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Evaluation of antibacterial activity and cytotoxic effects of green AgNPs against Breast Cancer Cells (MCF 7)

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Abstract. The present work reports a facile, rapid and an eco-friendly method for the synthesis of silver nanoparticles using *Luffa acutangula (L. acutangula)* leaves extract and their antibacterial and cytotoxic effects. The synthesized silver nanoparticles (AgNPs) were characterized by UV-Visible spectroscopy (UV-Vis), Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction analysis (XRD). Additionally the topography, morphology and the elemental composition of the particles were determined by Scanning Electron Microscopy (SEM) and Energy dispersive spectrophotometric (EDS) technique and the measured particle sizes from SEM micrographs are in the range of 12.5 to 24.5nm. The *in-vitro* antimicrobial activity of the synthesized nanoparticles was high against gram positive *Staphylococcus aureus* and moderate against gram negative *Escherichia coli* and *Pseudomonas aeruginosa* strains. Further, the cytotoxic effects of synthesized AgNPs were evaluated against Human Breast Cancer (MCF 7) cell line.

Keywords: silver nanoparticles; Luffa acutangula; antibacterial activity; cytotoxic effect; cancer cells

1. Introduction

Nanotechnology is a science and engineering of functional systems at nanoscale usually ranging from 1 to 100 nanometers. Nanoparticles are being viewed as fundamental building blocks of nanotechnology. Smaller nanoparticles exhibit larger surface area to volume ratio (Indhumathy *et al*. 2014, Sulaiman *et al*. 2013, Gurunathan *et al*. 2009). Plants have the potential to reduce metal ion into metal nanoparticles (Praksah *et al*. 2013). Nanoparticles have received considerable attention due to their wide range of applications in the field of catalysis, optoelectronics, chemical sensing, bio sensing and biotechnology (Krasteva *et al*. 2002). Silver nanoparticles (AgNPs) have been widely used in many consumer goods, such as medical devices, cleaning agents, and textile (Qian *et al*. 2014) due to its unique antimicrobial properties and exhibit low toxicity (Geetha *et al*. 2013). Nanoparticles synthesis is usually carried out by various physical and chemical methods using various hazardous and toxic chemicals. However, green synthesis approaches of producing nanoparticles are an alternative source of conventional methods (Gopinath *et al*. 2013). Biological

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synthesis of metal nanoparticles was performed by using bacteria, fungi and plant extract (Subba Rao *et al*. 2013, Husseiny *et al*. 2007). Plant mediated synthesis of nanoparticles has great importance due to its simplicity and an eco- friendliness (Garima *et al*. 2011) and moreover plants extract act as reducing and capping agents for the synthesis of nanoparticles, Several plants such as *Momordica charantia* (Sunil *et al*. 2012) , *Memecylon umbellatum* (Arunachalam *et al*. 2013), *Mangifera indica* (Phillip 2010), *Trigonella foenum-graecum* (Aswathy Aromal and Philip 2012), *Carthamus tinctorius* (Nagajyothi *et al*. 2012), *Teraxacum officinale* (Tetty *et al*. 2012), *Citrus reticulate* (Nagajyothi *et al*. 2013), *Alstonia scholaris* (Prabha Shetty *et al*. 2014) and *Boswellia ovalifoliolata* (Supraja *et al*. 2015) have been successfully used for efficient and rapid extracellular synthesis of silver, gold and zinc nanoparticles which is more advantageous than chemical, microbial synthesis (Ashok kumar *et al*. 2014).Biosynthesized AgNPs from leaf extract of *Vitex negundo* L. proved to be an antitumor agent against human colon cancer cell line HCT15 (Prabhu *et al*. 2013). In vitro cytotoxicity effect was analyzed by AgNPs synthesized using *Sesbania grandiflora* leaf extract against human breast cancer (MCF-7) (Jeyaraj *et al*. 2013). The potential silver nanoparticles synthesized from calli extract of *Citrullus colocynthis* was investigated on human epidermoid larynx carcinoma cell line (Satyavani *et al*. 2011). Govender *et al*. 2013, studied cytotoxic activity of *Albizia adianthifolia* (AA) mediated silver nanoparticles and showed mechanistically the activation of AA AgNP in the intrinsic apoptotic pathway in A549 lung carcinoma cells. Earlier, researchers have reported on the synthesis of silver nanoparticles using leaf extract of *L. acutangula* (Moideen *et al*. 2014) but thorough investigations on the antimicrobial activity, MTT assay in particular is lacking.

In this paper, we present a simple and rapid biosynthesis of silver nanoparticles using *L. acutangula* leaf extract, which belongs to the family of cucurbitaceae. As prepared silver nanoparticles were characterized using various techniques, such as UV Vis spectroscopy, FT-IR, XRD, DLS, SEM-EDS. This work provided a potential approach for the production of silver nanoparticles without the involvement of additional chemicals and physical steps. In this work, we report the green synthesis of AgNPs using leaf extract of *L. acutangula* at room temperature, Antibacterial activity and cytotoxicity assay with MTT cells. *L. acutangula* leaf used in the traditional medicine for jaundice, hypoglycaemic, skin diseases, the plant leaves, root, fruit and seeds are mainly used for stomach pain, dysentery, malarial fever etc (Tupe *et al*. 2013).

2. Materials and measurements methods

Silver nitrate (99 % pure) was purchased from Sigma- Aldrich, India. Nutrient broth, nutrient agar plate, was supplied by Hi-Media, India. MTT (4, 5 Dimethyl thiozol bromide) was purchased from Invitrogen, USA.

