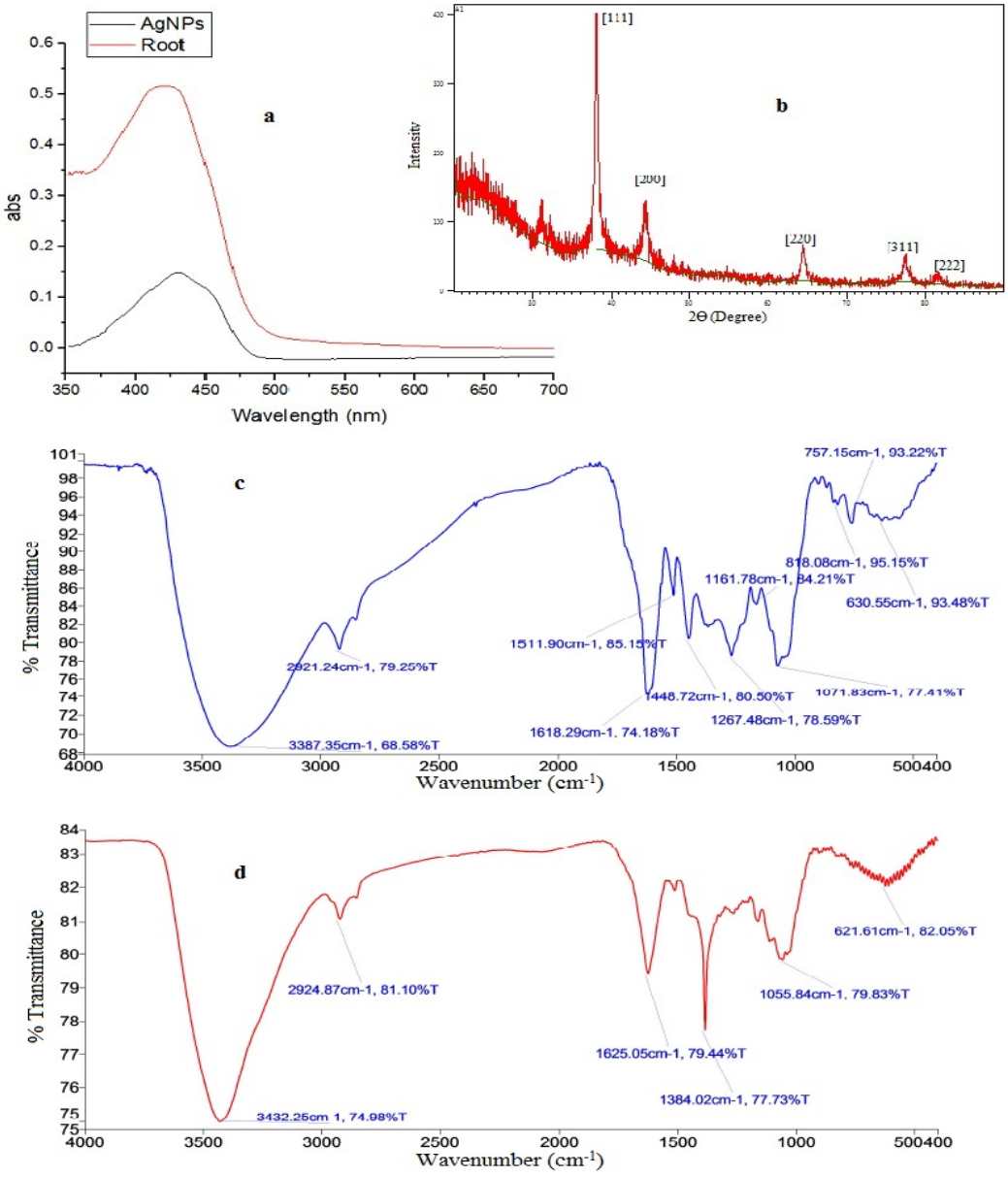

Figure 2: (a-b) TEM micrograph of synthesized Ag NPs, (c-d) FESEM micrograph of synthesized Ag NPs showing spherical shape and (e) EDX shows strong metal peaks of silver

