ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt
International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

Biosynthesis of Silver Nanoparticles Using *Ganoderma Lucidum* and Assessment of Antioxidant and Antibacterial Activity

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Abstract

There is an increasing interest these days in the green route of synthesis of metal nanoparticles using plant extract, fungus and bacterial- mediated eco-friendly materials. Silver nanoparticles were synthesized using an intracellular extract of *Ganoderma lucidum*, a mushroom from Nepal then characterization of silver nanoparticles was performed. The Ag-NPs thus formed show surface plasmonic resonance with a maximum absorption band at 420 nm. Their crystalline nature was confirmed as a face-centered cubic structure by the XRD, Furthermore, SEM revealed that they were in the size range of 10-30 nm and were spherical in shape. The possible biomolecule involved in the reduction and stabilization of Ag-NPs were believed to be oxidized polyphenol, and carbonyl group, amino acid residue. Ag-NPs exhibit good antioxidant activity but showed low antioxidant in comparison to the fungal extract alone, which was studied using DPPH antioxidant assay. The effect of the colloidal silver nanoparticles solution against six human pathological bacteria was carried out by Disc diffusion method. The zone of maximum inhibition was seen in *Bacillus subtilis* (17.0 ± 0.13 mm) and least effective against *Escherichia coli* (10.1 ± 0.2 mm). Further, the results showed that Ag-NPs in combination with antibiotics have better antibacterial effect as compared with Ag-NPs alone. The maximum effect with a 3.2 and 5.3 fold increase was seen in Gentamicin and Streptomycin respectively providing the synergistic role of Ag-NPs. The results of antimicrobial studies indicated that the Ag-NPs are the metal of choice and can be effectively used in combination with antibiotics in order to improve their efficiency against various pathogenic microbes.

Keywords: silver nanoparticles (Ag-NPs); *Ganoderma lucidum*; X-ray diffraction; FTIR-ATR; antimicrobial activity; synergistic effect

Introduction

Nanotechnology is an unprecedented multidisciplinary field merging the physical, chemical, biological and engineering science aspect to understand and manipulate the realm of atoms and molecules. The ideas and concepts behind nanoscience and nanotechnology started with a talk entitled "There's Plenty of Room at the Bottom" by physicist Richard Feynman at an American Physical Society meeting on December 29, 1959 (Feynman, 1960). After a decade, Professor Norio Taniguchi coined the term 'Nanotechnology' in his explorations of ultra-precision machining (Taniguchi, 1974). Due to the unique

Article may be cited as:

M. Poudel et al. (2017) Int. J. Appl. Sci. Biotechnol. Vol 5(4): 523-531. DOI: 10.3126/ijasbt.v5i4.18776

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Peer reviewed under authority of IJASBT

physicochemical characteristics of nanoparticles including optical, electronic, magnetic, catalytic and antibacterial properties, the Nobel method of synthesis has come to be in the interest of scientist (Natsuki *et al.*, 2015).

It holds a huge promise for the design and development of many types of novel products for its potential medical applications for early disease detection, treatment, and prevention (Marambio-Jones and Hoek, 2010). Besides, metal nanoparticles can be modified with various chemical functional groups which allow them to conjugate with antibodies, ligands, and drugs of interest (Patri *et al.*, 2005). Among the noble metals, silver has been used since the ancient time for burn wound treatment, dental work, catheters and bacterial infection control, in forms of metallic silver, silver nitrate, and silver sulfadiazine (Tran *et al.*, 2013). Due to unique properties, silver nanoparticles have shown effective antimicrobial activities against pathogenic bacteria and viruses (Karwa *et al.*, 2011) so, it has been used in many biomedical devices, wound dressing, and antimicrobial coating as it releases continuously low amount of silver ions that provide protection against pathogens (Yang and Li, 2013). In present, for the production of monodisperse nanoparticles, physical and chemical methods are used though nanoparticles produced by these methods are less stable and give toxic chemical as a byproduct (Ghorbani *et al.*, 2011). The use of toxic chemicals and nonpolar solvents in synthesis leads to the inability to use nanoparticles in clinical fields (Reidy *et al.*, 2013). Therefore, the non-toxic and eco-friendly method for synthesis of nanoparticles is the need of the present world. Thus, there is an increasing demand for 'green nanotechnology' (Iravani *et al.*, 2014). Despite the fact that biological synthesis of nanoparticles is considered as safe, eco-friendly and reasonable, it has different drawbacks as well. The particles are not monodisperse and the rate of production is slow but optimization of factors involved like p^H, temperature, metal ion concentration in a rightful amount provide large-scale application of biological synthesis (Verma and Mehata, 2015).

