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# Antibacterial and antioxidant activity of biosynthesized silver nanoparticles from the high-altitude medicinal plants *Rhododendron anthopogen, Neopicrorhiza scrophulariiflora* and *Rheum australe* of Nepal

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***ABSTRACT***
Silver nanoparticles have been massively applied in the medical field including microbial population controls. Green synthesis of AgNPs is more intriguing domain due to possessing environmentally and economically friendly properties. For this experiment, we prepared AgNPs taking the methanolic extract of high altitude medicinal plants namely *Rhododendron anthopogen, Neopicrorhiza scrophulariiflora* and *Rheum australe* and antibacterial and antioxidant activity were observed. The biosynthesized AgNPs was confirmed using techniques UV-Vis spectroscopy, FTIR and XRD. Thus, prepared AgNPs demonstrated surface plasmon peak at wavelength nearly 430 nm in UV-Vis spectroscopy. FTIR spectroscopy date explained the role of functional groups in forming stable AgNPs and XRD pattern revealed that the silver nanoparticles were face-centered, cubic and crystalline in nature. The green synthesis of AgNPs working as an antibacterial agent was assessed by zone of inhibition against four readily available bacterial strain *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* and DPPH method is used to calculate antioxidant activity. Taken all together, the highest antioxidant activity $IC_{50}$ 69.84±0.03 µg/mL was exhibited by the silver nanoparticles of *R. australe* and the highest inhibition zone measuring16 mm was calculated by *N. scrophulariiflora against S. aureus.*

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## 1. Introduction

Silver nanoparticles (AgNPs) have become a potential therapeutic tool for the healing of several malaises and parasites because of exhibiting potent anti-plasmodial, anti-bacterial and anti-fungal activities [1]. Using antibiotics haphazardly consequences developing multidrug resistant pathogens and the several contagious ailments which is a crucial reason of rocketing mortality rate globally [2]. Infections caused by multidrug resistant bacteria may create various impacts including suppression in mortality and morbidity rate, monetary problem along with hospitalization for a longer period [3]. There are different kinds of physical, chemical and biological methods established for the synthesis of nanoparticles. Synthesizing nanomaterials using chemical process generates an immense quantity of hazardous by-products and the physical method requires high amount of energy, high pressure and high temperature. So, the green chemistry is today's requirement because it corporates pollution free technologies. Biological processes are assumed as sound safe and environmentally friendly. Biological methods employ either microorganisms or plant extracts and these methods have been popular in the present time due to being cost effective, simple and a viable alternative to the chemical and physical synthetic methods [4]. Microorganisms like fungi and bacteria have been used widely in the synthesis of AgNPs [5].

The effectiveness of antibiotics seems weaker than the earlier time due to increasing microbial resistivity, that is possible by either deteriorating immunity power of human or evolving a noble microbial strain by a mutation. Hence, a new antibacterial drug molecule is always demanding. The green synthesis of AgNPs among other metals could be a promising agent because they are notable for their action towards both gram-positive and negative bacterial and fungal species [6, 7]. Much studies have been done on the synthesis of green AgNPs using several medicinal plants [8, 9]. Nevertheless, there is still a lack of synthesizing high altitude medicinal plant based AgNPs by adopting environmentally clean, economically stable and commercially viable protocols [10]. A large number of medicinal plants are being consumed industrially for making various herbal medicine and nutritional products using their bioactive ingredients.