2.1 Collection of plant material

Fresh leaves of *L. acutangula* were collected from Aranthangal village, Serkkadu, Vellore district, Tamil Nadu. Healthy leaves were washed with distilled water to remove dust 10 g *L. Acutangula* leaves were added to 100 ml of distilled water and boiled for 40 minutes at 80℃. The leaf extract was filtered through whatman's No.1 filter paper and the obtained filtrate was stored at 4℃ for further use.

2.2 Collection and Isolation of bacterial sp

The bacterial strains (*E.coli, S. aureus* and *Pseudomonas aeruginosa*) were collected from CMC (Christian Medical College, Vellore, Tamil nadu, India), For pure cultures isolation the bacteria were placed on nutrient agar medium Further, it is maintained in nutrient agar slants (bacteria) for onward analysis.

2.3 L. Acutangula leaf extract mediated synthesis of silver nanoparticles

To prepare the AgNPs, a 90-mL aqueous solution of 1.0×10^{-3} M silver nitrate was mixed with a 10-mL of 5 % aqueous solution of *L. acutangula* leaf extract. The *L. acutangula* Ag solution was colorless after adding 5minutes the color of the solution was changed to dark brown which visually confirms the formation of nanoparticles. These *L. acutangula* silver nanoparticles were characterized by using the techniques such as X-ray diffractometry (XRD), Fourier transform infrared spectrophotometry (FT-IR), UV-Vis spectrophotometry, Dynamic light scattering (DLS) (Particle size, zeta potential), Scanning electron microscopy (SEM), Energy dispersive spectrometer (EDS) and Transition electron microscopy (TEM).

2.4 Characterization of silver nanoparticles

2.4.1 UV-visible spectrum for synthesized nanoparticles

Preliminary characterization of AgNPs was carried out by UV-Vis spectroscopy. The UV-Vis spectra was recorded on a Shimadzu (model UV-2450) spectrophotometer and the sample was measured in the wavelength range of 250-800 nm.

2.4.2 FTIR analysis for synthesized nanoparticles

The synthesized AgNPs were characterized by FTIR (Affinity-1, Schimadzu) spectrometer using KBR pellet in the range of 500-4000 cm^{-1}. The spectrum of synthesized AgNPs and the spectrum of leaf extract were studied.

2.4.3 X-ray diffraction (XRD) analysis for synthesized nanoparticles

The product obtained was analyzed for the formation of AgNPs by Bruker D8 Advance *X*-ray diffractometer (cukα radiation, λ=1.54A^{o}), the diffraction peaks were obtained in 2θ and the scanning range was done between 10^{o}-90^{o}.

2.4.4 Dynamic light scattering analyzer for synthesized nanoparticles

The aqueous suspension of the synthesized nanoparticles was filtered through a 0.22-lm syringe-driven filter unit, and the size and distribution of the nanoparticles were measured using dynamic light scattering technique (Nanopartica, HORIBA, SZ-100).

2.4.5 Scanning electron microscopy (SEM) and EDS analysis

The size and morphological characterization of the synthesized AgNPs were studied using scanning electron microscopy (FEI Quanta FEG 200-High Resolution Scanning Electron Microscope) equipped with X- ray energy dispersive spectrometer. The nature of the elements was analyzed by EDS.

2.5 Assay for Antibacterial activity of L. acutangula leaf extract silver nanoparticles

The antibacterial activity of *L. acutangula* silver nanoparticles was examined on the basis of colony formation by *in-vitro* Petri dish assays (disc diffusion). Each bacterial isolates was cultured on growth media that induced prolific bacterial production. Bacterial cultures were collected from cultures that were incubated at 37℃ for 2 days for (bacteria) and diluted with sterile, deionized water to a concentration of 100 spore's ml-1. Aliquots of the bacterial suspension were mixed with serial concentrations of silver preparations to a final volume of 1 ml and were also mixed with sterile, deionized water as control. A 10 μl subsample of the conidia and *L. acutangula* silver mixture stock was taken at 30, 60 and 90 ppm after silver treatments and diluted 100-fold with the deionized water.

Three NA plates for bacteria per each combination of exposure *L. Acutangula* silver concentration were tested. The filter paper disc dipped in different ppm and inserted on mediums (NA), and then, the plates were incubated at 37℃ for 2 days respectively. The average number of colonies from silver-treated bacterial suspensions was compared with the number on the water control (percent colony formation). The zone size was determined by measuring the diameter of the zone in mm (Aneja 2003).

2.6 MTT Assay

Human breast cancer cell line was used to evaluate the percentage of cell inhibition. MCF 7 cells were sub-cultured and seeded, different concentrations (10-60 μg/ml) of cytotoxic drug (Synthesized AgNPs) was diluted in maintenance medium (Dulbeccous Modified Eagle Medium) and it was added to each well. After 4 hours, the fresh medium containing MTT dye was add and incubate for 4-8 hours at 37℃. The medium containing dye was removed and DMSO (Dimethyl sulphoxide) was added to dissolve the formazan crystals. Mitochondrial dehydrogenases of viable cells reduce the yellow water soluble MTT to water insoluble formazan crystals, which were solubilized by DMSO. The absorbance was recorded at 570nm using ELISA reader. Each experiment was done in triplicate.

