Antibacterial studies of silver nanoparticles obtained from greenery *Annona reticulata* leaf extract

*Shinde S.A. and Kote J. R.

School of Life Sciences Swami Ramanand Teerth Marathwada University Nanded

Sharad Chandra Arts, commerce and Science College Naigaon Dist- Nanded

Abstract

The present investigation, Ag NPs were synthesized by using an aqueous *Annona reticulata* leaf extract (ARLE) to form an *Annona reticulata* leaf silver nanoparticle (ARLEAgNPs) and corroborated from the X-ray diffraction spectrum. As-synthesized AgNPs were spherical with an average particle size of ~22 nm. Fourier transform infrared spectrum suggested capping of the phyto constituents, probably polyphenols which might appear from the ARLE. The results obtained from the Antibacterial activity assays suggested that these green ARLEAgNPs were more potent against three *E.coli*. Minimum inhibitory concentration value of the ARLEAgNPs was within the range of 10-20 µg/ml.

Keywords: *A. reticulata*; Silver Nanoparticles; Antibacterial, MIC.

1. Introduction

Tuberculosis (TB)is one of the commonly known diseases worldwide where statistically 9.4 million new cases have been registered and 1.7 million people died of TB in 2009 (WHO, 2010). Deadly bacterial pathogen of *M. tuberculosis* is the causative agent of TB. TB is a chronic infectious disease caused by several species of mycobacteria. More than 40 years into the evolution of anti-TB chemotherapy. Due to multidrug resistant strains of mycobacteria and to a high prevalence of tuberculosis in patients who have Acquired Human Immunodeficiency Syndrome (AIDS) and in addition, treatment of HIV-related TB is complex by overlapping drug toxicities and drug-drug interactions between antiretroviral treatment and anti-TB treatment, the risk for development of immune reconstitution inflammatory disease. The more of people

infected with the disease in the world. Development of targeted drug delivery system for tuberculosis is essential to improve patient's compliance. Functionalized silver NPs based drug delivery system which has high affinity towards infected tubercle cells than normal cells [1], might achieve the synthesized ARLEAgNPs have been extensively applied in surgical instruments, wound dressings, bond prostheses and heart valves, electronics, and biosensing [2, 3]. Routinely, ARLEAgNPs are used in textiles, laundry additives, room sprays, water cleaners, and food storage containers [4], cancer [5], catalysis [6], ambient temperature [7]. Colorimetric ensing [8], endophytic fungus [9], how AgNPs interact with the cell membrane of bacteria , Ag^{+} ions bind to sulfur, oxygen, and nitrogen of essential biological molecules and inhibit bacterial growth [10]. Literature studies and fight against specific bacteria like two gram positive as well as two gram negative bacteria [11,12,],

Present investigation, in the plant contain is a valuable source for new antitubercle agents. The present article focuses on how synthesized AgNPs can be targeted on three different *Mycobacterium* species to develop novel antituberculosis agents. The literature reports that many plant based NPs show better results as compared to the chemical based NPs. [10-12]

2. Materials and method

2.1. Materials

All used needful requirement were of analytical grade, Silver nitrate (99.9%), phosphate buffers, streptomycin , were purchased from Himedia, Latur, and Maharashtra, India.

2.2 Collection of pathogen

The *E. coli* were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh (PB), India, were sub cultured and maintained into Lowenstein-Jensen media.

2.3 The Biosynthesis of AgNPS

A. reticulata is a high amount of alkaloids containing belonging to the family Leguminoceae. The leaves of *A. reticulata* used in this study were collected from the Swami Ramanand Teerth Marathawada University Nanded campus, Maharashtra India. The leaves were primarily cleaned with Millipore Deionised (DI) water, washed and dried by pressing with blotting paper. They were then shade-dried and chopped into small pieces. 10 g of leaves in 100 ml of distilled water were microwave-irradiated for 5 min, and the extract was filtered through Whatman filter paper and stored at 4ºC for further experiments. A 100 ml portion of aqueous 0.1 M $AgNO_3$ was move to a conical flask, and 10 ml of aqueous *A. reticulata* extract was added with vigorous magnetic stirring for 10 min. The reduction of Ag^+ ions into Ag NPs at room temperature was completed in 30 min, as observed by the changed color of the liquid extract from greenish-yellow to dark brown to indicate the formation of AgNPs (Fig. 1).For the purification of ARLEAgNPs, the reduced solution was centrifuged at 5000 rpm for 15 min. The supernatant liquid was discarded, and the residue was dispersed in distilled water. The samples were centrifuged about five times to wash distilled water off any nonessential materials from that had been absorbed onto the surface of the ARLEAgNPs.

