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BIOGENIC SYNTHESIS OF SILVER NANOPARTICLES USING HAPLOPHYLLUM GILESII LEAF EXTRACT AND EVALUATION OF ITS BIOLOGICAL ACTIVITIES

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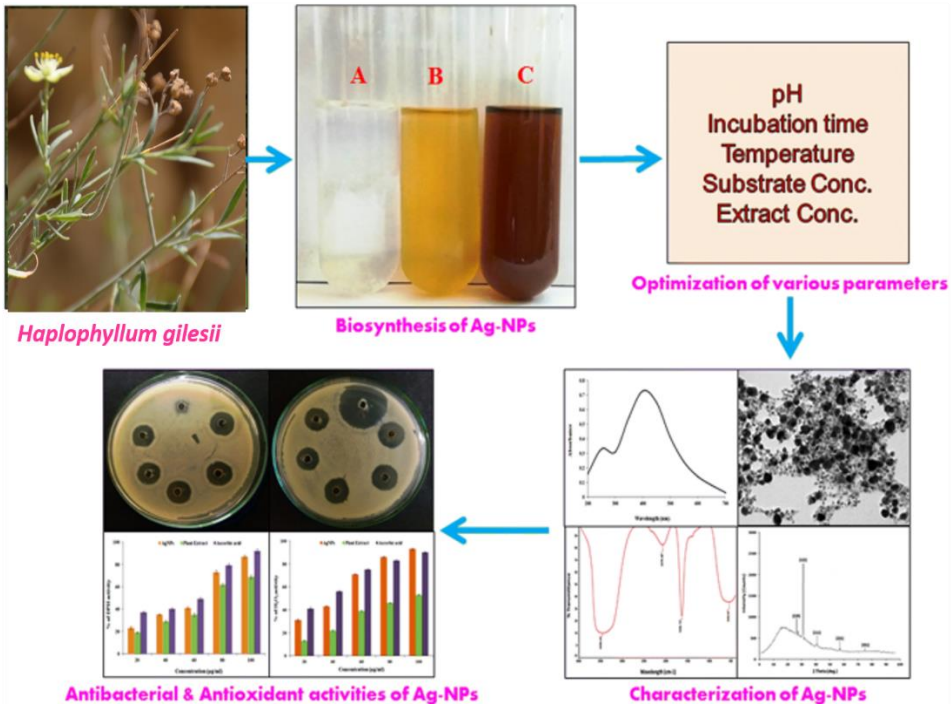
Abstract
Nanotechnology has got significant importance in biomedical era in the last two decades. Nanoparticles(NP) being smaller in size are acquiring worldwide attention in the field of science and technology. Moreover, the biosynthesis of nanoparticles from various plant extracts is is profitable and eco-friendly. In the present study *Haplophyllum gilesii* (Hemsl.) C.C. townsend a narrow endemic plant species reported from Northern Pakistan (1500-4000 meters) was screened first time for synthesis of silver nano-particles(AgNPs) from the leaf extract. The prepared metal nanoparticles (MNPs) were further characterized by using X-ray diffraction (XRD) analysis, UV–visible spectroscopy and Scanning Electron Microscopy (SEM). A comparative antibacterial potential of raw methanolic extract and prepared silver nanoparticles(AgNPs) was evaluated which revealed that Nps exhibited the most pronounced antibacterial potential against Gram- negative and Gram- bacteria positive i-e AgNPs of *H. gilesii* exhibited significant zone of Inhibition against *S. aureus*, *B. subtiis*, *E. coli, S. typhi, P. aeruginosa* and than the crude extract alone. Similarly, the results of the antioxidant activity of AgNPs of *H. gilesii* were more significant than crude extract. Hence, the present study proved that *H. gilesii* might be a good source of potential antibiotics and valuable antioxidants. Moreover, further research is needed to isolate essential bioactive compounds to devise anti-cancerous drugs.