High-altitude medicinal plants are the rich sources of bioactive compounds due to their life cycle completed in the harsh climatic conditions [11]. In this study, we selected locally used three high altitude medicinal plants *R. anthopogen, N. scrophulariiflora* and *R. australe* considering their potent bioactive ingredients. *R. anthopogen* is a store house of camphene, thujene, pinene, δ-cadinene and α-cadinol etc that are involved in anti-flammatory, anti-microbial, anti-fungal and anti-proliferative activities [12]. *N. scrophulariiflora* contains many chemical constituents which include jatamansinone, jatamansic acid, volatile oil and supercritical fluid [13]. *R. australe* is rich in biologically potent secondary metabolite anthraquinones (emodin, chrysophanol, physcion, aloe-emodin and rhein) and stilbenoids (piceatannol, resveratrol). The root of *R. australe* is generally applied as an expectorant and appetizer and also practiced for healing cuts, wounds, muscular swellings, tonsillitis and mumps and as anti-bacterial, anti-cancer, anti-diabetic, anti-fungal and anti-oxidant agent [14]. Thus, we selected these three medicinal plants whose anti-bacterial and anti-oxidant activities are not reported using plant based AgNPs and their biological activities would be a new approach reporting from Nepal originated plants.

## 2. Material and methods

Fresh and healthy plant materials were collected from the low base camp, Mardi Himal, Kashi District of Nepal in October, 2018. Details are shown in **Table 1**. Plants identification and authentication was done by Dr. Ram Chandra Poudel, taxonomist, Molecular Biotechnology Unit, Nepal Academy of Science and Technology. Chemicals and reagents utilized in the experiment are of molecular grade. The Bacterial strains were brought from Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal. Milli-Q water

(PCR grade; Conductivity = 0.056 μS/cm, 25 °C) was used throughout the experiment.

**Table 1**: Description of collected plants species for the study

| Taxonic name | Indigenous name | Collected sites | Elevation | Used plant parts | Yield in percentage |
|---|---|---|---|---|---|
| *Neopicrorhiza scrophulariiflora* | Kutki | Low camp, Mardi Himal | 3650 m | Leaves | 26. 5 |
| *Rheum austral* | Padamchal | Low camp, Mardi Himal | 3500 m | Leaves and stem | 7.1 |
| *Rhododendron. anthopogen* | Sunpati | Low camp, Mardi Himal | 3300 m | Leaves and stem | 9.7 |
| Taxonic name | Indigenous name | Collected sites | Elevation | Used plant parts | Yield in percentage |
| *Neopicrorhiza scrophulariiflora* | Kutki | Low camp, Mardi Himal | 3650 m | Leaves | 26. 5 |
| *Rheum austral* | Padamchal | Low camp, Mardi Himal | 3500 m | Leaves and stem | 7.1 |
| *Rhododendron. anthopogen* | Sunpati | Low camp, Mardi Himal | 3300 m | Leaves and stem | 9.7 |

## 2.1 Silver nanoparticle synthesis

100 mg of the dried methanolic extract was mixed in 1mM silver nitrate solution up to 100 mL volume. Thus prepared solution was incubated at room temperature for 24 hours and observed for changing colors from brown to yellow. Spectrophotometer (Agilent technologies, Cary-60, Singapore) was used to analyze conversion of Ag+ to Ag(0). The sample was scanned in the absorbance ranging 350-700 nm at minimum scanning rate. Baseline correction of the spectrometer was carried out using distilled water as a blank reference. The fully reduced solution that shows the peak in the absorbance 400-450 nm on UV-Vis spectrophotometer. Twenty-four hours later, the mixture was centrifuged (9000 rpm, 20 min, 25 °C). The pellet was redispersed in Milli Q filtered water (Conductivity = 0.056 μS/cm, 25 °C). Thus, synthesized AgNPs were oven dried at 25-26 °C. After cooling, the samples were stored in a vial at 4 °C till further usage [15].

## 2.2 Characterization Technique

### *2.2.1 UV-VIS spectra analysis*

The solution of AgNPs in deionized water were characterized in UV-Vis spectrophotometer (Agilent technologies, Cary-60, Singapore). The spectrograph of the AgNPs was observed in a quartz cuvette where baseline correction was taken with water as a reference [16]. Absorbance was obtained at the range of 400-450 nm wavelengths.

