Life Science Informatics Publications
Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences
Journal Home page http://www.rjlbpcs.com/
RJLBPCS
ISSN 2454-6348

Original Research Article **DOI - 10.26479/2018.0402.15**

EXTRACELLULAR BIOSYNTHESIS OF SILVER NANOPARTICLES USING *MORINGA OLEIFERA* LEAVES EXTRACT AND ITS ANTIMICROBIAL EFFICACY IN PACKAGING MATERIALS

B. Narwade[1], N. Prasad[2], S.M. Lokhande[1]*, A.B. Madavi[3], A. K. Sahoo[1]

1. Department of Food Technology, Department of Technology, Shivaji University, Kolhapur, India

2. Department of Nanoscience and Technology, Shivaji University, Kolhapur, India

3. Department of Chemical Technology, Department of Technology, Shivaji University, Kolhapur, India

ABSTRACT: Colloidal nanosilver was biosynthesized by reduction method using *Moringa oleifera* leaves extract as a reducing agent. The sizes of the nanoparticles were controlled by different parameters such as precursor concentration and reducing agent concentration. The synthesized nanoparticles were analyzed by UV-vis spectrophotometer, Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and X-ray diffraction (XRD). The sizes of poly-dispersed Ag NPs were obtained within the range of 1-56.9 nm, 2-448.1nm and 3-4705.0 nm. The nanosilver showed effective antimicrobial efficacy towards *Escherichia coli*, *Staphylococcus aureus, Salmonella typhi and Bacillus subtilis* were checked by agar disc diffusion method. Synthesized nanosilver was used for preparation of nanocomposite.

KEYWORDS: Ag NPs, *Moringa oleifera*, Characterization, Antimicrobial Activity

***Corresponding Author: Dr. S.M. Lokhande** Ph.D.
Department of Technology, Shivaji University, Kolhapur, India
* Email Address: sml_tech@unishivaji.ac.in

1. INTRODUCTION

Nanoparticles having the length within 1-100 nm scale [1] synthesized by chemical or biological methods, intends to increase the surface to volume ratio of particle [2]. Chemical synthesis methods have been showed critical effects due to the various toxic natures of chemicals absorbed or adsorbed on the surface material [3]. Synthesis of nanoparticles via biological methods promoted the cost

effective and eco-friendly methods in the field of nanoscience and technology without any high pressure, energy, temperature and toxic chemicals [4].Recently, green synthesis methods developed by yeast, fungi, bacteria and plant extract [5,6,7] use for the biosynthesis of different nanoparticles. Singaravelu *et al* [8] have synthesized the metallic nanoparticles by *Sargassum*. Fungi such as *Verticillium, Fumigatus, Trichoderma, Asperellium,* and *Phapnerochaete Chrysosporium* have been explored for noble metallic nanoparticles synthesis. The microorganisms continue to be investigated for bio-mineralization and metal nanoparticles synthesis. However maintenance of cell culture for synthesis of nanoparticle is expensive and requires advanced skills. The use of plant extracts for nanoparticles are exciting possibility in biosynthesis methodologies and relatively unexplored and under-exploited [9].Consequently various plant extract is reported such as Murrayakoenigii leaf [10], Tansy fruit[11], Jatropha curcas [3],Cinnamomum zeylanicum leaf[12] , Camellia sinensis[13], Aloe vera [14], Mushroom [15], Azadirachta indica[16], Capsicum annuum[3] and Carica papaya[17] etc for the evolution of physicochemical properties of metal(silver, gold) nanoparticles [18]. Moreover, the nanosilveris increasing the involvement in the field of food industry due its strong antimicrobial efficacy against the bacteria and microorganisms owing to attachment with the cells surface of microorganism which breaks the permeability, respiration function of cells[19], also used as in different consumer products such as deodorants, clothing, bandages, cleaning solutions and as antimicrobial agents [20]. Nanosilver can be produced either intra or extra-cellularly by using biological routes. It has been reported in literatures that parallel to the chemical process, it is also possible to synthesize gold nanoparticles of uniform size extra-cellularly using extremophilic actinomycete *Thermomonospora* species [21] Jose-Yacaman and co-workers[22] showed that live alfalfa plants when supplied with Au^{3+} ions reduces into Au^{0} state and absorb them resulting in the internal formation of gold nanoparticles. Jose-Yacaman's groups [23] have observed the synthesis of silver nanoparticles using alfalfa sprouts, undergoing the nucleation of silver molecule due to the accumulation in inside the cells of alfalfa plants. Kharisov et al.[24] used the extract from *Coffeaarabica*, and *Cymbopogon citrus* as green reagents for Ag NPs synthesis which reduces the silver nitrate solution by tea extract or epicatechin of varied concentrations, the controlled spherical size of silver nanoparticles were formed which depends upon the concentration of tea extract and epicatechin. Shinde et al. [25] studied the antimicrobial properties of CuO nanosheets for the development of antibacterial agents against bacillus subtilis. For the development of packaging material, nanotechnology plays important role for maintaining the quality of the product, increasing the shelf life of food with retarding the growth of microorganism [20]. In this study to extend the green chemistry approaches for synthesizing the nanoparticles from plant extracts at 60 ^{0}C. The reductions of silver precursor's solution was due to the presence of different content of carbohydrate, fat, proteins, enzymes& coenzymes, phenols flavanoids, terpenoids, alkaloids, gumetc compounds in the different extract and stabilize nanoparticles prevents the agglomeration of nanoparticles [26].S.

