JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 1131-1140

Evaluation of Improved Antibacterial Activity of Chitosan Coated Silver Nanoparticles- Azithromycin Nanoconjugate against Clinical Isolate of Human Pathogenic Bacteria

S. Karthick Raja Namasivayam* and S. Ganesh

Department of Biotechnology, Sathyabama University, Chennai, India.

(Received: 17 September 2012; accepted: 15 November 2012)

The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas ofsolar energy conversion, catalysis, medicine, and water treatment. In present situation, silver nanoparticles are in great use in the medicinal, pharmaceutical, agricultural industry and in water purification.. In the present study,enhanced antibacterial activity of chitosan coated silver nanoparticles-Azithromycin nanoconjugate against clinical isolate of *Pseudomonas aeruginosa* was studied. Synthesized nanoconjugate was characterized by Fourier Transform Infrared Spectroscopy (FT-IR),scanning electron microscopy and Energy-dispersive X-ray spectroscopy (EDX) which reveals the formation of silver nanoparticles- Azithromycin nanoconjugate.Anti bacterial activity against clinical isolate of *E.coli* and *Pseudomonas aeruginosa* showed distinct activity The highest increase in inhibitory zone was observed with silver nanoparticles-azithromycin nanoconjugate and showed 60 to 100 fold increase of antibacterial activity. This result suggests that the synergistic activity of nanoparticles with antibiotics which will lead to new generation of antimicrobial agents with highest efficacy to prevent pathogenic infection.

Key words: Silver nanoparticles, azithromycin, nanoconjugate, *Pseudomonas aeruginosa*, antibacterial activity.

The discovery of antibiotics in the 20th Century marked a watershed in the treatment of infections. The ability to treat the serious infections of the pre-antibiotic era stimulated advances in medical fields and enlarged the scope of medical care. However, while a drastic change has taken place in the causes of fatal infections, they are still a major cause of death the world over[1] .While demographic changes and drug access issues are important reasons in the developed and developing worlds, respectively, "relentless and Dizzying Rise of Antimicrobial Resistance" has contributed in a large measure to the persistence of infections as a major cause of morbidity and mortality[2]. The rapid emergence of resistance to antibiotics amongst pathogens generates visions of the 'potential post-antibiotic era threatening present and future medical advances[3,4].

Nanotechnology is significant on account of its pre-eminence upon the comprehension, use, and control of matter at magnitudes of a minute scale, akin to approaching atomic levels, with which to manufacture new substances, instruments, and frameworks[5,6]. Nanoparticles possess exceptional physical and chemical properties, which lead to rapid commercialization. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging,

* To whom all correspondence should be addressed.
E-mail: biologiask@gmail.com

sensing, targeted drug delivery, gene delivery Systems and artificial implants. Hence, nanosized organic and inorganic particles are catching increasing attention in medical applications due to their amenability to biological functionalization[7,8] .Synthesis of noble metal nanoparticles for applications such as catalysis, electronics, optics, environmental, and biotechnology is an area of constant interest. Gold, silver, and copper have been used mostly for the synthesis of stable dispersions of nanoparticles, which are useful in areas such as photography, catalysis, biological labeling, photonics, optoelectronics and surface-enhanced Raman scattering (SERS) detection[9].In present situation, silver nanoparticles are in great use in the medicinal, pharmaceutical, agricultural industry and in water purification[10,11]. Since noble metal nanoparticles such as silver, gold nanoparticles are widely applied to human contacting areas. Silver nanoparticles have been reported to have antimicrobial activity against a wide range of microorganism[12].Taken together, this compound as a highly safe compound may be considered for combination therapy against pathogenic microorganism due to its potential synergistic effect with important antibiotics[13]. These nanoparticles exhibit tolerable monodispersity and in the case of particles synthesized extracellularly, exhibit excellent long term stability. Silver nanoparticles have been reported to have antimicrobial activity against a wide range of microorganisms[14]. In the present study the improved antibacterial activity of chitosan stabilized silver nanoparticles-Azithromycin nanoconjugate was evaluated against *Pseudomonas aeruginosa*

MATERIALS AND METHODS

Synthesis and characterization of silver nanoparticles

Silver nanoparticles (AgNPs) were synthesized by chemical reduction of 0.1 M silver nitrate with 0.1 M tri sodium citrate with 0.1 M sodium borohydride as reducing agent. Synthesis of silver nanoparticles was confirmed by the conversion of the reaction mixture into brown colour and further characterization of the synthesized silver nanoparticles was carried out with determination of Plasmon absorption maxima with UV-Vis spectroscopy and particle morphology with Transmission Electron Microscopy (TEM) and Fourier Transform Infrared Spectroscopy (FT-IR) analysis was done which helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanoparticles

Antibacterial activity of chemogenic silver nanoparticles

The antibacterial activity of silver nanoparticles (AgNPs) was tested against pathogenic bacteria *E.coli* and *Pseudomonas aeruginosa* was obtained from Chrompet general hospital, Chennai. The strains were maintained on Brain - Heart Infusion Agar slants. The inoculum was prepared in Nutrient broth. After 24 hours, the inoculum was spread with sterile cotton swab on Nutrient agar plate. Wells (8mm) were made using sterilized cork borer and 0.25mg/ml, 0.50mg/ml, 0.75mg/ml and 1mg/ml of different concentration of silver nanoparticles were added separately. The seeded plates were incubated at 37^0C for 24 hours and the plates were observed for zone of inhibition. After the incubation period, the diameter of the zone was recorded.

Evaluation of antibacterial activity of free azithromycin

Active pharmaceutical ingredient (API) of azithromycin was originally obtained from Jubilant life sciences, New Delhi was used in the present study. Different concentration of azithromycin was prepared in deionised water and the antibacterial activity was studied by well diffusion method as described earlier.

Preparation of chitosan coated silver nanoparticles ˘azithromycin nanoconjugate

Chitosan was obtained from Rolex chemical industries, Mumbai and refined twice by dissolving it in dilute HOAc solution. The solution was filtered, the chitosan was precipitated with aqueous sodium hydroxide, and the precipitate was dried in vacuum at room temperature[7]. chitosan stabilized azithromycin-silver nanoparticles conjugate was prepared with aqueous solution of Azithromycin 3mg and 0.25mg/ml, 0.50mg/ml, 0.75mg/ml, 1.00mg/ml of synthesised nanoparticles plus 0.2% of chitosan solution. The mixture was stirred under magnetic stirrer for 3hrs to get the complete homogenous mixture. The slurry thus

obtained was freeze dried and used for further studies. Characterisation of nanoparticles loaded antibiotic was carried out with FT-IR and Scanning electron microscope (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX) used for quantitative detection and localization of elements in the nano specimens.

Evaluation of antibacterial activity of chitosan stabilized Ofloxacin-silver nanoparticles conjugate

Different concentration of chitosan stabilized azithromycin-silver nanoconjugate was evaluated against *E.coli* and *Pseudomonas aeruginosa* by well diffusion assay as described earlier.

RESULTS AND DISCUSSION

Synthesis and characterization of silver nanoparticles

Silver nanoparticles (AgNPs) synthesis adopting chemical reduction was primarily confirmed by colour change of the reaction mixture from pale yellow to brown clearly indicating the formation of silver nanoparticles (Fig-1). The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation. Brown colour formation was observed within 10 minutes after the drop wise addition of sodium borohydride to the silver nitrate and tri sodium citrate solution. Synthesized silver nanoparticles characterized by UV-Vis spectroscope which reveals a strong broad surface Plasmon peak located at 420nm (Fig-2). Particle morphology size and shape with transmission electron microscopy reveals spherical particles with the size of 20- 40nm (Fig-3).

Evaluation of antibacterial activity of silver nanoparticles

All the tested concentration of silver nanoparticles (AgNPs) showed antibacterial activity against *E-coli*. No inhibition was recorded in *Pseudomonas aeruginosa* at all the tested concentration (Table-5 and Fig-11). In *E-coli* the zone of inhibition recorded at respective concentration 0.0025mg/ml, 0.0050mg/ml, 0.0075mg/ml and 0.0100mg/ml was 20, 23, 26 and 30mm respectively (Table-2 and Fig-8).

Antibiogram of free azithromycin

Different concentration of azithromycin

Table 1. Zone of inhibition (mm) of free azithromycin with different concentration (mg/ml) against *Escherichia coli*

S. No	Concentration (mg/ml)	Zone of inhibition(mm)
1.	0.25	34
2.	0.50	37
3.	0.75	39
4.	1.00	41
5.	0.025	32
6.	0.050	34
7.	0.075	37
8.	0.100	39
9.	0.0025	30
10.	0.0050	32
11.	0.0075	35
12.	0.0100	33
13.	0.0050	32
14.	0.0100	34
15.	0.0150	36
16.	0.0200	39
17.	0.0075	31
18.	0.0150	34
19.	0.0225	37
20.	0.0300	39
21.	0.0025	27
22.	0.0050	31
23.	0.0075	34
24.	0.0100	37
25.	0.00125	23
26.	0.00250	26
27.	0.00375	30
28.	0.00500	32
29.	0.00025	19
30.	0.00050	22
31.	0.00075	25
32.	0.00100	28
33.	0.0003	20
34.	0.0006	26
35.	0.0015	28
36.	0.003	30
37.	0.0045	32
38.	0.006	34
39.	0.00015	16
40.	0.0003	20
41.	0.00075	23
42.	0.0015	25
43.	0.00225	28
44.	0.003	30
45.	0.00003	00
46.	0.00006	10
47.	0.00015	18
48.	0.0003	21
49.	0.00045	23
50.	0.0006	25