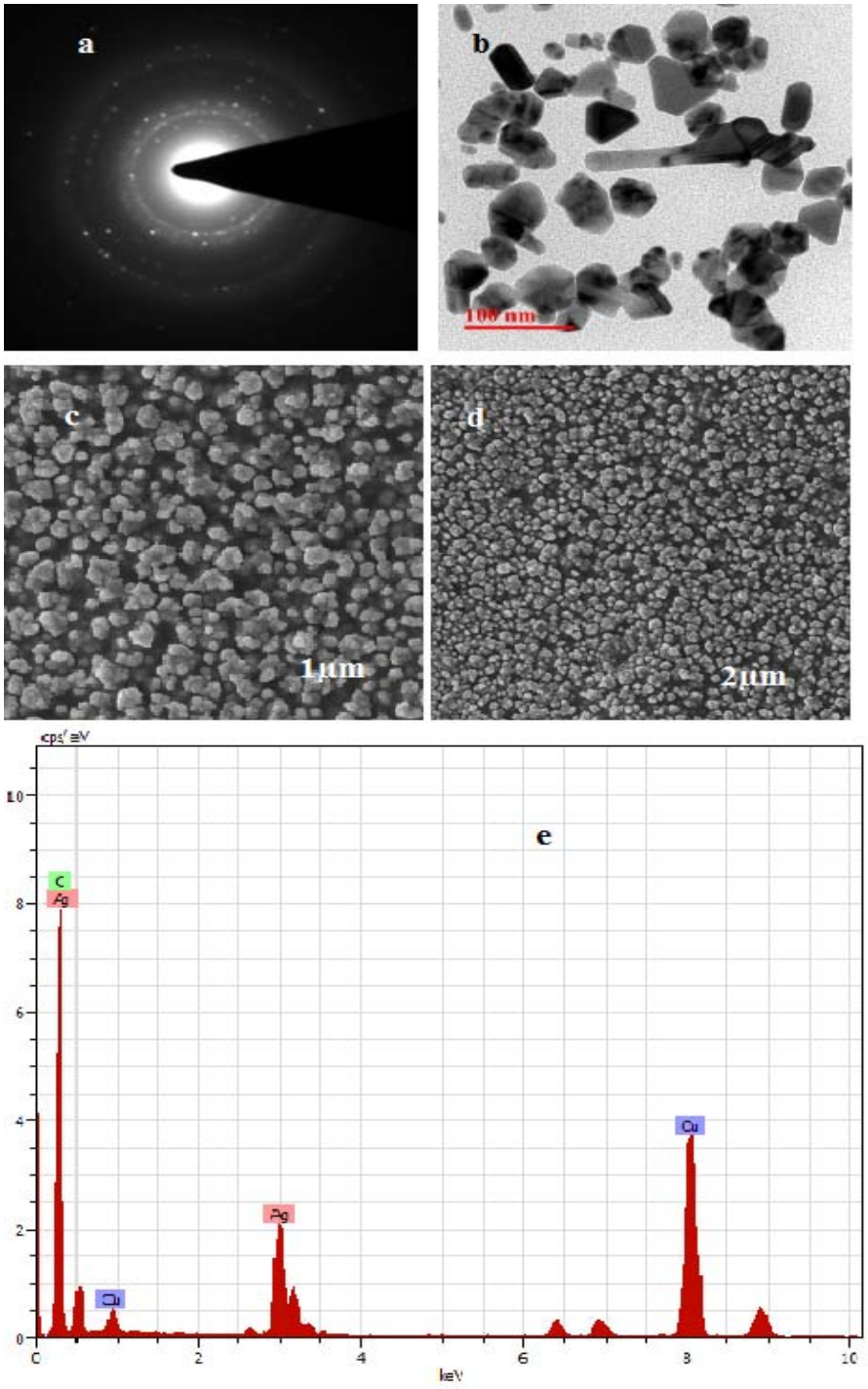

Figure 3: Antimicrobial and antifungal activity of synthesized Ag NPs using *R.emodi* root crude extract (A1) and *R.emodi* root crude extract (A) against different human pathogens and fungi's (a) *Streptomyces griseous*, (b) *Bacillus subtilis* (c) *Staphylococcus aureus* (d) *E.coli,*(a1) *Aspergillus niger*, (b1) *Fusarium oxysporum*. Concentration range 50 and 100mg/ml.

