Life Science Informatics Publications
Research Journal of Life Sciences, Bioinformatics,
Pharmaceutical and Chemical Sciences

Journal Home page <http://www.rjlbpcs.com/>

Original Research Article

DOI: 10.26479/2019.0502.69

**ASSESSING THE ENZYME MODULATING AND ANTIMICROBIAL
EFFICIENCY OF *MORINGA* CAPPED SILVER NANOPARTICLES FOR
THEIR POTENTIAL USE AS FODDER SUPPLEMENT**

S. Gupta, S. A. Bisht, V. Kelkar-Mane*

Department of Biotechnology, University of Mumbai, Vidyanagari, Kalina,
Santacruz (E), Mumbai 98, India.

ABSTRACT: Feed and fodder supplements like antibiotics and digestive enzymes have been used since decades to enhance animal yield and productivity. The use of antibiotics has resulted not only in the micro flora developing resistance, but has also affected the reproductive cycles and breeding performance of the animals. Metal nanoparticles, especially silver, have been used as antibiotic substitutes due to its effective antimicrobial effect on wide spectra of microorganisms. Silver nanoparticles (AgNPs) especially in the range of 10-50nm are not only known to have increased stability, biocompatibility but also significant enzyme modulating activity. A known tropical, nutritional plant, *Moringa oleifera* which has intrinsic antimicrobial activity and is also known to improve the nutritional quality of animal feed because of its minerals, amino acids which might also serve as reducing agent for synthesis of silver nanoparticles used in this study. The nanoparticles thus synthesized were found to be 11nm in size with spherical shape and uniform dispersion. Synthesized nanoparticles were characterized using Ultraviolet (UV) spectrophotometer, FT-IR (Fourier transform infrared spectroscopy), DLS (dynamic light scattering), and XRD (X-ray diffraction). Their antimicrobial spectra was compared with commercially procured, chemically synthesized silver nanoparticles against *Escherichia coli* MTCC45, *Pseudomonas aeruginosa* MTCC1748 as well as *Candida albicans* MTCC227. Maximum activity was observed at 0.012µg/ml in *Moringa* synthesized nanoparticles as compared to the chemically synthesized nanoparticles. The same concentration of the phytosynthesized nanoparticles was found to enhance the cellulolytic activity, thus establishing its dual benefit as a probiotic as well as a substitute for antibiotics in animal feed supplement.

KEYWORDS: Silver nanoparticles, Cellulases, *Moringa oleifera*, Plant extract.

Corresponding Author: Dr. V. Kelkar-Mane* Ph.D.

Department of Biotechnology, University of Mumbai, Vidyanagari, Kalina,
Santacruz (E), Mumbai 98, India.

1. INTRODUCTION

In rural regions, agricultural waste is mostly used for livestock as feed. In order to enhance the productivity and growth of livestock, antibiotics as well as probiotics in addition to elements like sulfur, have been used [1]. Recently, exogenous enzymes like cellulases, pectinases and xylanases have been used for enhancing the nutritive value of the feeds [2]. However, the search for substitutes especially antibiotics lead into the possible use of nanoparticles as additives. Antibiotics in poultry as feed additives have been banned since 2003 as per EC Regulation No. 1831/2003 as they pose a risk to human health by development of resistant pathogens [2-5]. Silver nanoparticles can be used as preservatives in cosmetics and anti-acne preparation [6,7]. Their chemical as well as biochemical synthesis have been well established. The biochemical synthesis uses different plants extract such as *Solidago altissima* [8], *Solanum xanthocarpum*, *Ocimum sanctum* (Tulsi leaf) [9-10], *Camellia sinensis* (tea leaf) [11], *Terminalia chebula* [12], *Callicarpa maingayi* stem bark etc. [13-14]. These plant extracts not only serve as reducing agents but also aid in capping the nanoparticles, leading to a uniformly dispersed biocompatible suspension. The present work uses one such multi-dimensional tropical plant, *Moringa oleifera* to synthesize silver nanoparticles (AgNPs). This tropical plant has been the choice for the said study since it is known to improve the amylolytic as well as cellulolytic activity of rumen micro flora of cattle [1]. In addition to the plants intrinsic nutritional value, its different parts such as flowers, leaves, roots, gums, fruits, and seeds have also been used in the treatment of cardiovascular dysfunction, renal malfunction, inflammation, hematological and liver diseases [15]. Rubanza et.al reported that *Moringa oleifera* and enzyme improves the digestibility of animal feed because of their crude proteins, amino acids, and mineral content [16]. Toxicity reports show that the leaf extract of *Moringa oleifera* has no significant effects in humans, rabbits, poultry and rats [17]. It was thus hypothesized that the silver nanoparticles synthesized using *Moringa* would not only have antimicrobial properties but would also contribute in enhancing the cellulolytic activity. Results indicated a potential antimicrobial activity against *Escherichia coli* MTCC45, *Pseudomonas aeruginosa* MTCC1748 as well as *Candida albicans* MTCC227 which are known to be opportunistic pathogens. A concentration dependent effect of nanoparticles on cellulase activity was also assayed. Thus phytosynthesized nanoparticles have the potential to be used as fodder supplements.