P.Dubey [11] said the possibility of Ag^{+} reduction in to Ag^{0} was due to the conversion of terpenes groups $> C = O$ in to– $C(O) = O$. In the present work, the synthesis of silver nanoparticles has been carried out using aqueous extract of *Moringa oleifera* leaves, and also studied their characterization, inhibitory effect against Gram-negative and Gram-positive bacteria. Then the synthesized nanoparticles were coated over LDPE packaging material for improving the antimicrobial activity.

2. MATERIALSAND METHODS

Materials

Silver Nitrate purity (99.95%) was procured from Sigma Aldrich, Kolhapur, India and used without further any purification. *Moringa oleifera* leaves were collected in the month of September from the premises of botany department of Shivaji University, Kolhapur, India. All other chemicals used were of analytical grade and all solution prepared in the double distilled water.

Preparation of plant extract

The collected fresh leaves of *Moringa oleifera* are as shown in Figure 1 were washed in tap water to remove adhered dust particles. Then, it is dried in ambient temperature to remove residual moisture. After removal of moisture it is grinded into fine powder. Firstly, the leaves powder is thoroughly dissolved in de-ionized water i.e. 11.11(w/v). And the resulting solution is heated at 60 ^{0}C for about 30 minutes. Secondly, the extract solution was filtered using filter paper having pore size 50 mm. This filtrate can act as efficient reducing agent.

Figure 1: Picture of *Moringa oleifera* leaves.

Synthesis of silver nanoparticles

For the synthesis of Ag NPs using *Moringa oleifera* leaves extract, the aqueous solution of 0.2 M silver nitrate was prepared. Then, in above solution, plant extracted was drop wise added in the ratio of 9:1.This reaction mixture was heated up to 60-80 ^{0}C in hot water bath with continuous stirring for about 20 minutes. The color of solution gradually changes from brown to dark brown, which indicates the formation of Ag nanoparticles as shown in Figure 2(a).The synthesized nanoparticles were separated by centrifugation at 1500-2000 rpm for 8 minutes. The supernatant solution was discarded and collected the settled nanoparticles from the bottom, then washed with ethanol and

dried in hot air oven at 105ºC for 3 hours and finally obtained the moisture free Ag nanoparticles. Then it is crushed in mortar, finally granular size of Ag nanoparticles was obtained as shown in Figure 2(b). These samples are used for characterization and applications in food packaging [27].

Figure 2: (a) Colloidal silver nanoparticles (b) Granular silver nanoparticles.

Antimicrobial activity

Antimicrobial activity of Ag NPs was investigated by agar well diffusion method against strains of microorganisms and pathogenic bacteria i.e. *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Bacillus subtilis* with different concentrations (25, 50,100 µL) of silver nanoparticles solutions by national committee for clinical laboratory standards. The microorganisms were incubated in petri plate at 37 ˚C for 24 hrs using nutrient agar media. Microorganisms were spread over the solidified plates and bored the wells by sterile borer (4 mm diameter). The wells was filled with 25, 50,100 µl of silver solution. Streptomycin (1mg/1ml) used as antibiotic control for comparative study [28].

3. RESULTS AND DISCUSSION

Characterizations of Ag NPs

The absorbance spectra of Ag NPs were monitored in UV-Vis spectrophotometer UV-2450 (Shimadzu) within the range of 200-800 nm. X-ray diffraction was used to confirm the formation of crystalline nano-materials and also their nature. Scanning electron microscope (Hitachi S-4500) used to studied the surface morphology of nanoparticles, machine operated with silver powder deposited on a carbon strip,(1,000-3,000) magnification, 800×800 μm^2 surface area at 18kV.The size distributions of silver nanoparticles were determined by DLS Zetasizer range (Malvern) based on the laser diffraction method with multiple scattering techniques.