Many biological entities such as microorganisms (like bacteria, fungi, etc.), biochemical components (like enzyme, polysaccharide etc.) and plants are being used for the biological synthesis of nanoparticles (Mukherjee *et al.*, 2001; Singh *et al.*, 2016). However, the use of microorganisms especially the fungi is potentially exciting since they secrete plenty of enzymes and are also easy to handle. These properties of fungi have made them an ideal biological candidate for the synthesis of various metal nanoparticles (Dhillon *et al.*, 2012). Comparatively, the higher fungi such as mushrooms in this regard have not received the much attention. Mushrooms are the fleshy fruiting bodies of the Basidiomycetes fungi, typically found above ground in soil, rotten woods or trees. *Ganoderma* has been reported to present throughout the world and several reports have been made of their distinctive biological properties such as antibacterial, antioxidant, anti-inflammatory, antiproliferative, anticancer, antitumor, cytotoxic, anti-HIV, antidiabetic, hepatoprotective and others (Kao *et al.*, 2013).

Therefore, in the present study, the *Ganoderma lucidum* an important medicinal mushroom has been explored for the synthesis of silver nanoparticles (Gurunathan *et al.*, 2013; Kannan *et al.*, 2014). These fungi constitute a very favorable object of Nano-biotechnological studies, as they can accumulate large amounts of reduced nanoparticles (Arun *et al.*, 2014). Hence, biosynthesis of Ag-NPs by medicinal mushrooms i.e. the *Ganoderma lucidum* will be more safe and eco-friendly. Here, we have demonstrated the biosynthesis of Ag-NPs using the wild medicinal mushroom *Ganoderma lucidum* and studied its antimicrobial potential against human pathogens and antioxidant properties. The nanoparticles being synthesized were characterized, evaluated for their antioxidant and antimicrobial activities.

Materials and Methods

Materials

Silver nitrate ($AgNO_3$) was purchased from Fisher Scientific Pvt. Ltd, India. The six bacterial cultures were obtained from Department of Microbiology, NIST College. Nutrient agar, Mueller-Hinton (MH) agar and antibiotics disc for the antibiotics susceptibility test were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India.

Collection of Sample

Medicinal wild mushroom *Ganoderma lucidum* was collected from Chandragiri hill near Kathmandu valley in November 2016. The identification of sample was confirmed by senior mycologist Pof. Dr. Mahesh Kumar Adhikari.

Fungal Extract Preparation

After collection of the sample, a fruiting body was washed several times with deionized water and dried at 40ºC in the oven for 3 days. The dried sample was grounded into powder form using mortar and pestle. 5 gram of powdered sample was extracted using water (200 ml) via Soxhlet extractor at 80ºC for 8 h. Thus obtained extract was filtered through Whatman No: 1 filter paper and then concentrated to 100 ml under 60ºC using rotary evaporator. The extract was stored at 4ºC in the refrigerator until further use.

Synthesis of Silver Nanoparticles

For the synthesis of silver nanoparticles, 10 ml of mushroom extract was added into 150 ml of a conical flask containing 90 ml of 1 mM silver nitrate solution and incubated at 60ºC in dark, also the stirring of the reaction solution was done in a different time interval. The consequent reduction of silver ions (Ag^+) was monitored periodically for 24 h. After 4 hours of incubation, the color of the reaction mixture changed from light yellow to pale

yellow color, further the color was changed into dark brown indicating the formation of Ag-NPs (Gurunathan *et al.*, 2013).

Purification of Silver Nanoparticles

The Ag-NPs formed were collected by centrifugation at 10,000 rpm for 30 min at 4ºC. The clear supernatant was discarded and the pellet of colloidal silver was washed three times with double distilled water to remove impurities and the unbound extract components. Finally, Ag-NPs were dried at 60ºC in the hot air oven and were used for further characterization.

Characterization of Silver Nanoparticles

UV-VIS spectroscopy

Ultraviolet spectra have been proved to be quite sensitive to the emergence of silver colloids because silver nanoparticles exhibit an intense absorption peak due to Surface Plasmon excitation. Synthesized silver nanoparticles were confirmed by sampling the reaction mixture at regular interval of time (6 h, 12 h, 18 h and 24 h) and the absorption spectra were scanned at the wavelength of 250-600 nm.