2. MATERIALS AND METHODS

Materials

Analytical Grade (AR) Silver Nitrate (AgNO_3 , 99.99%) was procured from Sisco research Laboratory Pvt. Ltd (SRL), Mumbai, India. AR grade Ethanol absolute (99.9%) was procured from S.D Fine chemicals. Nutrient agar, Nutrient broth, Sabouraud's agar, Sabouraud's broth, Fetal bovine serum (FBS) were procured from Hi-Media Laboratories, Mumbai, India. AR grade Trisodium citrate, Calcium chloride, Tris HCl, were sourced from Loba chemie, Mumbai, India. Chemically synthesized Silver nanoparticles were procured from Nano research lab Jharkhand, India.

Collection of plant material

Fresh leaves of *Moringa oleifera* (Drumstick) were collected from the premises of University of Mumbai, Santacruz (E), India.

Preparation of aqueous extract

Moringa leaves were washed twice with two volumes of double distilled water and dried at 40°C in a hot air oven. The dried leaves were then crushed into fine powder and used as required. 10 grams of this crushed powder was suspended in 100 ml of double distilled water and kept on a rotary shaker at 30°C for 12 hours. The extract so obtained was stored at 4°C until further use [17, 18].

Synthesis of silver nanoparticles

Silver nanoparticles (AgNPs) were prepared by reducing 90ml of 1mM silver nitrate solution with 10ml of plant extract obtained as mentioned in 2.3. This mixture was maintained between 60°C - 80°C for 20 minutes till its colour changed from pale brown to dark brown. The dark brown solution thus obtained indicated the formation of silver nanoparticles. The synthesized nanoparticles were stored at 4°C till further uses [18].

Analytical characterization of silver nanoparticles

Phytosynthesized silver nanoparticles formation was measured using UV-Vis spectrophotometer (Thermo scientific multiskan go) operated at 1nm resolution with a wavelength ranging from 400nm to 700nm at 30°C . A Sharp peak obtained in the visible region (420nm) of the electromagnetic spectrum indicated the presence silver nanoparticles. The particles size of nanoparticles synthesized was obtained by X-ray diffraction using XRD-6100 (Shimadzu analytical, INDIA pvt. Ltd) as well as its hydrodynamic size determined using dynamic light scattering (DLS) technique using Microtrac S3500 (Malvern instruments, UK). Fourier Transform Infra-Red Spectroscopy (FT-IR) using FrontierTm (PerkinElmer, Inc) was used to characterize the functional group of the capped nanoparticles [19-21].

Nano catalytic activity

Nanocatalytic activity was determined as described by Bipin et.al [23]. 50 μl of bacterial

cellulase (1mg/ml) was mixed with 200 µl Carboxymethyl cellulose (CMC) (1%) and incubated at 37°C for 20mins. *Moringa* synthesized nanoparticles were added in increasing concentration from 0.003ppm -0.06ppm in each tube. The tubes were incubated at 28°C in a shaker incubator for 15min and formation of reducing sugars was estimated using the 3,5-Dinitrosalicylic acid (DNS) method [24].

Antimicrobial activity and Germ tube inhibition

The antimicrobial activity of silver nanoparticles was tested against *Escherichia coli* MTCC45 and *Pseudomonas aeruginosa* MTCC1748 by agar cup diffusion method. 10⁶ colony forming units (CFU) of the test organism were spread on sterile Mueller Hinton agar (MHA) plates. Gentamycin at a concentration of 1 mg/ml was used as positive control. The aqueous extract of *Moringa oleifera* leaves was also assayed for its antimicrobial activity. Commercially available chemically synthesized AgNPs were also evaluated for their antimicrobial activity. The plates were incubated at 37°C for 24 hours and zone of inhibition were observed, indicating the antimicrobial activity [13,19]. Method described by Acharya et.al was used to determine the pathogenicity of *Candida albicans* MTCC227 [25]. Colonies of *Candida albicans* of size of about 2mm were pre-exposed to 0.5 ml of Flucanazole (1mg/ml), phytosynthesized AgNPs, commercially available silver nanoparticles and aqueous extract of *Moringa oleifera* in separate tubes for 2 hours followed by incubation in 0.5 ml fetal bovine serum. All the tubes were incubated at 37°C for 3 hours for induction of germ tube. *Candida albicans* grown in fetal bovine serum was maintained as a control.

