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Original Article

Synthesis of Silver Nanoparticles Stabilized With Phytochemicals and its Application towards *Invitro* Antioxidant and Antibacterial Activities

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Abstract:
Medicinal plants are widely used by the Indian population since it has no harmful side effects and low cost compared to other treatments. In the 21st century, nanotechnology field is expected to be the base for all the important technological innovations. From that, green synthesis of silver nanoparticle is gaining more momentum due to its commercial demand besides; it plays a significant role in the medical field and biomedical applications. In the present study silver nanoparticles were synthesized from *Kigelia africana* leaf extract (KAL) and characterised by UV-visible, XRD, FESEM, HRTEM and crystalline structure was studied by SAED. The green synthesized Ka Ag NPs considerably inhibited the growth of human pathogenic both Gram-positive *Staphylococcus aureus* (23 mm) and Gram-negative bacteria *Escherichia coli* (19 mm).

Key Words:*Kigelia africana*, Phytochemicals, Ag NPs, Antibacterial, HRTEM.

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1. INTRODUCTION

Nanotechnology is a most promising field to generate new applications in several fields includingbiotechnology and nanomedicine and the nanomaterials could be used widely due to their fascinating applications in many fields[1,2]. Nanoparticles enhanced reactivity, strength and electrical characteristics[3]. Recently, biosynthesis of nanoparticles received considerable attention due to its unique physicochemical characteristics, electronic, magnetic, optical, catalytic properties[4] as well as antibacterial[5],antioxidant and antiproliferativeproperties [6]. Plant mediated synthesis of metal nanoparticles are cost-effective, environmental amicable, safe to handle and offers broad range of biomolecules which was kenned to mediate synthesis of nanoparticles [7]. Recently, a number of reports were reported regarding biosynthesis of silver nanoparticles using plant extracts as a reducing agent[8], [9], [10], [11], [12], [13] and [14].

AgNPs synthesised from leaf extract of *Iresine herbstii* exhibited, higher free radical scavenging ability, total antioxidant activity and reducing potential than the ethanolic leaf extract [15]. Green synthesis of AgNPs depends on rudimental requisites of solvent medium, reducing agent and non-hazardous stabilizing agent[16].*Kigelia africana* (Lam), belongs to the family Bignoniaceae. It is widespread across India, Africa, Ghana, Sierra Leone, Gambia, Sudan and Nigeria [17][18], have reported sexual complaints about infertility, poor libido, sexual asthenia and impotence were treated with medicines containing the fruits, roots or leaves of *K. africana*. The present study mainly focused in synthesis of silver

nanoparticles by green chemistry approach and to evaluate its antibacterial and antiproliferative activity by *in vitro* approach

2. MATERIALS AND METHODS

2.1 Materials

Silver nitrate ($AgNO_3$) from Merck (Germany), Nutrient agar were purchased from Hi Media (Mumbai, India), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), methanol, hydrogen peroxide, sulfanilamide, sodium nitroprusside, phosphate buffer, phenazine methosulphate (PMS),Nitroblue tetrazolium (NBT).

2.2 Methods

2.2.1 Preparation of K. africana leaf broth

Fresh leaves of *K. africana* were accumulated and washed exhaustively in tap water for 30 minutes in order to abstract the dust particles. Finely cut leaves were placed in 500 mL Erlenmeyer flask along with 100 mL of distilled water and then boiled the coalescence at 60 °C for 10 minutes. After boiling, extract was filtered through Whatmann filter paper and stored at 4 °C for further experiments.

2.2.2 Preliminary screening of phytochemicals

Screening of phytochemicals werecarried out usingstandard procedures [22].

2.2.3 Test for alkaloids

To 2 mL of leaf extract, 2 mL of conc. hydrochloric acid was added, to this few drops of Mayer's reagent was added. Formation of green colour or white precipitate indicated the presence of alkaloids.

2.2.4 Test for anthraquinones

To 1 mL of leaf extract few drops of 10% ammonia solution was added, appearance of pink colour precipitation indicated the presence of anthraquinones.

2.2.5 Test for carbohydrates

To 2 mL of extract, 1 mL of Molisch's reagent and few drops of conc. sulphuric acid was added. Formation of purple or reddish colour indicated the presence of carbohydrates.

2.2.6 Test for flavonoids

To 2 mL of leaf extract, 1 mL of 2 N sodium hydroxide was added. Formation of yellow colour indicated the presence of flavonoids.