X-ray diffraction analysis (XRD)

The dried powdered of silver nanoparticle was used to determine crystalline structure of silver and the XRD was done at NAST, Khumaltar, Nepal using D2 phazer machine of Bruker Company with Cu-Kα (λ=1.54056 Å) radiation. The diffraction pattern was recorded from diffraction angle range of 20º to 80º at a 2θ pattern. The image obtained was compared with the standard JCPDS data (file no: 87-0717). The average crystalline size was calculated from the width of the XRD peaks by assuming that they are free from non-uniform strains (Monshi *et al.*, 2012) using the Scherrer formula:

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

Where,

λ is the X-ray wavelength in nanometer (nm)
β is the peak width of the diffraction peak profile at half maximum height resulting from small crystallite size in radians
θ is the angle of incidence of X-ray and
K is a constant related to crystallite shape normally taken as 0.9.

Scanning Electron Microscopy (SEM)

The surface morphology and structural analysis were studied by U-4800, Hitachi Co. Ltd operating at 10 kV in National Institute of Materials Science, Japan. The scanning was done up to 100 nm.

FTIR Spectroscopy

Fourier transformed IR spectroscopy (FTIR-ATR) was done in Department of Plant Resource (DPR), Thapathali, Kathmandu. Scans in the range of 250–5000 cm^{-1} were collected for each spectrum at a spectral resolution of 4 cm^{-1}.

DPPH Radical Scavenging Assay

The capability of Ag-NPs for scavenging DPPH (2, 2-diphenyl-1picrylhydrazyl) radical was evaluated by the method described by (Brand-Williams *et al.*, 1995) with slight modification. 4 mg of DPPH was dissolved in 100 ml of methanol to prepare 0.1 mM DPPH solution. A series of dilution of Ag-NPs and *Ganoderma* extract was formulated in distilled water to make the different concentration of 10, 20, 40, 60, 80, 100 µg/ml. To 1 ml of sample 2 ml of DPPH was added and incubated in the dark for 30 minutes. The absorbance at 517 was measured against a blank (methanol), where ascorbic acid was used as the standard. The decrease in absorbance of the reaction mixture indicated a higher free radical scavenging activity. The scavenging activity of the sample was expressed as the percentage and calculated using formula as:

$$\text{Percentage of inhibition (\%)} = (A_c - A_o)/Ac \times 100$$

Where,
A_c is the absorbance of the DPPH solution without sample
A_o is the absorbance of the test sample (DPPH + sample)

Antibacterial Assay

Antibacterial activity of the extracellular silver nanoparticles, synthesized by *Ganoderma lucidum* was tested by disc diffusion method. Bacteria including *Klebsiella pneumoniae, Escherichia coli, salmonella typhi, Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* were used as hosts to test the antimicrobial activity. For the preparation of disc, Whatman filter paper (No:1) was used to prepare four discs approximately of 6 mm in diameter, which were placed in hot air oven at 180ºC for sterilization.

Pure cultures of bacterial pathogens were subcultured in the nutrient broth for overnight and turbidity of the culture was maintained by comparing with 0.5% McFarland standard. Overnight cultures of each bacterial strain were swabbed uniformly onto individual plates using sterile cotton swabs then each sterilized discs were loaded with different test samples in each cultured agar plates. After incubation at 37ºC for 24 h, the diameter of the zone of inhibition which appeared as a clear area around the disk was measured using Vernier caliper.

Two set of antimicrobial experiments were performed:

Effect of Silver Nanoparticles and Other Chemicals

To observe the effect of different test solutions on the microbial strain, sterile paper disks were placed on the agar plates of microbial strains and 20 µl of 200 µg/ml (w/v) samples were applied to the disks. Gentamicin (10 µg per disk) was used as the positive control. All the plates were incubated at 37ºC for 18–24 h. The zone of inhibition,

which appeared as a clear area around the disks, was measured after 24 h.

Synergistic Effect of Silver Nanoparticles

To study the synergistic effect of Ag-NPs with antibiotic (Gentamicin and streptomycin) against the microbial strain, a disc with 20 µl of 100 µg/ml Ag-NPs and Ag-NPs incorporated with freshly prepared 100 µg/ml of gentamicin and streptomycin were placed on the agar plate of microbial strains. After incubation for 24 h at 37ºC, the diameter of the zone of inhibition on the microbial lawn was measured.