### *2.2.2 FTIR spectra analysis*

From FTIR data confirms several functional groups found in the sample. For this purpose, prepared AgNPs dispersed in distilled water was registered in FTIR spectrometer. At first, potassium bromide (KBr) sample was scanned as a background. A few drops of AgNPs dispersed in the distilled water were dribbled on the KBr and scanning was carried out. The spectra obtained from FTIR demonstrating many frequencies at different wavelength were compared with standard infrared and Raman spectroscopy table. FTIR (IR Tracer-100, Shimadzu, Japan) in the absorbance ranging 4000–400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ was used for the analysis.

### *2.2.3 XRD spectral analysis*

AgNPs pellet was prepared by centrifuging the sample at 10,000 rpm for 15 min. Thus made pellets were dehydrated completely in an oven at 50 $^{o}$C and XRD was performed to find out the particles size. During the experiment, Bruker D2 Phaser Diffractometer (Germany) with 2θ angles ranging from 20° to 80° and monochromatic Cu Kα radiation source (λ = 0.15418 nm) generated by applying 30 kV and 10 mA to examine the particles nature.

## 2.3 Antioxidant activity

*DPPH assay*

Ascorbic acid with the concentration 10, 20, 60, 80, 100, 120 and 150 µg/mL was taken as a reference. Crude extract and AgNPs having 2000, 1500, 1000, 500, 250, 125 and 62.5 µg/mL solution were dissolved in dimethyl sulphoxide (DMSO). 0.01 mM concentration of DPPH was prepared in 100% methanol. Thus, prepared 100 mL of DPPH was mixed to the equal volume of different sample concentration and ascorbic acid solution. Total 200 mL of each solution was prepared in an Elisa plate. The plate was hold for 30 minutes under dark environment. Similarly, 0.1 mM DPPH measuring 200 µL was taken as a control. The absorbance was measured at the wavelength 517 nm after 30 minutes. The following equation was applied to calculate the scavenging capacity in percentage:

$$\text{Scavenging capacity} = \frac{A_0 - A_t}{A_0} * 100$$

Where, $A_0$ = the absorbance of the control and $A_t$ = the absorbance of the extractives/standard

The scavenging capacity was determined by calculating $IC_{50}$ value. A plot is designed taking concentration versus regression equation for $IC_{50}$ determination [17].

## 2.4 Antibacterial properties

*2.4.1 Bacterial strains*

Active cultures of four standard bacterial strain namely *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603) *and P. aeruginosa* (ATCC 27853) were used to know the antibacterial activities using both synthesized AgNPs and plant extract.

*2.4.2 Inoculation*

A single colony of a bacterial strain was scratched and inoculated in a 5 ml inoculated bottle containing nutrient broth. The inoculation was allowed until the growth noted similar to the Mac-Farland (0.5%) as the standard suggested by WHO. Then the bacterial growth was further incubated (37 °C, 3-4 h). The cloudiness of bacterial suspension was set at 0.5 McFarland standards. The McFarland standards was prepared the day before antibacterial tests. Antimicrobial effect was observed after swabbing inoculums on MHA plate.

*2.4.3 Agar well diffusion method*

Antibacterial activity of different extracts was determined by well diffusion method on Muller Hinton agar (MHA) medium. Required wells were built on MHA medium surface with a cork borer (6 mm diameter). Inoculums with $10^6$ CFU/mL of bacteria were scattered on the MHA medium coated plates with a sterile swab moistened. 20 µL extract having the concentration of 100, 50, 25 and 12.5 mg/mL and same volume of extraction solvents (MeOH and DMSO) was taken as a negative and streptomycin (1mg/mL) as a positive control. Likewise, biosynthesized AgNPs measuring 20 µL was added into the wells containing negative and positive controls. The Plates were left and allowed to disperse the sample on MHA solution. The plate was covered and incubation was performed (37 °C, 24 h). In the next day, sample inhibited zone against microbial was calculated by measuring the diameter in mm [18]. The AgNPs showing the zone of inhibition above or equal to 8 mm were validated as the sample working effectively.