UV-vis spectra analysis of nanoparticles

The synthesis Ag nanoparticles were monitored using UV-Vis spectrophotometer. Figure 3 shows the UV–vis absorption spectrum of Ag nanoparticles before and after addition of *Moringa oleifera* leaves extract. Without reducing agent, two absorbance peaks of silver precursor at 360 nm and 420

nm were observed, which should be assigned to the ligand–to–metal charge transfer (LMCT) of palladium complex [29]. Reducing the metal ions causes a decrease in the plasmon band intensity and a flattening of the peak. Absorption peaks are flattened after the addition of reducing agent indicating that the metal ions vanish. The absorbance peak depends upon particle size of the synthesized nano-particles. A bathochromic shift is observed with decrease in particle sized where normal position of plasmon band shifts toward a shorter wavelength and vice a versa. These effects can be explained by the fact that absorption band is closely dependent on the particle size. Also, with the more reduction of the metal ions, the absorption band is broadened (due to the d–d interband transitions) and then vanished [30].

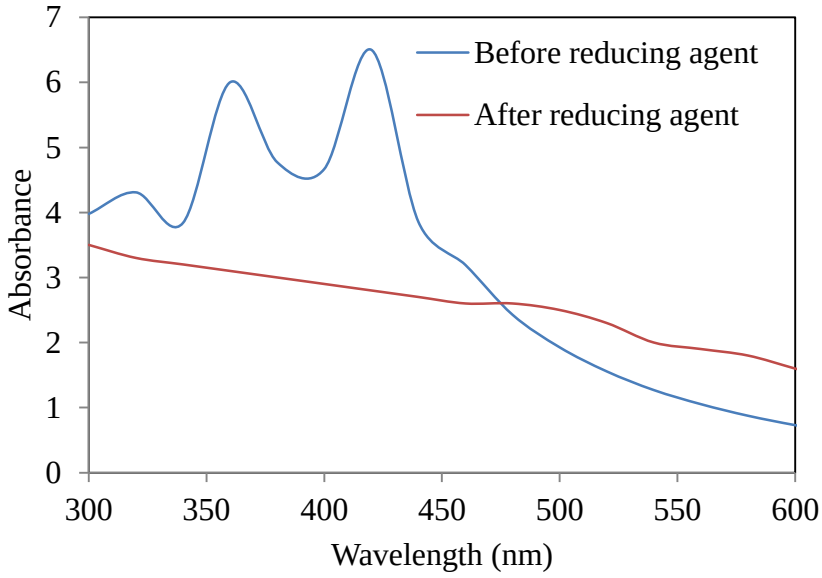

Figure 3:UV-Vis absorption spectrum of addition of (a) Before reducing agent; and (b) After reducing agent solution in $AgNO_3$ solution.

Effect of reducing agent on nanoparticles

Moringa oleifera leaves extract was effectively used as reducing agent for synthesis of silver nanoparticles. The effect of *Moringa oleifera* leaves extract on the formation of Ag nanoparticles was studied by varying the amount (3, 6, 9, 12, 15 gm) at constant 0.2 M silver nitrate solution without use of any surfactant. This is depicted as shown in Figure 4. It was observed that the maximum absorption spectra (3.2 abs) of silver nanoparticles at 420 nm in 9 gm reducing agent [31] and enhanced the reduction rate favours the generation of much more nuclei and formation of smaller nanoparticles. The atoms formed at the latter period were used mainly to the collision with the nuclei already formed instead of the formation of new nuclei, leading to the formation of larger particles [32]. A minimum absorbance spectrum was observed when the filtrate used for reduction which was prepared with 3gm of dry leaves. This is because only few nuclei were formed at the early period of the reduction.