Results and Discussions

Visual Analysis

The formation of silver nanoparticles in the reaction mixture was visually identified by the change in color. After 4 hours of incubation, the color of the reaction mixture changed from light yellow to pale yellow color, in the end of reduction process dark brown color was observed (Fig. 1). No change in color was observed in case of silver nitrate solution.

Fig. 1: Color change produced in the silver nitrate solution on addition of Ganoderma extract.

UV-VIS Spectra Analysis

After addition of *Ganoderma* extract to silver nitrate solution, a UV-VIS scan was taken from 250 to 600 nm at different time interval. The fixed ratio of extract to metal ion solution led the change of color from pale yellow to dark brown due to the formation of silver nanoparticles. This change in color is due to the Surface Plasmon Resonance phenomenon.

The sharp bands of silver nanoparticles were observed around 420 nm (Fig. 2). The band around 420 nm suggests that the particles were well dispersed without aggregation (Zhang *et al.*, 2016). Further, we examined the synthesis of silver nanoparticles with respect to increase in time and the results suggest that with an increase in the reaction time, the intensity of its peak increases due to increase in the concentration of nanoparticles.

XRD Analysis

X-ray diffraction (XRD) was carried out to confirm the crystalline nature of particles synthesized using the fungal extract of *Ganoderma lucidum* and the XRD pattern obtained. Braggs reflection indicating four diffraction peaks at the 2θ value of 38.11, 44.30, 64.45 and 77.40 were observed corresponding to (111), (200), (220), (311) planes respectively, for silver. By comparing the XRD pattern of synthesized Ag-NPs with the joint committee on powder diffraction standard (JCPDS) file no: 87-0717 (Fig. 3) the Ag-NPs synthesized were indexed as face-centered cubic (FCC) structure of silver.

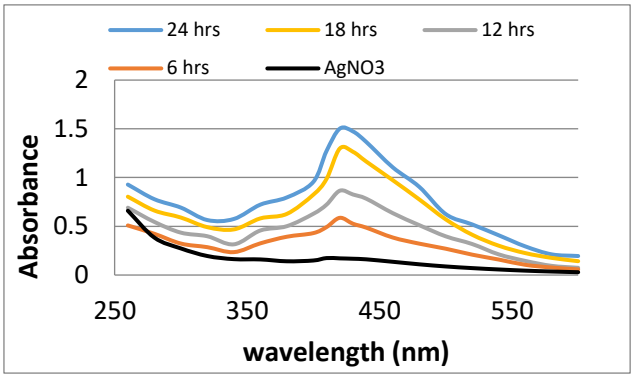

Fig. 2: UV-VIS spectra of silver nanoparticles recorded at the function of time.

The peak corresponding to (111) plane is more intense than that of other planes indicating prevailing growth of silver nanoparticles along (111) direction. In addition, unassigned peaks (*) were observed. These peaks are due to the organic compound present in the surface of Ag-NPs which are present in the extract as amorphous form and some impurities present in the sample (Philip, 2009).

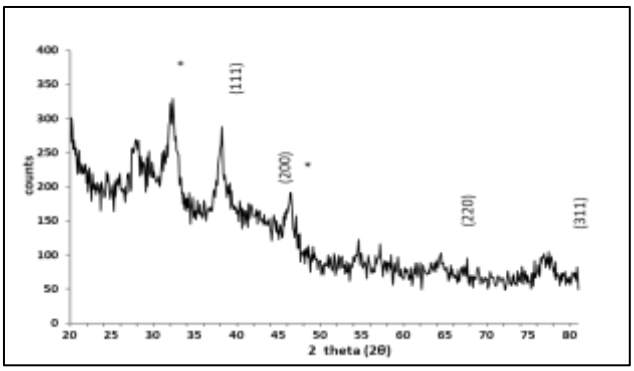

Fig. 3: XRD pattern of silver nanoparticles synthesized from the fruiting body of Ganoderma lucidum.

The average crystalline size of the silver nanoparticles was estimated using the Debye Scherrer equation.