Calculation

Percentage of cell inhibition (%) =100 - (Sample absorbance/Control absorbance)×100

Fig. 1 *Luffa acutangula* leaves

(a) (b) (c)

Fig. 2 Change of color of the colorless to dark brown solution confirms the formation of silver nanoparticles by *Luffa acutangula* leaves extract

3. Results and discussion

3.1

In this present study, silver nanoparticles were synthesized from *L. acutangula* leaves extract, Fig. 1. The fresh suspension of *L. acutangula* was green in colour, after addition of $AgNO_3$ the suspension turns dark brown within 15 minutes of incubation period from colorless making it one of the fastest bio-reducing methods to produce silver nanoparticles and there was no significant change afterwards Fig. 2. In previous study using *Psidiumguajava* (Kuldeep *et al*. 2014) and *Ceratoniasiliqua* leaf extract (Awwad *et al*. 2013), the silver nanoparticles was obtained within 2 minutes of reaction time.

3.2 UV-Visible spectral analysis

UV-Vis absorption spectrum of AgNPs with a broad surface Plasmon resonance (SPR) peak at 436nm Fig. 3, has confirmed the reduction of silver ions to metallic silver. A similar result was observed by previous study (Selvaraj *et al*. 2014). The frequency and width of the surface plasmon absorption depends on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium (Bijanzadeh *et al*. 2012).

3.3 FT-IR analysis and identification of the functional groups

FT-IR measurement was used to find out the possible bio-molecules responsible for the reduction of Ag^+ ions and capping the biosynthesized AgNPs. Fig. 4(a) shows the spectrum of *L. acutangula* leaf extract peaks at 3363 cm^{-1} (due to O-H stretching of alcoholic groups), 2943 cm^{-1}

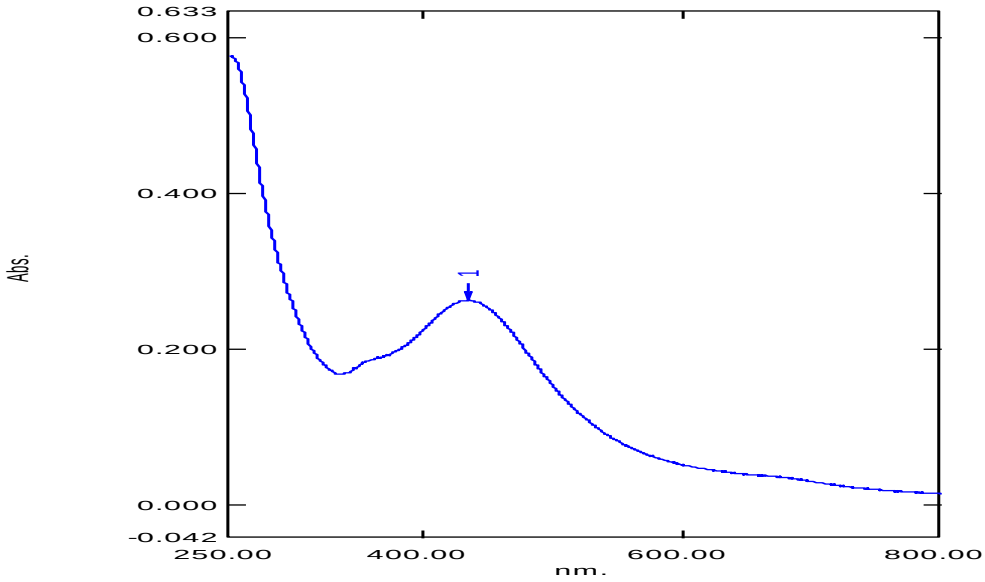


Fig. 3 Vis absorption spectrum of silver nanoparticles after 1 hour of reaction

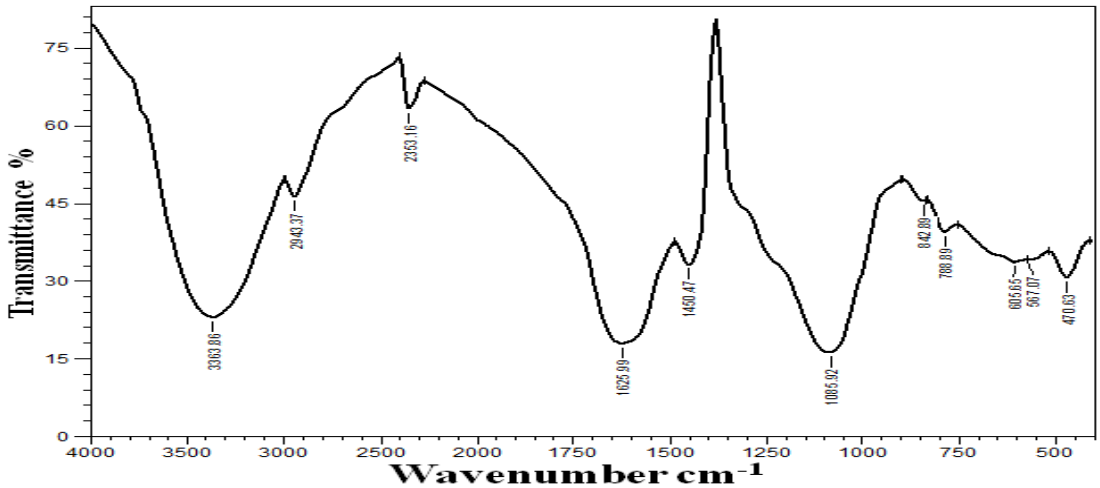


Fig. 4(a) FT-IR spectrum of *Luffa acutangula* leaf extract

(C-H groups), 2353 cm^{-1} (C≡N group of nitriles), 1625 cm^{-1} (carbonyl groups of carboxylic acid), 1450 cm^{-1} (methylene compounds), 1085cm^{-1} (due to N-H aliphatic amines), 842, 788 cm^{-1} (C-Cl group of alkyl halides), 605 cm^{-1} (C-H group of alkynes), 567, 490 cm^{-1} (C-Br group of alkyl halides), FT-IR study of biosynthesized AgNPs showed absorption peaks at 3304 cm^{-1} O-H stretching vibration of carboxylic acids, 3088c m^{-1}. =C-H stretching vibration of alkenes, 3003 cm^{-1} C-H stretching vibration of alkanes, 2667, 2341cm^{-1} H-C=O stretching vibration of aldehydes, 2025 cm^{-1} -C≡C- stretching vibration of alkynes, 1764 cm^{-1} C=O stretching vibration of carboxylic acids, 1695 cm^{-1} -C=C- stretching vibration of alkenes, 1525 cm^{-1} N-O asymmetric stretching vibration of nitro compounds, 1386 cm^{-1} C-H rock stretching vibration of alkanes, 1068 cm^{-1} =C-H