2.2.7 Test for glycosides

To 2 mL of leaf extract, 3 mL of chloroform and 10% ammonia solution was added. Formation of pink colour indicated the presence of glycosides.

2.2.8 Test for phenols

To 1 mL of the leaf extract, 2 mL of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green colour indicated the presence of phenols.

2.2.9 Test for quinones

To 1 mL of leaf extract, 1 mL of conc. sulphuric acid was added. Formation of red colour indicated the presence of quinones.

2.2.10 Test for saponins

To 2 mL of leaf extract, 2 mL of distilled water was added and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm foam layer indicated the presence of saponins.

2.2.11 Test for tannins

To 1 mL of leaf extract, 2 mL of 5% ferric chloride was added. Formation of dark blue or greenish black indicated the presence of tannins.

2.2.12 Test for triterpenoids

To 1.5 mL of leaf extract, 1 mL of Libermann-Buchard reagent (acetic anhydride + conc. sulphuric acid) was added. Formation of blue green colour indicated the presence of triterpenoids.

2.2.13 Test for steroids and phytosteroids

To 1 mL of leaf extract equal volume of chloroform was added along with few drops of conc. sulphuric acid, appearance of brown ring indicated the presence of steroids and bluish brown ring formation indicated the presence of phytosteroids.

2.3 Biosynthesis of silver nanoparticles

The aqueous leaf extract of *K. africana* was used for the bio-reduction process. To synthesize nanoparticles 2, 5, 10 and 15 mL of aqueous leaf extract were carefully added to 98, 95, 90 and 85 mL of 1 mM aqueous $AgNO_3$ solution in 250 mL Erlenmeyer flasks. The flasks containing extract were incubated in a shaker at 150 *g*in dark conditions. The bio-reduction of the silver ions in the solution was monitored periodically by measuring the UV–vis spectroscopy of the solutions at (200–800 nm).Silver nanoparticles obtained from the solution were purified by repeated centrifugation at 12,000*g* for 20 minutes followed by dispersion of the pellet in deionized water three times to remove the water soluble biomolecules such as proteins and secondary metabolites [19].

2.4 Characterization of silver nanoparticles

The UV-visible analysis was performed in the absorption wavelength of 200 – 800 nm. The synthesized silver nanoparticles were studied using FTIR (Perkin-Elmer GX FT-IR spectrometer). Electron Microscopy was used to observe the size, shape and morphology of the synthesized nanoparticles. After freeze drying, the structure and composition were studied by SEM (scanning electron microscopy), EDAX (energy-dispersive X-ray spectroscopy) and XRD (X-ray diffraction). The synthesized AgNPs were also characterized using High Resolution Transmission Electron Microscope (HRTEM). Samples for HRTEM observation were prepared by casting a drop of the silver nanoparticles on a carbon coated copper grid and the excess solution was removed by tissue paper and allowed to air dry at room temperature for overnight.

2.5 In vitro antioxidant activities

3. RESULTS

2.5.1 Free radical scavenging assay using DPPH

The ability of AgNPs and aqueous extracts to scavenge the free radical was investigated[20]. Briefly, 0.1 mM solution of DPPH in methanol was yare. Initial absorbance of DPPH in methanol was quantified at 517 nm and did not transmute throughout the period of assay. An aliquot (20 – 100 µL) of samples was integrated to 3 mL of methanol DPPH solution. Methanol alone accommodated as blank and DPPH in methanol without the plant extracts served as positive control. After 30 minutes, discolouration of the purple colour was measured at 517 nm and percent radical scavenging activity was calculated as follows:

$$FRSA = [(A_c - A_s) / A_c] \times 100$$

Where A_c= absorbance of control;A_s = absorbance of test sample after 30 minutes.

2.5.2 Hydrogen peroxide radical scavenging (H_2O_2) assay

Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (50 mM, pH 7.4) and the concentration of hydrogen peroxide was determined by spectrophotometer absorption at 230 nm. Different concentrations of samples (20 – 100 µg/mL) were added to hydrogen peroxide solution and the absorbance at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide[21]. The percentage of hydrogen peroxide scavenging was calculated as follows:

$$H_2O_2 \text{scavenging } (\%) = (A_o - A_1/A_o) \times 100$$

Where A_o= absorbance of control;A_1 = absorbance of test sample

2.5.3 Superoxide radical scavenging activity

The effect of *K. africana* leaf extract and synthesized AgNPs on superoxide generated a non-enzymic system was measured spectrophotometer[22]. The reaction mixture (20–

100 µg/mL) was added with 1 mL of 60 µM phenazine methosulphate (PMS) and 1 mL of 150 µM nitroblue tetrazolium (NBT). The above mixture was incubated at ambient temperature for 5 minutes and discolouration was read by spectrophotometer at 560 nm.Ascorbic acid was used as a positive control.