SEM Analysis

The size and shape of Ag-NPs deduced through scanning electron micrograph at various magnifications ranging 300 nm to 100 nm. Nanostructure and surface morphological studies of SEM images of silver nanoparticles show highly aggregated crystalline spherical SNPs of varied particulate size about 10-30 nm (Fig. 4). Our result has good resemblance with (Nazeruddin *et al.*, 2014) result.

The estimated average size of crystalline nanoparticle is 24.12 nm.

Table 1: XRD particle size evaluation of Ag-NPs using scherrer equation

SN	2θ	θ	hkl	FWHM	$\beta = {}^{\pi}/_{180} \times FWHM$	Particle size (D) (nm)
1.	38.11	19.05	111	0.809	0.0041	10.39
2.	44.30	22.15	200	0.303	0.0052	28.78
3.	64.45	32.22	220	0.304	0.0053	31.51
4.	77.40	38.70	311	0.394	0.0048	25.82

FWHM full width at half maximum

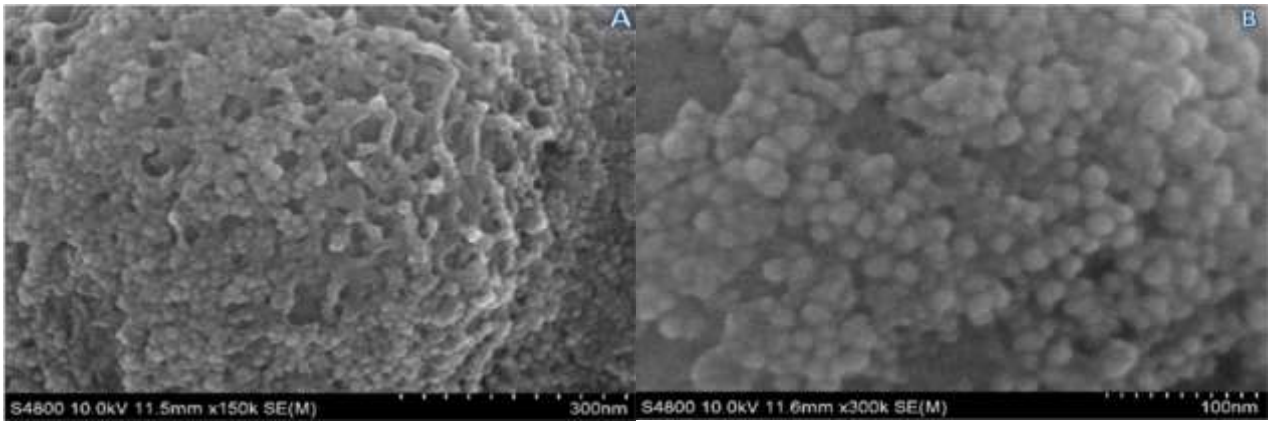

Fig. 4: SEM image of Silver nanoparticles at (A) 300 nm (B) 100 nm scale.

FTIR Analysis

FTIR analysis was carried out to identify possible biomolecules present in the extract which are responsible for the reduction of silver ion and stabilization of the bio reduced Ag-NPs which are shown in Fig 5a. The ATR-FTIR spectrum of aqueous extract of *Ganoderma* showed a prominent peak at 3263.56 cm^{-1}, 1635.64 cm^{-1} and 362.62 cm^{-1} (Fig. 5a). The broadband at 3263.56 cm^{-1} corresponds to -OH stretching H-bonded alcohol and phenolic compound and the band at 1635.64 cm^{-1} represents carbonyl N-H stretch of primary amine due to vibration in amide linkage of the protein (Bunghez *et al.*, 2011). The ATR-FTIR spectrum of Myco-synthesized silver nanoparticles shows a band at 3278.99 cm^{-1}, 1635.64 cm^{-1} and 354.9 cm^{-1} (Fig. 5b) the band at 3278.99 cm^{-1} corresponds to -OH stretching alcohol and phenolic compound and the band at 1635.64 cm^{-1} represents the stretch mode of carbonyl group coupled to the amide linkage (Kharat and Mendhulkar, 2016).

Therefore, the synthesized nanoparticles were surrounded by proteins and metabolites like terpenoids containing hydroxyl functional groups. From the analysis of FTIR studies, we can confirm that the proteins and carbonyl groups from the amino acid residues have the stronger ability to bind metal indicating that the proteins could possibly act as a capping agent and prevent agglomeration, thereby stabilize the nanoparticles (Niraimathi *et al.*, 2013). This suggests that the biological molecules could possibly accomplish dual functions of formation and stabilization of silver nanoparticles in the aqueous medium.