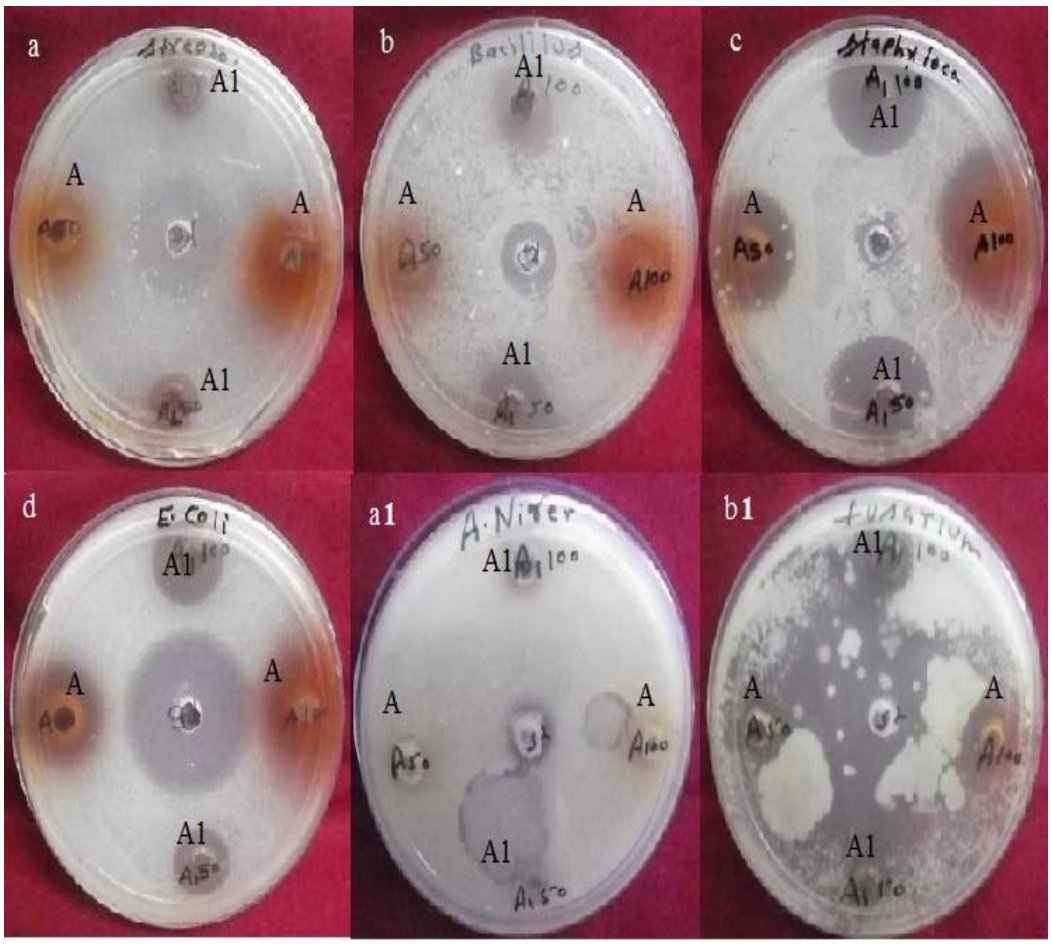

Figure 4: Anticancer activity of Ag NPs and crude extract (a) Control (b) Positive control (c) *R.emodi* root crude extract – 250 µg (d) Synthesized AgNP's – 250 µg. Effect of cell viability 0020and cytotoxicity of (e) *R.emodi* root crude extract and (f) synthesized Ag NPs of *R.emodi* root crude extract in MCF-7 cancer cells