Fig. 1. Color change during the phytoreduction Ag^+ to Ag NPs, white to brown after 24 h. incubation. a) $AgNO_3$ b) *A. reticulata* leaf extract c) $AgNO_3$ with leaf extract.

2.4 Structural and morphological characteristics

XRD measurement was carried out using a Structural elucidation X-ray diffraction spectrum obtained from X-ray Diffractometer (Rigaku/MAX 2500 V, Cu Kα, λ = 1.5418 ºA) Morphological and optical studies were confirmed from the images and spectra obtained using field-emission scanning electron microscopy (FE- SEM, Hitachi S-4200).The functional groups present in the phyto-constituents in the leaves extract of *A. squamosa* and their involvement in the synthesis of ARLEAgNPs were determined by the FT-IR studies. The ARLEAgNPs were mixed with KBr to make pellet, and the FT-IR analysis was carried out by Nicolet IS-10 (Thermo Fischer Scientific Instruments) FTIR.

2.5. Antimycobacterial activity of the test drug

As per experimental standardization, initially 176mg/100ml(1M) concentration of ARLEAgNPs was used for antimicrobial analysis and it was further taken up to only 20ul/ml add into the well, however the clear zone of inhibition was observed under the experimental condition. The sensitivity test of three different strains of *M.tuberculosis, M.Pheli, and M.avim* was studied by agar diffusion method. In short a sterile cork borer of 7mm diameter was used to bore holes into the inoculums seeded solidified Lowenstein Jensen medium. A 20µl volume of each of the ARLEAgNPs was loaded into the well using a sterile pipette. From the $AgNO_3$ solution(176mg/100ml), 20µl was added in agar well plates.The plates are pre-diffusion of the sample and incubated at 37º C for 48 h. Growth of *M.tuberculosis, M.Pheli, and M.avim* was observed after the diameter of the inhibition zone measured the well size. Rifampicin (10µg/ml) was used as reference standard.

2.6 *In-vitro* stability studies

1ml of ARLEAgNPs was incubated at 37ºC for 48 h. with 0.5 ml of each of, 2-4M saline, PBS pH 4.5,4.7 and sample reserved at specific time intervals such as 2, 4, 6,12, 24, 48 and 72 h. were analyzed spectrophotometrically.

2.7 Temperature dependent stability

To study the stability of ARLEAgNPs, the particles to develop were stored in a refrigerator and analyzed UV-spectra for every week for stability. We observed that under refrigerated condition ARLEAgNPs were more stable for nearly 3 months.

2.8 Minimum inhibitory concentration (MIC)

The MIC determination for the antimycobacterial activity of ARLEAgNPs was studied by employing a microdilution method by Lowenstein-Jensen medium (Broth). The inoculum was prepared by using the method [13]. The ARLEAgNPs to a concentration of 20µl against mycobacterium species. Serial dilutions were studied by by adding culture broth to reach concentrations ranging from 20 µl of each dilution was distributed in 96-well microtitre plates, along with a sterility control and growth control (containing culture broth plus DMSO without antimicrobial substances). Each test and growth control well was inoculated with 5 µl of a bacterial suspension (102 CFU (colony focusing unit) per ml or 98 CFU per well). The present study were performed in equal, and the micro-dilution trays were incubated at 36ºC for 18 h. Bacterial growth was detected by measuring the optical density (ELISA reader, Thermo Multiscan EX Ref:51118170) and by adding an equal concentration of ARLEAgNPs and solvent. The microtitre plates were again incubated at 36 ºC for 30 min, in those wells where bacterial growth occurred, the ARLEAgNPs changed from yellow to brown.

2.9 Viability study

The three *Mycobacterium* species an aliquot of 10 µl of cell suspension from each well was spread on Lowenstein Jensen Medium. The plates were incubated for 48 h at 37 ºC and observed for the presence of colonies on the Lowenstein Jensen medium. MIC of lowly concentration to need kill 99.9% of Mycobacterium *species* [14].

