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RESEARCH ARTICLE

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING OCIMUM AMERICANUM AQUEOUS LEAF EXTRACT AND STUDY OF ITS ANTIMICROBIAL ACTIVITY AGAINST ESBL AND MBL PRODUCING PATHOGENS

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ABSTRACT

The present study reports the synthesis of silver nanoparticles (Ag-NPs) using aqueous extract of *Ocimum americanum* (L.) leaf and silver nitrate ($AgNO_3$) solution. The synthesized Ag-NPs were analysed using UV-Visible Spectroscopy (UV-Vis), Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The UV-Vis spectrum ofAg-NPs in aqueous solution showed an absorbance peak around 424nm, which is a characteristic property of nanoparticle formation. The antimicrobial property of Ag-NPs was analyzed by qualitative methods like disc and well diffusion method against Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) producing E.coli, Pseudomonas, Proteus, Citrobacter and Klebsiella species. The mean zones of inhibition for Ag-NPs and AgNO3were found to be in the range of 14-20mm for the test pathogens. Also the nanoparticle coated dressings showed antibacterial activity against the same, indicating its potential to control wound bio-burden and hence reduce the risk of further infection.

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INTRODUCTION

The increasing prevalence of antibiotic resistant bacteria in hospitals and the community has significantly limited the effectiveness of current drugs resulting in treatment failure. In recent years, global spread of Extended Spectrum β-Lactamase (ESBL) producing pathogens is exclusive, and has been reported from many parts of the world (Szijarto *et al.,* 2014). ESBLs are enzymes that are produced by pathogenic bacteria, making them resistant to 3rd generation cephalosporins e.g. cefuroxime, cefotaxime and ceftazidime, which are the most widely used antibiotics for treatment of common infections in many hospitals (Rawat and Nair, 2010). Metallo β-Lactamases (MBLs) are bacterial zinc enzymes that are able to hydrolyse most β-lactam antibiotics (Yong *et al.,* 2009). In addition, they also show a high degree of resistance to other groups of antibiotics (Queenan and Bush, 2007). Looking at the severity of problem and present situation, the development of new antimicrobial compounds or, the modification of those available, in order to improve antimicrobial activity for therapy, antisepsis or disinfection, is currently a high priority area of research (Duran *et al.,* 2010). There is a concern regarding the lack of discovery of new effective antibiotics especially for multidrug-resistant gram-negative bacteria which produce ESBL enzyme. One of the recent efforts in addressing this challenge lies in exploring antimicrobial activity of Silver Nanoparticles (Ag-NPs), to combat the problem of widespread drug resistance (Huh and Kwon, 2011). Nano-medicine offers the potential to manipulate materials and therapeutic agents at the scale of molecules, so that the medications can be precisely delivered, to the target area. Nanoparticles (NPs) exhibit entirely new or improved properties compared to the original material, and present a higher surface area to volume ratio, which is related to the enhancement of antimicrobial activity (Bhirde *et al.,* 2014). Use of silver has been severely limited by the toxicity of silver ions to humans. However, nanotechnology facilitates the production of smaller silver particles with increased surface area to volume ratios, increasing the efficacy of bactericidal action at a very low concentration (Sladkova *et al.,* 2009). As a result, Ag-NPshave been subjected to a wide range of applications, most importantly as antimicrobial agents to prevent infections such as in burn and traumatic wound dressings, diabetic ulcers, coating of catheters, dental cavities,