Table 2. Zone of inhibition (mm) of Silver nanoparticles (AgNPs) with different concentration (mg/ml) against *Escherichia coli*

S. No	Concentration (mg/ml)	Zone of inhibition (mm)
1.	0.0025	20
2.	0.0050	23
3.	0.0075	26
4.	0.0100	30

inhibited both the tested strains expect 0.00003mg/ml in *E.coli* (Fig-7(K)) and 0015mg/ml,0.0003mg/ml,0.00045mg/ml,0.006mg/ml in *Pseudomonas aeruginosa* (Fig-10(k)). In *E-coli* maximum inhibition was recorded in 1mg/ml and 0.1mg/ml as 41 and 39mm (Table-1, Fig-7(A) and Fig-7(B)). In *Pseudomonas aeruginosa* highest zone of inhibition was observed in 1mg/ml and 0.1mg/ml as 40 and 40mm (Table-4, Fig-10(a) and Fig-10(b)).

Table 3. Zone of inhibition (mm) of chitosan stabilized azithromycin + Sliver nanoconjugate (mg/ml) against *Escherichia coli*

S.	Azithromycin		Silver nnaoparticles		Chitosan stabilized Azithromycin-silver nanoconjugate	
No	Concentration (mg/ml)	Zone of inhibition (mm)	Concentration (mg/ml)	Zone of inhibition (mm)	Concentration (mg/ml)	Zone of inhibition (mm)
1.	0.00015	18	0.0025	20	0.00015 + 0.0025	45
2.	0.0003	21	0.0050	23	0.0003 + 0.0050	45
3.	0.00045	23	0.0075	26	0.00045 + 0.0075	45
4.	0.0006	25	0.0100	30	0.0006 + 0.0100	45

Preparation and characterisation of chitosan stabilized azithromycin -silver nanoparticles conjugate

Chitosan stabilized azithromycin -silver nanoparticles conjugate was primarily confirmed by colour change of the reaction mixture from dark brown to pale yellow, scanning electron microscopy analysis and FT-IR. Scanning electron microscopy (SEM) images were recorded by using a Carlzeiss Supra 55 field emission scanning electron microscope equipped with an energy-dispersive spectrum (EDS, oxford instruments) capability. In a SEM setup, the nanoparticulate sample, coated to be conductive (e.g. gold, palladium), is scanned in a high vacuum chamber with a focused electron beam. The scanning electron microscopy study reveals chitosan stabilized azithromycin-silver nanoparticles conjugate as spherical particles with the size range of 190 to 200nm (Fig-4). FT-IR analysis helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanoparticles. The profiles of FTIR spectroscopy of silver nanoparticles reveals the main adsorption 3904.98cm^{-1}, 3661.30cm^{-1}, 2092.27cm^{-1}, 1638.22cm^{-1} and 867.02cm^{-1}(Fig-5). A new revealed adsorption was recorded in chitosan stabilized azithromycin-silver nanoparticles conjugate 3096.73cm^{-1}, 3463.11cm^{-1}, 2368.34cm^{-1}, 2083.27cm^{-1}, 1638.16cm^{-1} and 705.17cm^{-1} (Fig-6).

The SEM analyzer built-in with an EDX micro-analyzer allows a quantitative detection and localization of elements in the nano specimens. The EDX images illustrated the presence of larger amount of carbon, oxygen in a range of 53.09 and 25.75% respectively; in addition, elements like nitrogen, fluorine and silver were identified in a range of 15.36, 1.08 and 3.72% respectively (compound percentage) in azithromycin-silver nanoparticles conjugate (Fig-4).