Superoxidescavenging (%) = $(A_o - A_1/A_o) \times 100$

2.5.4Nitric oxide radical scavenging assay

Sodium nitroprusside 0.1 mL (10 mM), 0.2 mL of phosphate buffer (0.2 M, pH 7.8) was mixed with 0.5 mL of different concentrations of aqueous extract, AgNPs and standard (BHT) and incubated at room temperature for 150 minutes.After incubation, 0.2 mL of Griess reagent (1% Sulphanilamide, 2% phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine hydrochloride) was added and all readings were taken in triplicate. The absorbance was read at 546 nm. The percent inhibition was calculated by following equation[21].

Nitric oxide scavenging (%) = $(A_{control} - A_{sample})/A_{control} \times 100$

2.6In vitro antibacterial activity

The antibacterial activity of green synthesized AgNPs was tested against six human pathogens by Agar well diffusion method [23].The bacterial cultures such as *Bacillus subtilis* (6633), *Escherichia coli* (ATCC 25922), *Micrococcus luteus* (10240), *Salmonella typhimurium* (13111),*Pseudomonas aeruginosa* (ATCC 27583) and *Staphylococcus aureus* (ATCC 25923) were maintained in Nutrient Agar (NA) slants and used prior to assay.Nutrient agar plates were inoculated with 100 µL of standardized bacterial culture (1.5×10^6 CFU/mL) and spread with sterile swabs. Wells of 6 mm size are made in the agar using cork borer. Different concentrations of synthesized AgNPs (25, 50, 75 and 100 µg/mL)was poured into the wells. The plates thus prepared were left at room temperature for 10 minutes for diffusion of samples into the agar plates. After incubation for 24 hours at 37 ºC, the zone of inhibition was measured and expressed in millimetres

3.1 Screening for phytochemicals of K. africana

Phytochemical screening was carried out with the aqueous extract and the results were summarised (Table. 1). Preliminary screening revealed the presence ofaromatic acids, alkaloid, cardiac glycosides, flavonoids, phenols, steroids, tannins, terpenoids and triterpenoids.

Table 1: Screening of aqueous extract of *Kigelia africana* for phytochemicals

S. No	Name of Phytochemicals	Inference
1	Alkaloids	++
2	Carbohydrates	++
3	Flavonoids	+++
4	Glycosides	+
5	Phenols	++
6	Quinones	+
7	Saponin	++
8	Steroids	++
9	Tannins	+++
10	Triterpenoids	++

+++ - Strongly Positive, ++ - Positive + - Trace - - Not detected

3.2 Spectral characterization of nanoparticles

Synthesis of silver nanoparticles wasachieved using aqueous extract of *K. africana*. The aqueous silver nitrate solution turned to brown colour was noted by visual observation in few hours of incubation. The absorption spectrum of *K. africana* leaf extract and AgNPs was read at 200 – 800 nm and surface plasma resonance observed at 452 nm which confirmed the formation of AgNPs (Fig. 1A, B and C).

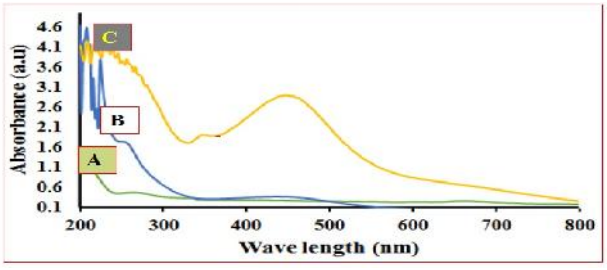

Fig 1. Absorption peak of silver nanoparticles from K. africana under UV–vis absorption spectroscopy: (A) AgNO3, (B) Aqueous extract and (C) Ag NPs