Keywords: Silver Nanoparticles, Antibacterial, Antioxidant, *Haplophyllum gilesii.*



Graphical Abstract

1. Introduction

Nanotechnology has got the special attention of researchers due to its vast scope and application in various industrial, physiochemical, and biological fields (Riaz *et al*., 2022). A metallic nanoparticle can be synthesized radially by various physical and chemical processes but they are not profitable and environment-friendly as they may damage the biological system. (Sondi *et al.* 2004; Sotiriou *et al.* 2010). Plant-mediated silver nanoparticles are attaining worldwide attention because of the strong toxicity of silver against various microorganisms. Moreover, silver nanoparticles exhibit promising anti-cancerous, antiviral and anti-inflammatory properties. (Kalishwarala *et al.* 2010; Safaepour *et al.* 2009 ; Salama *et al*., 2020; Tariq *et al*., 2022;)

Silver has got significant antibacterial activity as the silver ions react with the thiole group of various respiratory enzymes and proteins to inhibit their activity in the transport of substances across the cell membrane(Cho *et al.* 2005). Nowadays medicinal plants are extensively being used for the biosynthesis of nanoparticles being free of chemical toxins and providing natural capping agents. Synthesis of nanoparticles by crude plant extracts is cost-effective over the synthesis of nanoparticles by microorganisms (Tariq *et al*., 2022). Silver nanoparticles are long been known for their significant importance in the medicine, biology, and pharmaceutical industry and have proven as broad-spectrum antibacterial agents to combat various infections (Shankar *et al.* 2005). A brief pharmacological survey of some other members of the genus Haplophyllum showed significant biological activities that are antimicrobial, antioxidant, cytotoxic, cardiovascular, and anti-inflammatory properties. Moreover, various species of *Haplophyllum* have widely been used traditionally for the cure of several diseases through local community. Paste of the whole plant of H. acutifolium is used in Iran to treat dermal injuries and inflammations (Ghorbani, 2005). Similarly, *H. myrtifolium* is used to cure warts, herpes, lichens, erysipelas, diarrhea and some kind of tumors (such as testicular cancer) (Varamini *et al.* 2007). Likewise, in Saudi Arabia H. tuberculatun has been consumed for the treatment of malaria, rheumatoid arthritis, headaches, and some pre-birth problems, additionally to eradicate lumps, treat skin discoloration, toxicities, and parasitic infections (Al-Yahya et al. 1992).

Haplophyllum gilesii (Hemsl.) C.C. Townsend belongs to the family Rutaceae, herbs to semi-shrub up to 3 feet in height. Leaves simple with creamy yellow flowers. Confined to the Karakoram Himalayan range of Pakistan, in dry localities. (Alam, 2009; Alam and Ali 2010). Traditionally it is used for various skin diseases, ulcers, and as stomachic. Besides these it has got a strong antimicrobial and cytotoxic potential, Ashfaq *et al.* (2020).

Therefore, the present study was focused on the synthesis AgNPs via biologically active *H. gilesii* leaf extract.

2. Materials and Methodology

2.1 Plant and Chemicals

Haplophyllum gilesii samples were collected from Dassu region District Kohistan, Khyber Pakhtunkhwa, Pakistan. Taxonomic identification of the plant material was confirmed by the senior taxonomist Prof. Dr. Manzoor Hussain and Associate Prof. Dr. Jan Alam Departmentof Botany, Hazara University Mansehra, Pakistan. The voucher (Voucher Specimen No: HUP 9081) has been deposited at the Herbarium, Department of Botany of Hazara University. AR-grade silver nitrate (Merck KGaA, 64271 Darmstadt, Germany) was purchased from Musaji Adam & Sons Chemicals, Karachi, Pakistan.

2.2 Silver nanoparticles(AgNPs) Synthesis

AgNPs were prepared from particular plant extracts by using the green chemistry route. The crude plant sample was washed thrice with deionized water. 10g fine powder of the dried part of plants was dissolved in deionized water (100 ml). The plant extracts were incubated at room temperature for one day after treatment with 1 mM o $AgNO_3$ aqueous solution and. To prevent photoactivation of silver nitrate the incubation of material was performed in the dark system. The solution color is transformed from yellow to radish tanned after ten minutes, demonstrating a reduction of Ag ions from $AgNO_3$. In addition, AgNPs aqueous solution was processed to centrifuge for 20 min on 10,000 rpm followed by washing with water. To eliminate boundless material from AgNPs, the process was continual three times. Finally, the mixture was dried out and stored in at 4 ^{0}C for further research.

