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Research Article

Green synthesis of Ag-nanoparticles by roots-extract of *Rumex nepalensis* and their biological potentials

Authors & Affiliation

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Abstract

The current study has reported the silver-nanoparticle's green synthesis of silver-nanoparticles using silver ions from silver nitrate and by aqueous-root-extract of *R. nepalensis*. Initial clue of Ag-nanoparticles (AgNPs) formation was change in the color of solution from light brown to grey followed by broad band of UV-Visible spectrum obtained at 447 nm. Average size of the resulting AgNPs was 17.15 nm and X-Ray Diffraction (XRD) confirmed their crystalline cubic structure. SEM showed high intensity, spherical and cubic shaped AgNPs in addition to some irregular morphologyof AgNPs' FT-IR analysis indicated incidence of natural products which reducedthe silver and stabilized the resulting NPs and that is also evident by the rich phenolic (149 mg RE/g) and flavonoids (143 mg GAE/g) contents in methanolic rootextract of *R. nepalensis.* These phenolic and flavonoid contents were also found responsible for antioxidant potency (IC_{50}= 1.533) shown by the same extract. Antibacterial screening of methanolic extract, aqueous extract and biosynthesized AgNPs of *R. nepalensis* showed inhibition zone (8.0, 6.0 and 8.0mm) and (10.0, 11.0 and 8.0 mm) against *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. In this study, antibacterial activities of aqueous rootextract of *R. nepalensis* was found to be the most potent against *Pseudomonas aeruginosa*.

Keywords: *R. nepalensis;* Methanolic-extract; Aqueous-extract; Phenols; flavonoids; AgNPs.

Introduction

Nanoparticles possess size of 1-10 nm and are foundation of modern nanotechnology, which have intensified R&D for exploring the preparation of nanoparticles. Among all, green synthesis of these metallic nanoparticles by eco-friendly, economical and reproducible approaches used plant materials, have become most attractive. Actually, plant materials are diverse reservoirs of wide-variety of natural products due to their complex enzymatic machineries and abundant starting materials. Thus, especially medicinal plants provide unique opportunities for exploring the variety of nanoparticles synthesis for meeting the unmet needs. Also, these variety of natural products of medicinal plants not only synthesize these NPs but also some of them synthesize with very small sizes which is coupled with the fact these natural products also help the stabilization of synthesized NPs by chelation.

With respect to the applications of NPs in biological systems, Ag is among the most tolerated and least toxic metals to human biology thus, it is of unique R&D interest nanostructures of biocompatible metal and semiconductors[1]. This is also evident by the centuries-old use of silver in various traditional products for various curative effects including treating both internal and external infections by micro and unicellular organisms[2,3]. Along with the biomedical application, silver-NPs have other broad range industrial applications of high value for example, reactions-catalysis,high-sensitivity-sensors, production, and material industry[4]. Silver-NPs have also provided positive results for tackling malignant tumor-growth[5,6].

Rumex nepalensis spreng(Family: *polygonaceae*, common name: Shalkhay or Hoola) is widely distributed in the temperate Himalayas at altitudes between 1200-4300m. *Rumex nepalensis* is used in traditional medicines to cure skin sores, colic, and syphilitic ulcers. The pounded root is given to animals in case of diarrhea and dysentery while leaves are applied to treat scabies. *Rumex nepalensis* is pharmacologically used as a wash for reducing body pain, shows purgative properties, analgesic, antipyretic, anti-inflammatory activities, and is also used as a skeletal muscle relaxant[7]. Therefore, in the light of the above importance, we decided to investigate biochemical properties and synthesized AgNPs by aqueous extract of the root of this plant.

Materials and Methods:

Roots of *Rumex nepalensis* (Hoola) were obtained from Muzaffarabad-AJK, Pakistan. Professor Dr. M. Q. Khan, Depart. of Botany University of AJK, Pakistan, (voucher specimen kept as; MR-MA-04-BOT-2017) had verified the sample. These roots were dried and ground to powder for preparing the aqueous- and methanolic extracts. $AgNO_3$, aluminum chloride, sodium nitrite, Na-carbonate, methanol, DMSO, acetone, DPPH, ascorbic acid, gallic acid, rutin-hydrate, Folin-Ciocalteu Reagent & nutrient-agar were purchased from Sigma Aldrich.

