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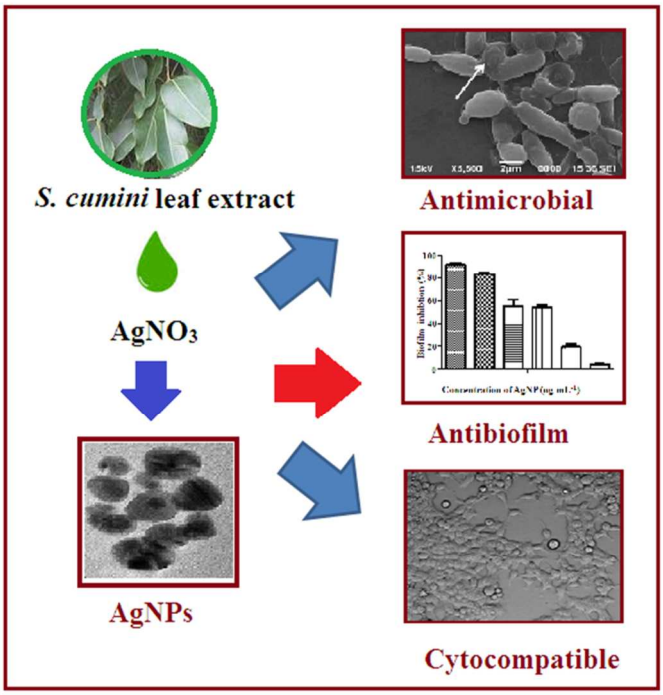

Synthesis of green and biocompatible silver nanoparticles with high anti-microbial and anti-biofilm activities.

Green silver nanoparticles: enhanced antimicrobial and antibiofilm activity with effect on DNA replication and cell cytotoxicity

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Abstract

Biofabricated metal nanoparticles are biocompatible, inexpensive and eco-friendly. They find immense utility in the domain of biomedical and material science. The present work focuses on 'green' synthesis of silver nanoparticles (AgNPs) using the methanolic extract of *Syzygium cumini* leaf. AgNPs showed the characteristic surface plasmon resonance peak at 442 nm. The XRD pattern confirmed the formation of face centered cubic AgNPs. The nanoparticles were uniformly distributed within a narrow size range of 10-20 nm. The particle exhibited significant antimicrobial activity against a panel of pathogens like *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis, Trichophytonrubrum, Aspergillus sp* and *Candida albicans*. Alterations in membrane permeability of AgNPs treated microbial cells were evident from scanning electron microscope images. The replication fidelity of small (1500 bp) DNA fragments in the presence of AgNPs was compromised in a dose-dependent fashion and addition of bovine serum albumin (BSA) to PCR reactions reversed the effect of AgNPs. Besides, the prepared nanoparticles inhibited biofilm formation in a wide range of AgNPs concentration. Significantly, cytotoxicity assays showed good compatibility of AgNPs with human embryonic kidney cells (HEK 293).In summary, the study suggests an eco-friendly, cost effective and biocompatible approach for synthesizing AgNPs, which may act as a potential template for designing novel antibacterial, antifungal and antibiofilm agent.

Keywords: Silver nanoparticles (AgNPs), *Syzygium cumini*; Antimicrobial; Anti-biofilm; PCR amplification; cytotoxicity

Introduction:

The major worries of the public Health Departments around the world are the increasing incidences of microbial infectious diseases.[1] The severity of the issue has been enhanced by the emergence of multi-drug resistant microbial strains. Incidentally, around 80% of the infectious diseases are caused by biofilm forming organisms.[2] Bacteria within the biofilm 'become' up to 1000-fold more resistant to antibiotics.[3] Efforts to disrupt biofilm formation have led to the identification of bioactive molecules of diverse origins.[4] In recent years, nanoparticles (NPs) have shown potential for use in chronic disease diagnostics, bacterial infection prevention, resisting bacterial colonization on medical devices and in the food and clothing industries.[5-9-] Synthesis of noble metal nanoparticles with enhanced activity for applications such as drug delivery, catalysis, electronics, optics, environmental, and biotechnology is an area of constant interest.[10-14] The commonly employed methods for synthesis of metallic NPs involve physical and chemical approaches which utilize toxic chemicals, hazardous conditions, costly apparatus etc.[15,16] Therefore, the development of protocols to synthesize nontoxic, eco-friendly metal NPs is currently of great interest.[17]Several methods have been used for the green synthesis of metal NPs using various biological materials as reducing agents such as microorganisms, plant extracts etc.[18-22]Among the most important bioreductants, plant extracts are relatively easy to handle, readily available, cost effective and have been well explored for synthesizing metal NPs. Different parts of plants such as fruit, bark, fruit peels, root and callus are utilized in this regard.[23-27] Silver nanoparticles (AgNPs) prepared as mentioned above are known for their antimicrobial activity against a diverse group of microorganism and have been used for many years as an antimicrobial substance in the biomedical domain.[28,29]Antimicrobial activity of AgNPs can find applications including reduction of infections on the burns, prevention of

microbial biofilms on catheters, elimination of microbes from fabrics and treatment of water.[30-36] Apart from these anti-bacterial activities, AgNPs have been found to be effective as anti-fungal, anti-inflammatory, anti-viral, anti-angiogenesis and anti-platelet agents.[37-39] A strong cyto-protective activity towards human immunodeficiency virus infections using AgNPs have also been demonstrated.[40]