2.3 Characterization of Nanomaterial

Double Beam UV/Vis spectrophotometer was used for confirmation of prepared AgNPs in the wavelength ranges of 300 to 800 nm at a resolution of 1 nm. While, the morphology of surface and determination of particle average size were examined through the use of field emission-scanning electron microscope FESEM (JSM-5910- JEOL-JAPAN). JEOL JDX 3532 XRD was employed to check the purity and crystallinity of the synthesized nanoparticles in a centralized laboratory of of Peshawar University, Pakistan.

2.4 Antibacterial Activity of Nanomaterial

The bacteriocidal assesment of NPs was evaluated for Gram-negative (*P. aeruginosaE. coli,* , and *S. typhi)* and Gram-positive bacteria (*B. subtilis* and *S. aureu*) by adopting Muller Hinton agar and agar well diffusion assay with incubation for 72 hours at 37°C. 7mm well were formed by using a sterile cork borer on each Petri plate. 10 µL AgNPs in various concentrations i.e. 10mg/mL, 20mg/mL, 30mg/mL, and crude extract 5mg/mL was incubated for one day at 37°C after pouring into the wells. 5µL standard antibiotic streptomycin (10 µg/mL) was acclimated as positive control while DMSO and $AgNO_3$ were for negative control. After 24 hours, zone of inhibition(ZOI) were calculated in mm.

2.5 Antioxidant Activity of Nanomaterial

The synthesized silver nanoparticles (AgNPs) and the extract underwent evaluation for antioxidant activity using the DPPH method. 250µg/mL, 500 µg/mL, 1000µg/mL of crude extract, and synthesized AgNPs was combined with 3 mL of a methanolic solution containing DPPH radicals (0.1 mM). Following a 1 hour incubation period, the absorbance was measured at 517 nm (Chang *et al*. (2002). The percentage of inhibition activity was computed using the formula:

$$\% \text{ Antioxidant potential} = \frac{A_0 - A_e}{A_0} \times 100$$

where Ao represents the absorbance without extract, and Ae represents the absorbance with the extract.

2.6 Statistical Analysis

Results are revealed as mean ± standard deviations. ANOVA (One-way) was used for statistical analysis by using student's t-test and Graph Pad Prism (version 9.4.0). P values less than the 0.05 were considered to be statistically significant.

3. Results

Reduction of Ag^{+} ions into silver nanoparticles is accomplished by using crude plant extract. Gradual change in the color reaction was due to the size of the AgNPs, different concentrations of the extract used and the formation of the Ag NPS was due to the bio-reduction of Ag+ ion by *H. gilesii* extract. Color transposed from colorless to yellowish red and finally to reddish brown with constant stirring confirmed the formation of stable Ag NPs (**Fig. 1**).

AgNPs synthesis and gradual change in the UV spectra of reaction mixture is brought about by the oscillation and conduction of free electrons which is induced by the interaction of the electromagnetic field called surface Plasmon peak or Surface Plasmon Resonance (SPR) Smitha *et al.* (2009); Shankar *et al.* (2003) and Mulvaney, (1996) revealed that Ag nanoparticles shows yellowish to the reddish brown color in aqueous solution and the change of color is the due reduction of AgNO3 to AgNPs and Surface Plasmon Resonance effect. The present study revealed that *H. gilesii* exhibited durable reducing and strong capping agents due to the presence of varities of bioactive compounds for the synthesis of NPs.

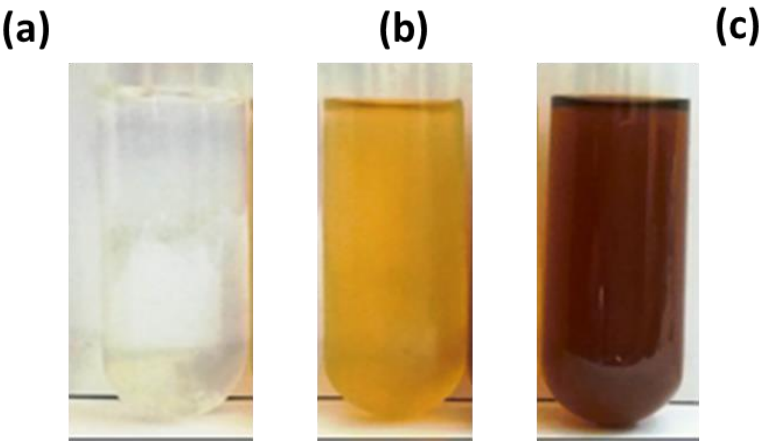


Fig. 1: Silver Nitrate solution (a), Gradual change in reaction mixture (b), and Synthesized AgNPs (c).