Antibacterial activity of chitosan stabilized azithromycin -silver nanoparticles conjugate

It can be seen that the chitosan stabilized antibiotic nanoparticles conjugate retarded

Table 4. Zone of inhibition (mm) of Azithromycin with different concentration (mg/ml) against *Pseudomonas aeruginosa*

S. No	Concentration (mg/ml)	Zone of inhibition(mm)
1.	0.25	32
2.	0.50	35
3.	0.75	37
4.	1.00	40
5.	0.025	30
6.	0.050	34
7.	0.075	36
8.	0.100	40
9.	0.0025	24
10.	0.0050	26
11.	0.0075	29
12.	0.0100	31
13.	0.0050	27
14.	0.0100	30
15.	0.0150	33
16.	0.0200	36
17.	0.0075	30
18.	0.0150	33
19.	0.0225	35
20.	0.0300	38
21.	0.0025	21
22.	0.0050	27
23.	0.0075	30
24.	0.0100	34
25.	0.00125	20
26.	0.00250	24
27.	0.00375	27
28.	0.00500	32
29.	0.00025	19
30.	0.00050	23
31.	0.00075	27
32.	0.00100	29
33.	0.0015	17
34.	0.003	21
35.	0.0045	24
36.	0.006	30
37.	0.00075	14
38.	0.0015	19
39.	0.00225	23
40.	0.003	27
41.	0.00015	00
42.	0.0003	00
43.	0.00045	00
44.	0.0006	00

Fig. 1. Silver nanoparticles synthesised by chemogenic method

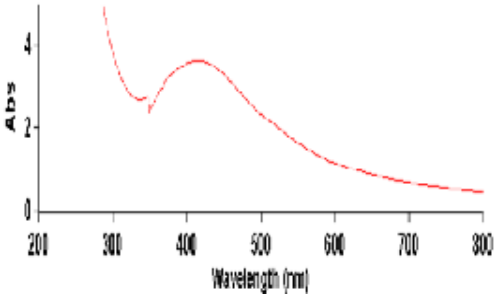


Fig. 2. UV-vis absorption spectra of silver nanoparticles

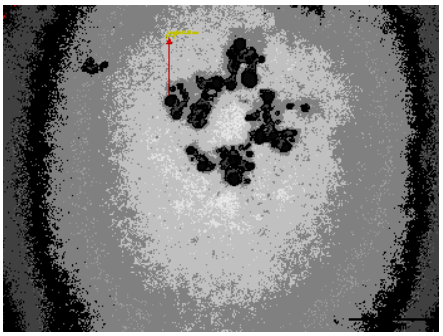


Fig. 3. Scanning electron micrograph of Silver nanoparticles

Table 5. Zone of inhibition (mm) of Silver nanoparticles (AgNPs) with different concentration (mg/ml) against *Pseudomonas aeruginosa*

S. No.	Concentration (mg/ml)	Zone of inhibition (mm)
1.	0.0025	00
2.	0.0050	00
3.	0.0075	00
4.	0.0100	00

Table 6. Zone of inhibition (mm) of chitosan stabilized Azithromycin - Sliver nanoconjugate against *Pseudomonas aeruginosa*

S. No	Azithromycin		Silver nnaoparticles		Chitosan stabilized Azithromycin-silver nanoconjugate	
	Concentration (mg/ml)	Zone of inhibition (mm)	Concentration (mg/ml)	Zone of inhibition (mm)	Concentration (mg/ml)	Zone of inhibition (mm)
1.	0.00015	00	0.0025	00	0.00015 + 0.0025	20
2.	0.0003	00	0.0050	00	0.0003 + 0.0050	27
3.	0.00045	00	0.0075	00	0.00045 + 0.0075	31
4.	0.0006	00	0.0100	00	0.0006 + 0.0100	43

bacterial growth to a degree comparable to that demonstrated by the free antibiotic. When the free antibiotic conjugated with nanoparticles the diameter of the zone of inhibition were increased by 60 to 100 folds.Chitosan stabilized azithromycin -silver nanoparticles conjugate reveals distinct increase in antibacterial activity against both the tested strain (Table 3 and 6, Fig-9 and 12). In *E-coli* the maximum inhibition was recorded in 0.00015+0.0025mg/ml, 0.0003+0.0050mg/ml,

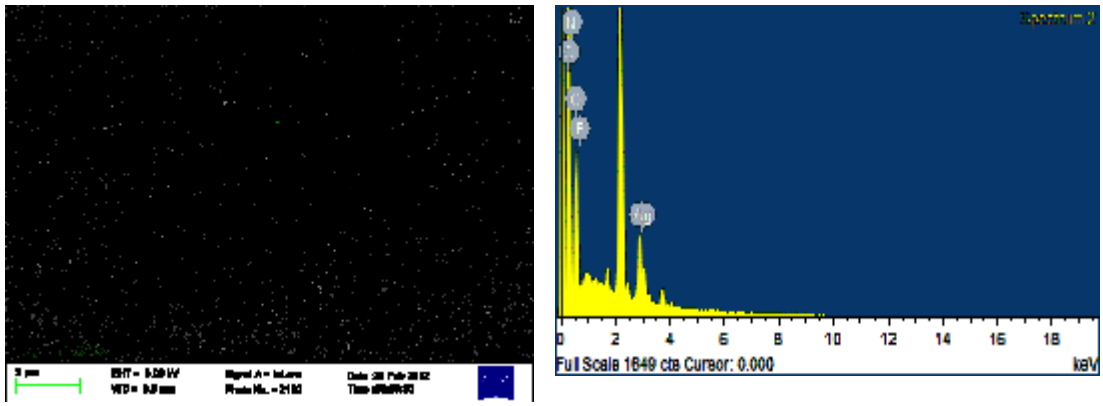


Fig. 4. Scanning electron micrograph and EDX of Chitosan stabilized azithromycin-silver nanoparticles conjugate