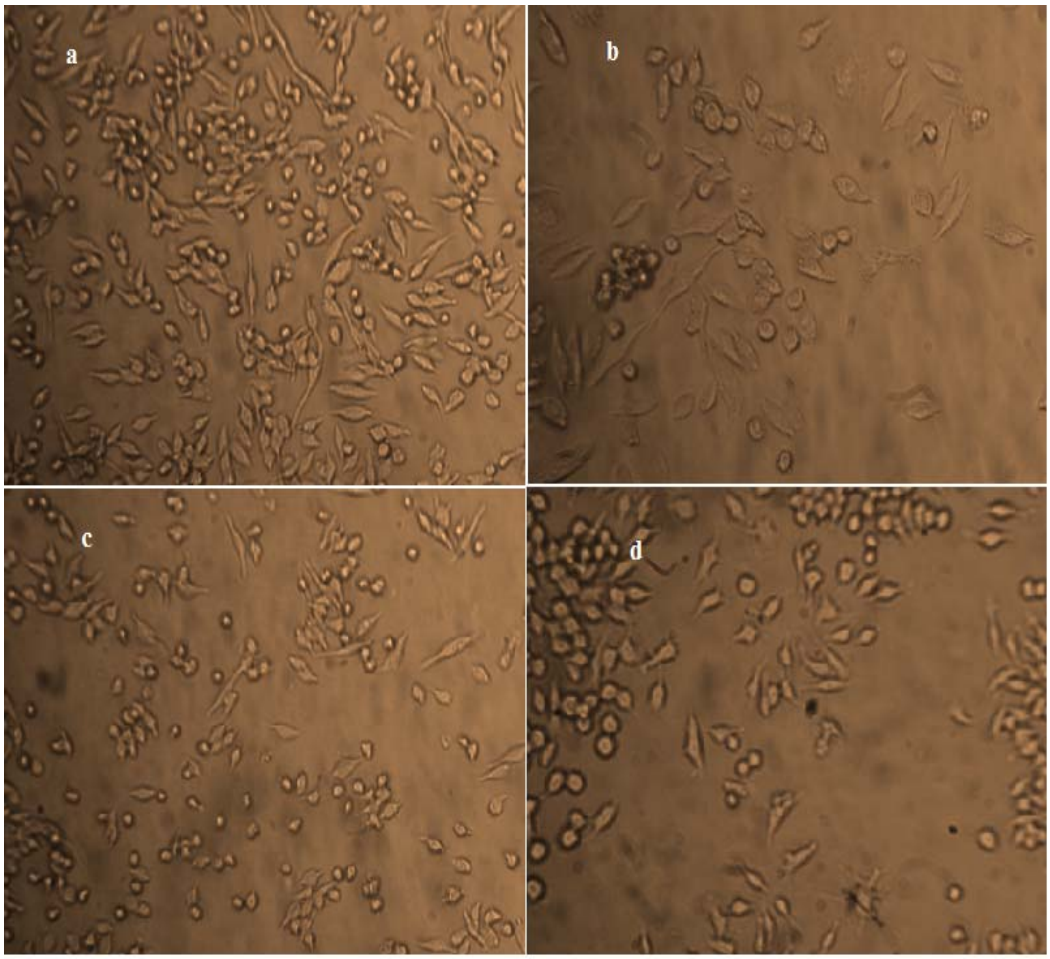

Table 1: Inhibitory action of synthesized Ag NPs and *R.emodi* crude root extract against human pathogenic bacteria & fungi