3.1 Characterization of Nanomaterial

3.1.1 UV-Vis

Reduction of AgNO3 into AgNPs loaded in *H. gilesii* is confirmed by UV-Visible spectroscopy. UV-Vis spectra gives an intense peak in the range of 400-450nm. Each concentration gave a different absorbance peak recorded at 435 nm as shown in **Fig. 2a**, absorbance mainly depends upon the dielectric medium and size of nanoparticles. From the current study, it is observed that biomolecules in the respective plant are responsible for the reduction and the formation of AgNPs.

3.1.2 XRD

XRD patterns are generally used to confirm the crystalline nature and size of synthesized silver nanoparticles. A large number of Bragg reflections were shown by XRD patterns that are established on the face-centered cubic(fcc) structure geomatery of AgNPs . In the current study distinct peaks had been shown by *H. gilesii* nanocrystals i-e at 2theta 37.93, 44.18, 64.46, and 77.23 which corresponds to the (111), (200), (222), and (311), Ag crystals reflection planes respectively. Average size of the

NPs was calculated by utilizing Debye-Scherrer equation. The estimated mean size of the particle was 13 nm as shown in **Fig. 2b.**

3.1.3. SEM

FSEM is used for determining the shape, distribution and size of the synthesized AgNPs. In the current study AgNPs of the aerial parts of *H. gilesii* were detected through SEM analysis at 20,000 X magnification, which was spherical with a uniform size of 10-20µm and distributed equally over the medium position as shown in **Fig. 2c**.

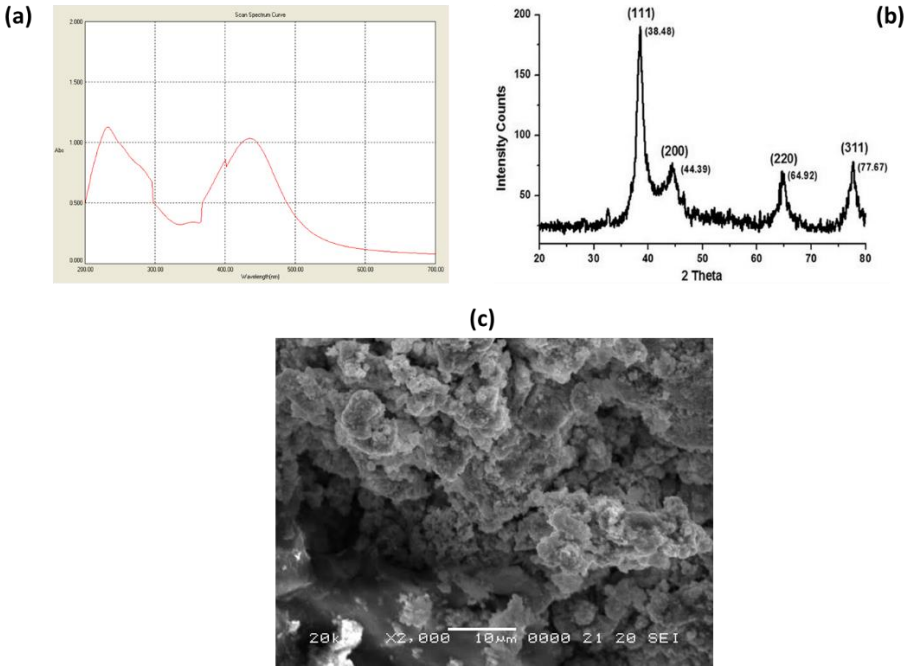


Fig. 2: UV-Visible spectrum (a), XRD spectrum (b), and SEM image of the synthesized AgNPs of *H. gilesii*