In the study, plants with significant medicinal values were chosen from the high-altitude of Nepal regarding their importance in local areas. Plants found in high altitude are rich sources of bioactive compounds because they have to battle with the harsh climatic conditions for survival. Climatic condition, plants component used, extraction time and

procedure, solvent types and temperature play a pivotal role in separating biologically potent compounds [19]**.**

3.1.1 UV-vis Spectra Analysis

The AgNPs of selected plants namely *R. anthopogen*, *N. scrophulariiflora* and *R. australe* extracts observed absorption peaks at the wavelenght 400-450 nm. *R. australe* exhibited the peak at 420 nm, *R. anthopogen* at 430 nm and *N. scrophulariflora* at 450 nm in this analysis. The occurrence of the peak in these range indicates the formation of AgNPs [20, 21, 33]. **Figure 1** shows UV-Vis spectra of the plants based AgNPs.

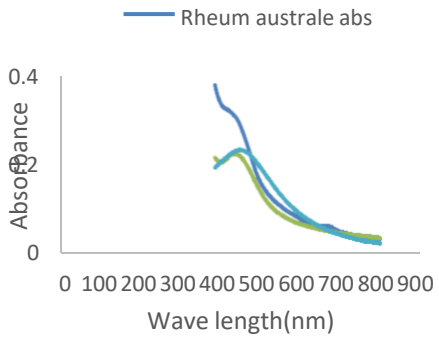


**Fig. 1:** UV-vis spectra of AgNPs from *R. austral, R. anthopgen* and *N. scrophulariflora.*

### 3.1.2 FTIR Spectroscopic Analysis

In FTIR spectroscopy, the absorption of AgNPs was carried out in the wavelenth between 4000 to 500 $cm^{-1}$. The sample showed the peak at various wavelengths. The peak detected in between 3400 to 3700 $cm^{-1}$ predicted the formation of hydroxyl group (O-H) and the next observable peak at 1690-1630 $cm^{-1}$ informs carbonyl group (C=O). Presence of these two functional groups indicates the formation of nanoparticle by the reduction of silver nitrate. The FTIR spectrum of *N. scrophulariiflora* synthesized AgNPs (**Fig 2)** observes the primary peak at 3442 $cm^{-1}$ which is possible having O-H stretching vibration occurs in alcoholic and phenolic groups and the peak at 1635 $cm^{-1}$ indicates the formation of carboxy groups [22]. Similar peaks of *R. anthopogen* (**Fig 3**) and *R. australe* (**Fig 4)** were appeared in the FTIR spectra.