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scaffold, and medical devices (Rai *et al.,* 2009). The chemical and physical methods of nano-silver production are extremely expensive and also involve the use of toxic chemicals, which may pose potential environmental and biological risks. It is an unavoidable fact that the Ag-NPssynthesized, have to be handled by humans, and must be available at cheaper rates for their effective utilization; thus, there is a need for an environmentally and economically feasible way to synthesize these nano-particles. The quest for such a method has led to the need for biomimetic production of Ag-NPs whereby biological methods are used recently for the same (Prabhu and Poulose, 2012). Plant extracts are rich sources of secondary metabolites. Most of the extracts contain several compounds that can easily reduce silver nitrate ($AgNO_3$) to Ag-NPs. Biological methods for the production of nano-particles are considered as a safe and environment friendly alternative to the conventional physical and chemical methods as it is cost effective, and the usage of high pressure, energy, temperature and toxic chemicals is completely eliminated (Prabhu and Poulose, 2012). The genus Ocimum belonging to Lamiaceae family, has worldwide distribution and consists of 160 species with 24 species native to India (Gupta, 1994). *Ocimum americanum* is a traditional aromatic medicinal plant distributed all over India mostly on waste lands, river banks and sides of paddy fields. The essential oils of Ocimum contain compounds such as euginol, linalool, geraniol, 1, 8-cineol, citral and camphor (Selvi *et al.,* 2012). These essential oils are being used as pharmaceutical agents because of their anti-cancer, anti-asthamatic, anti- stress, antimicrobial (Nascimento *et al.,* 2011), anti-diabetic (Nyarko *et al.,* 2002) and anti-oxidant properties (Behera *et al.,* 2012). This studyreports the synthesis of Ag-NPs using *Ocimum americanum* aqueous leaf extract and its antimicrobial activity against ESBL and MBL producing pathogens.

MATERIALS AND METHODS

Plant material

Ocimum americanum leaves was obtained from local garden and authenticated by an expert botanist.

Test organisms

Gram-negative pathogens isolated and characterized for ESBL and MBL production in a previous study were used as test organisms (Aruna and Mobashshera, 2012). About 50 ESBL producing pathogens that included representative isolates of each of the following genera, i.e., Klebsiella, Escherichia, Pseudomonas, Proteus and Citrobacter and 7 MBL producing pathogens were used in the current study. These isolates were maintained on Luria-Bertani (LB) Agar slants supplemented with 100µg/ml of ampicillin and stored at refrigerated conditions.

Preparation of Plant Extract

Fresh leaves of *Ocimum americanum* (20g) were washed thoroughly with distilled water and then cut into small pieces. These finely cut pieces were mixed with 100ml distilled water, and kept for boiling for a period of 5mins. After cooling, it was filtered through Whattman Filter paper no.1 and this filtrate was stored at 4°C before further use.

Synthesis of Ag-NPs

The above aqueous extract (10ml) was mixed with 90ml of 0.001M $AgNO_3$ solution. These solutions were allowed to react at room temperature in dark and checked for appearance of brown colour after 15mins at room temperature. Periodic sampling was carried out to monitor the formation of Ag-NPs (Ramteke *et al.,* 2013; Anuradha *et al.,* 2014; Jayapriya and Lalitha, 2013).

Characterization of Silver Nano-particles

The synthesized Ag-NPs were analysed using UV-Visible Spectroscopy (UV-Vis), Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR).

UV-Vis Spectral Analysis

The reduction of pure Ag^{2+} ions was monitored by measuring its UV visible spectrum, after diluting a small aliquot of the Ag-NP solution in distilled water. All readings were taken in triplicates to avoid instrumental or practical errors. Periodic sampling was carried out to monitor the formation of Ag-NPs.

SEM analysis

Scanning electron Microscopicanalysis was carried out to generate an image of the nano-particles and determine itsapproximate size (Ramteke *et al.,* 2013; Anuradha *et al.,* 2014; Jayapriya and Lalitha, 2013).

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The binding properties of Ag-NPs synthesized by *Ocimum americanum* leaf extract were investigated by FTIR analysis. Dried Ag-NPs and dried *Ocimum americanum* leaf extract were mixed well with Potassium Bromide (KBr) to form pellets. The spectra were recorded in the wave range of 450–2500cm^{-1}.

Assessment of Antibacterial Activity

The evaluation of the antibacterial activity of the Ag-NPs against test pathogens was done with the help of disc diffusion and well diffusion method.

Disc diffusion method

The log phase bacterial inoculum (10^8cfu/ml) was standardized using MacFarland's standard and swabbed onto Mueller and Hinton agar plates. Sterile filter paper discs (Whatmann No.1, 6 mm diameter) impregnated with 10µl of Ag-NPs, Ocimum leaf extract and $AgNO_3$solution were placed onto these plates with the help of sterile forceps and incubated at 37°C/24 h. A disc soaked in10µl of ampicillin (100µg/ml) was used as a control. Negative results were read only after 24h, as longer or shorter incubation periods may give misleading results.