3. RESULTS AND DISCUSSION

Biosynthesis of silver nanoparticles (AgNPs)

Silver nitrate changed color from pale to dark brown solution upon addition of plant extracts indicating the formation of stable silver nanoparticles [26].

Characterization of silver nanoparticles

UV-Vis spectra analysis of silver nanoparticles

Phytosynthesized silver nanoparticles were subjected to UV-Vis spectroscopy. The absorbance of the diluted (1:10) sample was recorded from 300-600nm range. Maximum absorbance was observed at 420nm, indicating the formation of silver nanoparticles. A Surface Plasmon Resonance (SPR) in the range of 410 to 450nm was indicative of AgNPs synthesis. The single peak obtained thus suggested that the synthesized nanoparticles could be spherical in shape [7,14,26].

Figure 1: UV-Visible spectrum of silver nanoparticles. SPR observed at 420nm indicating the formation of silver nanoparticles in the solution.

Fourier Transform Infra-Red Spectroscopy (FT-IR) analysis of silver nanoparticles

The reducing as well as capping biomolecules in AgNPs synthesis was identified using FT-IR. FTIR spectra of silver nanoparticles were in the range of $500\text{--}4000\text{ cm}^{-1}$. Three absorption peaks around 1010 cm^{-1} could be assigned as the absorption peaks of --C--N stretching vibrations of the amine group, --C--O--C or --C--O groups, respectively. The functional groups or bonds such as --C--O--C-- , --C--O-- , and --C=C-- derived from heterocyclic compounds such as alkaloid or flavones and the amide (I) bond derived from the proteins that are present in the leaves extract act as a capping ligands of the nanoparticles. The broad and strong bands at $3385\text{--}2913\text{ cm}^{-1}$ were due to bonded hydroxyl (--OH) or amine groups (--NH) and aliphatic group (C--H) of the *Moringa oleifera* extract, respectively. The 1630 cm^{-1} peak is recognized to the carboxyl group (--C=O) stretching vibration. The adsorption at around $1300\text{--}1500\text{ cm}^{-1}$ notably showed that --NO_3 existed in residual amounts. The peak in 1010 cm^{-1} and 920 cm^{-1} were related to Ag-NP bending with oxygen from hydroxyl groups of *Moringa oleifera* compounds [14,28].

Figure 2: Fourier-Transform Infrared Spectra analysis of silver nanoparticles synthesized from *Moringa oleifera*

Dynamic light scattering and XRD analysis of silver nanoparticles

The hydrodynamic diameter of phytosynthesized silver nanoparticles was found to be 147nm and polydispersity index (PDI) of 0.343. X-ray diffraction pattern (Fig.2) was used to analyze the structural properties, crystalline nature and phase purity of the silver nanoparticles. The nanoparticle structure was confirmed by the characteristic peaks observed in the XRD graph. All scanning lines were observed at 2θ angle 27.66° , 32.06° , 37.82° , 45.02° , 64.46° , and 76.92° . XRD patterns were investigated to determine peak intensity, position and width, full-width at half-maximum (FWHM). The average crystal size of the silver nanoparticles was calculated using Debye–Scherrer equation. XRD pattern obtained indicated a cubic and hexagonal arrangement with an average estimated crystal size of 11nm as resulting from the Full width at half maximum (FWHM) peak [13].

Figure 3: Particle size distribution (DLS) of silver nanoparticles synthesized from *Moringa oleifera*. The hydrodynamic diameter of silver nanoparticles was found to be 147nm and polydispersity index (PDI) of 0.343

Figure 4: X-Ray Diffraction Patterns of silver nanoparticles (Ag-NPs) synthesized from *Moringa oleifera*. Average estimated crystal size of silver nanoparticles is 11nm.