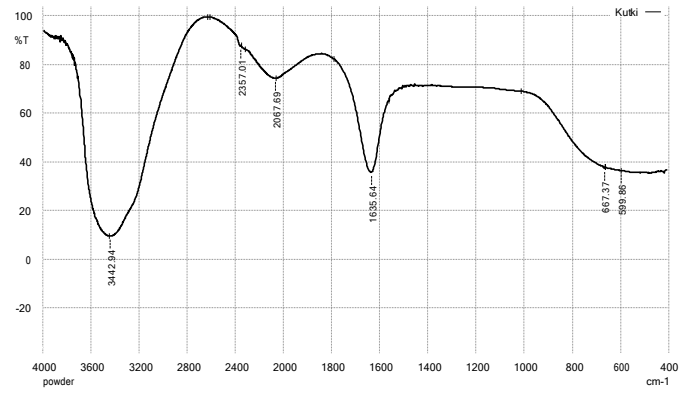


**Fig 2:** FTIR spectrum of the silver nanoparticle using *N. scrophulariiflora.*

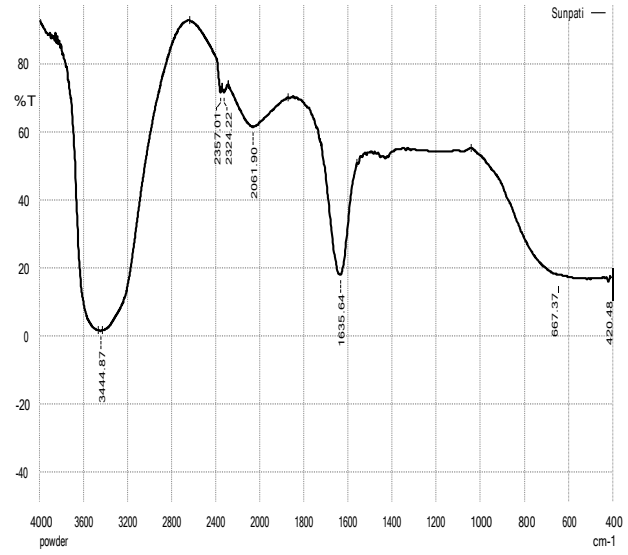


**Fig 3:** FTIR spectrum of the silver nanoparticle using *R. anthopogen.*

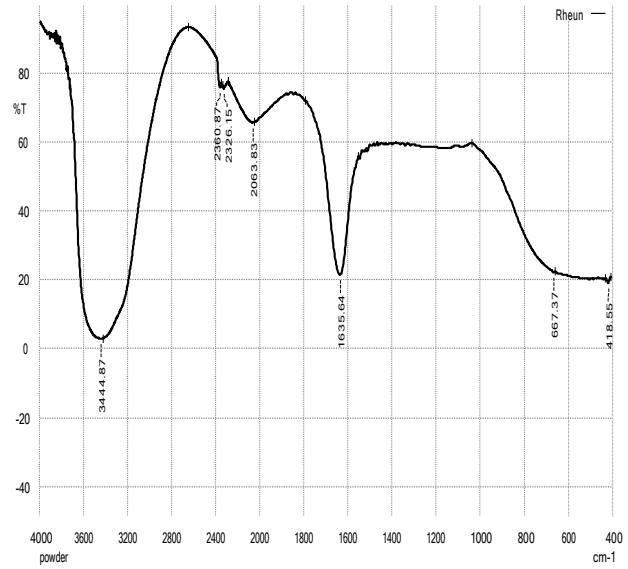


**Fig 4:** FTIR spectrum of the silver nanoparticle using *R. australe.*

### 3.1.3 X-ray diffraction analysis

XRD was used to examine crystallize size and structure of the biosynthesized AgNPs was examined by XRD. AgNPs of *N. scrophulariiflora* (**Fig 5c**) showed the diffraction peak at 2$\theta$ = 30.5, 37.8, 44.0, 64.3 and 77.7, *R. anthopogen* (**Fig 5a**) showed at 30.3, 37.7, 43.9, 64.14 and 77.12 and *R. australe* (**Fig 5b**) demonstrated at 32.08, 37.9, 44.3, 64.4 and 77.2. These peaks formation in XRD supported AgNPs having nanocrystal and crystalline shape which were compared with the standard. The observed peaks can be assigned to the planes (122), (111), (200), (220) and (311) facet of silver crystal [23]. Spectra showing such a few peaks which were cumbersome to specify because of the bioorganic compounds/protein(s) contamination [24, 25]. The crystalline size was calculated by analyzing peak intensity, position and full width at half maximum (FWHM). The size of crystalline particles was determined by Debye–Scherrer's equation.

$$D = 0.9\lambda / \beta \cos\theta$$

where, $\lambda$ = x-ray wavelength, $\beta$= diffraction line broadening measured as half of its maximum intensity in radians and

$\theta$ = Diffraction angle

The crystal size of the sample was estimated taking the line width of the peak (111) of all three samples. The size of AgNPs prepared from *R. anthopogon* (Fig. 5a), *R. austral* (Fig. 5b) and *N. scrophulariiflora* (Fig. 5c) was found 9, 11 and 16 nm respectively.

