

Research Article



Green Synthesis of Silver Nanoparticles Using Stem of *Ruta graveolens* L: Antibacterial, Cytotoxic and Antioxidant Activity

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ABSTRACT

The green chemistry approach using plants involves phytoconstituent mediated reduction process for synthesis of nanoparticles. Biogenic Silver nanoparticles (AgNPs) were synthesized using aqueous stem extract of *Ruta graveolens*. The adopted biological method is compatible with green chemistry principles because the plant extract acts as a dual functional molecule: as reductant and a stabilizing agent for the synthesis of AgNPs. The UV-visible spectra confirmed that reduction of silver ions for Ruta stem extract at 485 nm on 24-hour incubation. FT-IR spectroscopic spectrum showed the peaks at 3507, 2829, 1579, 1010, 755 which are characteristic of flavonoids and terpenoids present in the stem. The zeta potential of Ruta AgNPs was found to be -35.4 mV which indicate that the nanoparticles are quite stable due to charge accumulation on its surface. The TEM micrograph reveals that the particles were of spherical shape & monodispersed, the average size of the particles is around 15 nm. Ruta AgNPs showed cytotoxicity against cancer cells and the results indicated that with the increase in Ruta AgNPs concentration there was a significant ($p < 0.05$) decrease in cell viability of HepG2 as compared to control. The antioxidant activity of Ruta AgNP was confirmed by ABTS radical scavenging activity. Ruta AgNPs may provide novel resources for the evaluation and development of newer, safer, and effective antimicrobial and antiproliferative drug formulations.

Keywords: *Ruta graveolens*, silver nanoparticles, characterization, Cytotoxicity, antimicrobial, antioxidant.

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INTRODUCTION

Nanoparticles, are particles with a size ranging from 1 to 100 nm in two or three dimensions according to the American Society for Testing and Materials (ASTM) standard definition. Noble metal nanoparticles have also received much interest in recent years because they have immense applications in medicine, biology, material science, physics, and chemistry. Silver nanoparticles (AgNPs) have garnered considerable attention because of their special properties, which include good electrical conductivity, chemical stability, and catalytic and antibacterial properties¹. AgNPs have been used widely in the health industry, food storage, in cloth coatings, and also in a range of environmental applications as antimicrobial agents. AgNPs are being produced in a multitude of ways, including physical and chemical processes. To obtain AgNPs, green chemistry has emerged as a clear and viable alternative to more complex chemical synthesis. Green synthesis of metallic nanoparticles using plant extract is based on ability of plant metabolites to reduce metal ions and stabilize them. The following

properties can be considered as advantages of using plants for nanoparticle synthesis; availability, safety in handling, and presence of a variety of phytoconstituents. Plant crude extracts contain novel secondary metabolites including phenolic acid, flavonoids, alkaloids, and terpenoids, which are mainly responsible for the reduction of ionic metal into bulkmetallic nanoparticles².

Ruta graveolens L. is a small shrub or evergreen herb with a strong odour. It belongs to the Rutaceae family. *Ruta graveolens* L. (Rutaceae) is traditionally used in Indian herbal medicine to treat rheumatism, arthritis, and other inflammatory conditions³. The roots and aerial parts of the plant contain over 120 natural compounds. Acridone alkaloids, coumarins, volatile substances, terpenoids, flavonoids, and furoquinolines are some of the most common phytochemical compounds contained in *R.graveolens*⁴. Hence it is expected that silver nanoparticles synthesized using stem extract may be useful in medicine. The main mechanism considered for the nanoparticle synthesis process is reduction due to phytochemicals. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method, there is no need to use high pressure, energy, temperature and toxic chemicals. AgNPs are one of the most promising antimicrobial materials applied in nanomedicine. AgNPs can interact with the microorganism cell wall, generating reactive oxygen species that ultimately leads to cell death⁵.



This is the first study to use *Ruta graveolens* stem aqueous extract in the synthesis of AgNPs. It is important to analyse the prepared nanoparticles before application in order to resolve the safety problem and use the full potential of any nano material in the purpose of human welfare, nanomedicines, or the health care industry. Techniques such as, UV-visible spectroscopy, TEM (Transmission Electron Microscopy), FTIR (Fourier Transform Infra-Red spectroscopy), and DLS (Dynamic Light Spectroscopy) were used to characterize the synthesized silver nanoparticles. The synthesized *Ruta graveolens* mediated silver nanoparticle were evaluated for antibacterial property, the cytotoxic effect and antioxidant activity.