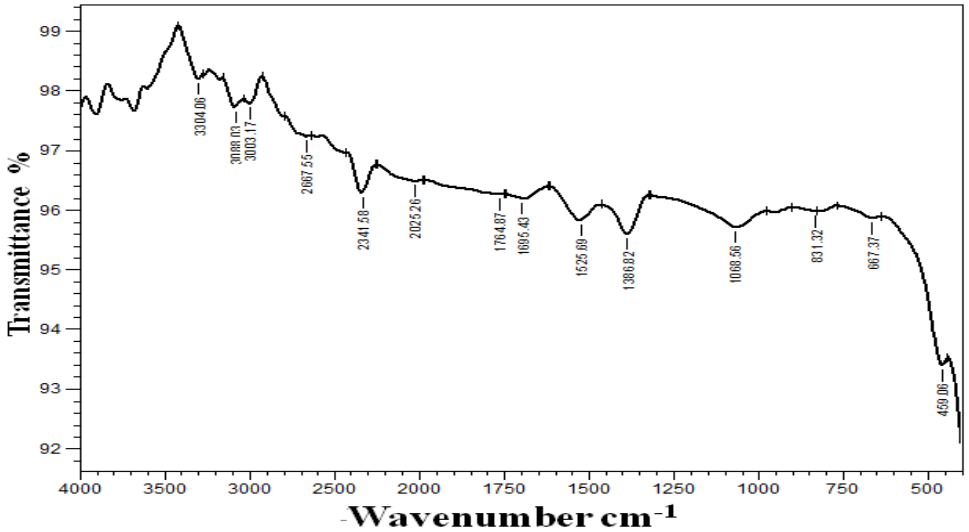


Fig. 4(b) FT-IR spectrum of silver nanoparticles synthesized by *Luffa acutangula* leaf extract

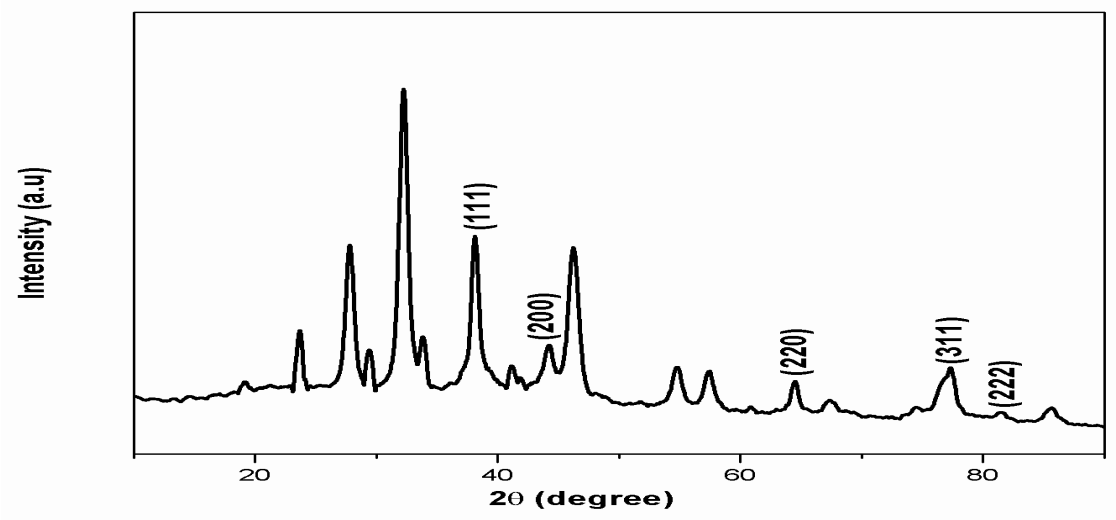
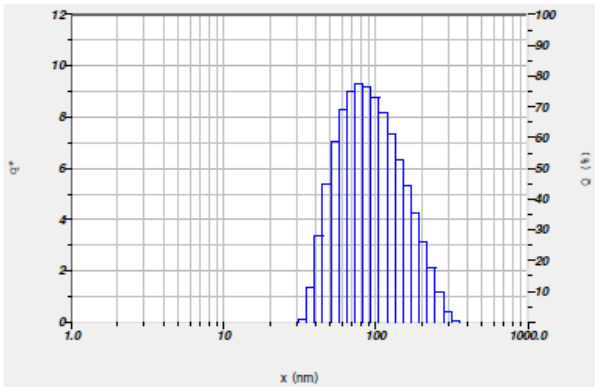


Fig. 5 XRD spectrum silver nanoparticles synthesized by *Luffa acutangula* leaf extract