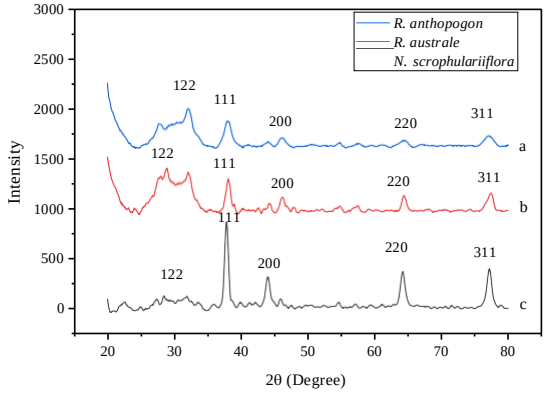


**Fig 5:** XRD diffraction pattern of AgNPs synthesized from (a) *R. anthopogon*, (b) *R. austral* and *N. Scrophulariiflora.*

### 3.2 Antioxidant Activity of AgNPs

Among three medicinal plants, AgNPs synthesized using *R. australe* showed significant antioxidant potential having $IC_{50}$ 69.84±0.03 µg/mL but crude methanolic extract exhibited 100±0.54 µg/mL. Similarly, the AgNPs of *R. anthopogen* and *N. scrophulariflora* had antioxidant activity $IC_{50}$ 98.17±0.02 and 74.56±0.05 µg/mL respectively. While comparing antioxidant activity between crude extract and their AgNPs, the former showed remarkably less potent with higher $IC_{50}$ (Table 2). Several articles have reported significant antioxidant properties of biologically prepared AgNPs using *P. pinnate* plant [26] and *E. suberosa* [27, 33] than their crude and so on. The results suggested that biosynthesized silver nanoparticle is more beneficial than its crude extract using as an organic antioxidant. Antioxidant compounds protects our health from various oxidative stresses which is connected to degenerative diseases. AgNPs can be taken into another screening test on animal models and then clinical trials to validate its effectiveness.

**Table 2:** Antioxidant properties ($IC_{50}$) of crude extract and AgNPs

| S.N. | Plant name | Extract in methanol $IC_{50}$ (µg/mL) | Methanolic AgNPs $IC_{50}$ (µg/mL) |
|---|---|---|---|
| 1. | *R. australe* | 100±0.54 | 69.84±0.03 |
| 2. | *R. anthopogen* | 140±0.39 | 98.17±0.02 |
| 3. | *N. scrophulariiflora* | 120±0.78 | 74.56±0.05 |

### 3.3 Antimicrobial Activity of Silver Nanoparticles

AgNPs have been using several important fields like medicine, cosmetics and environment and so on [28]. The

biosynthesized AgNPs of the medicinal plants were examined their antibacterial properties by measuring diameter of inhibition area with different bacterial strain (**Fig. 6 and Table 3**). The maximum inhibitory zone measuring 16 mm was exhibited by *N. scrophulariflora* silver nanoparticles against *S. aureus* in **Fig 6(E)** and the minimum inhibition below 6 mm was showed by the AgNPs of *R. australe* against *K. pneumoniae* depicted in **Fig 6(K)**. From the studies, it is cleared that the AgNPs synthesized biologically has shown higher potential than its extract because of having wide surface region increases interaction to the cell wall of a bacterium [29]. Several similar findings were cited describing effectiveness of AgNPs testing on *E. coli* with *Mentha piperita* [30], *Acalypha indica* [16] and *Berberis asiatica* (33). The mysterious working principle between AgNPs and its effective microbial activity is developing an electrostatic force of the positively charged Ag+ ion and negative charges generated on phospholipids bilayers of bacterial cell wall [31, 32].