Agar well diffusion method

In this method, 50µlof above mentioned test compounds (Ag-NPs, Ocimum leaf extract and $AgNO_3$) were added to the wells punched in Mueller and Hinton agar plates seeded with test organisms. The resulting zones of inhibition was measured after incubation at 37°C/24 h

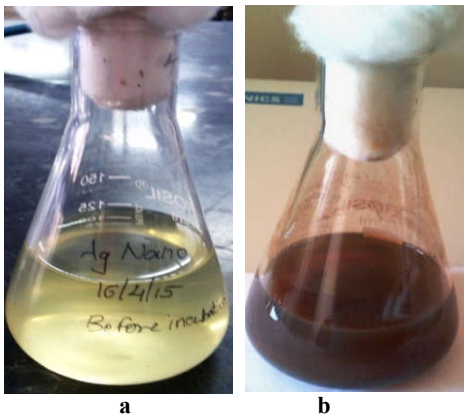
Antibacterial activity of nanoparticle coated hospital dressing material

White colour hospital dressing cloth of 1.5cm x 1.5cm dimension was autoclaved. The cloth was then soaked in nanoparticle solution. The nanoparticles coated cloth was allowed to dry in laminar air flow and its antibacterial activity was tested. Log phase bacterial inoculum (10^8cfu/mL) was swabbed onto Mueller and Hinton's agar plates and the above dressing was placed onto these plates with the help of sterile forceps and incubated at 37°C for 24h.Uncoated cloth was used as control (Venkatrajah *et al.,* 2012; Augustine and Rajarathinam, 2012)

RESULTS AND DISCUSSION

Synthesis of Silver nanoparticles

Reduction of silver to Ag-NPs is marked by acolor change of the solution from yellow to dark brown as indicated in Fig. 1. Ag-NPsexhibit dark yellowish brown colour due to surface Plasmon resonance.

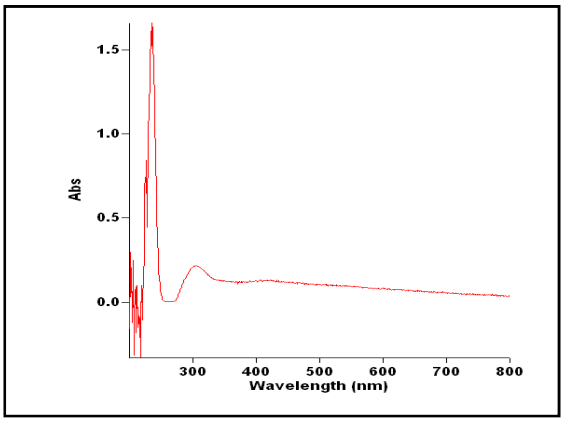


a b

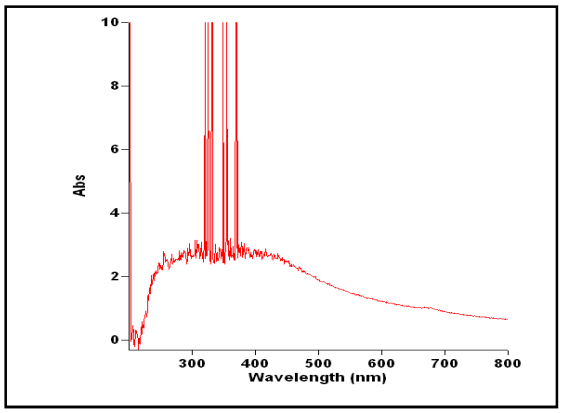
Fig. 1. Silver nanoparticles obtained using *Oscimumamericanum*leaf extract
a.Mixture of $AgNO_3$and plant extract before incubation
b.Silver nanoparticle formation after 15min incubation

Characterization of Silver nanoparticles

The formation of the Ag-NPswas confirmed by UV-visible spectroscopy. UV-visible spectra showed no evidence of absorption in the range of 400-800nm for $AgNO_3$ (Fig 2a.). Fig, 2b shows UV-Vis absorption peaks of the aqueous plant extracts in the range of 200-300nm indicating the presence of several organic compounds, which are known to interact with silver ions. An absorption band at 270nm is attributed to the aromatic amino acids of proteins. It is well known that the absorption band at 270nm arises due to electronic excitations in tryptophan and tyrosine residues in the proteins. This observation indicates the release of proteins into *Ocimum americanum* leaf extract and, suggests a possible mechanism for the reduction of the metal ions present in the solution. Fig. 3 represents the absorption spectra of nanoparticle formation at different time intervals.