3.2 Biological screening of AgNPs

3.2.1 Antibacterial activity

Nanotechnology exhibitsa significant role in the production of plant-mediated metallic NPs which could be a source of innovation in the field of medicine and microbiology (Riaz *et al*., 2022)**.** The results obtained from the antibacterial activity of extract and AgNps were effective against various Gram-positive (*Bacilllus subtilis Staphylococcus aureus*) and Gram-negative bacterial strains (*Pseudomonas aeruginosa, Salmonella typhi and Escherichia coli*). DMSO and silver nitrate were used as *the* negative control while as a positive control streptomycin was utilized. The ZOI in mm is given in (**Table-1**). The ZOI of AgNps of *H. gilesii* against *S. aureus* was 9 mm, 13 mm, and 16mm at 10mg/mL, 20mg/mL, and 30mg/mL while in the case of crude sample and streptomycin solely it was 7mm and 18mm respectively. However, *B. sabtilis* showed a 7mm, 11mm, and 13mm ZOI at 10mg/mL, 20mg/mL, and 30mg/mL of AgNps while in the case of streptomycin and extract alone it was 5mm and 16mm respectively. Similarly, for *E. coli* it was observed 9mm, 11mm, and 13mm for AgNps and 6mm and 17mm for crude extract and streptomycin alone. *P. aeruginosa* showed 6mm, 9mm, and 11 mm ZOI after treatment with 10mg/mL, 20mg/mL, and 30mg/mL of AgNps and 5mm and 13mm for extract and Streptomycin alone. In the *S. typhi* ZOI observed were 8mm, 10mm, and 13mm for 10mg/mL, 20mg/mL, and 30mg/mL of AgNps while 6mm and 14mm for extract and streptomycin respectively. (**Fig. 3**).

Table-1 Antibacterial potential of *H. gilesii* (HG) extract, HG-AgNPs, and Streptomycin against Gram-negative and Gram-positive strains.

Microorganism	Zone of inhibition in mm				
	HG-AgNPs (10mg/mL)	HG-AgNPs (20mg/mL)	HG-AgNPs (30mg/mL)	HG Extract (5mg/mL)	Streptomycin (10 mg/mL)
Gram-positive Bacterial strains					
S. aureus	9 ± 1.00^{a}	$13 \pm 0.51a^{b}$	16 ± 1.5^{c}	7 ± 0.11^{d}	18 ± 1.00^{e}
B. subtilis	7 ± 0.21^{b}	11 ± 0.46^{a}	13 ± 0.98^{bc}	5 ± 1.15^{c}	16 ± 0.95^{c}
Gram-negative Bacterial strains					
E. coli	9 ± 0.69^{a}	11 ± 0.92^{c}	13 ± 1.00^{d}	6 ± 0.25^{e}	17 ± 1.50^{ab}
P. aeruginosa	7 ± 0.92^{cd}	10 ± 0.81^{c}	12 ± 0.84^{e}	6 ± 0.59^{d}	15 ± 0.89^{b}
S. typhi	8 ± 0.59^{d}	10 ± 0.76^{a}	14 ± 1.39^{b}	6 ± 0.32^{a}	16 ± 0.67^{e}

Data are expressed just as mean ± SD (n = 3). represent Significant differences appeared at $p < 0.05$ (by Student's t-test) showing due to different letters in the same column.