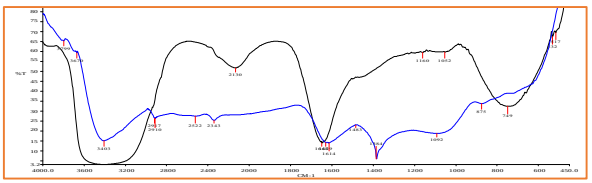

Fig 2. FTIR spectra of (A) Aqueous extract of K. africana (B) synthesized Ag NPs

The major factor liable for reduction of silver ions (Ag^+) into silver nanoparticles (AgNPs) were identified using FTIR spectroscopy (Fig. 2). Biologically synthesized AgNPs were mixed withpotassium bromide to make a pellet. The spectrum of leaf extract alone showed distinct peak in the range of3412 cm^{-1} whichis assigned to O-H stretching vibration of phenolic compounds. The medium absorption peak located at 2130 cm^{-1}-C=C- stretch (alkanes), 1648 cm^{-1} C=C stretch identified as alkenes, 1160 cm^{-1}C-H alkyl halides, 1052 cm^{-1}C-N stretch (aliphatic amines), 749 cm^{-1}C-H aromatics and a peak at 532 cm^{-1}C-Br stretch denotes alkyl halides.Biological components are known tointeract with metal salts *via* these functional groups and mediatetheir reduction to nanoparticles.

3.3 Structural analysis

XRD patterns of green synthesized silvernanoparticles from aqueous extract of *K. africana* was clearly explained. Anumber of Bragg reflections with 2 θ values of 32.16°, 38.28°, 42.39°and 64.62° sets of lattice planes are observedwhich can be indexed as (111), (121), (200) and (220) facets of silver respectively. XRD patternthus clearly illustrates that the synthesized AgNPs are crystalline in nature (Fig. 3).The pattern showed numbers of Bragg reflections that might be indexed on the basis of face centred cubic (fcc) structure of silver.

3.4 Morphological characterization

Morphology of the synthesized silver nanoparticles were in well resolved cubic, spherical and oval in structure. EDX spectrum along with the FESEM was known to provide chemical analysis of the fields and itdisplayed the strong presence of elementary nature as shown in Fig. 4.HR-TEM images showed that the synthesized silver nanoparticles are mono-dispersed with 13– 55 nm size and shape of the nanoparticles are spherical in shape with varying size as shown in the (Fig. 5).

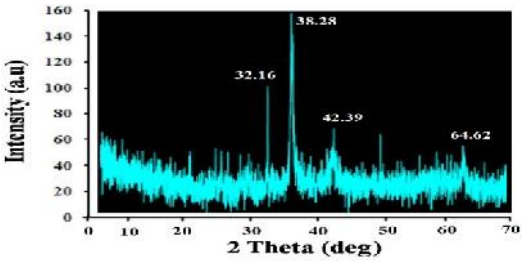

Fig 3. X-ray diffractogram of synthesized Ag NPs by *K. africana*

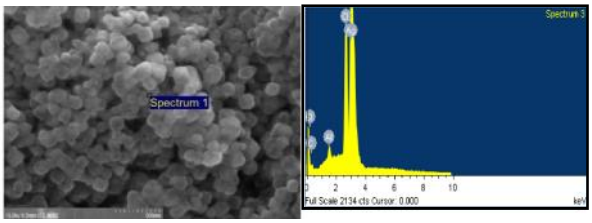

Fig 4. FESEM analysis and Energy Dispersive X-ray spectrum of synthesized Ag NPs

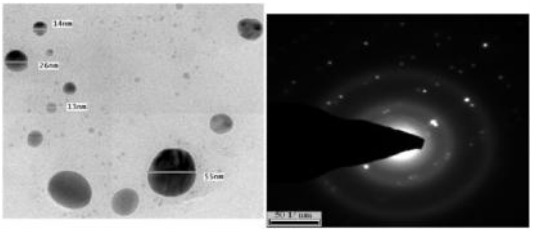

Fig 5. TEM micrograph and SAED analysis of AgNPs synthesized from 1 mM AgNO3

3.5*In vitro* antioxidant activity

3.5.1 Free radical scavenging assayusing DPPH

The aqueous extract and AgNPs showed impressive scavenging activities at (20-100 µg/mL).It exhibited dose dependent activity from 21.04 – 64.35% in Aqueous extract, 27.38 – 79.87% in AgNPs and in standard 33.56 – 79.08%. (Fig. 6a). The medicinal value of plants has become more evident which contain a diverse array of secondary metabolites with antioxidant potential[24].