METHODS

Chemicals, Reagents and Media

The plant material, *Ruta graveolens* were collected from Kerala, India and identified taxonomically. Silver nitrate (AgNO_3), Mueller Hinton agar and Mueller Hinton broth and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), nutrient media were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India and used without any further purification. Cell lines (HepG2) were obtained from National centre for cells Sciences (NCCS), Pune, India.

Collection of plant samples and preparation of *R. graveolens* extract

The stem extract was prepared from the freshly collected plant, *Ruta graveolens*. They were surface cleaned thoroughly with running tap water, followed by sterile distilled water. 5g stem were cut into small pieces and boiled with 100ml deionized water for 5 min. The prepared plant extract was filtered through Whatman No. 1 filter paper and the aqueous filtrate was used further to synthesize AgNPs.

Biosynthesis of AgNP

Biological reduction of AgNO_3 was carried out as follows. A stock solution of 10mM AgNO_3 was used for the preparation of varying AgNO_3 concentration (0.1 – 1.0 mM). For all the preparations, 5 ml plant extract was used. The prepared flasks were kept in dark for 24 hours at room temperature for biosynthesis of silver nanoparticles to take place.

Characterization by UV-Visible spectrophotometry

The formation of AgNPs was primarily observed by monitoring the change in colour of the extract after treatment with AgNO_3 (0.1 to 1 mM). The bio-reduction of silver ($\text{Ag}^+ \rightarrow \text{Ag}^0$) ions in stem aqueous extract was monitored with the UV-visible double beam spectrophotometer (Model no: UV 1800, Shimadzu, Japan) in the wavelength range from 300 to 700 nm. Distilled water was used as the blank.

Transmission electron microscopy (TEM)

The particle size and microstructure were studied by transmission electron microscopy PHILIPS CM 200 operating at 200kv. Synthesized silver nanoparticles were sonicated for 30 minutes to 1 hour in 100% ethanol to obtain a homogenous suspension of nanoparticles. A drop of the AgNP suspension was placed on to carbon-coated copper grid, dried under IR light and loaded in to the TEM microscope to obtain image.

Zeta Potential

Zeta Potential is used as an indicator of stability of nanoparticles. The analysis of the biologically synthesized silver nanoparticles from *R. graveolens stem extract* was performed using Horiba Scientific Nanopartica Nanoparticle Analyser SZ-100 (Japan). About 100 μl of sample was diluted with 1 ml of sterile deionized water and loaded in to the folded capillary cells. The measurements were made at 30.5°C with the electrode voltage of 3.3 V.

Dynamic light scattering (DLS)

The DLS method is a special method of measuring particle size in the range of 1 – 5000 nm, where a laser beam is made to incident on the nanoparticle suspended in the liquid (NPs are under brownian motion). The scattered beam intensity (fluctuation) with time (microsecond) was measured. The analysis of the biologically synthesized silver nanoparticles was performed using Horiba Scientific Nanopartica Nanoparticle Analyser SZ-100 (Japan).

FT-IR spectroscopy

Synthesized nanoparticles were subjected to FTIR spectroscopy, Bruker 3000 Hyperion Microscope with vertex 80 FTIR in the range of 400–4000 cm^{-1} at Spectral resolution of 0.2 cm^{-1} . The AgNPs were grinded with KBr and pressed to form pellets of around 0.5 to 1.0 mm thickness. The pellets were later placed in the IR path and the spectrum was analysed.

Antimicrobial analysis

The AgNPs were separated by centrifugation at 10,000 rpm for 15 minutes, the pellet thus obtained was redispersed in sterile distilled water. The synthesized AgNPs of *Ruta graveolens* was tested for antibacterial activity by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia Coli* and *Klebsiella pneumoniae* and fungus *Candida albicans*.