Aqueous and methanol-extracts preparation

Fresh roots of *R. nepalensis* were washed three times with d-water, shade-dried (12 days), and powdered. 120 g of this powder material was extracted with 500mL d-water by dipping for 16days which was passed through Whatman paper-1 and then was used for the biosynthesis of AgNPs. The methanolic extract was prepared by dissolving100 g of the powdered sample in 500 mL of methanol, incubated for 16 days, and filtered through Whatman filter paper No.1. The solvent was evaporated using a rotary evaporator to get crude methanolic extract for the biological activities.

This study took place at the Laboratory of SA-CIRBS, IIUI and Chemistry Department, U-AJK.

Biosynthesis of AgNPs

Silver nitrate solution weighing 100 mL of 4 mM strength has been added to 100 mL aqueous extract of *R. nepalensis*. The brown color of this mixture after 1 h, was turned into grey and that indicated silver-NPs formation. For completion of the reaction, this mixture was further incubated at room temperature in dark for 24 h, and then UV-Vis spectrophotometry was employed to confirm the NPs formation. Repeated centrifugation by 3000 rpm around 15 min with washing was employed for further purification and then were dried for advance studies.

AgNPs characterization using UV-Visible, XRD, SEM, FT-IR

The formation of AgNPs was indicated by the color change of the solution and then was verified by UV-Vis. spectroscopy (200-800 nm) using Analytik Jena SPECORD 50 UV-Visible Spectrometer with d-water as a reference. Dried powder of AgNPs was used for X-ray analysis by Bruker D8 powder X-ray diffractometer using Cu Kα radiation employing 40 KV as well as 30 mA. Scanning was performed within 10-80 region at 2θ diffraction angle. XRD spectrum obtained has been compared with standards (JCPDS) library for crystalline structure in addition to the size of these AgNPs determination with Debye–Scherrer equation:

$$D=K\lambda / \beta\cos\theta,$$

in which, D - Mean size of synthesized AgNPs,
K - Constant (K = 0.89),
λ - Wavelength of X-ray (0.154 nm),
β - Maximum peak width at half the height and
θ - Diffraction angle measured in degrees.

Photograph for morphological characterization and the size distribution of these AgNPs was taken by

SEM (JEOL JSM-6490A). Sample for the SEM analysis was prepared, by placing a drop of these AgNPs on a carbon-coated copper grid. FT-IR analysis was employed for the functional group's identification of natural products in *R. nepalensis* which are also responsible for the formation and chelation of AgNPs(Perkin-Elmer-Spectrum 100 FT-IR Spectrometer).

Antibacterial activities

Bio-synthesized AgNPs, water, and methanolic extracts of *R. nepalensis* were analyzed compared to *P. aeruginosa* and *E. coli* by the method of Well-diffusion[8]. These strains were sub-cultured at the temperature of 35 ºC on Muller–Hinton broth then incubated in a shaker (200 rpm). Sterile-cotton-swab was used in swabbing every strain on an individual plate. Wells with 6 mm diameter were made in agar by metal cork-borer which is sterile. A micropipette was used to add, 30 µL of every sample to the respective well on agar plates. DMSO has been employed as a negative control. Once incubated at 35 ºC about 24 h, zones of inhibition were measured by calculating the inhibition zone diameter in mm around well.

Antioxidant activity

DPPH assay was employed for determining the antioxidant potential of the methanolic extract of *R. nepalensis*[9]. 7.98 mg of DPPH was dissolved in 100mL of methanol for preparing the stock solution. Multiple concentrations (10-30 µg/mL) of methanolic extract were treated with DPPH solution and incubated for 1 h in absence of light. UV-absorbance of each sample was taken at 517 nm and ascorbic acid was used as a positive control. These trials were worked out in triplicate. %Agar radical scavenging abilities were assessed using the formula.

$$\text{Radical Scavenging (\%)} = (A_{control}-A_{test})/A_{control} \times 100$$

Where $A_{control}$ is the absorbance of ascorbic acid and A_{test} is the absorbance of the test sample.

The readings of various extract concentrations were plotted *Vs* their %age scavenging activities employing standard-linear regression (MS-excel) for finding IC_{50}.

Determining the total phenolic contents

The study on total phenolic contents (TPCs) of the MeOH-root extract was carried out by Folin–Ciocalteuprotocol[10]. The volume of 200 µL of MeOH extract (1 mg/mL) of *R. nepalensis* was made up to 3 mL using d-water, and then was assorted with 0.5 mL Folin–Ciocalteu reagent and after incubating for 3 min, 2 mL of 20% Na_2CO_3 was added into it. The resulting mixture was further incubated for 60 min in the absence of light and UV-absorbance was taken at 650 nm. TPCs of in this MeOH-extract of *R. nepalensis* were determined employing curve of calibration: y = 1.56x - 0.075, with R^2=0.998 of gallic acid, then stated in mg of gallic acid alike every g dry weight.