Test strain	Concentration of crude extract 50mg/ml , 100mg/ml		Concentration of Ag NPs 50mg/ml, 100mg/ml	
	Zone of inhibition in mm		Zone of inhibition in mm	
Bacillus subtilis	9 ±0.5	12 ±0.5	18±0.5	22 ±0.5
Staphylococcus aureus	28 ±0.5	38 ±0.5	32±0.5	34 ±0.5
Streptomyces griseous	9 ±0.5	12 ±0.5	14 ±0.5	16 ±0.5
Escherichia coli	18 ±0.5	22 ±0.5	20 ±0.5	30 ±0.5
Aspergillus niger	Nil	12 ±0.5	Nil	14 ±0.5
Fusarium oxysporum	16 ±0.5	22 ±0.5	13 ±0.5	20 ±0.5

Table 2: Percentage of cell viability and cytotoxicity of *R.emodi* root crude extract and synthesized AgNPs

Test	Root extract Concentration (µg/ml)					Ag NPs Concentration (µg/ml)					PC	C
	50	100	150	200	250	50	100	150	200	250		
% of Viability	83.78	74.94	65.35	55.73	46.57	77.32	64.76	53.70	42.40	34.13	26.11	100
% of cytotoxicity	16.21	25.05	34.64	44.26	55.43	22.67	35.23	46.29	57.59	65.87	73.89	0

2. METHOD

2A. Chemicals

Silver nitrate (AgNO3), A.R. and methanol, A.R. used in this study were procured from Merck (India) All other analytical reagent were purchased from Merck (Germany).

2B. Extraction of root extract and Synthesis of AgNPs

Roots of *R. emodi* were shade dried and pulverized in Wiley mill to powder form. The air dried and crushed root (250g) of *R. emodi* was extracted with methanol solvent in soxhlet extractor and the extract was concentrated under the reduced pressure. The same methanolic extract was used as a crude extract for further analysis. For the synthesis of AgNps; 10ml of root extract were dissolved in 500ml of double distill water and then boiled for 7-8minutes before decantation. The 10ml of obtained supernatant was mixed with 50ml of 1mM $AgNO_3$. The reaction mixture was incubated until colour changes from dark brown to orange at the room temperature.

2C. Characterization of AgNPs

The reduction of silver ions was monitored by UV-Visible (LAMBDA 750 Perkin Elmer) spectrophotometer at different time intervals (0h, 12h, 24h) between 300-700nm wavelength range. FTIR (Compact Perkin Elmer) spectral analysis in the range between 4000 cm^{-1} and 400 cm^{-1} was carried out to identify the possible biomolecules present in the *R. emodi* root extract which are responsible for reduction and stabilization of AgNPs. The shape and size of AgNPs were described by Transmission electron microscopy (TEM; TecnaiG^2 T20 ST) with EDX equipment. EDX analysis showed a strong elemental silver peak and morphological measurements which were observed by Field emission electron microscope (FESEM; Nano Nova 450). X-ray diffractometer (Panalytical X-Pert Pro) analysis describes crystallinity and size of synthesized AgNPs. [29]

2D. Antimicrobial Assay

i) Antibacterial activity

The antibacterial activity of root extract and AgNPs against Gram +ve (*Bacillus subtilis, Streptomyces griseous* and *Staphylococcus aureus*) and Gram –ve (*Escherichia coli*) human pathogens were studied by agar well diffusion method. All pathogenic microorganisms were grown at 37ºC for overnight in Mueller- Hinton agar (Hi Media India) broth. Wells Mueller-Hinton agar plates were prepared and overnight grown bacterial suspension (100µl) was swapped uniformly on the surface of a Mueller-Hinton agar plates. After 24h incubation at 37ºC, zone of inhibition were measured [31].

ii) Antifungal activity

Anti-fungal activity of the root extract and AgNPs were investigated by agar well diffusion method. The fungi were grown on Sabouraud's Dextrose Agar (SDA) for 72h at 37ºC. Fungi were spread on Sabouraud's Dextrose Agar (SDA) plates. Four discs were placed on each agar plates. *R. emodi* root crude extract and AgNPs (50 and 100mg/ml) were kept in each discs of each Petri plates with respective pathogens. After incubation of 72 h, diameter of zone of inhibition around each disc and the results were recorded [16, 32].