3. Results and discussion

3.1. Structure, bonding morphology studies

In present investigation, AgNPs were successfully synthesized from plant leaves. A change in the color of solution from white to brown after biotransformation during the biosynthetic process [16-18]. Further, the overnight incubation in dark room condition the white color reaction mixture was turned into a dark brown color solution that indicates the ARLEAgNPs synthesis (Fig. 1). The size, shape, morphology, composition and of the prepared NPs [19]. The X-ray diffraction pattern of the biosynthesized silver nanoparticles produced by the leaf *A.reticulata* extract was further studied and confirmed the presence of Ag NPs (Fig. 2-a). A study of our XRD spectrum confirmed that AgNPs were present as nanocrystals, as determined by the XRD peaks at the 2q values 27.70º, 32.48º, 38.30º, 41.10º, 44.30º, 46.80º, 49.34º, 54.31º, 57.15º, 64.4º corresponding to the 110, 120, 022, 122, 032, 041, 112, 023, 132 and 221, planes for Ag respectively. A similar report for XRD was shown in *Morinda citrifolia* leaf extract for synthesized Ag NPs [20] (Fig: 2-a). FTIR result showed that they became valuable tool in indulgent the participation of functional groups in interactions between metal particles and biomolecules [20-22]. In the present study, FTIR measurements were performed to identify the biomolecules responsible for capping and stabilizing the Ag NPs (Fig: 2-b).The FTIR spectrum (Fig: 2-b) of ARLEAgNPs showed a very strong peak at 1011.39 cm^{-1} corresponding to –OH stretching in alcohols and phenolic compounds [20-22]. Peaks observed around 2951.81 cm^{-1}, 1581.71 cm^{-1} were assigned to the C–H stretching vibration of methyl, methylene, and methoxy groups. The medium intense peaks at 2848.12 cm^{-1} and 823.33 cm^{-1} were assigned to the stretching vibrations of C-O and C-C, respectively [20, 23-25]. The vibrational bands corresponding to bonds such as – C-C and C-O from the compounds such as flavonoids and terpenoids in *A.reticulata* leaf hence, it was assumed that these biomolecules could responsible for capping and stabilization.