(a)



(b)

Fig. 2. UV-Vis spectrum of Silver nitrate (a) and Plant extract (b)

Scanning electron Microscopicanalysis

SEM analysis was carried out to generate an image of the nanoparticles and determine its size. The SEM micrograph (Fig.4) revealed the size of AgNPs to be in the range of 41-184nm.

Fourier Transform Infrared Spectroscopic analysis

Figures 5, 6 and 7 represent the FTIR analysis of$AgNO_3$, AgNPs and *Oscimum* plant extract respectively. Fourier Transform Infrared Spectroscopy (FTIR) of pure $AgNO_3$was used for comparison (Augustine and Rajarathinam, 2012). The FTIR spectra of pure $AgNO_3$ and reduced $AgNO_3$showed considerable variation in the peaks of spectra. About 23 peaks were reported in $AgNO_3$solution and 14 peaks were observed for purified Ag-NPS. Prominent peaks reported in case of nanoparticles were 3295.1647,1079,2928,1033 and 825cm^{-1} (Table 1). FTIR spectrum reveals two bands at 1647 and 1525 cm^{-1} that correspond to the bending vibrations of the amide I and amide II bands of the proteins respectively; while their corresponding stretching vibrations were seen at 2928 cm^{-1}. The presence of the signature peaks of amino acids supports the presence of proteins in cell-free filtrate as observed in UV-Vis. spectra.