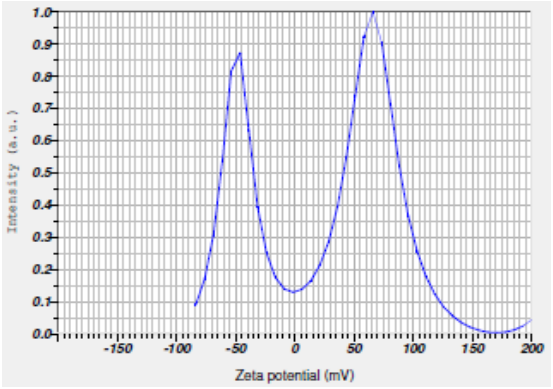
stretching vibration of alkenes, 831 cm^{-1} C-Cl stretching vibration of alkyl halides, 667 cm^{-1} C-H stretching vibration of alkynes, 459 cm^{-1} C-Br stretching vibration of alkyl halides. The FT-IR analysis indicates the involvement of alkenes, carboxyls, alkynes and alcoholic groups involved in synthesis of AgNPs Fig. 4(b). These compounds could attribute to the reduction of silver nanoparticles.

3.4 X-ray diffraction analysis

The crystalline nature of the AgNPs was confirmed by the analysis of XRD pattern. Fig. 5



(a)



(b)

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	102.9 nm	51.2 nm	77.6 nm
2	—	--- nm	--- nm	--- nm
3	—	--- nm	--- nm	--- nm
Total	1.00	102.9 nm	51.2 nm	77.6 nm

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	65.5 mV	0.000509 cm2/Vs
2	-49.0 mV	-0.000380 cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : 11.1 mV
Electrophoretic Mobility mean : 0.000086 cm^2/Vs

Fig. 6(a), (b) the histogram (size distribution) of silver nanoparticles (dynamic light scattering) and zeta potential (11.1 mV) of silver nanoparticles synthesized using *Luffa acutangula* leaf extract

showed four distinct diffraction peaks at 2θ degree of 38.11°, 44.21°, 64.47°, 77.41° and 83.23° corresponding lattice planes indexed at (111), (200), (220), (311) and (222) of Face Centred Cubic (FCC) structure of AgNPs respectively which are in the agreement with the database of Joint Committee on Powder Diffraction Standards (JCPDS No. 01-087-0597).

3.5 Dynamic Light scattering (DLS) analysis

Dynamic light scattering technique has been used to measure hydrodynamic diameter of the hydrosol (particle suspension). $AgNO_3$ was found to be 77.6 nm Fig. 6(a). The recorded value of zeta potential of the silver nanoparticles was 11.1 mV, Fig. 6(b), which resulted in the agglomerated state of the formed AgNPs. If the hydrosol has a large negative or positive zeta potential (C30 mV), then the particles tend to repel with each other and show no tendency to agglomerate resulted in polydispersed particles.

3.6 SEM analysis

The morphology and size of the AgNPs was viewed by SEM. The synthesized nanoparticles were found to be highly scattered due to its spherical nature and the diameter of the particles was12.5 to 24.4 nm, Fig. 7. In this study, small AgNPs were attached to the surface of large bio-molecules. The aggregation of the nanoparticles indicates that they were in the direct contact, but stabilized by a capping agent (Panneer selvam *et al*. 2011).

3.7 EDAX analysis

The elemental composition of powdered sample was determined by EDAX detector, Fig. 8

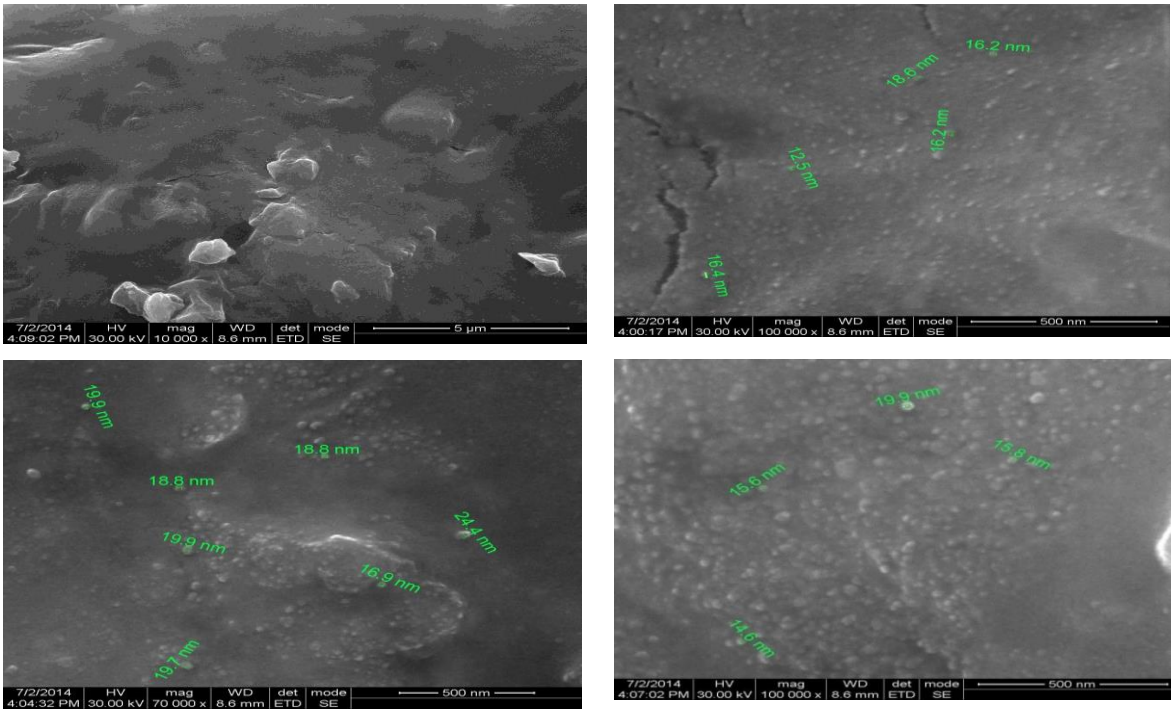


Fig. 7 SEM imagesof silver nanoparticles synthesized by *Luffa acutangula* leaf extract