Determining the total flavonoid contents

The investigation of total flavonoids contents (TFCs) of MeOH-extract of *R. Nepalensis* was performed by the colorimetric protocol of aluminum chloride[11]. 50 µL of MeOH-extract (1 mg/mL of MeOH) has been added to 4 mL of d-water & 0.3 mL of 5% sodium nitrite solution which later of 5 min incubation, was added with 0.3 mL of 10% aluminum chloride solution and then further incubated for 6 min. Afterward, 2 mL of 1 M sodium hydroxide solution has been added then using d-water the volume was brought to 10 mL. After incubating for 15 min, the UV-Vis absorbance was taken at the wavelength of 510 nm. The calibration curve: y = 0.404x - 0.026, R^2= 0.997 was employed in order to measure TFCs as mg rutin equivalent dry weight of samples per gram (mg RE/g) and experiments were for repeated three times.

Results and Discussion

Observation

The light brown color of the aqueous root-extract of *R. nepalens* turned into a grey color (**Fig. 1**) upon mixing with aqueous $AgNO_3$ solution which was the preliminary indication for the formation of Ag-NPs.

UV-Visible spectroscopy

AgNPs formed by aqueous-roots-extract of *R. nepalensis* which induced reduction and stabilization of the resulting Ag-NPs from silver nitrate., were verified by UV-Vis. spectroscopy showing broad absorption band at 447 nm after 24 h. The peak broadening in the spectrum indicated that the nanoparticles were stable and polydispersed (**Fig. 2**).

These results were in agreement with the previous literature[2-12].

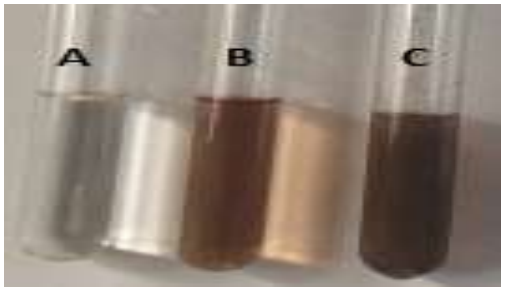

Fig. 1: $AgNO_3$ solution **A**, *R. nepalensis* extract **B**, AgNPs formed **C,** by mixing *R. nepalensis* extract with $AgNO_3$ solution.

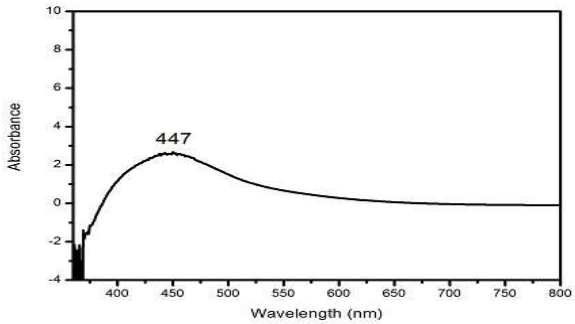

Fig. 2: UV-Visible spectrum of AgNPs biosynthesized by aqueous roots-extract of *R. nepalensis*.

XRD analysis

The crystal structures of bio-synthesized AgNPs from silver ions by reduction using aqueous roots-extract of *R. nepalensis* were long-established using characteristic peaks of X-ray diffraction (XRD)-pattern (**Fig. 3**). These sharp diffraction peaks at 2θ values of 38.197°, 44.357°, 64.490°, and 77.512° corresponding to (111), (200), (220), and (311) planes, respectively in the spectrum represents face-centered cubic (fcc) structure of Ag^0 because of excellent concurrence with the JCPDs (89-3722) card. Additionally, a few minor peaks (represented by triangle in the graph) were observed at 27.77°, 30.33°, 32.23°, and 46.21°, which indicated the hexagonal phase of Ag_2O_3 present in trace amounts, which has been formed in addition to cubic Ag^0 and have concurrence with JCPDs (77-1829) standard card. The average AgNPs particle size and lattice constant 'α' corresponding to (1 1 1) plane was found to be 17.15 nm and 4.086 A^0 respectively[3-13].