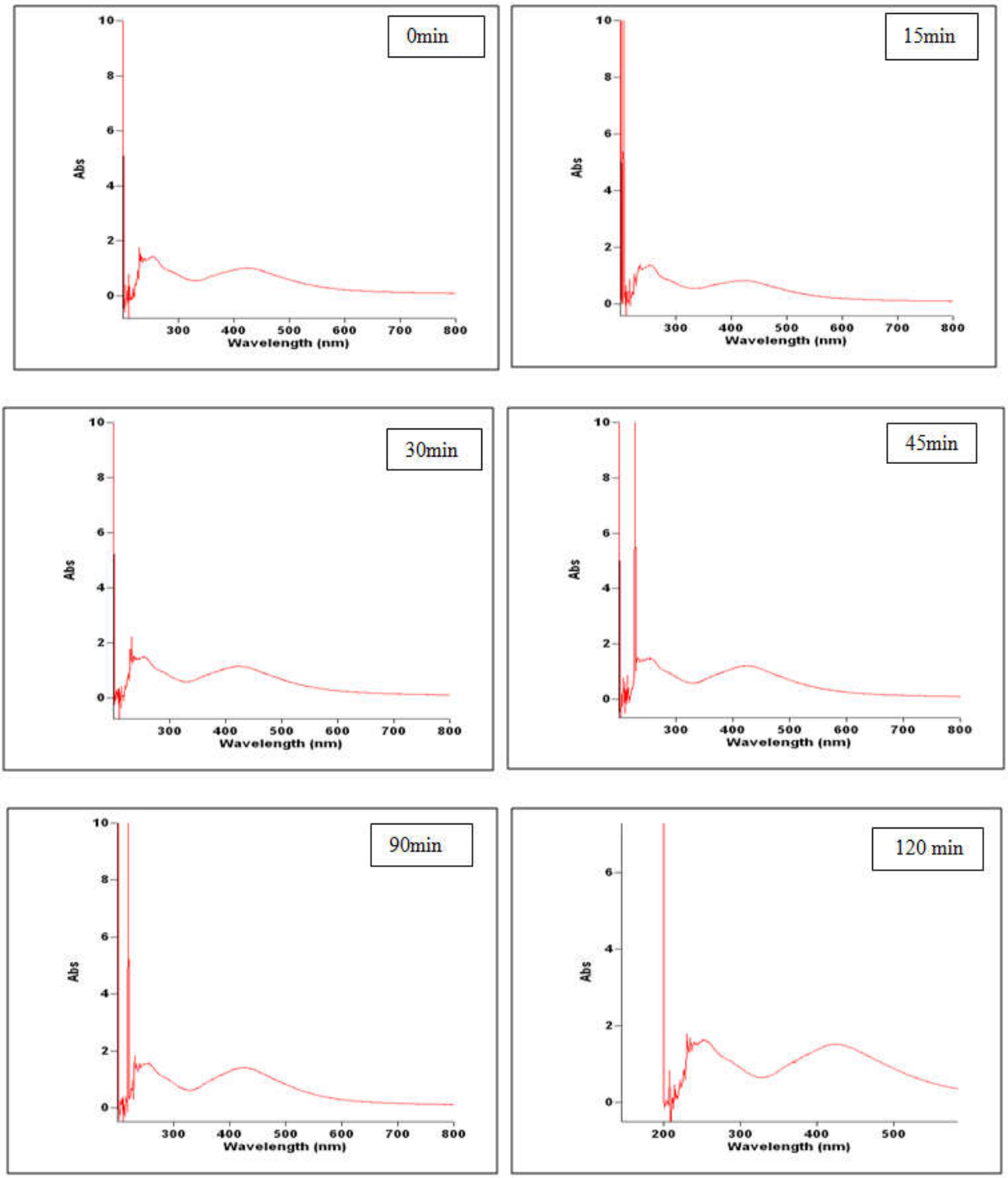


Fig. 3. Spectral scans of Silver nanoparticles at different time intervals showing peak in the range of 390-450 nm after 120min

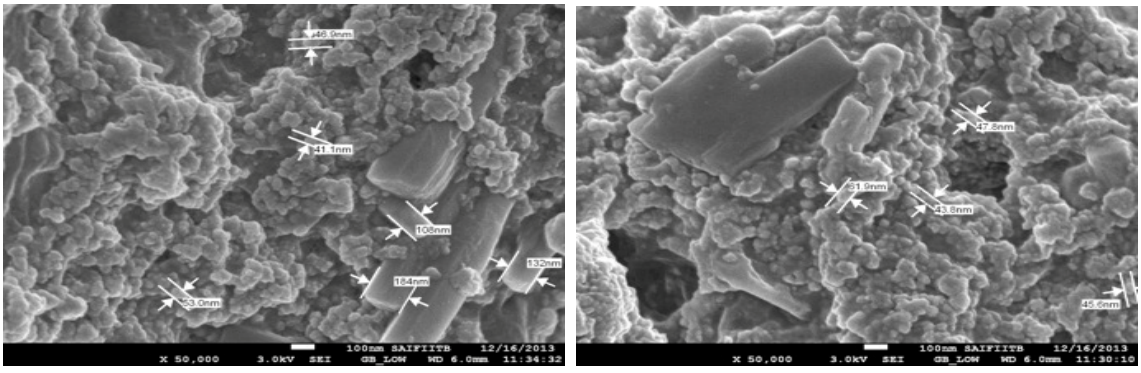


Fig. 4. SEM analysis of silver nanoparticles

Table 1. FTIR bands assignment

Wave number	Assignment
3295 cm-1	O-H stretching of H-bonded alcohols and Phenols
2928 cm-1	O-H stretching of carboxylic acids
1647 and 1525 cm-1	N-H bending of primary amines
1246-1033 cm-1	C-C streching of alcohols, carboxylicacids, ethers and esters

Table 2. Zones of inhibition obtained against ESBL and MBL producers using silver nanoparticles by disc diffusion method

Test pathogens	No. of isolates	Mean zones of nanoparticles (mm)	Mean zones of 1 mM $AgNO_3$ (mm)
ESBL producers			
E.colispp	9	13.0	13.6
Pseudomonas spp	10	14.1	13.7
Klebsiellaspp	8	14.5	13.8
Citrobacterspp	8	12.2	12.6
Proteus spp	8	11.2	11.2
MBL producers			
E.colispp	3	12.6	12.3
Pseudomonas spp	1	12.0	12.0
Klebsiellaspp	2	14.5	14.5
Citrobacterspp	1	10.0	10.0

Note: No zone of inhibition was observed for plant extract and ampicillin coated disc

Table 3. Zones of inhibition obtained against ESBL and MBL producers using silver nanoparticles by agar well diffusion method

Test pathogens	No.of isolates	Mean zones of nanoparticles (mm)	Mean zones of 1 mM $AgNO_3$ (mm)
ESBL producers			
E.colispp	9	17.6	17.3
Pseudomonas spp	10	19.4	18.6
Klebsiellaspp	8	19.7	19.1
Citrobacterspp	8	14.9	15.5
Proteus spp	8	13.4	14.1
MBL producers			
E.colispp	3	15.4	15.0
Pseudomonas spp	1	14.8	14.0
Klebsiellaspp	2	16.5	16.5
Citrobacterspp	1	15.2	15.0