2E. Cytotoxicity Potential

i) Cell culture
Breast cancer cell lines (MCF-7) were maintained in MEM medium, supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine at 37ºC with 5% CO_2.

ii) Measurement of cytotoxicity by MTT assay
Cytotoxicity effect of synthesized AgNPs and root crude extract were performed on breast cancer cell lines (MCF-7 cells) measured using MTT assay. Briefly cultured MCF-7 cells (1.2×10^4 cells/ml) were plated on 96 flat-bottom well plates, then cells were exposed to different concentration of synthesized AgNPs and root crude extract (50, 100, 150, 200 and 250µg/ml) respectively and allowed for 24 h incubation at 37º C in 5% CO_2 atmosphere. After incubation, 10µl of MTT (5mg/ml) was added in each sample separately. After 4 hours of further incubation at 37 º C and 5% CO_2 atmosphere, 100 µl of DMSO was added to dissolve formazan crystals. Then, the absorbance was measured at 570 nm with reference filter as 655 nm in a microtitre plate reader. Cyclophosphamide was used as a positive control.

3. RESULTS AND DISCUSSIONS

3A. Synthesis of Ag NPs
Methanol extract of *R emodi* roots was used for the synthesis of silver nanoparticles which act as a reducing agent. The formation of AgNps was visibly characterized due to change of colour from dark brown to orange. After addition of root extract, the complete reduction of silver ions was achieved by 24 hours of incubation at room temperature because of the excitation of surface plasmon resonance of AgNps. The solution was observed extremely stable even after month of reaction, with no evidence of particle aggregation [41, 35].

The phytochemical analysis of *R emodi* roots revealed the presence of physcion, chrysophanol emodin, aloe emodin, rhein anthraquinones[26, 34]. These anthraquinones may be responsible for the formation of AgNps by reducing $AgNO_3$ and acting as a capping agent to provide stability to the medium and prevent agglomeration.

3B. Characterization of silver nanoparticles

i) UV-Visible Spectroscopic Analysis: The UV-Visible spectrum (Fig. 1: a) of synthesized AgNps showed intense peak at 425nm. It was due to excitation of longitudinal plasmon vibration in AgNps solution. The increase in intensity of silver nitrate solution with respect to crude root extract has indicated the formation of increased number of silver nanoparticles in solution [35, 36].

ii) XRD Analysis: The crystalline nature of synthesized AgNps was determined by XRD analysis (Fig.1: b). The diffraction pattern was recorded in the scanning mode at 40 KV and

30mA, with Cu Kα radiation. The XRD pattern indicated peaks at 2θ values of 38.17^0, 44.33^0, 64.44^0, 77.34^0 and 81.33^0 corresponding to the face centered cube(FCC) plane(111, 200, 220, 311) of silver crystals respectively which represents crystalline nature of AgNps. The presence of unpredicted peak at 31.84^0 may be due to anthraquinones belong to the *R. emodi* root extract. The mean particle diameter of the synthesized AgNps was found to be 27.5 nm using Debye-Scherrer equation

$$D = \frac{k\lambda}{\beta \cos\theta}$$

where: D is the mean crystalline size of the particle, *K* is a dimensionless shape factor. The shape factor has a typical value of about 0.9 but varies with the actual shape of the crystallite. λ is the wavelength(1.5418 A^0) of X- ray radiation source. β is the line broadening at half the maximum intensity (FWHM), after subtracting the instrumental line broadening, in radians and θ is the Bragg angle[35,37].