Minimum Inhibitory Concentration (MIC)

The MIC and MBC of green synthesized AgNPs were done using the method described in the guideline of [CLSI \(2012\)](#). The MIC of the biologically synthesizes silver nanoparticles was determined using sterile Mueller Hinton broth dilution method with silver nanoparticle concentration ranging from 0.5 $\mu\text{g}/\text{ml}$ to 500 $\mu\text{g}/\text{ml}$. 18 hours old bacterial culture was resuspended in sterile saline such that the



culture density reached that of McFarland's standard having approximate cell density of 1.5×10^8 CFU/ ml. The prepared bacterial culture was inoculated in the standard tubes of silver nanoparticles. A positive control was maintained by inoculating the culture in the sterile MH broth; whereas for negative control, the sterile MH broth was kept uninoculated. All the tubes were then incubated at 37°C for 24 hours. The MIC was noted by observing the tubes for turbidity. The lowest concentration of antimicrobial agent that inhibits the visual growth of the organism is noted as MIC.

Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration (MBC) is the lowest concentration ($\mu\text{g/ml}$) of the nanoparticles that kill the particular bacteria. It was determined by the spot inoculating the AgNP concentrations on the Sterile MH agar plates that did not show visible growth in minimum inhibitory concentration tubes. Suitable positive and negative controls were maintained. The plates that did not show visible growth after incubation at 37°C for 24 hours were noted as the minimum bactericidal concentration.

Table 1: Preparation of standard dilutions of silver nanoparticles

Tube no	Concentration ($\mu\text{g/ml}$)	Volume of AgNP (μl)	Volume of Nutrient broth (μl)	Volume of Culture (ml)	Total Volume (ml)
1	0.5	2.5	4997.5	0.1	5
2	2.5	12.5	4987.5	0.1	5
3	5.0	25	4975.0	0.1	5
4	10.0	50	4950.0	0.1	5
5	25.0	125	4875.0	0.1	5
6	50.0	250	4750.0	0.1	5
7	75.0	370	4625.0	0.1	5
8	100.0	500	4500.0	0.1	5
9	200.0	1000	4000.0	0.1	5
10	500.0	2500	2500.0	0.1	5
Positive Control	-	-	5000.0	0.1	5
Negative Control	-	-	5000.0	-	-

Cytotoxicity assay

Cells were maintained in DMEM media supplemented with 10% FBS. Confluent cells were passaged every third day and allowed to grow in T-flask kept in CO₂ incubator at 37°C. The MTT Assay⁶ is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. Cells were seeded at 37 °C for 24 h prior to treatment with AgNPs. Cells were treated with different concentration of AgNPs (200 and 400 $\mu\text{g/ml}$) and DMSO controls were also maintained. The viable cell percentage was calculated, taking into account the 100% viability of untreated cells.

% viability = [(Absorbance of treated cell – Absorbance of blank)/ (Absorbance of vehicle control – Absorbance of blank) × 100].

ABTS Free Radical Scavenging Assay

The ABTS radical scavenging activity of different concentrations of Ruta mediated silver nanoparticles was estimated by a standard protocol⁷. 2mM ABTS was dissolved in 50ml of distilled water. 70mM Potassium per sulphate was prepared by dissolving 0.0378gm in 2ml of distilled water. Stock solution was prepared by mixing 200 μl of potassium per sulphate in 50ml of ABTS and stored in dark for 12 hours. Prior to use the solution was diluted with ethanol to get an absorbance of 0.7 at 734nm. Free radical scavenging activity was assessed by mixing 20 μl (50, 100 and 200 $\mu\text{g/ml}$) of Ruta AgNPs with 4ml of ABTS working standard. The mixture was left for 30 min at room temperature and the absorbance was measured using a spectrophotometer at 734 nm. Ascorbic acid was taken as a standard. ABTS free radical scavenging percentage activity was calculated using the following formula:

ABTS radical scavenging (%)

= [(Absorbance of control - Absorbance of sample)/Absorbance of control] × 100



Statistical analysis

Statistical analyses were performed using one way analysis of variance (ANOVA) followed by Dunnett's test using SPSS software package (version 18.0) and p values less than 0.05 were considered significant. The results are denoted as mean \pm standard deviation of six experiments.