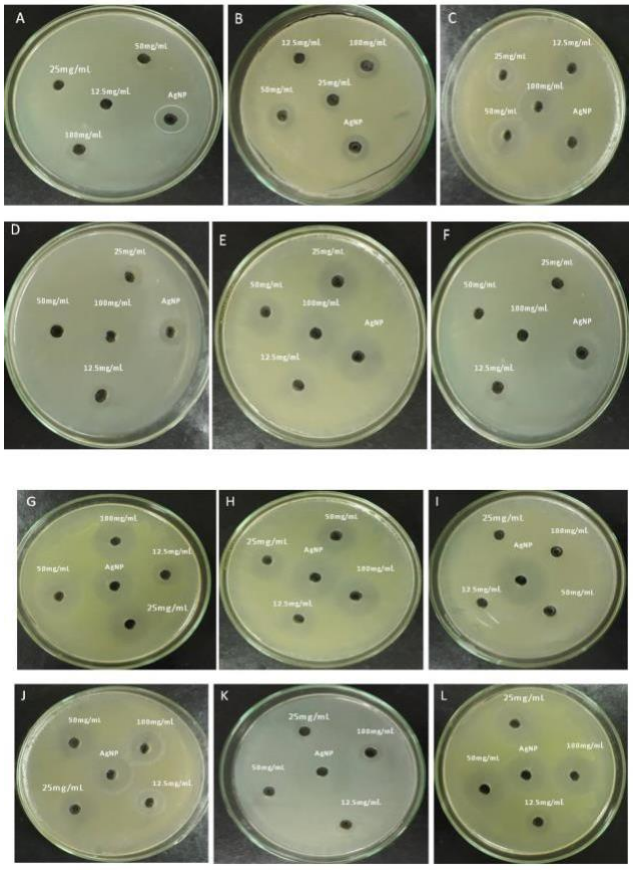

**Fig 6:** Crude extract and AgNPs in several concentrations showing antibacterial properties of *R. australe* (A) *S. aureus*, (B) *K. pneumoniae*, (C*) P. aeruginoa* and (D) *E. coli*, *N. scrophulariflora* (E) *S. aureus*, (F) *K. pneumoniae*, (G) *P. aeruginoa* and (H) *E. coli* and *R. anthopogon* (I) *S. aureus*, (J) *K. pneumoniae*, and (K) *P. aeruginoa* and (L) *E. ceoli*.

**Table 3:** Crude extract and AgNPs exhibiting zone of inhibition (in mm) against bacteria.

| Plants | Concentration mg/mL | Zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | *S. aureus* | *K. pneumoniae* | *P. aeruginoa* | *E. coli* |
| R. australe | Crude (50) | 6 | 10 | 12 | 6 |
| | AgNPs (12.5) | 11 | 11 | 10 | 9 |
| *N. scrophulariflora* | Crude (50) | 14 | 6 | 14 | 9 |
| | AgNPs (12.5) | 16 | 9 | 12 | 13 |
| *R. anthopogon* | Crude( 50) | 6 | 8 | 6 | 12 |
| | AgNPs (12.5) | 15 | 12 | 6 | 12 |

## Conclusions

Methanolic extract of AgNPs was synthesized using high altitude medically profound plants namely *R. australe, N. scrophulariflora* and *R. anthopogon*. Biosynthetic AgNPs formation was confirmed by using UV-Vis spectrophotometer where the sample showed the peaks in the wavelength ranging 400-450 nm. FTIR data added more evidences for the formation of AgNPs in the context to the functional groups by providing the peaks at 3400-3700 $cm^{-1}$ and for 1690-1630 $cm^{-1}$ wavelength for hydroxyl group (O-

H) and carbonyl group (C=O) respectively. Additionally, obtained XRD spectral peaks corresponding to the standard provided the synthetic sample in nanocrystal and crystalline in shape. *R. australe* AgNPs exhibited the highest antioxiant propertis ($IC_{50}$ 69.84±0.03 µg/mL) among the samples. The most effective antimicrobial activity was calculated from the AgNPs of *N. scrophulariiflora* showing zone of inhibition 16 mm against *S. aureus*. Regarding our studies, it is cleared that the importance of biosynthesized AgNPs to their extracts is high. These biosynthesized AgNPs might be useful in the several domains in the coming times after performing additional experiments

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