3.5.2Hydrogen peroxide radical scavenging assay

The scavenging ability of *K. africana* aqueous leaf extract and the synthesised AgNPs on hydrogen peroxide radical was represented in Fig. 6b. At 100 µg/mL, aqueous extract exhibited 21.75 - 74.62%, followed by AgNPs 39.48 - 87.62%and BHT (standard) showed 41.40 – 83.03%activity respectively.

3.5.3 Superoxide radical scavenging activity

The synthesized silver nanoparticles and aqueous extract exhibited concentration dependent scavenging of superoxide radicals. Among this, AgNPs showed better activity at 100 µg/mL(23.68 – 71.02%), followed by aqueous (18.34 – 63.45%) and in standard (37.42 – 75.80%).The order of radical scavenging activity was in the following order: Rutin >AgNPs>Aqueous extract (Fig. 6c).

3.5.4 Nitric oxide scavenging activity

The scavenging ability of synthesised AgNPsand aqueous leaf extract on nitric oxide radical was representedin (Fig.6d) and compared with BHT as standard. All the samples were capable to scavenge radicals in a concentration dependent manner.At 100 µg/mL, aqueous extract and AgNPsrevealed28.98 – 81.37% and 19.36 – 67.25%of nitric oxide scavenging. On the other hand,BHT exhibited 35.17 – 84.50% of activity on nitric oxide which is greater than the aqueous extract and AgNPs.

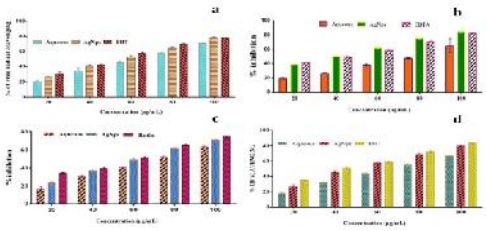

Fig 6. *In vitro* antioxidant activities of silver nanoparticles

3.6Antibacterial activity

The antibacterial assay of AgNPs was performed against six human disease causing pathogenssuch as Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*and*Micrococcus luteus*) and Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*and*Salmonella typhimurium*) by well diffusion method. The concentration of silver nanoparticles varied between 25, 50, 75 and 100 µg/mL (Fig. 7). The highest antibacterial activity was found against *P. aeruginosa* (31) and *S. typhi* (28 mm), respectively.The moderate activity was obtained in *S. aureus* (22 mm) followed by *M. luteus* (21 mm), *E. coli* (20 mm) and *B. subtilis* (19 mm) Table. 2.

Table 2: Antibacterial activity of Ag NPs against human pathogens

S. no	Human pathogens	Concentration (µg/mL)				Imipenem
		Zone of inhibition (mm)				
		25	50	75	100	
1	*Bacillus subtilis*	7.0	11.0	15.0	22.0	25.6
2	*Escherichia coli*	9.0	13.5	17.0	20.0	28.5
3	*Micrococcus luteus*	10.0	12.0	15.0	21.0	22.9
4	*Pseudomonas aeruginosa*	14.0	17.5	24.0	31.0	27.0
5	*Staphylococcus aureus*	12.0	16.5	19.0	22.0	27.5
6	*Salmonella typhimurium*	11.5	14.0	18.0	28.0	29.4

4. DISCUSSION

The absorption spectra of metal nanoparticles were dominated by surface plasmon resonance (SPR) particle size, dielectric medium and surface adsorbed species [26,20]. According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles whereas, anisotropic particles could elevate to two or more SPR bands [26]. The number of SPR peaks increases as the symmetry of nanoparticle decreases [27]. Significant colour change of the solution before two hours indicated fast reduction of $AgNO_3$ by the aqueous extract [28].