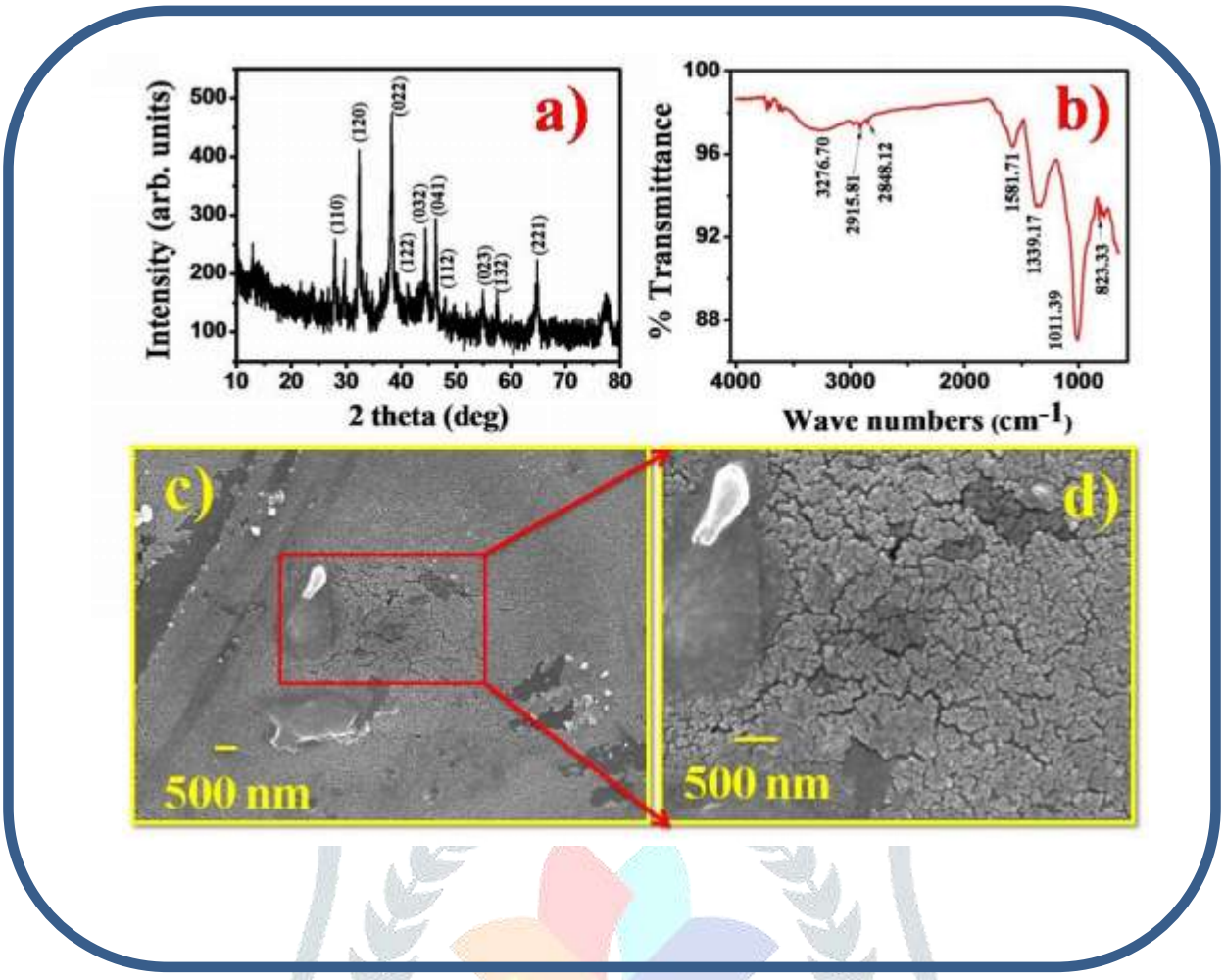

Fig.2. a) XRD, b) FTIR, c) and d) low and magnified SEM images of- ARLEAgNPs

The absorption band at 1339.17 $cm^{-1}$was characteristics of amide II (N-H) from proteins. This result suggests that proteins are interacting with the biosynthesized Ag NPs. The result also proves that ARLEAgNPs to inhibit the growth of three species *E.coli*. FESEM (Fig.2.C and D) clearly shows the presence of the synthesized Ag NPs with magnification with 500nm.

3.2. The antibacterial activity of test drugs

The antimycobacterial activity of three *E.coli* was performed against biosynthesized ARLEAgNPs. The three *E.coli* of are resistant to most new antibiotics resulting in new research into the well-known silver-based nanomaterials including ARLEAgNPs. This effect was the size and dose dependent and more prominent activity against selected *Mycobacterium* strains [26, 27]. To assess the antimicrobial activities of the synthesised NPs, only the nanoparticles that exhibited significant antibacterial activities compared to $AgNO_3$

and standard TB drug, such as, sreptomycin are shown in [Fig 4 a-c].The antimycobacterial activity zone inhibition around the disk of approximately 6 mm each with an $AgNO_3$ also inhibited the growth of *E.coli*.

Triterpenoid, diterpenoid major constituents present in ARL, and these compounds were most likely responsible for the antibacterial efficacy of the plant [28-29]. The inhibition results show that triterpenoid diterpenoid from *A.reticulata* plants leave responsible for the increased antimycobacterial activity of ARL capped AgNPs. Both the ARLEAgNPs test drugs showed only moderate antimycobacterial activity. The antimycobacterial study results for ARLEAgNPs and the standard drug sreptomycin when tested at two concentrations against three different *E. coli* [Fig-1.1- a),b),c)], it is clear that ARL with $AgNO_3$ are shows novel antituberculosis drugs. As compared with the $AgNO_3$ in [Fig-1.1- a),b),c)] were also good antimycobacterial agent. Standard drug Rifampicin showed moderate activity against three species at concentration of 20µg/ml; both ARLEAgNPs, at concentration 20µg/ml and $AgNO_3$ at concentration 20µg/ml to inhibit the growth of three *E.coli*. These results proved that ARLEAgNPs kill the growth of *Mycobacterium* species 99.9% as compared to $AgNO_3$ and Standard drug Rifampicin. This finding indicated that the larger surface area of the NPs could act more effectively on the bacterial cells through the membrane and its charge-related aspects [30]. The antimycobacterial effects of the $AgNO_3$ solutions were measured by determining the minimum concentration needed to inhibit the growth of tested microorganisms. The MIC values of the ARLEAgNPs and the standard against test microorganisms are given in Table 1. The tested microorganisms were completely inhibited at concentrations of 20 µg /ml of ARLEAgNPs and the standard. The $AgNO_3$ solution at a concentration of 20 µg /ml of ARL, $AgNO_3$, and the standard Rifampicin showed inhibition at10 µg /ml. Thus, we can conclude from the results of this study that the $AgNO_3$ inhibited the growth and multiplication of all tested mycobacterium species and also showed potential activity against three mycobacterium species.

Table 1. Minimum inhibitory concentration (MIC) of ARLEAgNPs, $AgNO_3$ and the standard tested against three *E.coli* (µg / ml).