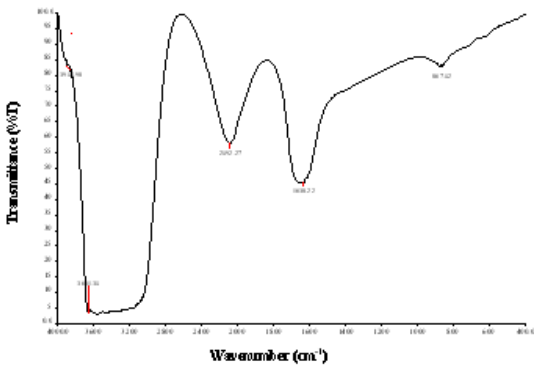


Fig. 5. FT-IR spectra for Silver nanoparticles

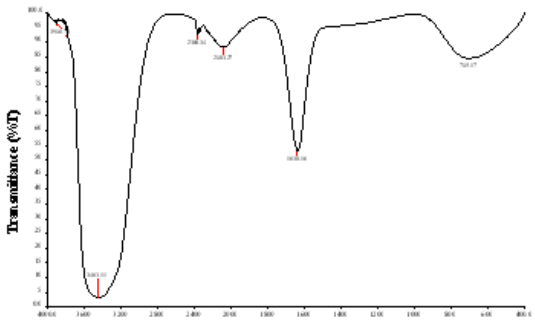
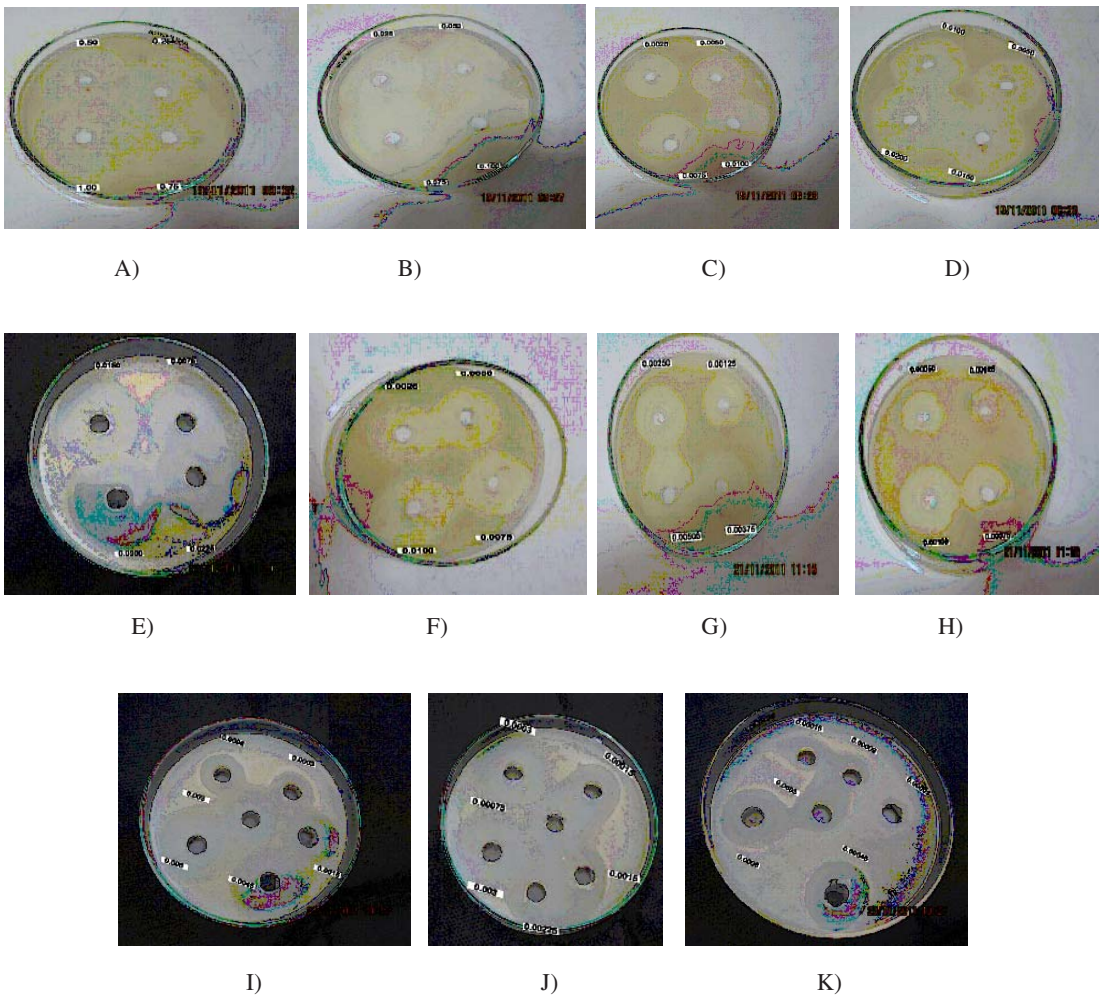