RESULTS

Biologically synthesized silver nanoparticles

In biological synthesis of AgNPs (Fig.1) after addition of Ruta stem extract to the solution of silver nitrate, the solution changed from colourless to dark orangish-brown. The appearance of color in reaction flasks was the indication of AgNPs formation due to bio-reduction process. The colour intensity increased as a function of time due to the reduction of Ag⁺.



Figure 1: Synthesis of silver nanoparticles using ruta shoot extract (left – 0 hours) and (right – after 24 hours of incubation)

UV-Visible spectrophotometry:

The absorption spectra of Ruta AgNP (Fig.2) showed the characteristic surface-plasmon of silver nanoparticles. The UV-visible spectra confirmed that reduction of silver ions at 485nm on 24 hour incubation.

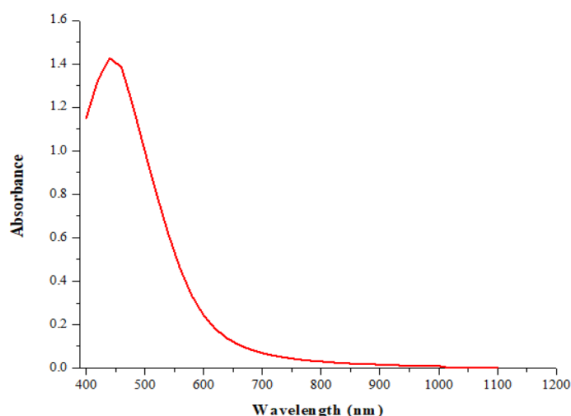


Figure 2: UV-visible spectrum of Silver nanoparticles synthesized using 0.5 mM AgNO₃ solution

Transmission electron microscopy

The shape and size of the AgNPs were elucidated with the help of TEM (Fig.3). The particles were of spherical shape & monodispersed. The TEM micrograph & the particle size distribution suggest that the size of the particles were around 15 nm.

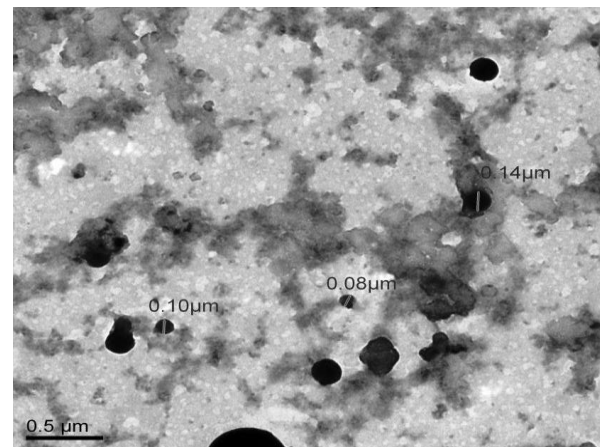


Figure 3: TEM micrographs of Ruta stem AgNPs

Zeta Potential

The nanoparticles are most stable if the zeta potential values are higher than +30 mV or lower than -30 mV. The zeta potential of silver nanoparticles synthesized from ruta stem (Fig.4) was found to be -35.4 mV (which indicates that the nanoparticles are quite stable due to charge accumulation on its surface. This coulombic repulsion helps in keeping the nanoparticles dispersed, prevents agglomeration and thus remains stable for months together.

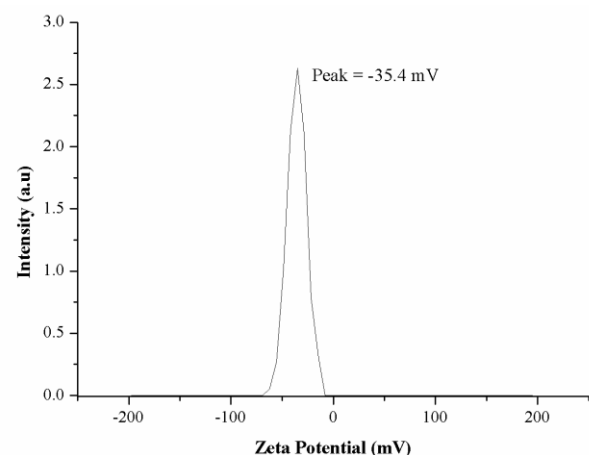


Figure 4: Zeta Potential of Ruta stem AgNPs

Dynamic light scattering (DLS)

The particle size as obtained by DLS for ruta stem AgNPs (Fig. 5) was found to be 130nm. The values from DLS are much higher than those from TEM. The DLS intensity shows values around 5-7 times higher than the results obtained from TEM. These results may be due to broad distribution formed by mixing two particles.