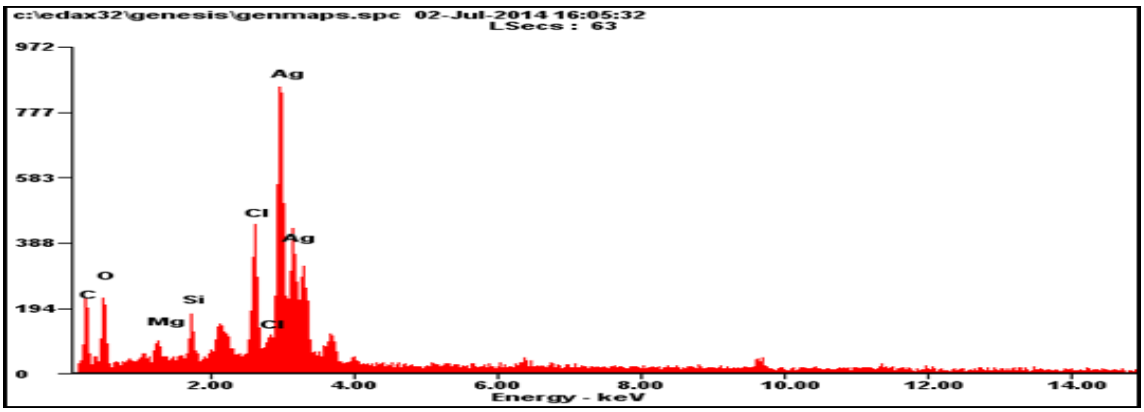


Fig. 8 Energy dispersion X-ray spectrum (EDS) micrograph of bio-synthesized AgNPs showing the elemental presence of Ag, C, O, Si and Mg

revealed the strong signal in the silver region and confirmed the formation of AgNPs. They were also observed spectral signals for carbon and oxygen indicated that the extracellular organic moieties from leaf extract of *L. acutangula* were adsorbed on the surface or in the vicinity of the metallic nanoparticles.

3.8 Antibacterial activity

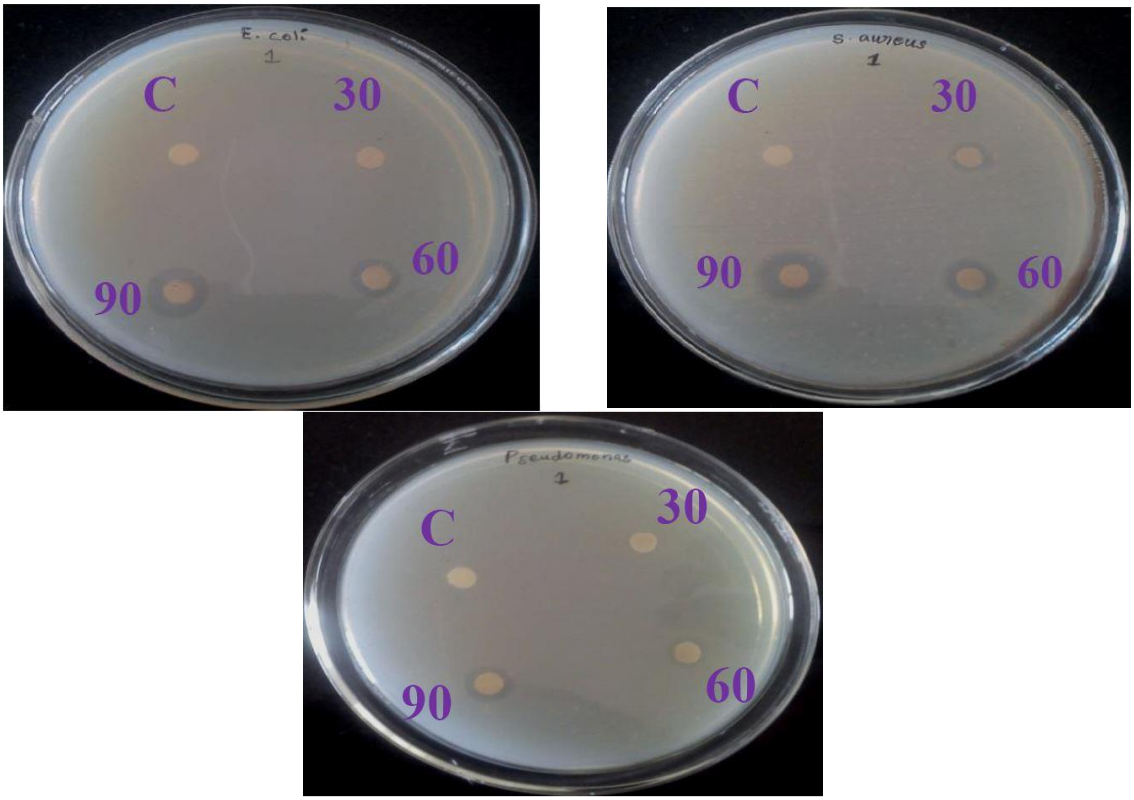


Fig. 9 Antibacterial activity of synthesized AgNPs at different ppm concentrations against gram-positive and gram-negative bacteria

1. Control
2. 30ppm of synthesized AgNPs
3. 60ppm of synthesized AgNPs
4. 90ppm of synthesized AgNPs

Silver nanoparticles obtained from *L. acutangula* have very strong inhibitory action against Gram-positive and Gram-negative bacteria Fig. 9. These isolates were collected from CMC, Vellore. Three concentrations of AgNPs (30, 60, 90 ppm) were prepared and were applied against an array of bacterial species *viz, S. aureus* (Gram positive), *E.coli* and *P. aeruginosa* (Gram negative). The higher concentration (90 ppm) of AgNPs showed significant antibacterial effect Table. 1 compared with other concentrations (60 and 30 ppm). But when compared to *E. coli* and *P. aeruginosa* (Gram negative) *S. aureus* (Gram positive) shown effective zone of inhibition in all concentrations.