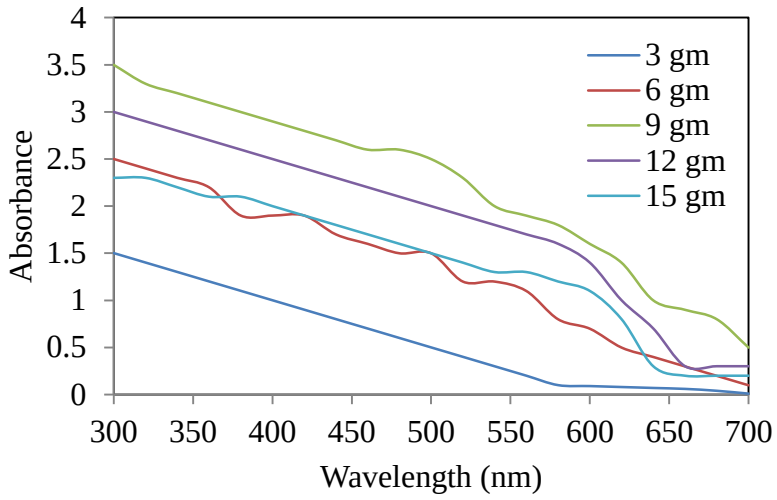

Figure 4: UV–Vis absorption spectra of silver nanoparticles at 0.2M $AgNO_3$ with different amount of (3, 6, 9, 12, 15gm) *Moringa oleifera* leaves extract powder.

Effect of $AgNO_3$ solution on nanoparticles

Also, it is tried to study the effect of concentration of silver nitrate on the formation of Ag NPs. For such study keeping the leaves concentration constant i.e. 9 gm, the concentration of silver nitrate solution was varied from 0.2 to 1.M. The experimental results are depicted in Figure 5. It is observed that with increase in concentration of silver nitrate solutions, the absorbance goes on increasing upto 0.6M again the absorbance goes on decreasing. Thus, the maximum absorbance is observed at 0.6M concentration. Qui-li *et al.* [33] observed that the increases the reaction conversion rate of Cu^{2+} concentration leading to the amount of copper nuclei rises and small sizes of particle were obtained. The average particle size increased as the collision frequency of the particles formed increased, and also the protection from the adsorption of surfactant molecules on the particle surface was weakened due to a lower surfactant concentration compared with the precursor concentration. Thus, the tiny particles agglomerated to form larger particle [34].

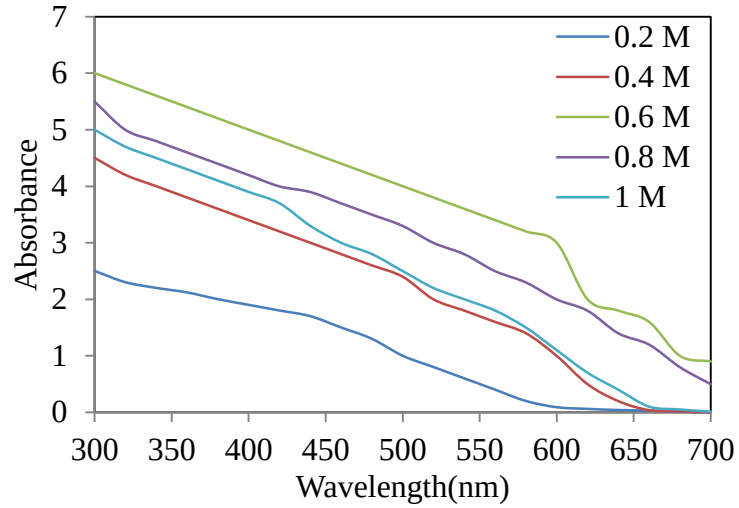

Figure 5: UV–vis absorption spectra of Ag NPs have different concentrations (0.2, 0.4, 0.6, 0.8, 1 M) of $AgNO_3$ solutions at constant 9 gm of reducing agent.

Scanning electron microscope analysis of nanoparticles

The morphology of biosynthesized silver nanoparticles was analyzed by Scanning Electron Microscope (Hitachi S-4500). The Scanning Electron Microscopy image reveals that the synthesized nanoparticles are poly-dispersed in nature. The synthesized nanoparticles exhibit irregular shapes and having no certain fixed geometry. The agglomerate structure seems to be pebble like structure. The formation of poly-dispersed nanoparticles takes places due to unequal agglomeration of nanoparticles up to certain extent. The Scanning Electron Microscopic image is depicted in Figure 6.