			Minimum inhibitory concentration (µg/ml)
Sr.no	**Test drug**	**Concentration (µg/ml)**	***E.coli***
1	AgNPs	20	*05*
2	AgNPs+ARL	20	*04*
3	Streptomycin	20	*04*

4. Conclusions

In present investigation green route synthesis of bio-functionalized $AgNO_3$ using *A.reticulata* plant extracts in the presence of sunlight to produce ARLEAgNPs. These synthesized ARLEAgNPs are good antibacterial agent. Still date, there have been no reports on the as. *E.coli* against ARLEAgNPs cause the 99.9% cell death. A direct connection between green approach ARLEAgNPs stability and antimycobacterial effects were also demonstrated. The present investigation suggest that apart from its usual biogreenery and biocompatible properties, bio-aspects of green synthesized ARLEAgNPs are also a ecofriendly, easily synthesized, less toxic, targeted oriented. potential antibacterial agent.

References

[1] M J. Hajipour, K. M. Fromm, A. A. Ashkarran, D. J. de Aberasturi, I. R. de Larramendi, T. Rojo,V. Serpooshan, W. J. Parak and M. Mahmoudi, Antibacterial properties of nanoparticles, Trends in Biotechnology 30 (2012) 499.

[2] S.T. Dubas, V. Pimpan. Humic acid assisted synthesis of silver nanoparticles and its application to herbicide detection, Materials Letters 62 (2008) 2661–2663.

[3] X.Y. Xu, Q.B. Yang, J. Bai ,T.C. Lu, Y.X. Li, X.B. Jing, Fabrication of biodegradable electrospun poly (l-lactide-co-glycolide) fibers with antimicrobial nanosilver particles, J Nanosci Nanotechnol. 8 (2008) 5066–70.

[4] C.F. Chau, S.H. Wu, G.C. Yen, The development of regulations for food nanotechnology, Trends Food Sci Technol.18 (2007) 269–80.

[5] V. Raju, T. Ramar, M. Krishnasamy, G. Palani, K. Krishnasamy, k. Soundarapandian, Green biosynthesis of silver nanoparticles from Annona squamosa leaf extract and its in vitro cytotoxic effect on MCF-7 cells, Process Biochemistry 47(2012) 2405–2410.

[6] N. Muniyappan, N.S., Nagarajan Green synthesis of silver nanoparticles with Dalbergia spinosa leaves and their applications in biological and catalytic activities, Process Biochemistry 49 (2014) 1054–1061.

[7] Salprima Yudha S, Doni Notriawan , Eka Angasa , Totok Eka Suharto , John Hendri , Yuta Nishina, Green synthesis of silver nanoparticles using aqueous rinds extract of Brucea javanica (L.), Merr at ambient temperature, Materials Letters 97 (2013) 181–183.

[8] V.V. Kumar, S. Anbarasan, L. R. Christena, N. S. Subramanian, S. P. Anthony, Bio-functionalized silver nanoparticles for selective colorimetric sensing of toxic metal ions and antimicrobial studies, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 129 (2014) 35–42.

[9] B. S. Fathima, R. M. Balakrishnan, Biosynthesis and optimization of silvernanoparticles by endophytic fungus Fusarium solani, Materials Letters 132 (2014) 428–431.

[10] M. Prathap, A. Alagesan, B. D. Ranjitha Kumari, Anti-bacterial activities of silver nanoparticles synthesized from plant leaf extract of Abutilon indicum (L.) Sweet, Nanostruct Chem. 4 (2014) 2-6.

[11] N. Muniyappan, N.S. Nagarajan, Green synthesis of silver nanoparticles with Dalbergia spinosa leaves and their applications in biological and catalytic activities, Process Biochemistry 49 (2014) 1054–1061.

[12] Y. Zhanga, X. Chenga, Y. Zhanga, X. Xuea, Y. Fu, Biosynthesis of silver nanoparticles at room temperature using aqueous aloe leaf extract and antibacterial properties, Colloids and Surfaces A: Physicochem. Eng. Aspects 423 (2013) 63– 68.

[13] Juan, L. et al, Deposition of silver nanoparticles on titanium surface for antibacterial effect, Int. J. Nanomed. 5 (2010) 261–267.

[14] D. M. Alia, N. Thajuddina, K. Jeganathanb, M. Gunasekaranc, Plant extract mediated synthesis of silver and gold nanoparticles and its antibacterial activity against clinically isolated pathogens, Colloids and Surfaces B: Biointerfaces 85 (2011) 360–365.