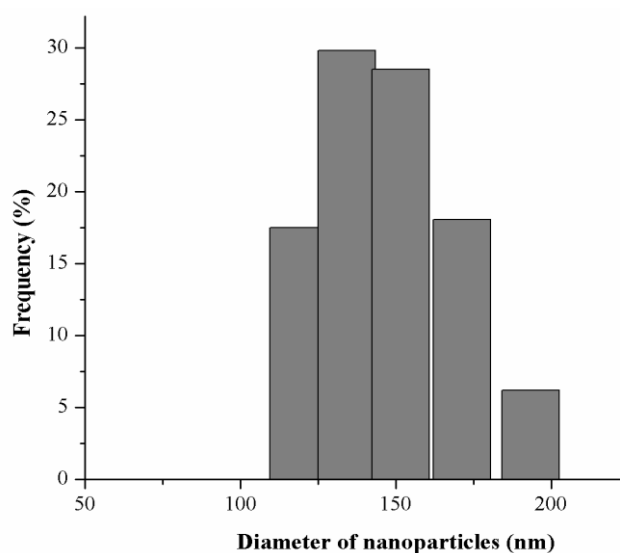


Figure 5: DLS of Ruta stem AgNPs

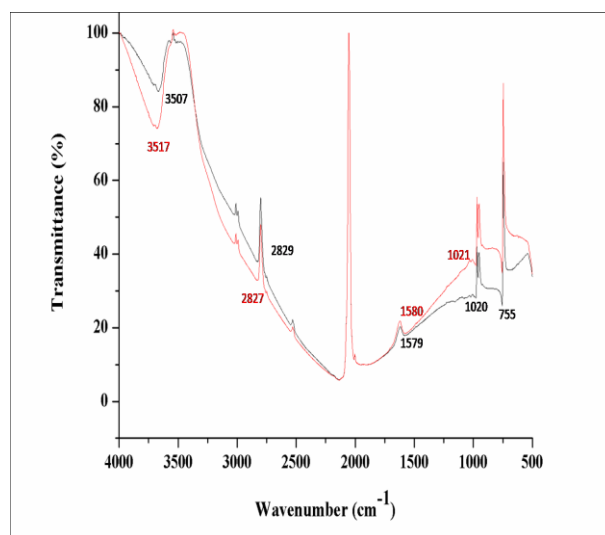


Figure 6: FTIR of Ruta stem AgNPs

Fourier transform-Infrared (FT-IR) spectroscopic analysis

To characterize and identify the biomolecules that were bound specifically on the synthesized Ag NPs, FT-IR spectroscopic analysis was utilized. The spectrum so obtained, as shown in Fig. 6 shows the peaks at 3507, 2829, 1579, 1020, 755 are characteristic of flavonoids and terpenoids present in the stem. These bands corresponding to the stretching vibration of methylene, antisymmetric stretching, vibration of methylene, C-H stretching, C-C stretching vibration of aromatic, C=O stretching, C-H stretching vibration and C-N stretching of amines. The band position between 500 and 700 are due to C-C and C-H phenyl ring substitution as expected for this plant. FTIR results suggest that *Ruta graveolens* acts as a good bioreductant for biosynthesis of silver nanoparticles.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Different concentrations of Ruta AgNPs were tested (Table. 2) against *E. Coli*, *S. aureus*, *C. albicans* and *K. pneumonia*. For *E. coli*, after incubation at 37°C for 24 hours, turbidity was observed from concentration 0.5 – 10 µg/ml tubes of silver nanoparticles, since no growth was observed from concentration 25 µg on wards, the MIC was found to be 25 µg/ml. The suspension from tubes 25 - 500µg/ml was inoculated on to sterile MH agar plates of the same concentrations and incubated at 37°C for 24 hours, no growth was observed from 50 – 500 µg/ml and hence the MBC was found to be 50 µg/ml. Similarly, the MIC and MBC for *S. aureus* was found to be 10 µg/ml and 25 µg/ml, for *K. pneumonia* it was found to be 25 µg /ml and 50 µg/ml and for *C. albicans* it was found to be 25 µg/ml and 50 µg/ml respectively.