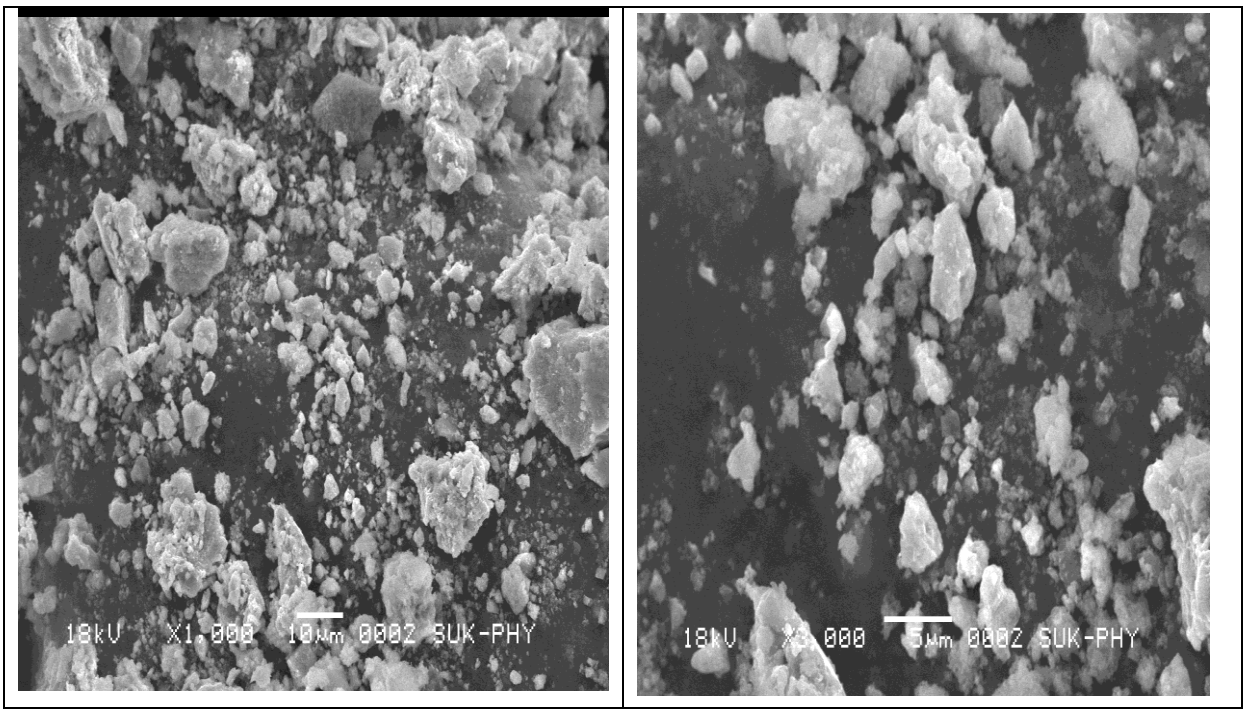

Figure6: SEM images of biosynthesized Ag NPs.

Dynamic light scattering and XRD analysis of nanoparticles

From Figure 7(a) DLS histogram, it has been seen that hydrodynamic diameters of Ag nanoparticles within the range of 1-56.9 nm, 2-448.1nm and 3-4705.0 nm. XRD analysis of Ag nanoparticles showed intense peak corresponds to the 2 theta (degree) as shown in Figure 7 (b). A number of Bragg's reflections were present which can be indexed on the basis of FCC structure of silver. Particle size was calculated by Debye-Sherrer formula [31] and measured size of particle i.e.18.43 nm, 19.32 nm and 20.95 nm and confirmed that the Ag nanoparticles were formed in crystalline in nature.