Fig. 6. FT-IR spectra for chitosan stabilized azithromycin-Silver nanoparticles conjugate



A) B) C) D)

E) F) G) H)

I) J) K)

Fig. 7. Zone of inhibition of azithromycin with different concentration (mg/ml) against *Escherichia coli*

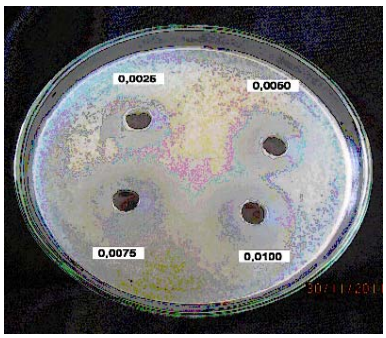


Fig. 8. Zone of inhibition of Silver nanoparticles (AgNP) with different concentration (mg/ml) against *Escherichia coli*

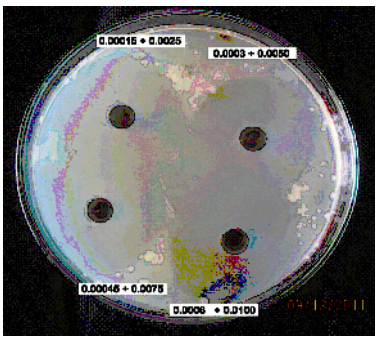


Fig. 9. Zone of inhibition of azithromycin + Sliver nanoparticles (AgNPs) with different concentration (mg/ml) against *Escherichia coli*