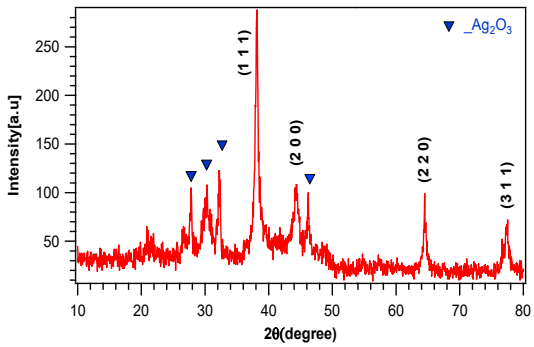

Fig. 3: XRD pattern of biosynthesized AgNPs using *R. nepalensis* aqueous roots-extract

Scanning electron microscopy (SEM) analysis of biosynthesized AgNPs

The morphological characteristics of AgNPs biosynthesized using *R. nepalensis* extract were confirmed by SEM, which showed high intensity, spherical and cubic shaped AgNPs along with some irregular morphology because of aggregation (**Fig. 4**). These results are in accordance with the previous literature[4-15].

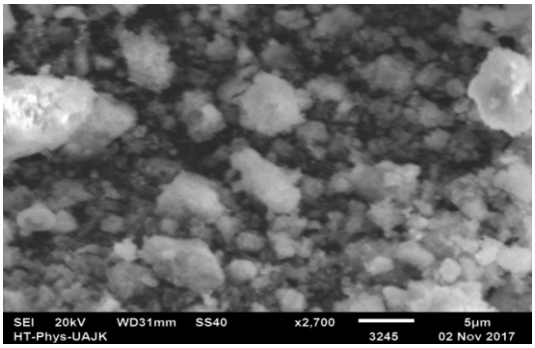

Fig. 4: SEM image of AgNPs synthesized by aqueous roots-extract *R. nepalensis*.

FTIR analysis

R. nepalensis extract mediated AgNPs showed nine absorption peaks in FT-IR spectrum located at 3413, 2982, 2024, 1581, 1414, 1103, 1019, 876 and 648 cm^{-1} (**Fig. 5**). Absorption at 3413 cm^{-1} indicated the presence of hydrogen-boned hydroxyl moieties from phenols and alcohols. A weak and a strong peaks at 2982 and 1414 cm^{-1} signposted the C-H stretching & bending of saturated hydrocarbons, respectively. Peaks at 2024 & 1103 cm^{-1} showed the presence of amine (C-N bend & stretch), correspondingly. A frail band on 1650 cm^{-1} indicated the amide C=O stretch (not labeled) whereas, a bending peak at 1581 cm^{-1}

was due to N-H bend of amine functional groups. Two peaks at 1103and 1019 cm^{-1}revealed the C-O bond of alcohols and phenol's moieties. Absorption at 876 and 648 cm^{-1} were due to the aromatic C=C bonds and C-X bonds from alkyl halides, respectively[3-13,15]. Overall, the FT-IR spectrum revealed the phenol, amines, amide, protein, aromatic, and saturated-hydrocarbon containing natural products in the extract and these had played role in green synthesis (reduction) and stabilization of Ag-NPs.

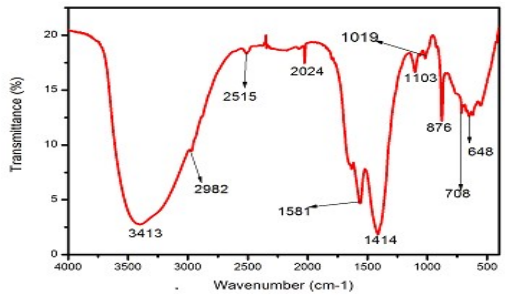

Fig. 5: FT-IR spectrum of biosynthesized AgNPs using aqueous roots-extracts *R. nepalensis*.

Antibacterial activities

Antibacterial screening of methanolic extract, aqueous extract, and biosynthesized AgNPs of *R. nepalensis* showed 8.0, 6.0, and 8.0-mm inhibition zone against *E. coli* and 10.0, 11.0, and 8.0 mm inhibition zone against *P. aeruginosa*, respectively (**Fig. 6, 7**) (**Table 1**). The present study revealed the greater effect of aqueous root extract compared to the methanolic extract of *R. nepalensis* on microbial growth inhibition. These results are supported by the previous literature of *R. nepalensis* in which the aqueous extract was highly active against bacterial growth[15, 16].