Antibacterial activity and germ tube inhibition of silver nanoparticles

The antimicrobial activity of silver nanoparticles was assayed against *Escherichia coli* MTCC45 and *Pseudomonas aeruginosa* MTCC1748. The zone of inhibition was measured after incubating the plates for 24 hours at 37°C and gentamycin was used as a positive control. Phytosynthesized silver nanoparticle showed higher activity against *Pseudomonas aeruginosa* MTCC1748 as compared to *Escherichia coli* MTCC45. The zone of inhibition of phytosynthesized nanoparticles against *Escherichia coli* and *Pseudomonas aeruginosa* was 22mm and 27mm respectively. The activity of the phytosynthesized AgNPs was more than that of commercially available chemically synthesized nanoparticles that is 20mm and 22mm for *Escherichia coli*, *Pseudomonas aeruginosa* respectively. In present study the phytosynthesized silver nanoparticles showed greater activity against test organisms, this can be attributed to the smaller size than that of the commercially available nanoparticles. Pham van et.al reported that the antibacterial activity of AgNPs measuring 20–80nm was attributed to the release of silver ions [29-30].

**Figure 5: (a) Antibacterial activity of silver nanoparticles against *Escherichia coli*,
(b) Antibacterial activity of silver nanoparticles against *Pseudomonas aeruginosa***

Table 1: Antibacterial activity of silver nanoparticles synthesized from *Moringa oleifera*

	Zone of inhibition (mm)			
Test Culture	Gentamycin (1mg/ml)	Biogenic AgNPs (0.012µg/ml)	Chemical AgNPs (0.012µg/ml)	Aqueous extract of leaf of <i>Moringa oleifera</i> (200mg/ml)
Well Designation	A	B	C	D
<i>Escherichia coli</i> MTCC45	28 mm	22 mm	20 mm	0 mm
<i>Pseudomonas</i> <i>aeruginosa</i> MTCC1748	29 mm	27 mm	22 mm	0 mm

This study showed that the formation of germ tubes by *Candida albicans* MTCC227 was affected upon the pre-exposure to *Moringa oleifera* extracts as well as to the biologically synthesized silver nanoparticles. Formation of germ tube is one of the important virulence factors that contributes to the pathogenicity of *Candida albicans*. Inhibition of germ tube formation thus significantly reduces its pathogenicity. 92% germ tube inhibition was observed upon exposure to phytosynthesized AgNPs compared to 58% seen in case of commercially available AgNPs. 72% aqueous extract of *Moringa oleifera* also inhibited the formation of germ tube. This indicates that both aqueous extract of *Moringa oleifera* and silver nanoparticles synergistically inhibit the formation of germ tube in *Candida albicans*.

Figure 6: (a) Germ tube formation in FBS (negative control).98% of the *Candida* cells exhibited formation of germ tube.

(b) Oval *Candida albicans* cells budding in FBS containing phytosynthesized silver nanoparticles. This indicates that the cells are alive and AgNPs only induced a generalized inhibition of growth and decrease in pathogenicity.

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Peer review under responsibility of Life Science Informatics Publications

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Effect of silver nanoparticles on cellulase activity

The phytosynthesised AgNPs enhanced the activity of cellulase enzyme, thus acting as bionanocatalyst. The combination of cellulase enzyme with AgNPs showed double the activity at 0.012 $\mu\text{g/ml}$ concentration. Enzyme alone showed considerable activity. Previous studies reported that immobilization of enzymes enhances the enzyme activity. Suhartati et.al reported that combination of *Moringa* leaves with sulfur increases the cellulase activity but decreases the protease activity [1]. Dumri et.al reported that silver nanoparticles increase the lipase activity. The present work suggests using the nanoparticles to boost the enzyme activity for application in various industrial sectors [23].

Figure 7: Effect of silver nanoparticles on cellulase activity

4. CONCLUSION

The silver nanoparticles formed using *Moringa* extract were mono dispersed and showed surface plasmon resonance at 420nm. The phytosynthesized silver nanoparticles 11nm size enhanced not only the antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* that are known opportunistic pathogens but also boosted the cellulase activity in comparison to chemically synthesized nanoparticles. The success of the study lies in the fact that at one given concentration (0.012 $\mu\text{g/ml}$), the phytosynthesized silver nanoparticles potentially served as antibiotic as well as cellulase activity enhancer playing a dual role so as to be possibly used as feed supplements.

ACKNOWLEDGEMENT

The authors would like to acknowledge Department of science and technology (PURSE) for providing financial support.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication.

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