Note: No zone of inhibition was observed for plant extract and ampicillin coated disc

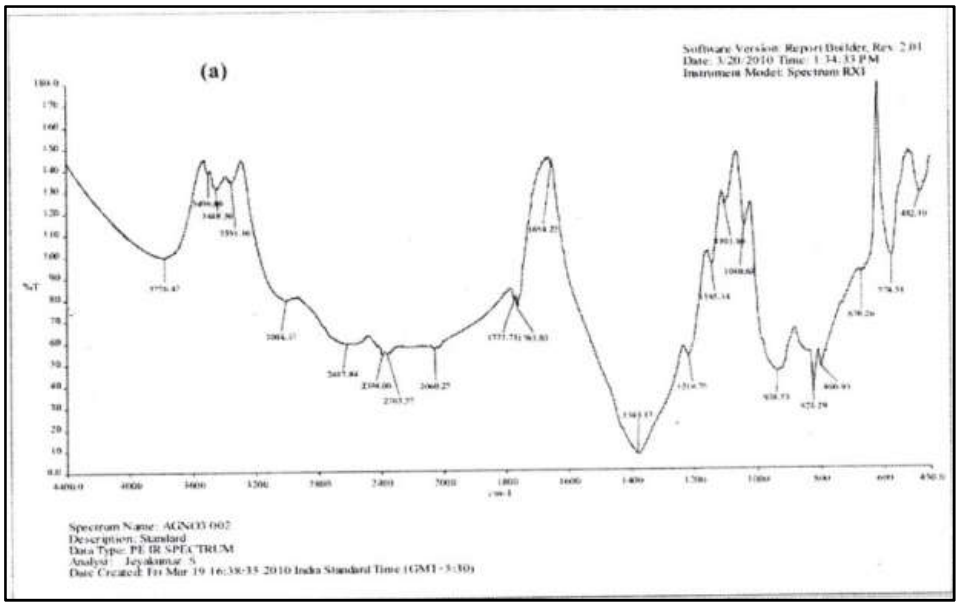


Fig. 5. FTIR analysis of silver nitrate solution

The two bands observed at 1388 and 1033cm^{-1} can be assigned to the C–N stretching vibrations of the aromatic and aliphatic amines, respectively. These observations indicate the presence and binding of proteins with Ag-NPs which can lead to their possible stabilization (Jain *et al.,* 2011; Ramana *et al.,* 2015).

Qualitative evaluation of antibacterial activity of nanoparticles: Nanobiotechnology is an important area of research that deserves all our attention owing to its potential application to fight against multidrug-resistant microbes. Fig. 8 illustrates the antibacterial activity of Ag-NPs using disc diffusion method.