Table 1: Antibacterial activities of *R. nepalensis* against *E. coli* and *P. aeruginosa*

Pathogens	Zone of inhibition in mm		
	Aqueous extract	Methanolic extract	AgNPs
E. coli	8.0	6.0	8.0
P. aeruginosa	10.0	11.0	8.0

Antioxidant activities

The methanolic root extract of *R. nepalensis* showed high antioxidant activity in terms of IC_{50} values (1.533) against DPPH indicating the presence of high phenolic and flavonoid contents (**Table 2**). Flavones are influential scavengers of highly oxidizing molecules (ROS), inclusive of singlet oxygen as well as extra free radicals which cause sicknesses including, oxidative stress and cancers. Generally, plants that are rich with conjugated-hydroxylated and aminated natural products possess very beneficial anti-oxidant potentials e.g., phenols, amine, hydroxylated polyketides with extended conjugation, and flavonoids as they possess powerful redox potentials and structural features[17,18].

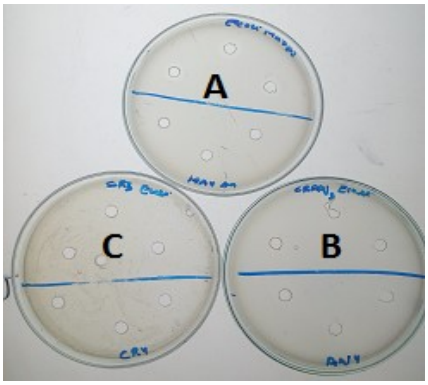

Fig. 6: Antibacterial activity of methanolic roots-extract **A**, aqueous extract **B** and AgNPs **C** of *R. nepalensis* against *E. coli*.

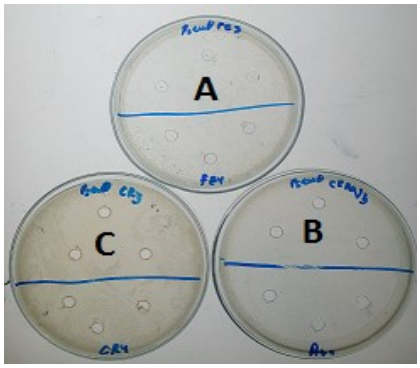

Fig. 7: Antibacterial activity of methanolic-extract **A**, aq. extract **B** and AgNPs **C** of *R. nepalensis* against *P. aeruginosa*.

Table 2: Antioxidant activities of methanolic roots-extracts of *R. nepalensis*

Extracts	Percent scavenging activity			IC_{50}
	10 µg/mL	20 µg/mL	30 µg/mL	
Methanolic	38.66	51.59	69.33	1.533

Determining total-phenolic and flavonoid-contents

The TPCs of the MeOH-squeeze out of roots of *R. nepalensis* was 149 mg Gallic acid equivalents/g and TFCs were 143 mg rutin equivalents/g where were determined by employing the calibration curve (R^2=0.998) and (R^2=0.997), respectively (**Table 3**). Plant's secondary metabolites like phenols and flavonoids have redox properties, which allow them to act as antioxidants. The antioxidant activities of these compounds depend on the presence of free OH-groups, especially 3-OH. Present results are in agreement with the previous literature[19, 20].

Table 3: Total phenolic and flavonoid contents of methanolic root extract of *R. nepalensis*

Extract	Total phenolic contents (RE mg/g DW)	Total flavonoid contents (GAE mg/g DW)
Methanolic	149	143

Conclusion

Nanotechnology has already become an unavoidable modern industrial need thus, it has become imperative to explore and develop economical, reliable, and eco-friendly processes for the synthesis of metallic nanoparticles. In this research, we report a simple green and low-cost approach for synthesizing stabilized Ag-NPs using bio-reduction of silver nitrate solution with aq-extract of the roots of medicinal *R. nepalensis*. Characterization of the obtained Ag-nanoparticles was performed by UV-Vis, XRD, FTIR, and SEM studies. The first indication of the formation of these Ag-NPs was the change in color of the solution from light brown to grey, which was also verified by the broad-band of UV- Visible spectrum, obtained at 447 nm. *R. nepalensis* proved to be a novel source for biosynthesizing AgNPs having an effective particle size of 17.15 nm determined by XRD. These biosynthesized AgNPs showed antibacterial activities and therefore can be used on a large scale to avoid bacterial infections. Further, the aqueous and methanolic extracts were also found to possess effective antimicrobial properties against *P. aeruginosa*. The present study emphasizes the use of *R. nepalensis* medicinal plant for the synthesis of AgNPs because its methanolic roots-extract possessed total phenolic contents of 149 mg Gallic acid equivalents/g and total flavonoid contents of 143 mg Rutin equivalents/g have played role in the synthesis of these AgNPs.

Acknowledgments

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Conflict of interest

This research work has no conflict of interest.

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