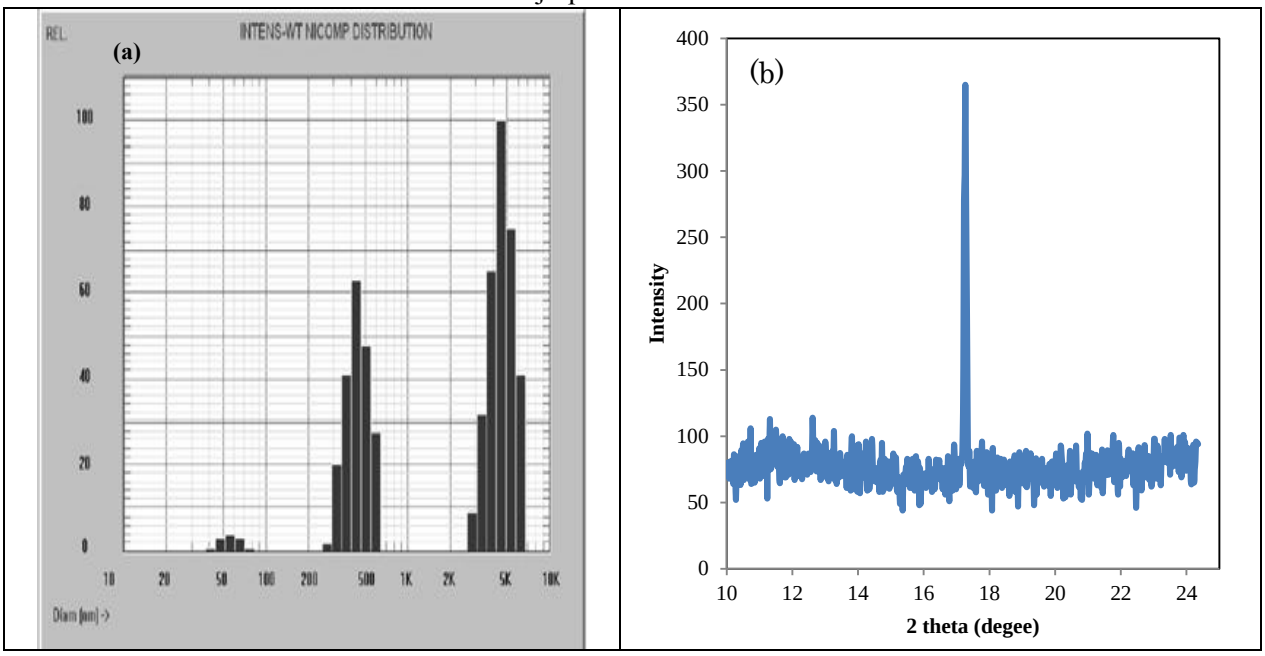

Figure7: (a) Particle size distribution histogram (b) XRD pattern of biosynthesized Ag nanoparticles.

Antimicrobial Studies of nanoparticles

Antimicrobial activity of silver nanoparticles against the *Escherichia coli, Staphylococcus aureus, Salmonella typhi and Bacillus subtilis* were carried out by disc diffusion method as shown in Figure 8 with reference of *Streptomycin*as described by the clinical laboratory standards. The antimicrobial activity was examined by measuring the zones of inhibition around each disc. It was found that the antimicrobial activity of silver nanoparticles were strongly effective against the microorganisms in 100µl of Ag NPs as shown in table 1, owing to the surface of smaller size nanoparticles to change the local electronic structure for the enhancement of chemical reactivity to control the bactericidal effect [35]. Ag NPs adsorb the surface of bacteria but in low concentration does not enter the cells of bacteria; actually respiration occurs across the cell membrane other than mitochondrial membrane [4].Table 2 shows that list of biosynthesized nanoparticles using different extracts and its antimicrobial activity against pathogens.

Table 1: The antimicrobial activity of silver nanoparticles and Ag nanocomposite synthesised from M. oleifera leaf extract

Microorganism	Amt. of Ag NPs (µL)	Zone of inhibition (mm)	
		Streptomycin	Ag NPs
Escherichia coli	25 µL	17	17
	50 µL	20	20
	100 µL	23	23
Staphylococus aureus	25 µL	15	15
	50 µL	18	18
	100 µL	20	20
Salmonella typhi	25 µL	17	14
	50 µL	20	15
	100 µL	21	20
Bacillus subtilis	25 µL	17	16
	50 µL	19	19
	100 µL	20	20