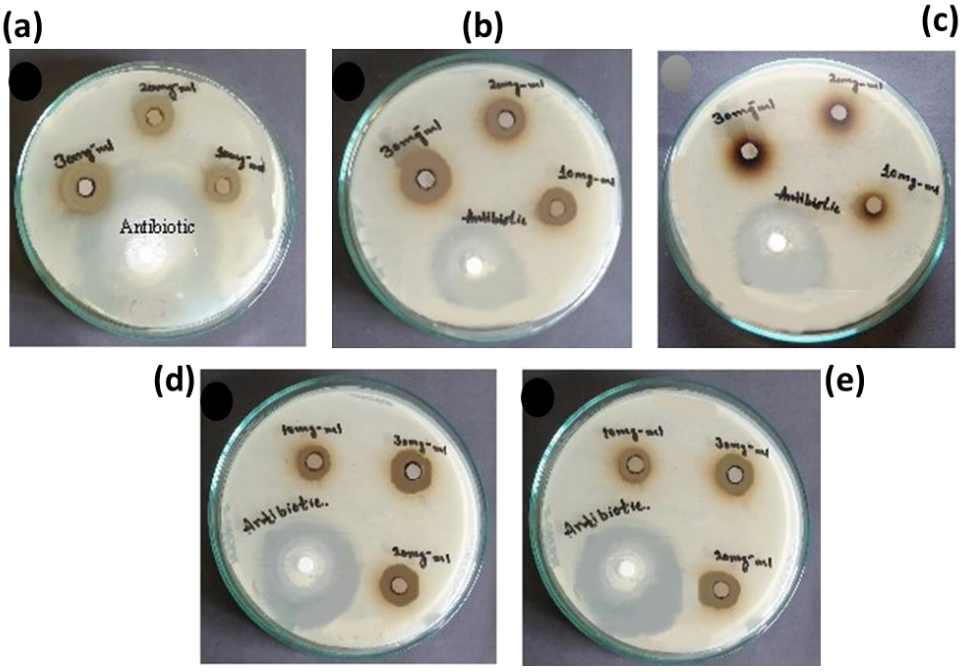


Fig. 3: Antibacterial activity of HG-AgNPs opposed to *Staphylococcus aureus* (a), *Bacillus subtilis* (b), *Escherichia coli* (c), *Pseudomonas aeruginosa* (d) and *Salmonella typhi* (e).

3.2.2 Antioxidant Activity

The antioxidant potential of medicinal plants is due to the presence of polyphenolic compounds which possess strong scavenging properties neutralizing the free radicals by donating electrons. The antioxidant potential of the silver nanoparticles of the aerial parts of *H. giles*ii was assessed against DPPH (free radical) at three different strength of solutions i.e. 250 µg/mL, 500 µg/mL, and 1000

µg/mL. The reducing potential was quantified by spectrophotometer and absorbance is analyzed by the change of color from purple to yellow. Plant-mediated AgNPs showed an excellent antioxidant potential at 1000 µg/mL under the incubation period of 30 minutes as compared to the extract alone. The AgNPs of *H. gilesii* exhibited 82% absorbance at 1000 µg/mL while crude extract had 57% absorbance. Ascorbic acid was used as standard (positive control) which exhibited 100% absorbance in all the concentrations of extract and AgNPs. DPPH was used as negative control against the plant extract and synthesized AgNPs which showed no activity as described in **Tabel-2** and **Fig. 4.**

Table-2 Antioxidant Potential of *H. gilesii*

Dilutions	**Acid (Positive Control)**	***H.gilesii Crude* Extract**	***H.gilesii* AgNPs**
1000(µg/mL)	100 ± 0.91^{a}	57 ± 0.32^{a}	82 ± 0.32^{ab}
500(µg/mL)	100 ± 0.68^{b}	49 ± 0.45^{b}	63 ± 0.51^{a}
250(µg/mL)	100 ± 0.72^{c}	35 ± 0.51^{c}	58 ± 0.28^{b}

Data are demonstrated as mean ± SD (n = 3). Dissimilar letters in the same column showed significant differences at $p < 0.05$ (by Student's t-test).

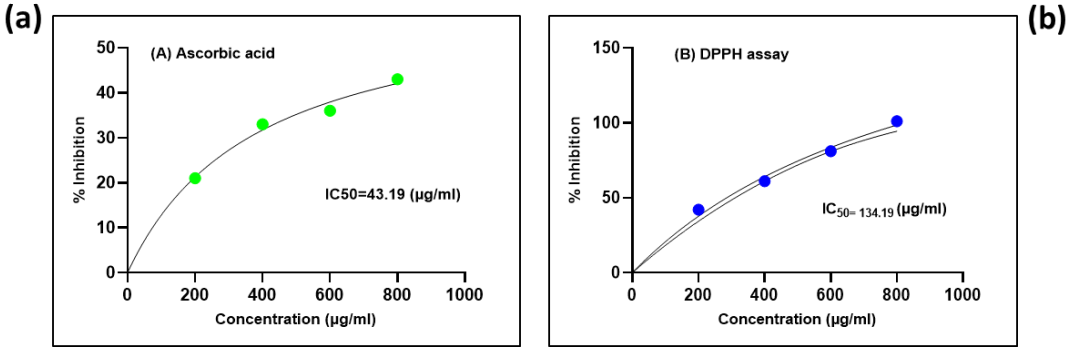


Fig.4: Antioxidant Potential of AgNPs of *H. gilesii* (a), and Ascorbic acid (b).