The inhibitory action of the microbes may be attributed to the loss of replication ability of DNA upon treatment with the silver ion, besides the fact that expression of ribosomal sub-unit proteins as well as some other cellular proteins and enzymes essential to ATP production becomes inactivated. When ppm concentration decreases the diameter of zone of inhibition was also decreased. The results indicated that antibacterial effect was dose dependent. The mechanism of inhibition of AgNPs on microorganisms is not well known. AgNPs binds with cytoplasmic membrane and killed the bacterial cell. This is because the electrostatic interaction between positively charged AgNPs and negatively charged cell membrane of microorganisms (Awal *et al.* 2010).

Table 1 Zone of inhibition of AgNPs from leaf extract of *Luffa acutangula* against various pathogenic bacteria

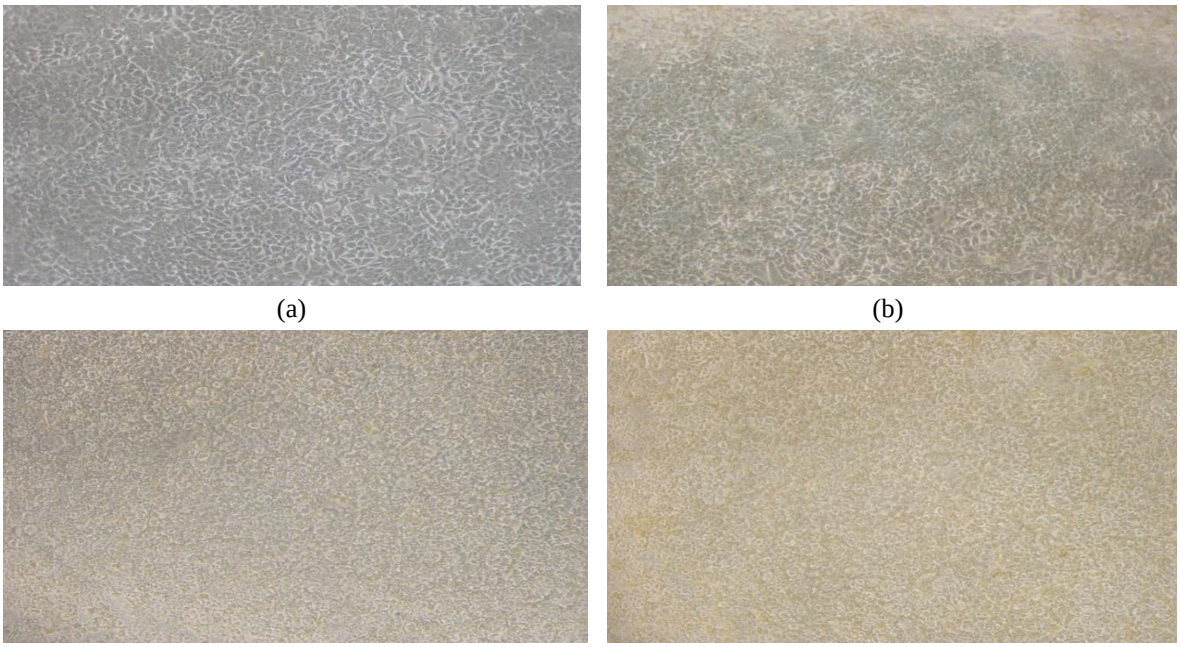
Pathogens	Gram nature	Zone of inhibition (mm)		
		AgNPs 30 ppm	AgNPs 60 ppm	AgNPs 90 ppm
E.coli	Negative	0.5±0.1	5±0.6	8±0.8
S. aureus	Positive	5±0.8	8±0.9	10±0.7
P. aeruginosa	Negative	0.5±0.1	5±0.4	7±0.3

The values represent the mean±standard deviation of three replicates of experiment

Table 2 Percentage of inhibition measured on MCF 7 cells after the treatment with silver nanoparticles for 48 hours by MTT assay

Synthesized silver nanoparticles treatment (µg/ml)	Cell inhibition (%)
MCF 7 + 10	25.02±0.28
MCF 7 +20	38.87±0.02
MCF 7 +30	42.36±0.02
MCF 7 +40	67.02±0.02
MCF 7 +50	90.87±0.02
MCF 7+60	91.71±0.03

The values represent the mean±standard deviation of three replicates of experiment

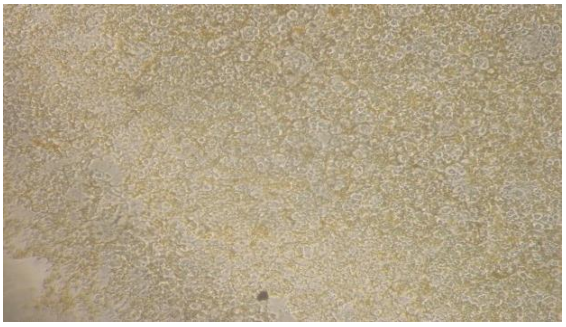


(a) (b)