iii) FTIR Analysis: FTIR spectra(Fig.1:c & d) of synthesized AgNps and methanolic crude *R emodi* root extract show transmittance at 3387 cm^{-1}, 2921 cm^{-1}, 1618 cm^{-1}, 1449 cm^{-1}, 1267 cm^{-1}, 10.27 cm^{-1}, 630 cm^{-1} and 3432 cm^{-1}, 2924.8 cm^{-1}, 1625 cm^{-1}, 1384 cm^{-1}, 1056 cm^{-1}, 621 cm^{-1} respectively. Variation in trasmission intensities of both synthesized AgNps and crude root extract were observed which is because of interaction of nanoparticles with biomolecules [35, 37].

FTIR spectrum of AgNPs synthesized uisng *R. emodi* shows the absorbance peaks at 3432 cm^{-1} indicating polyphenolic group along with 621 cm^{-1} aromatic C-H and the sharp peak at 2924 cm^{-1} may be due to C-H stretching of methylene group. Further, peaks at 1028 cm^{-1} - 1236 cm^{-1} indicate C-O single bond, peak at 1625 cm^{-1} represent carbonyl group (-C=O) from polyphenols. The band at 1384 cm^{-1} in AgNPs may be attributed to -C-O stretching mode. The band arising at 818 cm^{-1} is due to bending vibration of C-O-S. The absorption band at 1511 cm^{-1} corresponds to C=O stretching (carboxylic or amide) which is shifted (from 1511 cm^{-1} to 1625 cm^{-1}) after reaction. A strong peak at 3387 cm^{-1} represents primary N-H stretching. The bands at 1448 cm^{-1}, 1161 cm^{-1}, 1071 cm^{-1} are associated with respect to the non-conjugated C=C stretching, C-C stretching, and vibrations of the chain -C-O-C. The absorption peak at around 757 cm^{-1} indicates the aromatic banding. The majority of FTIR bands showed the characteristics functional groups of phenol, alcohol, aldehydes which reflects the presence of anthraquinones in *R emodi* roots. These anthraquinones may be involved in capping and stabilizing the AgNps.

iv) TEM with EDX: The size and morphology of synthesized AgNps were examined by EDX combined with TEM (Fig.2: a, b & e). The size of the AgNps was found to be in the range of 10-40 nm. TEM analysis indicated polydispersed shape of the particles. The EDX spectrum shows signals for silver and copper. The intense signals observed at 3KeV indicate that Ag was the major element of synthesized nanoparticles. It was because of the optical absorption of silver due to the surface plasma resonance in the range. The other signal of carbon indicates the presence of plant extract, which represents to the biomolecules capping over AgNp's. Copper metal peak is due to grid used for analysis. [41]

v) FE-SEM Analysis: The FE-SEM images (Fig.2: c & d) images further indicated that the synthesized AgNps were spherical in shape with mild agglomeration.

3C. Antimicrobial Investigation

Antibacterial and antifungal activity of the methanolic *R emodi* root extract and of the synthesized AgNPs were investigated (Fig.:3) The antimicrobial activity was examined by agar well diffusion assay which indicated well defined zones of inhibition, diameter in mm against three Gram +ve bacterial species namely *Bacillus subtilis, Staphylococcus aureus* and *Streptomyces griseous*; one Gram –ve *E coli;* and two fungal species *Aspergillus niger* and *Fusarium oxysporum.* It was observed that synthesized AgNps exhibit significant inhibitory activity against all bacterial and fungal species as compared to crude root extract. The synthesized AgNps showed prominent results against *Bacillus subtilis* (22±0.5 mm), *E Coli* (30±0.5 mm), *Staphylococcus aureus* (34±0.5 mm) and moderate activity against *Streptomyces griseous*(16±0.5 mm), *Aspergillus niger* (14±0.5 mm), *Fusarium oxysporum*(20±0.5 mm) at 100 mg/ml concentration on comparison to inhibition zone by crude extract(Table-1).