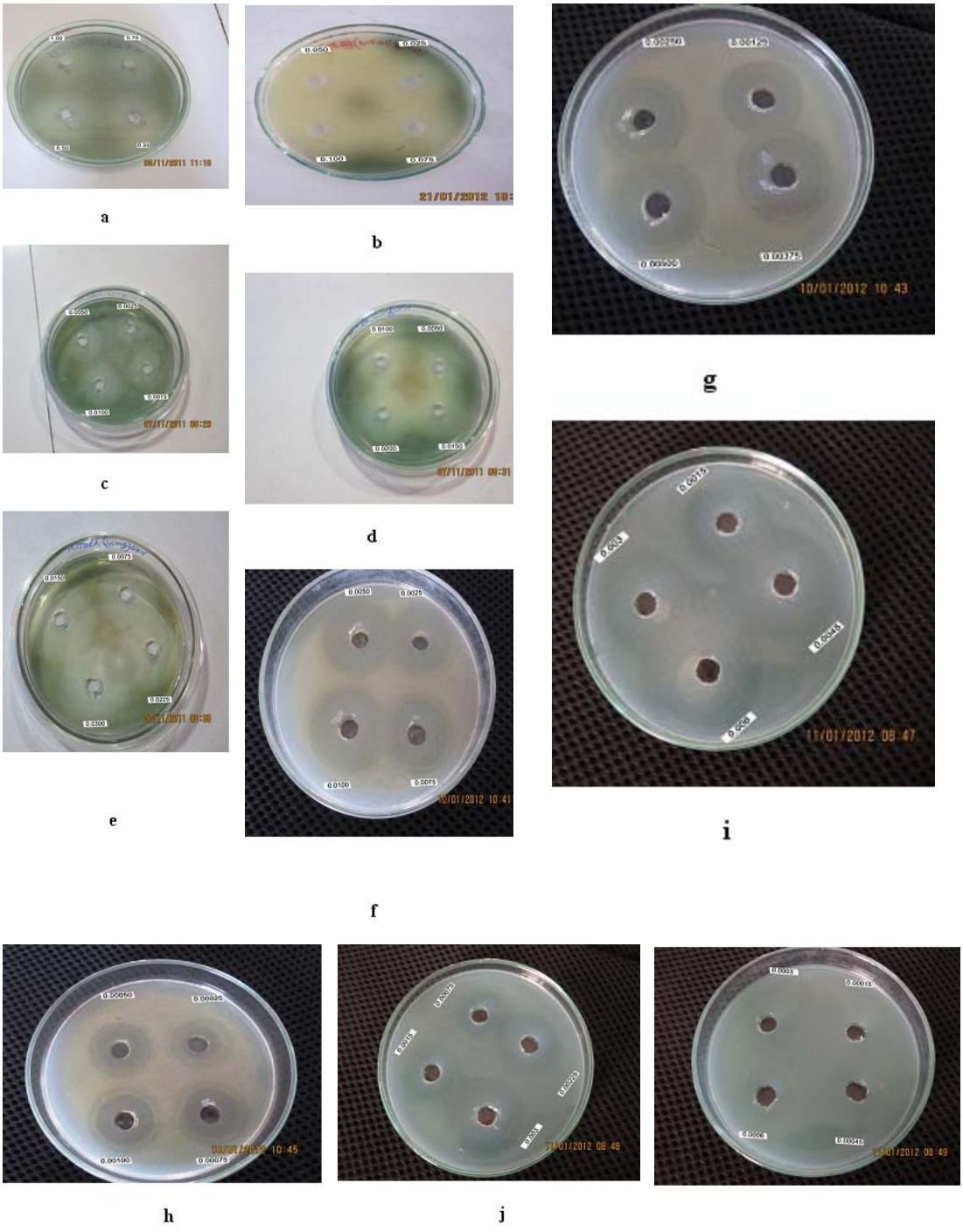


Fig. 10. Zone of inhibition of Azithromycin with different concentration (mg/ml) against *Pseudomonas aeruginosa*

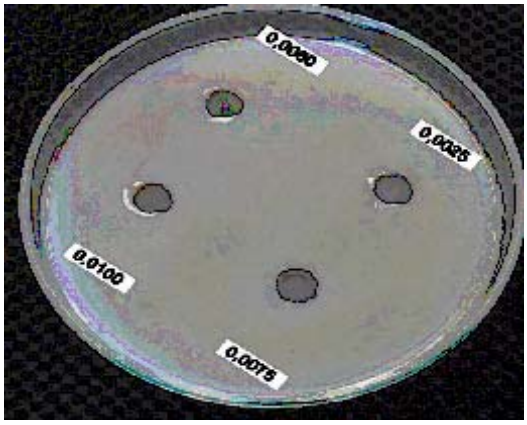


Fig. 11. Zone of inhibition of Silver nanoparticles (AgNP) with different concentration (mg/ml) against *Pseudomonas aeruginosa*

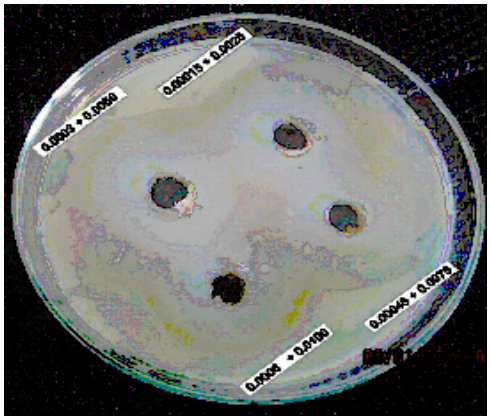


Fig. 12. Zone of inhibition of Azithromycin- Sliver nanoparticles (AgNPs) with different concentration (mg/ml) against *Pseudomonas aeruginosa*

0.00045+0.0075mg/ml and 0.0006+0.0100mg/ml Concentration with the zone of inhibition of 45mm (Fig-9) and improved efficacy was found to be 60,53.33,48.88 and 44.44% respectively. Similar improved activity of Azithromycin AgNP against *Pseudomonas aeruginosa* was recorded in 0.00015+ 0.0025mg/ml, 0.0003+ 0.0050mg/ml, 0.00045+0.0075mg/ml, 0.0006+ 0.0100mg/ml Concentration with the zone of inhibition of 20, 27, 31 and 43mm respectively (Fig-12) and improved efficacy was found to be 100% at all concentration respectively. Similar studies on improved activity of antibiotics with nanoparticles conjugate against pathogenic bacteria has been reported. Enhanced antibacterial activity of oflaxacin - biogenic silver nanoconjugate, gentamicin-gold nanoconjugate, Azithromycin-gold nanoconjugate, cloaxicillin and tetracycline-silver nanoconjugate against methicillin resistance *Staphylococcus aureus*,quinolone capped colloidal gold nanoparticles against *Staphylococcus aureus, Micrococcus luteus, Escherichia coli* and *Pseudomonas aeruginosa* studied by various workers clearly revealed the development of effective and alternative source of antimicrobials against human pathogenic bacteria[7,9,11,13-15] Synergistic antibacterial activity of anti bacterial antibiotics-silver nanoconjugate coated on cotton fabrics against *Staph.aureus* reported by Karthickrajanamasivayam *et al.*,[9] will lead to fabrication of effective antimicrobial dressing against human pathogems.The present study clearly reveals the enhanced activity of antibiotic with silver nanoparticles against pathogenic bacteria would suggest the possible use of nano formulated drug to fight against pathogenic bacteria.