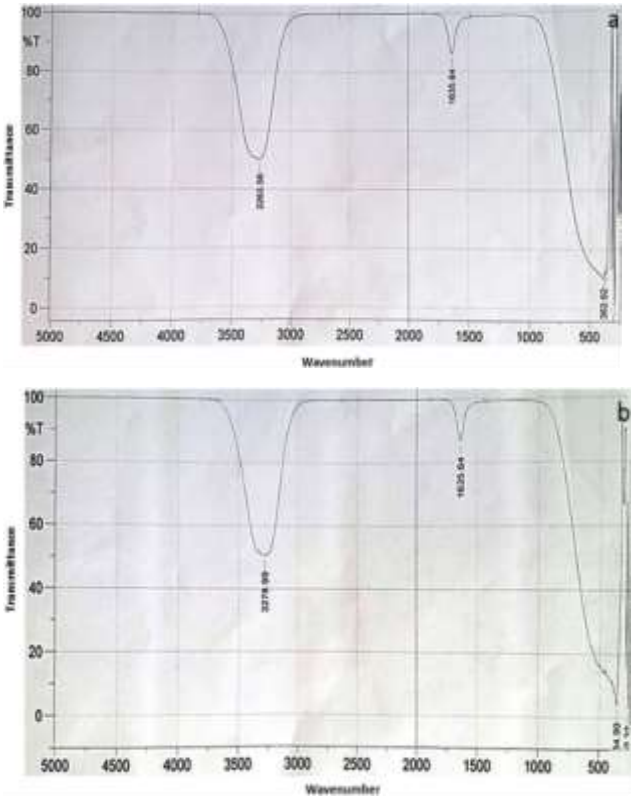

Fig. 5: FTIR spectra of Ganoderma extract (a) and silver nanoparticles (b) synthesized at 1mM concentration.

Antioxidant Analysis

The antioxidant activity of the *Ganoderma* extract and Ag-NPs was assessed using DPPH scavenging assay and compared with that of ascorbic acid. (Fig. 6) shows the

dose-dependent increase in the inhibition percentage of extract and synthesized Ag-NPs at 10, 20, 40, 60, 80, 100 µg/ml concentration. As the concentration of extract and Ag-NPs increases, the percentage of inhibition was found to be increased. Fungal extract exhibit higher activity at 100 µg/ml with 82.59% likewise lower activity observed at 10 µg/ml with 57% followed by silver nanoparticles showing maximum with 73.49% and minimum with 55.34% at 100 and 10 µg/ml respectively. In comparison to extract, Ag-NPs have shown less percentage of inhibition this may be due to the presence of less amount of functional group adhered to them. The FTIR result has suggested that hydroxyl and amine group were involved in synthesis and stabilization of silver nanoparticles thus the antioxidant property shown by silver nanoparticles is due to these functional groups (Bhakya *et al.*, 2015).

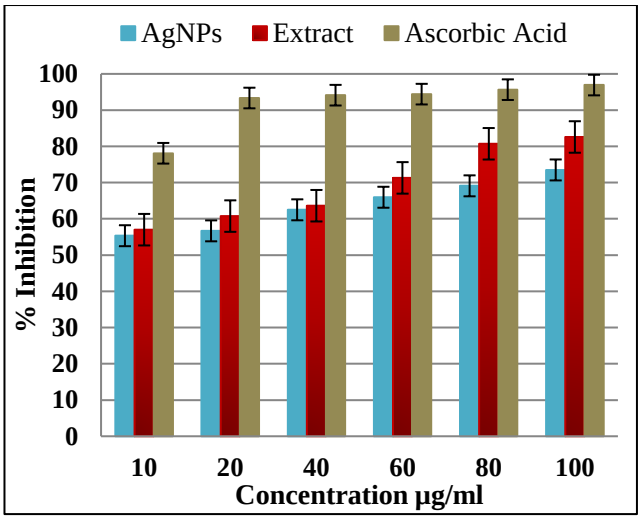

Fig. 6: DPPH free radical scavenging activity of Ganoderma extract, silver nanoparticles, ascorbic acid at 517 nm. Data represent the mean ± S.E of triplicate samples.