Table 2: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of Ruta stem AgNP on selected microorganisms

Tube	<i>E. coli</i>		<i>S. aureus</i>		<i>C. albicans</i>		<i>K. pneumonia</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
0.5µg/ml	+	+	+	+	+	+	+	+
2.5µg/ml	+	+	+	+	+	+	+	+
5.0µg/ml	+	+	+	+	+	+	+	+
10.0µg/ml	+	+	-	+	+	+	+	+
25.0µg/ml	-	+	-	-	-	+	-	+
50.0µg/ml	-	-	-	-	-	-	-	-
75.0µg/ml	-	-	-	-	-	-	-	-
100.0µg/ml	-	-	-	-	-	-	-	-
200.0µg/ml	-	-	-	-	-	-	-	-
500.0µg/ml	-	-	-	-	-	-	-	-
Key:	(+) Indicates growth (-) Indicates no growth							

Cytotoxicity assay

We observed that Ruta AgNPs suppressed growth of cancer cells and percentage cell viability were 48.5% in 200 µg/ml and 22.5% in 400µg/ml (Fig.7). The results indicated that with the increase in Ruta AgNPs concentration there was a significant ($p < 0.05$) decrease in cell viability of HepG2 as compared to control.

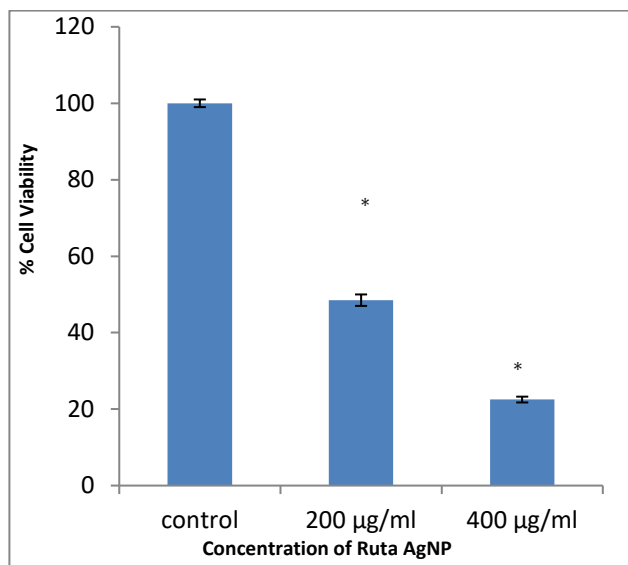


Figure 7: Cytotoxicity effect of Ruta AgNPs on viability of Hep-G2 cell lines. Each value is expressed as a mean \pm SD ($n = 6$). $P < 0.05$ was considered statistically significant compared to control.

ABTS Free Radical Scavenging Assay

The biologically synthesized Ruta AgNP showed good radical scavenging activity (Fig. 8) as the well-known antioxidant ascorbic acid. With the increase in the concentration of AgNP's ability to scavenge free radicals also increased on reaction with ABTS radicals.

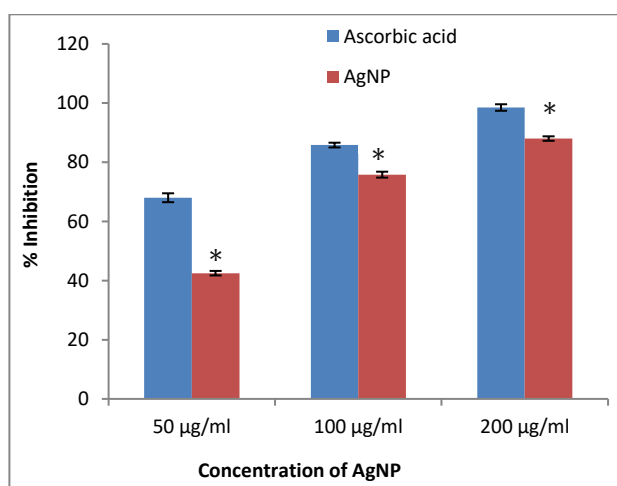


Figure 8. ABTS•+ radical-scavenging assay of Ruta AgNP. Ascorbic acid was used as the positive control. Each value is expressed as a mean \pm SD ($n = 6$). $P < 0.05$ was considered statistically significant compared to control.