The exact mechanism behind the elevated antimicrobial activity is still not clearly known and debatable. It can be assumed that the surface area to volume ratio of nanoparticles is playing a crucial role in furnishing antimicrobial activity against microbes. The effect of capping is due to the presence of phytochemicals around nanoparticles gives particular type of surface functionality to behave in a specific way to different cell types [37]. C. Raj Kuberan et al. [35] reported three possible mechanisms of AgNps against microbes, (i) it is believed that Ag^+ interferes with bacterial cell membrane synthesis, (ii) AgNps interfere with thio group of bacterial cell affecting respiratory chain reaction, cell division and finally leads to death, (iii) AgNps release silver ions that ill penetrate to the cell wall causing condensation of DNA damage and also by affecting the protein synthesis. Morones et al. [38] proposed that silver act as soft acid which acts upon the sulphur and phosphorous bases of DNA and inactivates its replication thus disabling the nuclear machinery of the cell. I. Sonadi et al. [39] mentioned that silver nanoparticles has ability to attach with the bacterial membrane causing structural changes in its membrane leading to the formation of 'pits' where they accumulate.

Hence, the involvement of above listed mechanisms might be responsible for elevated antimicrobial activity of AgNps.

3D. Cytotoxicity of silver nanoparticles

Crude root extract and synthesized AgNps were taken for cytotoxicity (Table-2) against MCF-7 cell lines using MTT [3-(4, 5- dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] assay. It was observed that synthesized AgNps induce cytotoxicity against MCF-7 cell lines in dose dependent manner [50, 100, 150, 200 and 250µg/ml]. Significant cytotoxicity was reported in biosynthesized AgNps than crude extract with LC_{50} 250µg/ml with respect to positive control. Morphological changes in cells treated with different

concentration of AgNps were observed due to apoptosis. The recorded and captured images of the same are mentioned in Fig.:4.

In earlier study AgNps synthesized using *Calotropis gigantean* latex has shown significant anticancerous effect against HeLa cell lines [35]. It was proved [40] that AgNps have antiangiogenic properties and exert ability to block the activity of abnormally expressing signal protein. D. Nayak [37] reported that biosynthesized silver nanoparticles synthesized using different plant origin *Cucurbita maxima* (petals), *Moringa oleifera* (leaves), *Acorus calamus*(rhizome) show significant antimicrobial and anticancer potential. It is because of nano size regime, the silver nanoparticles may directly bind to the DNA of the pathogenic bacterial strains leading to higher antimicrobial and anticancer potential. S. Arokiyaraj et al. [41] reported rapid green synthesis of silver nanoparticles from *Chrysanthemum indicum* L and its antibacterial and cytoxic effects. They have used MTT and LDH assays to observe cytotoxic potential of AgNps.

J.R. Nakkala et al. [42] mentioned cytotoxic effects shown by AgNps in HeLa and A549 cells involved apoptotic changes. These findings emphasize that synthesized nanoparticles may find their application in the field of nanomedicine.

4. CONCLUSION

AgNPs were synthesized using root crude extract of *R. emodi*. UV-Visible absorbance spectral analysis confirmed the surface Plasmon resonance of AgNPs. FTIR analysis reflects the presence of anthraquinones which are responsible for capping and stability of nanoparticles. XRD, FE-SEM, TEM with EDX analysis confirmed the crystalline nature of the synthesized biogenic AgNPs with spherical shape, size in the range 10-40 nm (average size 27.5 nm). Biogenic AgNPs had shown significant antibacterial and antifungal potential. Cytotoxic potential of synthesized AgNps was found better against breast cancer cell lines (MCF-7). The results obtained in this study advocate the use of biosynthesized nanoparticles in Nano medicine applications with more understanding of its mode of action.

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