The FTIR bands of 1160 and 1092cm^{-1} were due to the strong stretching vibrations of C-N aromatic and aliphatic amine. FTIR band intensities in different regions of spectrum for the synthesized AgNPs were analysed and the spectra showed respective peaks in the range 3403, 2910, 2343, 1614, 1384 and 1092 cm^{-1}. The FTIRspectrum of AgNPs shows bands at 3403 cm^{-1} (N-H stretch, 1°, 2° amines), 2910 cm^{-1} (C-H stretch, alkanes), 1614 cm^{-1} (N-H bend 1° amines), 1092 cm^{-1} (C-N stretch, aromatic amines). The comparison of FTIR spectrum between the leaf extract and silver

nanoparticles were observed only minor vicissitudes in the position as well as the absorption bands due to the silver nanoparticles O-H stretching vibration shifted from 3412 to 3403 cm^{-1}.The spectrum of IR peak at 3403 was referred as the strong stretching vibrations O-H functional group[29]. The vibrational mode at 2130 cm^{-1}corresponds to C=C variables present in the plant protein. The medium of the excruciating peak at of 749 cm^{-1} was mainly attributed to carboxyl group of C-C stretching vibration present in the plant [30].

XRD spectrum confirmed that,synthesized silver particles were in the form of nanocrystals as evidenced by peaks at 2θ values of 34.69^{o}, 38.40^{o}, 44.39^{o} and 64.62^{o} corresponding to facets of the face centred cubic crystalline structure. The sharpening peaks clearly indicated the obtained particles were in nanoregions [31].[32]described that, the more diminutive size particles perforate into the cell facilely. It is eminent to mention that the geometrically triangular nanoparticles are having very sharp vertexes and edges that would be more puissant to damage the target cells.

Scanning electron microscopy (SEM) of silver solutions, confirmed the existence of very small and uniform spherical nanoparticles. From the SEM images it can be observed that larger particles are formed due to aggregation of nanoparticles which might be induced by the evaporation of solvent during sample preparation. This could have contributed for the variation in particle size. Whereas,AgNPs from leaf extract of *Acalypha indica* found to be more than 1000 nm with spherical and cubic shapes [33].Most of the AgNPs are spherical in shape with moderate variation in particle sizes ranged from 8 – 90 nm.[34] stated that, silver nanoparticles obtained from *C. tropicum* were 20– 50 nm in size and the particles were poly crystalline and single orientation which formed a cluster of silver particles which was confirmed by TEM analysis[35].

Antioxidant activity

Free radical scavenging of AgNPs was found to increase in a dose-dependent manner. AgNPs exhibited higher antioxidant activity with more than 70 % scavenging of DPPH radicals when compare to standard (BHT). Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation [36]. The percent of radical scavenging was found to be high in silver nanoparticles, due to itscapability of good oxidant and electron loosing. More significantly, the presence of phenols in leaf extract of*K. africana*assists the reduction of silver ions to silver nanoparticles. The results indicated that minimal increase in antioxidant activity of AgNPs, compared to the plant extract.The percent of radical scavenging was found to be high in silver nanoparticles, due to itscapability of good oxidant and electron loosing. The antioxidant activity of AgNPs not only due to capping agents (phytochemical constituents), also the presence of elemental silver [37].

Antibacterial activity

Antibacterial activity of silver nanoparticles (AgNPs) was dose and size dependent,and alsomost prominenton gram negative and gram positive bacteria.[38] AgNPs interact with thiol groups in protein which inducedthe inactivation of bacterial protein synthesis as well as DNA replication. Nanotechnology allows exploitation ofantimicrobial properties of silver by using in the form of nanoparticles(Feng et al., 2000). The antimicrobial activity of AgNPs was due to the release of Ag^{+}relatively that too in low concentrations[40]. According to [41], the antibacterial activity <9 mm zone was considered as inactive; 9-12 mm as partially active; while, 13-18 mm as active and >18 mm as very active. Based on this valuable investigations, the present study considered as very active due to high susceptibility (*P. aeruginosa* - 31 mm and *S. typhi* 28 mm) of AgNPs which was valuable to treatvarious infectionscaused by the above mentioned two human pathogens.

5. CONCLUSION

The results obtained from this investigation was very interesting in terms of the identification of potential hill station plant for synthesis of silver nanoparticles. Thus, antibacterial screening results justify the traditional uses in various ailmentsincluding infectious diseases. The phytochemical analysis of aqueous extract indicated the presence of major phytocompounds, including alkaloids, flavonoids, phenolics, glycosides and triterpenoids which may have been responsible for the observed antioxidant activity.This simple and efficient methodcould open the gate for preparation of other kinds of nanoparticleswith wide range of applications in the field of life sciences as well assynthesizedAgNPs could be usedin biological applications. Therefore,further findingscan be extended to studythe mechanisms involved with antioxidant and antibacterial activity of thesilver nanoparticles.

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