ACKNOWLEDGEMENTS

We acknowledge Centre for Nanoscience and Technology, International Research Centre(IRC),Sathyabama University for the SEM and EDAS analysis assistance.

REFERENCES

1. Raghunath, D. Emerging antibiotic resistance in bacteria with special reference to India. *J. Biosci*., 2008; **33**(4)**:** 593–603
2. Dahl, J. A.,Maddux,B.L.,Hutchison,J.E.Towards greenter nanosynthesis.*Chemical reviews*., 2007; **107**(6): 2228-69
3. Hutchison, J.E.Greener Nanoscience: A Proactive approach to Advancing Applications and reducing implications of Nanotechnology. *ACS Nano.,* 2008; **2**(3): 395-402
4. Anastas, P.T., Warner, J.C.Green Chemistry-Theory and Practice,Oxford University Press, London; 1998; pp 30-36
5. Karthick Raja Namasivayam, S.,Chitrakala,K. Ecotoxicological effect of *Lecanicillium lecanii*

(Ascomycota:Hypocreales) based silver nanoparticles on growth parameters of economically important plants. *Journal of Biopesticides*, 2011; **4**(1): 97-101.

6. Karthick Raja Namasivayam, S.,Ganesh, S., Avimanyu,B. Evaluation of anti-bacterial activity of silver nanoparticles synthesized from *Candida glabrata* and *Fusarium oxysporum*. *Int J Med Res.,* 2011; **1**(3):131-136
7. Grace,D., Nirmala,A.,Pandian, K. Quinolone antibiotic-capped gold nanoparticles and their antibacterial efficacy against gram positive and gram negative organisms, *Journal of Biosciences*, 2007; **1**(2): 96-105
8. Karthick Raja Namasivayam S., Chandrasekar, S., Savitha V.A first report of antifungal effect of butanol cell free extract of *Streptomyces griseoaureofaciens*, *Journal of Pharmacy Research*.,2010 ;**3**(9): 2188-2189
9. Karthick Raja Namasivayam,S.,Avimanyu,B. Silver nanoparticle synthesis from *lecanicillium lecanii* and evalutionary treatment on cotton fabrics by measuring their improved antibacterial activity with antibiotics against *Staphylococcus aureus* (ATCC 29213) and *E. coli* (ATCC25922) strains, *Int J Pharm Pharm Sci*, 2011; **3**(4): 190-195
10. Zhang, L, Jiang, Y.,Ding,Y.,Povey.,M, York, D. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). *J Nanopart Res,* 2007; **9**(3): 479-489
11. Thati,V., Roy, A.S.,Prasad, M V N,. Shivannavar, C T, Gaddad, S M.Nanostructured zinc oxide enhances the activity of antibiotics against *Staphylococcus aureus*. *J. Biosci Tech*, 2010; **1**(2): 64 69.
12. Karthick Raja Namasivayam, S., Ghanadrakumar, E., Reepika, R. Synthesis of silver nanoparticles by *Lactobaciluus acidophilus* 01 strain and evaluation of its *in vitro* genomic DNA toxicity.*Nano Micro Letters,* 2010; **2**(3): 160-163
13. Burygin,G L., Khlebtsov,B.N.,Shantrokha, A.N., Dykman,L., Bogatyrev,V A.,Khlebtsov, N G. On the Enhanced Antibacterial activity of antibiotics mixed with gold nanoparticles, *Nanoscale Res Lett*, 2009; **4**: 794–801
14. Karthick Raja Namasivayam, S., Ganesh, S. Enhanced anti bacterial activity of chitosan stabilized Azithromycin-gold nanoconjugate against clinical isolate of *E.coli* and *Pseudomonas aeruginosa*.*J.Biotechnol.Biomate*, 2012; **2**(6): 86-89
15. Karthick Raja Namasivayam,S., Elamathy, K., Brijesh, K. Effect of biologically synthesized silver nanoparticles with plant products and chemotherapeutics against biofilm of clinical isolates of *Staphylococcus aureus* and *Candida tropicalis, Journal of Biotechnology and Biotherapeutics*, 2011; **1**(3): 17-21.