DISCUSSION

We observed formation of the AgNPs during the plant extract mediated reduction process, indicated by change in the color of the reaction solution from colorless to dark brown. The property of silver nanoparticles to exhibit surface Plasmon resonance (SPR) and related optical properties was utilized for analyzing the UV-Visible spectrum of synthesized nanoparticle⁸. The absorption peak in the range of 420–450 nm confirmed the formation of *Ruta graveolens L.* mediated silver nanoparticle formation. Due to the property of SPR the absorption of visible radiations imparts various colors to nanoparticles. The TEM micrograph & the particle size distribution suggest that the size of the nanoparticles was around 15 nm and they were of spherical shape & monodispersed.

The zeta potential of the biosynthesized AgNPs was found as a sharp peak at -35mV. The observed zeta potential value in the current study shows that silver nanoparticles synthesized is negatively charged and dispersed in the medium. Also, the negative value confirms the particles repulsion and demonstrates their stability, with higher magnitude potentials exhibiting increased electrostatic repulsion and therefore increased stability. It is generally argued that particles with zeta potentials greater than +30 mV and less than -30 mV are considered stable for colloidal dispersions in the absence of steric stabilization^{9,10}. DLS analysis found around 130 nm is based on the diffusive motion of particles in solution (Brownian motion). The hydrodynamic diameter measurement provides information on the aggregation state of nanoparticle solutions. In the current investigation, DLS for the synthesized nanoparticle is slightly higher than their TEM size which shows that our nanoparticle preparation is a stable unaggregated colloidal solution.

Results obtained for FTIR measurements were confirmatory to identify major functional groups in the Ruta extract and their possible involvement in the synthesis and stabilization of silver nanoparticles. The spectrum obtained, with peaks at 3507, 2829, 1579, 1020, 755 are characteristic of flavonoids and terpenoids present in the stem. FTIR results suggest that *Ruta graveolens* acts as a good bioreductant for biosynthesis of silver nanoparticles.

Antibacterial effect of Ruta silver nanoparticles was observed in tested microorganisms. One of the proposed mechanisms for antimicrobial activity of AgNPs reported¹¹ is that silver ions led to transformation of the bacterial DNA from the natural relaxed state to a condensed state in which the DNA molecule loses its replication ability. There are reports suggesting the action of AgNPs will damage the cell membrane, interrupt the metabolic activity, and subsequently lead to denaturation of protein and finally cell death^{12,13,14}. Also AgNPs could also produce several reactive oxygen species (ROS) which are toxic to the bacteria¹⁵. Reactive oxygen species are responsible for oxidation of cellular components i.e. cellular DNA and protein. Upon oxidation of these components, cell

becomes unstable at physiological and genetic level causing failure of metabolism and cell division process.

Free radical scavenging activity of Ruta AgNPs was proved by ABTS assay. The presence of important phytoconstituents such as flavanoids and terpenoids observed in FTIR spectrum also substantiates the ability of Ruta AgNPs to show antioxidant activity. The antioxidant potential can be attributed to the specific functional groups of phytoconstituents present in Ruta plant extract that are actually involved in the capping and stabilization of biologically synthesized silver nanoparticles. Given that this study is the first investigation on the cytotoxicity of Ruta stem AgNPs, an exact mechanism for its anticancer properties remains to be unclear and needs further evaluation. Free radical scavenging activity of the Ruta mediated silver nanoparticles can be correlated with cytotoxicity action in cancer cells. Several studies^{16,17} have shown that polyphenols and flavanoids are toxic to cancerous cells while being harmless to healthy cells.

CONCLUSION

The biological method of synthesis of silver nanoparticles can conveniently overcome the complications of biosafety of the chemicals used for the generation of nanoparticles. Recent reports show that phytonanotechnology is gaining great importance with more than 20 nanoparticle therapeutics available. This study adds another feature to the medicinal plant *Ruta graveolens*, for its ability to successfully formulate AgNPs, which could be used effectively for their antibacterial and antiproliferative properties.

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