[15] A. Ahmad, A. Khan, N. Manzoor, L. A. Khan, Evolution of ergosterol biosynthesis inhibitors as fungicidal against Candida, Microb Pathog. 48 (2010) 35–41.

[16] S. Sadhasivam, P. Shanmugam, K. Yun, Biosynthesis of silver nanoparticles by Streptomyces hygroscopicus and antimicrobial activity against medically important pathogenic microorganisms Colloids Surf. B: Biointerfaces 81 (2010) 358.

[17] L. Liu, T. Liu, M. Tade, S. Wang, X. Li , S. Liu, Less is more, greener microbial synthesis of silver nanoparticles, Enzyme and Microbial Technology. 67 (2014) 53–58

18] R. Pazhanimurugan, R. Balagurunathan, A study of the bactericidal, anti-biofouling, cytotoxic and antioxidant properties of actinobacterially synthesised silver nanoparticles, Colloids and Surfaces B: Biointerfaces 111 (2013) 680– 687.

[19] T.Y. Sumana, S.R. Radhika Rajasreea, A. Kanchanab, S. B. Elizabethc, Biosynthesis, characterization and cytotoxic effect of plant mediated silver nanoparticles using *Morinda citrifolia* root extract, Colloids and Surfaces B: Biointerfaces 106 (2013) 74– 78

[20] K.L. Kelly, E. Coronado, L.L. Zhao, G.C. Schatz, The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment,

J. Phys. Chem. B 107 (2003) 668-677.

[21] J. Kasturi, S. Veerapandian, N. Rajendiran, Biological synthesis of silver and gold nanoparticles using apiin as reducing agent, Colloid Surf. B: Biointerfaces 68 (2009)55- 60.

[22] J. Huang, Q. Li, D. Sun , Y. Lu, Y. Su, X. Yang, H. Wang, Y. Wang, W. Shao , N. He, J. Q. Hong and C. Chen, Biosynthesis of silver and gold nanoparticles by novel sundried Cinnamomum camphora leaf, Department of Chemical and Biochemical Engineering, Nanotechnology 18 (2007) 105104

[23] S.S. Shankar, A. Rai, A. Ahmad, M. Sastry, Rapid synthesis of Au, Ag, and bimetallic Au core-Ag shell nanoparticles using Neem (Azadirachta indica) leaf broth, J. Colloid Interface Sci. 275 (2004) 496-502.

[24] M.N. Nadagouda, T.F. Speth, R.S. Varma, Microwave-assisted green synthesis of silver nanostructures, Acc. Chem. Res. 44 (2011)469- 478.

[25] J. Puiso, D. Jonkuviene, I. Macioniene, J. Salomskiene, I. Jasutiene, R. Kondrotas, Biosynthesis of silver nanoparticles using lingonberry and cranberry juicesand their antimicrobial activity, Colloids and Surfaces B: Biointerfaces 121 (2014) 214–221

[26] V. Gopinath, D. Mubarak Ali, S. Priyadarshini, N.M. Priyadharsshini, N. Thajuddin, P. Velusamy, Biosynthesis of silver nanoparticles from *Tribulus terrestris* and its antimicrobial activity: a novel biological approach, Colloids Surf. B: Biointerfaces 96 (2012) 69-74.

[27]T. D. Thang , P.C. Kuo , G. J. Huang , N. H. Hung, B.S. Huang, M.L. Yang, N. X. L. and T.S. Wu. Chemical Constituents from the Leaves of *Annona reticulata* and Their Inhibitory Effects on NO Production, Molecules 18 (2013) 4477-4486

[28] P. Raveendran, J. Fua, S.L. Wallen, A simple and green method for the synthesis of Au, Ag, and Au–Ag alloy nanoparticles, Green Chem. 8 (2006) 34–8.

[29] L. Kvitek, A. Panacek, J. Soukupo, M. Kolar, R. Vecerova, R. Prucek, M. Holecova, R. Zboril, Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity, J Phys Chem C. 112 (2008) 5825–9.

[30] Ekaterina Gelman, John D. McKinney, and Neeraj Dhar, Malachite Green Interferes with Post antibiotic Recovery of Mycobacteria Antimicrob Agents Chemother. 56 (2012) 3610–3614.

[31] Aijaz Ahmad , Amber Khan , Nikhat Manzoor, Luqman A. Khan, Evolution of ergosterol biosynthesis inhibitors as fungicidal against Candida, Microbial Pathogenesis 48 (2010) 35–41.