4. Discussion

In the modern sciences NPs accounts for efficient drug delivery podum(Arokiyaraj *et al.* 2014; Riaz *et al*., 2022). Nanomaterials have been synthesized by using several microorganisms, enzymes and plant extracts Palanisamy *et al.* (2014). However, In the present work, *H. gilesii* was used to support AgNPs. Moreover, research was also performed to evaluate the antimicrobial potential of the plant-mediated Ag NPs and their crude extract. Results revealed that *H. gilesii* showed excellent antibacterial and antioxidant activity for AgNPs as compared to the extract alone as AgNps were in the size range of 10-200nm which bestow to penetrate the living system and account for antimicrobial potential. However, the route of action AgNPs in microorganisms system is still ambiguous. (Kim *et al.*, 2007, 2011). Characterization of the silver nanoparticles was carried out by XDR analysis, UV-visible spectroscopy and SEM studies. The results of the antibacterial studies, antioxidant studies, and characterization coincided with the work of a few other researchers. The UV-visible spectrophotometric analysis of the synthesized AgNPs of *H. gilesii* showed that intense peaks were recorded in the range of 400-450nm which could be due to the Surface Plasmon Resonance phenominan of nanomaterial. Krishnaraj *et al.* (2010) characterized the AgNPs of *Acalypha indica* leaf extract by UV-vis spectroscopy and confirmed the sharp peak at 420nm. Solgi and Taghizadeh (2012) analyzed the petal extract of *Rosa damascena* and the peel extract of *Punica granatum* by UV-Vis spectroscopy the intense peak recorded was 400-500 nm which was consistent with our findings. The diffraction peaks analyzed in the XRD analysis corresponded to the face-centered cubic geomatery of NPs with particle size between 5-50 nm (Erjaee *et al.* 2017). The

characterization of synthesized AgNPs by SEM studies ascertained the formation of silver nanoparticles with a particular size (10-20nm) and morphology. Jaina *et al.* (2009) analyzed silver nanoparticles of papaya fruit extract by SEM studies with an average particle size of 15 nm. Some other workers (Elumalai *et al.* 2010; Caroling *et al.* 2013; Pavani *et al*. 2013) reported a particle size of 10-50nm which coincided with our SEM analysis.

Antibacterial assay of the AgNPS showed significant effects against both Gram-negative and Gram-positive bacterial strains. Agar well diffusion method revealed that AgNPs of *H. gilesii* exhibited a larger ZOI at 30mg/ml in comparison to crude extract which is analogous to the results reported by (Pal *et al*. 2007; Ramamurthy *et al*. 2013; Nagati *et al.* 2012). The antibacterial efficiency of the AgNPs against individual bacterial strains can be determined by varying sizes of inhibition zones. Ashwani *et al.* (2014). Variation in the ZOI (mm) of AgNPs of the *H. gilesii* could be due to variable modes of action on different microorganisms.

Medicinal plants contain a special class of secondary metabolites which are known to stop oxidation by neutralizing the free radicals. AgNPs showed excellent antioxidant activity due to the enhanced surface area of nanoparticles because of the phenolic compounds present in the plant extracts which are absorbed on the surface, scavenge the free radicals (DPPH), and exhibit excellent antioxidant potential i-e maximum percent absorption at 1000µg/mL comparable to that of ascorbic acid (positive control). Some other researchers reported the antioxidant potential of AgNPs (Mittal *et al*. (2012); Abdel-Aziz *et al.* 2014; Kharat *et al.* 2016). Their results corresponded to our findings to a great extent.

5.0 Conclusion

Green synthesized AgNPs were confirmed and characterized by UV-vis spectroscopy, XRD and FSEM . Plant-mediated silver nanoparticles exhibited excellent antibacterial and antioxidant potential than the extract alone. The bactericidal potential of the plant-mediated AgNPs could be a source of innovation for pharmaceutical industries in the exploitation of alternative drugs.

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Conflict of Interest

The authors declare no conflict of interest regarding the data presented in the manuscript.

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