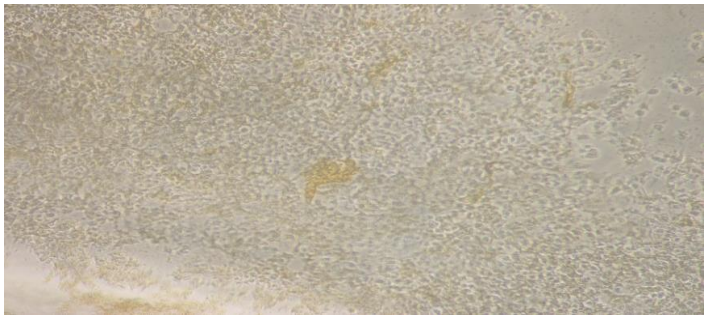
(c) (d)

Fig. 10 (a) control, (b) 10 µg/ml, (c) 20 µg/ml, (d) 30 µg/ml, (e) 40 µg/ml, (f) 50 µg/ml, (g) 60 µg/ml shows Breast Cancer Cells (MCF 7) treated with 10-60 µg/ml of synthesized AgNPs using *Luffaacutangula* leaf extract

(e)



(f)



(g)

Fig. 10 Continued

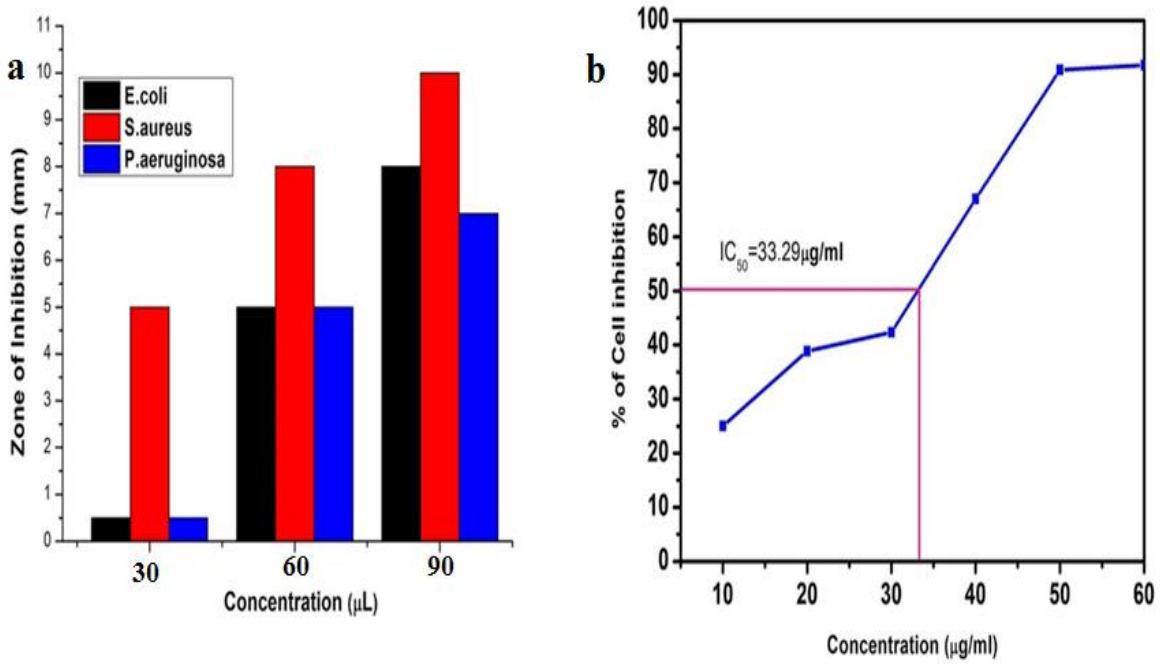


Fig. 11(a) Antibacterial activity histogram of AgNPs against *E. coli*, *S. aureus* and *P. Aeruginosa fluorescence*, (b) Cytotoxic activity of AgNPs against Human breast cancer cell line (MCF7)

3.9 MTT Assay

The *in-vitro* cytotoxic effects of AgNPs were screened against MCF 7 cell line and the percentage of cell inhibition was confirmed by MTT assay Table. 2. The silver nanoparticles were able to inhibit the MCF 7 cells in a dose dependent manner (Muthu Irulappan *et al*. 2010). After 48 hours of treatment, the AgNPs at concentration of 33.29µg/ml decreased the viability of MCF 7 cells to 50% of the initial level, and this was chosen as IC_{50} Fig. 10 and Fig. 11(a), (b). The cytotoxic effects of AgNPs are the active physico-chemical interaction of silver atoms with the functional groups of intracellular proteins, nitrogen bases and phosphate groups in DNA. Although, further studies are needed to fully understanding the mechanism involved in the anticancer activity (Moddab *et al*. 2011).

4. Conclusions

Herein, we have reported on the successful synthesis of silver nanoparticles by using *L. acutangula* leaves extract which is free from harmful reducing or capping agents.

- The result of UV-Vis spectrum, FT-IR, XRD, DLS, SEM and EDAX has confirmed the bio-reduction of Ag^+ ions.
- The antibacterial activity histogram of silver nanoparticles was demonstrated by clear zone of inhibition against gram positive and negative bacteria the histogram of % of cell inhibition AgNPs showed excellent cytotoxic effects against MCF 7 cell line in 33.29 μg/ml.
- This passes a way to use silver nanoparticles as anticancer drug in future. Moreover, the methodology employed here was simple, inexpensive and an eco-friendly.

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