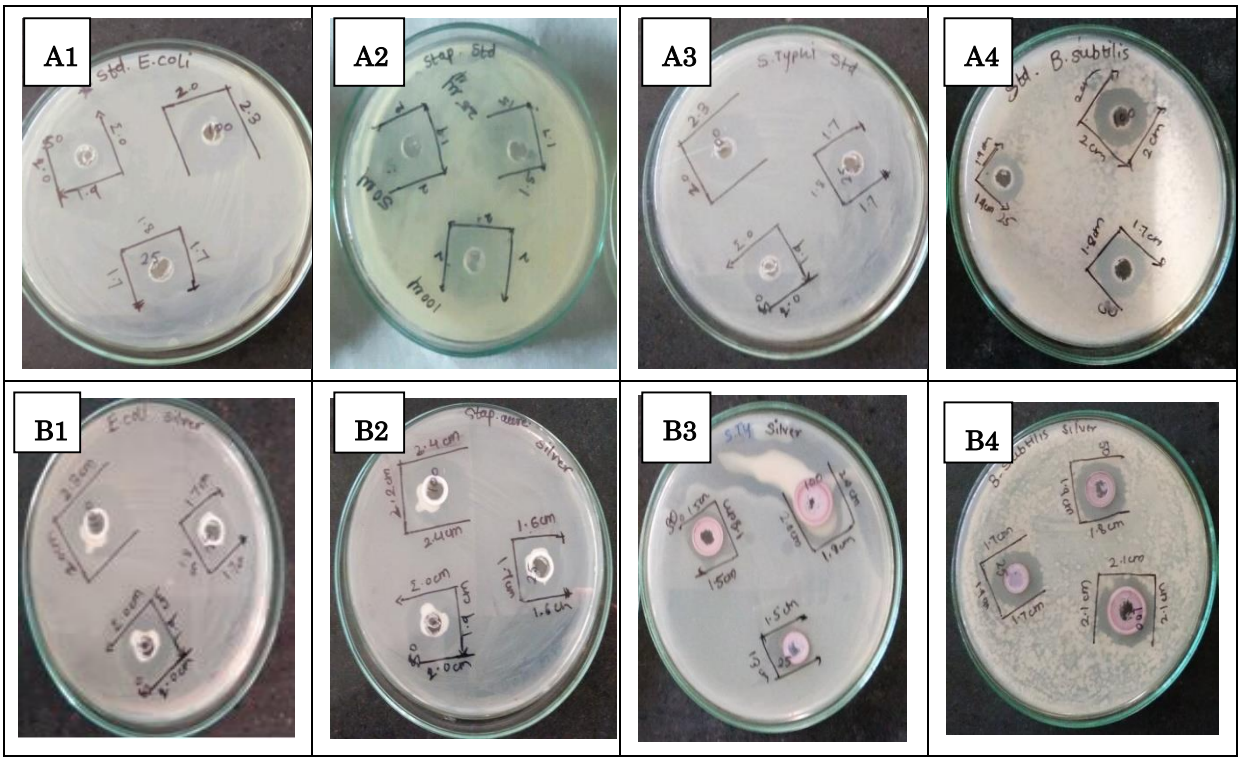

Figure8: Antimicrobial activities of silver nanoparticles against(B1:*Escherichia coli B2:Staphylococcus aureus,B3:Salmonella typhi and B4:Bacillus subtilis)with referenceStreptomycin* (A1:*Escherichia coli A2:Staphylococcus aureus,A3:Salmonella typhi and A4:Bacillus subtilis*).

Table2: List of biosynthesized nanoparticles using different extracts

NPs (Size)	Extracts	Conc. of Ag NPs	Pathogen	Results	References
Ag NPs (30 nm)	Argemone Mexicana	50mg/litre	*E. coli, P.syringae, A.flavus*	Zone of inhibition in mm (15 ± 0.4,10 ± 0.5, 10 ± 0.2)	[36]
Ag NPs (20-30 nm)	Acalypha indica	Vary conc. from 0.039 to 40 µg	*Escherichia coli, Vibrio cholerae*	Minimum inhibitory concentration at 20 µg/mL	[37]
Ag NPs (4-30 nm)	Ocimum sanctum (Tulsi)	Vary conc. from 5 to 100 µL/mL	*Escherichia coli, Staphylococcus aureus*	Minimum inhibitory concentration at0.314µL/mL, 1.25 µL/mL	[38]
Ag NPs Ag NPs (15-50 nm)	Polyalthia longifolia	10^{-3}M, 10^{-4}M	*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*	Zone of inhibition (mm) for 10^{-4}M was 8, 9.5, 16.4	[18]
Ag NPs (10-35 nm)	Citrus sinensis peel	50 µL	*Escherichia coli, Pseudomonas aeruginosa,Staphylococcus aureus*	Zone of inhibition (mm) was 16, 13.4, 9.2	[39]
Ag NPs (10-40 nm)	Lycopersico-n esculentum (red tomato)	Vary conc. from 0.2 to 100 µL/mL	*Escherichia coli*	Minimum inhibitory concentration at 50 µL/mL	[19]
Ag NPs (5-10 nm)	Saltmarsh plant, Sesuvium Portulacastru-m L.	50 µLl	*P.aeruginosa, K. pneumonia, S.aureus, L.monocytogenes, M. luteu*	Inhibition zone 23 mm diameter(Highest) against Staphylococcus aureus (Callus extract with PVA) and 8 mm (lowest) against Micrococcus luteu (leaf extract without PVA)	[4]
Ag NPs (40-50 nm)	Euphorbia - hirta	50 µL	*S.aureus,E. coli,K.pneumoniae, B.cereus,P.aeruginosa*	Zone of inhibition (mm) was 12.01 for S.aureus and 13 for B.cereus	[27]
Ag NPs	Ceratonia	0.5 µg/L	*Escherichia coli*	Zone of inhibition (mm) was 8 to	[31]