Antibacterial Activity

The antimicrobial activity of the colloidal solution of silver was compared to that of silver nitrate, *Ganoderma* extract and Gentamicin (positive control) after incubation for 24 h, Antimicrobial activities shown by different test solutions is shown in (Fig. 7). Synthesized silver nanoparticles have shown superior antimicrobial activity against all the tested human pathogens among which exhibited potent inhibitory activity against the pathogenic bacterium *Bacillus subtilis* with a clear inhibition zone of 17.0 ± 0.13 mm at the concentration 200 µg/ml. Similarly, silver nanoparticles significantly inhibited the growth of *S. typhi, K. pneumoniae, S. aureus* and *B. cereus* having to a zone of inhibition 12.2 ± 0.04 mm, 12.1 ± 0.16 mm, 13.9 ± 0.18 mm and 13.1 ± 0.06 mm respectively. Lowest antimicrobial activity was seen against *E. coli* with 10.1 ± 0.20 mm. Along with silver nitrate has shown high antimicrobial activity against *E. coli* and *S. typhi* having 7.3 ± 0.99 mm, 7.6 ± 0.80 mm ZOI respectively, lowest was seen against *K. pneumonia* with ZOI 6.4 ± 0.23 mm. The *Ganoderma* extract did not show any zone of inhibition in comparison to others which is presented as 6 mm. There was a slight difference in the susceptibility of gram-negative and gram-positive to silver nanoparticles. It is also possible that Ag-NPs not only interact with the surface of the membrane but can also penetrate the thick wall of Gram-positive bacteria (Morones *et al.*, 2005). This ensures that significantly large surface area of the particle is in contact with the bacterial cells surface which is expected to enhance the extent of bacterial exclusion (Seil and Webster, 2012).

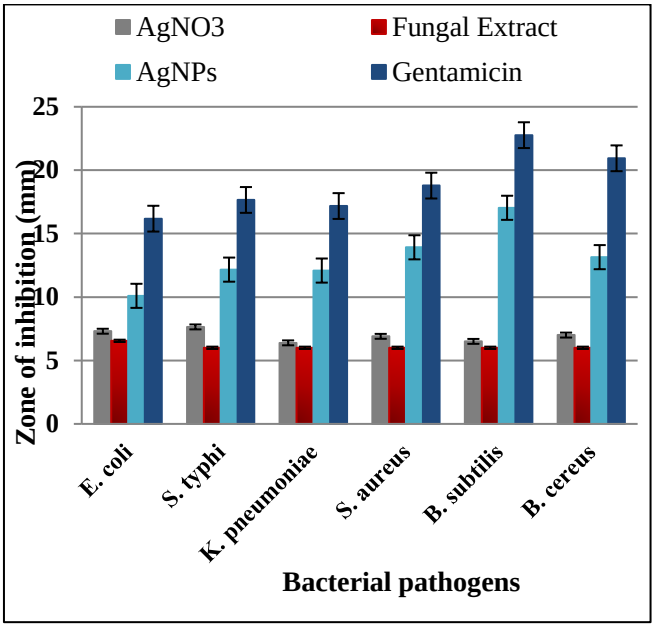

Fig. 7: Antibacterial activity of silver nanoparticles and other chemicals. Data represent mean ± S.E of triplicate samples from three identical experiments.

The effect of Ag-NPs alone and associated with the antibiotic Gentamicin and Streptomycin were investigated against pathogenic bacteria. The most effective antibiotic in combination with Ag-NPs was streptomycin (Fig. 8). It was observed that effect of antibiotics has increased in most of the case. Synergistic interaction of Ag-NPs with Gentamicin and Streptomycin has shown a minute increase in the inhibition zone against five pathogenic bacteria in the range 0.9 to 1.8 mm and 1.7 to 2.3 mm respectively with the exception of *Bacillus subtilis* where a 3.2 and 5.3 fold increase was seen in Gentamicin and Streptomycin respectively. The synergistic effect may be caused by a bonding reaction between antibiotics and Nanosilver. In synergism, the bactericidal effect is enhanced by the interaction between active groups like hydroxyl and amino groups present in these antibiotics with Ag-NPs by chelation. As a result, antibiotics–Ag-NPs conjugate is formed in which an Ag-NPs core is surrounded by antibiotic molecules and antibiotics molecules themselves can bind each other through van der Waals interaction and other weak bonds (Batarseh, 2004).