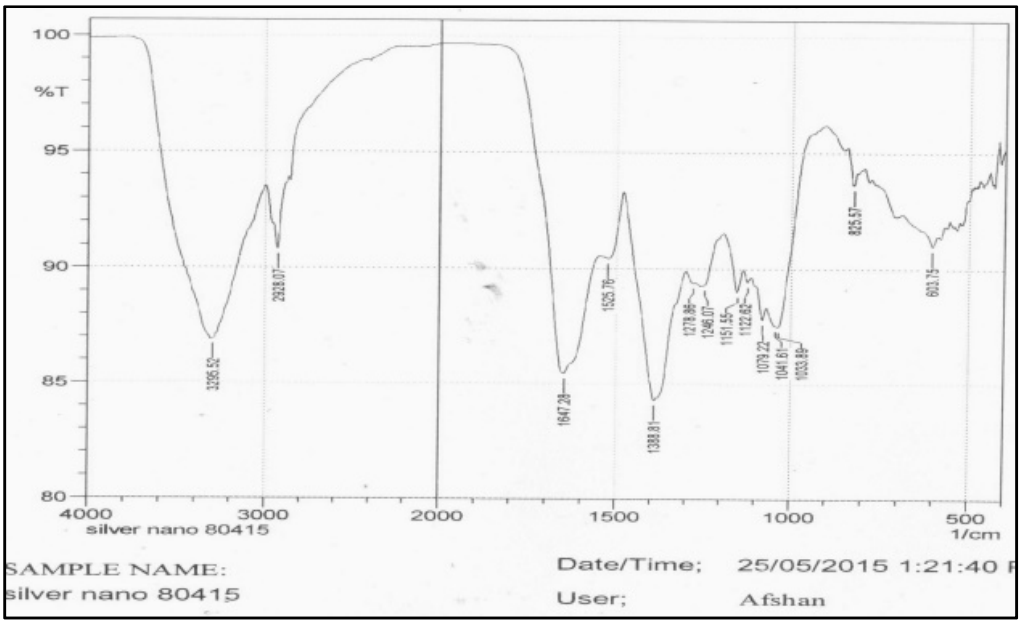
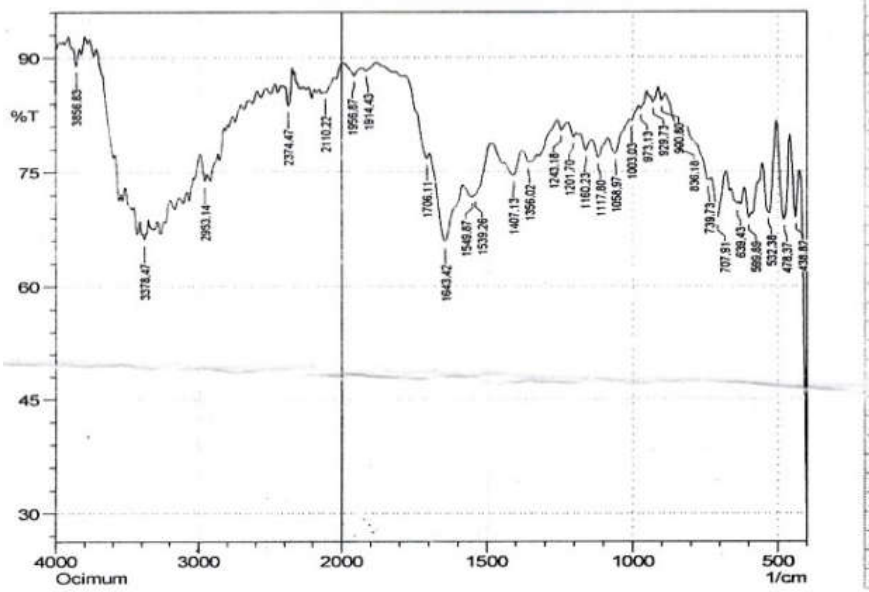


Fig. 6. FTIR analysis of Silver Nanoparticles



	Peak	Inten	Corr	Base	Base	Area	Corr
1	438.	68.9	8.14	458.	426.	4.28	0.76
2	478.	68.6	12.0	505.	458.	6.25	1.88
3	532.	69.5	0.3	552.	531.	3.02	0.1
4	599.	68.7	1.97	615.	591.	3.69	0.13
5	639.	70.6	0.78	660.	633.	3.97	0.1
6	707.	68.7	5.77	730.	678.	7.45	0.81
7	739.	73.8	1.11	817.	730.	9.7	0.05
8	836.	79.3	2.94	888.	817.	6.14	0.52
9	900.	84.3	1.36	912.	888.	1.7	0.08
10	929.	84.0	1.27	939.	912.	1.94	0.1
11	973.	83.2	0.82	978.	951.	2.05	0.09
12	1003	81.7	0.51	1008	978.	2.49	0.03
13	1058	77.3	2.9	1085	1008	7.62	0.51
14	1117	76.8	2.47	1140	1085	5.97	0.34
15	1160	77.8	1.76	1179	1140	4	0.18
16	1201	79.5	0.99	1221	1193	2.75	0.07
17	1243	80.4	1.25	1256	1221	3.17	0.11
18	1356	76.2	0.56	1377	1346	3.53	0.04
19	1407	74.4	2.53	1437	1377	7.34	0.45
20	1539	71.8	0.4	1542	1490	6.59	0.18
21	1549	71.6	0.5	1582	1543	5.59	0.08
22	1643	65.8	9.68	1695	1583	17.0	3.29
23	1706	76.7	1.87	1782	1696	7.38	0.28
24	1914	88.3	0.57	1927	1879	2.51	0.07
25	1956	87.6	1.3	1988	1927	3.26	0.16
26	2110	85.3	0.4	2120	2043	4.93	0.07
27	2374	83.6	0.34	2388	2372	1.18	0.02
28	2953	73.7	1.75	2989	2939	6.17	0.23
29	3378	66.0	0.93	3391	3365	4.44	0.07
30	3856	88.9	2.37	3879	3832	2.08	0.24