(5-40 nm)	siliqua			12.	
Ag NPs (2-448.1 nm) Polydispersed in size	Moringa Oliefera	25µL,50 µL,100 µL	*Escherichia coli Staphylococcus aureus, Salmonella typhi and Bacillus subtilis*	Zone of inhibition (mm) highest was 23, 20,20, 20 at 100 µL	This work

Preparation of Nano-composite package material

For preparation of nano-composite is becomes essential to coat the material. Firstly, the coating is achieved by dipping of LDPE in Ag NPs solution for 24 hours. Then, the substrate material i.e. LDPE is dried under the natural process of drying for 2-3 days at ambient temperature. The moisture content is removed and coating of Ag NPs on LDPE takes place which is widely used as packaging material in food industries. For the formation of composite materials, the LDPE can be dipped in colloidal solution of silver nanoparticles for different time duration i.e. for 3 hours and 24 hours. The nano-composite formed by dipping the polymer for 3 hours shows better activity than the nano-composite formed after dipping for 24 hours. When the dipping duration increases there is formation of black color on nano-composite. Thus, an attempt has been made to optimize the dipping duration for the formation of nano-composite material. The result is depicted in Figure 9.

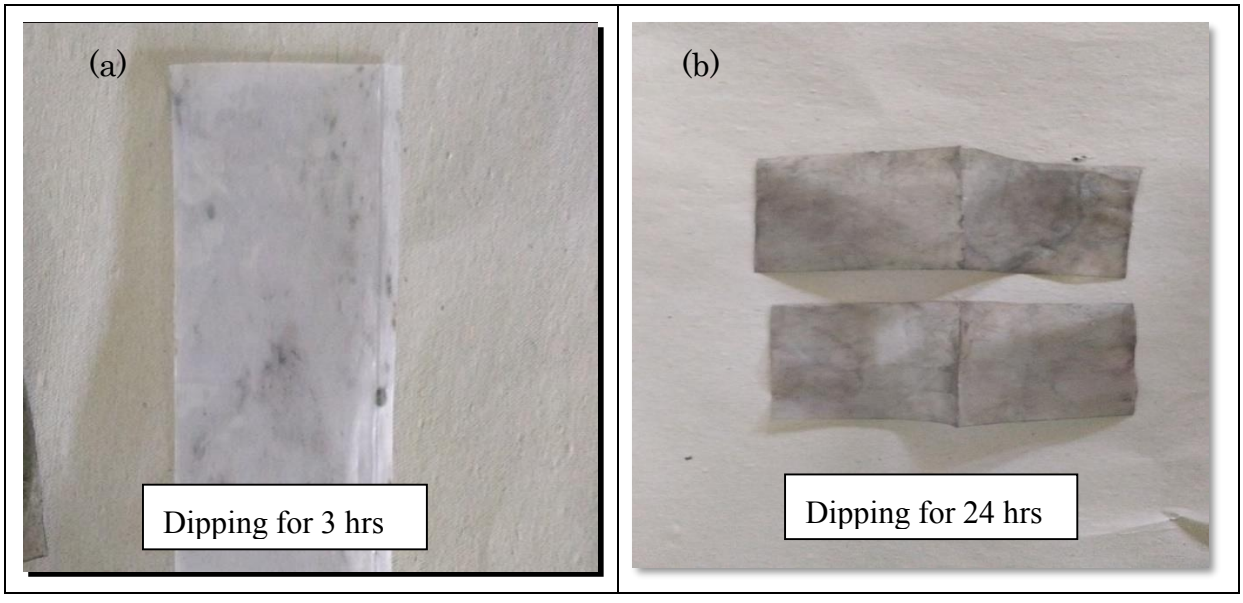

Figure 9: Photos of Ag nanocomposite.

4. CONCLUSION

Silver nanoparticles in the size range of 1-56.9 nm, 2-448.1nm and 3-4705.0 nm have been successfully synthesized using *Moringa oleifera* leaves extract which acts as efficient reducing agent. The antimicrobial efficacies of synthesized silver nanoparticles were more efficient for *Escherichia coli Staphylococcus aureus, Salmonella typhi and Bacillus subtilis.* The dipping of packaging

material (LDPE) in colloidal nanosilver solution was for 3 hour exhibit the betterment of colour of packaging material.

5. ACKNOWLEDGEMENT

The authors are thankful to Shivaji University, Kolhapur for providing research facilities and funding from TEQIP for financial assistance for analytical characterization.

6. CONFLICT OF INTEREST

The author declares that there is no area of conflicts.

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