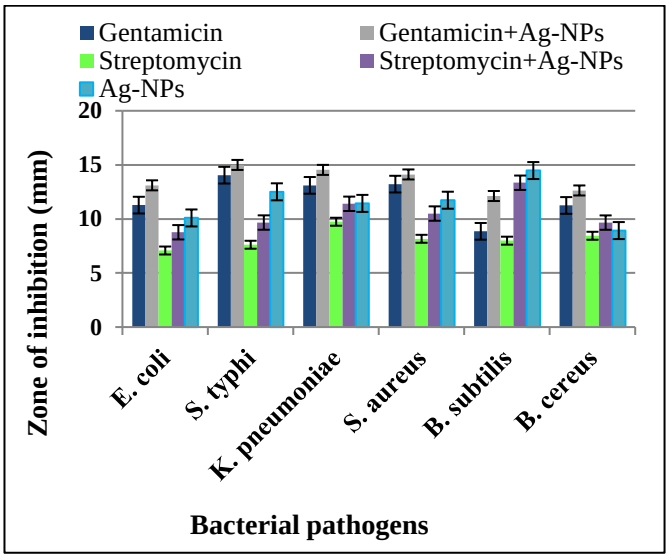

Fig. 8: Antibacterial activity of Ag-NPs alone and in combination with antibiotics. Data represent the mean ± S.E of triplicate samples from three identical experiments.

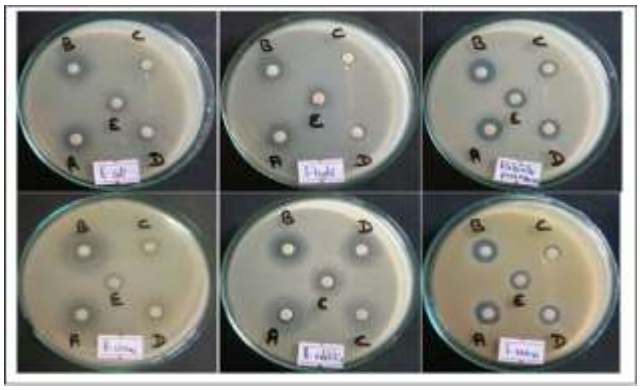

Fig. 9: Antibacterial activity of Ag-NPs alone and in combination with antibiotics; Gentamicin (A), Gentamicin + Ag-NPs (B), Streptomycin (C), Streptomycin + Ag-NPs (D).

Thus, the antimicrobial concentration is increased at the crucial site, which leads to increased destruction of bacteria. Thus, the process of antimicrobial group forming is actually that of increasing the antimicrobial agent concentration (Singh *et al.*, 2013). A more probable cause of the synergistic effect may be due to drug delivery of Ag-NPs to the cell. To our knowledge, cell membrane consists of phospholipids and glycoprotein, which are all hydrophobic groups. It is well-known that cell membrane consists of phospholipids and glycoprotein which are hydrophobic groups, Gentamicin and Streptomycin are hydrophilic but Ag-NPs are hydrophobic. Thereby, Ag-NPs, unlike antibiotics, can easily pass cellular membrane. It can be concluded that silver nanoparticles bonded antibiotics can be easily delivered to the cell (Hwang *et al.*, 2012).

Conclusion

There is an increasing trend of eco-friendly approaches of silver nanoparticles synthesis and likewise, we synthesized silver nanoparticles using fungus Ganoderma lucidum. The nanoparticle that we synthesized is Face-centered cubic crystalline, spherical shape and was characterized by existing instrumentation techniques. Extract of Ganoderma lucidum was found to contain phenolic, flavonoid and polysaccharides compound that seems to play a critical role in synthesis as well as stabilization of silver nanoparticles. Furthermore, we investigated the antioxidant and antibacterial activities of these silver nanoparticles and that have shown remarkable results. It is concluded that synthesized Ag-NPs have antioxidant activity due to capped phenolic and flavonoid compounds. Also, the synthesized Ag-NPs have shown potential bactericidal activity against human pathogenic bacteria. Synthesized Ag-NPs exhibited slightly equivalent or kin antibacterial activity as compared to gentamicin and streptomycin. These results suggest that in near future, silver nanoparticles can be selected as a potential antibacterial agent.

Acknowledgements

Authors are thankful to the Department of Biotechnology at SANN International College, Kathmandu for providing fund and allowing the use of laboratory facilities which were required for the completion of this research.

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