Fig. 7. FTIR analysis of Oscimum plant extract

The mean zones of inhibition for Ag-NPs and $AgNO_3$were found to be in range of 10-15mm (Table 2) for ESBL and MBL producing pathogens. Plant extract and ampicillin coated disc were used as a control and did not show any zone of inhibition. The mean zones of inhibition for both Ag-NPs and $AgNO_3$ were found to be in range of 13-20mm (Table 3) for ESBL and MBL producing pathogens by well diffusion method.

Antibacterial activity of nanoparticles coated hospital dressing material

The antimicrobial activity of the synthesized nanoparticles coated on cotton cloths was tested against test pathogens. There was no growth observed underneath the nanoparticle coated cloth (Fig.9).

Control plates showed good confluent growth underneath the hospital dressing without nanoparticle coating (Fig. 10). Thus silver-impregnated wound dressings have the potential to reduce both wound bio-burden and healing time. The antibacterial activity of silver is well documented (Feng *et al.,* 2000). The most ancient systems of medicine, Ayurveda also advocate the use of herbo-metallic drug preparations with silver metal for treatment of various diseases. Silver has far lower propensity to induce microbial resistance than antibiotics, and demonstrate oligo-dynamic bactericidal effect. Silver ions uncouple the respiratory chain from oxidative phosphorylation and lead to collapse of the proton motive force across the bacterial cytoplasmic membrane (Holt *et al.,* 2005). In a similar study, Bokaeian *et al.* (2014) reported antibacterial activity of Ag-NPs produced by *Plantagoovata* seed extract against antibiotic resistant *Klebsiella pneumoniae.*

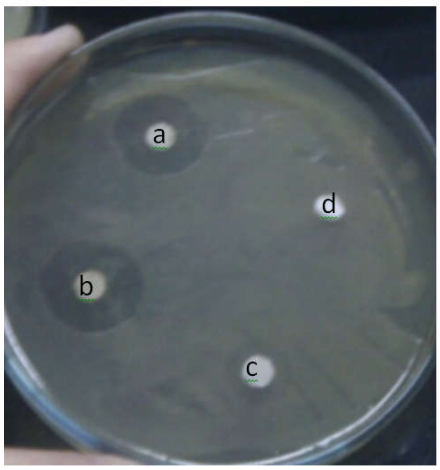


Fig. 8. Disc diffusion assay showing antibacterial activity of silver nitrate (a)and silver nanoparticles (b), comparatively less activity by plant extract (c) and resistance to ampicillin (d)

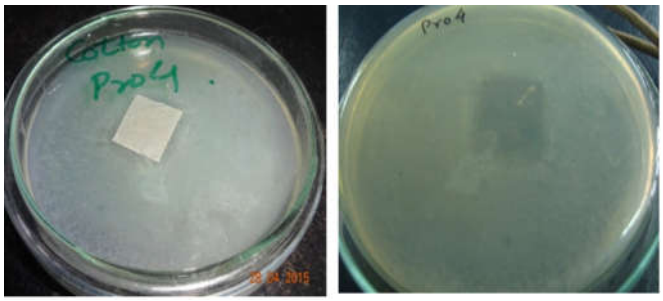


Fig. 9. The antibacterial activity of nanoparticle coated hospital dressing

Fig. 10. Control plate of hospital dressing without nanoparticle coating

Green synthesis of Ag-NPs using carob leaf extract and its antibacterial activity against *E.coli* was demonstrated by Awwad *et al.* 2013. Meena *et al.* (2012) synthesized Ag-NPs using aqueous leaf extract of *Lepisanthestetraphylla* as the reducing agent and evaluated its antibacterial activity against drug resistant bacteria isolates. The AgNPs at 30-50mcg (0.03-0.05mg) concentration significantly inhibited bacterial growth against multi drug resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* species. Rajawatand Qureshi, (2012) used tea extract to synthesize Ag-NPs which proved to be synergistic in antibacterial action with antibiotics against *Salmonella typhi*.

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