In addition to their direct bactericidal activity, AgNPs are also known to inhibit the biofilm formation of certain microorganism, possibly by inhibiting the microbial cells to colonize on the substratum such as medical devices (e.g. catheters) .[41-43] An *in vitro* study reported that AgNPs have a very good ability to inhibit biofilms, formed by *S. aureus*, coagulase negative *Staphylococcal* species, *Enterococcus*, *E. coli*, *P. aeruginosa* and *C. albicans* on plastic catheters.[44] It is evident from the previous findings that showed cytotoxic effects of various metallic nanoparticles preparation on mammalian cells. Gold nanoparticles showed no significant toxicity in HeLa cells,[45, 46] whereas significant size-dependent toxicity was observed in fibroblasts epithelial cells and melanoma cells.[47] Further, it was shown AgNPs that did not contain any capping or stabilizers exhibited significant toxic effect on mouse macrophage J774.A1,[48] whereas starch-stabilized AgNPs had no effect on cancer cell lines U251 and fibroblasts IMR-90.[49]

Syzygium cumini, commonly known as*jamun* is a well-known herbal drug in India. Its different parts are used to treat illness caused by bacterial and fungal pathogens.[50] *S.cumini* belongs to the *Myrtaceae* family, which is native to Asian continent. Hence, we report a clean, non-toxic and environmentally benign method for the synthesis of AgNPs using the reductive potency of the methanolic extract of dried leaf of *S. cumini*. A detailed analysis was undertaken here regarding

the antimicrobial activity and cytotoxicity of the synthesized particles. This endorses the synthesized AgNPs for various advanced biomedical applications related to microbial infections.

Materials and methods

Chemicals and Growth media

Silver nitrate ($AgNO_3$), Luria-Bertani (LB), Potato Dextrose Broth(PDB), Muller Hinton Broth (MHB), Trypticase Soya Broth, agar powder (bacteriological), crystal violet, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Gentamicin, nystatin and Clotrimazole were purchased from Himedia. Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin solution, L-glutamine, non-essential amino acid and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma.

Preparation of plant extract

Freshly collected leaves from the plant *S. cumini* were chopped into small pieces and dried. The dried leaves (250.0 g) were added to methanol (2.5 L) and continuously stirred for 72 h. The extract was filtered (Whatman no.1 filter paper), evaporated and dried under reduced pressure using a freeze dryer to give a dark brown powder. A small amount of the powdery extract (7.5 $mgmL^{-1}$) was used for the synthesis of AgNPs.

Synthesis of AgNPs

The reaction mixture was prepared by adding 10 mL of the plant extract (7.5 mg mL^{-1}) to 990 mL of 1 mM $AgNO_3$ solution in a 2.5 L beaker, equipped with a magnetic stir bar. The mixture was stirred 72 h at room temperature, where a colour change was observed (transparent to dark

brown). After 72 h, the mixture was centrifuged at 15,000 rpm for 5 min to remove any uncoordinated biological materials. After washing three times with distilled water, a black residue was obtained, which was dried followed by subsequent dispersion in sterile distilled water. Finally 100 mg mL^{-1} stock was prepared for conducting all biological experiment.

Microbial strains and their culture condition

Bacterial strains, *P. aeruginosa* MTCC 1688, *Staphylococcus aureus* MTCC 3160, *Escherichia coli* MTCC 40, *Bacillus subtilis* MTCC 121 *Klebsiella pneumonia* MTCC 618, *Mycobacterium smegmatis* MTCC 14468 and fungal strain, *Trichophytonrubrum, Aspergillus sp*, and *Candida albicans* MTCC 183, *Fusarium oxysporum* MTCC 284 were used in this study. All bacterial strains were cultured in Luria-Bertani (LB) broth at 37°C on a shaker at 180 rpm for 24 h and fungal strains were cultured in Potato Dextrose Broth (PDB) at 28°C on a shaker at 180 rpm for 24 h. All the MTCC strains were purchased from Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India.

Characterization of AgNPs

A Thermo Fischer Scientific Evolution 201- UV-Visible spectrophotometer was used to conduct optical measurements. The analysis was performed in quartz cuvette, using distilled water as a reference solvent. UV–Visible spectroscopic analysis was performed by continuous scanning from 350 to 700 nm and 1 mM $AgNO_3$ solution was used for the baseline correction. For UV-visible spectroscopic analysis, the AgNPs solution was diluted 10 times in each case. FTIR spectra of the aqueous leaf extract and purified AgNPs were analysed by FTIR spectrophotometer (PerkinElmer 1000 FT-IR spectrometer). To remove any free biomass residue or unbound extract on the surfaces of the NPs, AgNPs were repeatedly washed with distilled

water; subsequently the product was centrifuged at 15000 rpm for 5 min and dried. Purified AgNPs were mixed with KBr powder and pressed into a pellet for measurement. Background correction was made using a reference blank KBr pellet.XRD patterns were obtained on an X-ray diffractometer, 'Miniflex' (Rigaku Corporation Japan) at scanning rate 2.0 min^{-1} over the range of 2θ = 10–90°.TEM analysis was performed on a JEOL, JEM 2100 transmission electron microscope (JEOL, Tokyo, Japan) at operating voltage of 200 kV. AgNPs were drop-coated onto carbon film, and tested using an EDX spectrophotometer (JEOL-JSM 6390, Japan.) to confirm the elemental composition of the sample.

Antimicrobial activity and Minimum inhibitory concentration

The antimicrobial susceptibility of AgNPs was evaluated using the well diffusion methods according to CLSI standard.[51] 10^5 to 10^6 colony-forming units/mL (CFU mL^{-1}) of microorganism were spread on Muller Hinton Agar plates and methanol extract (75 µg), $AgNO_3$ (1mM) and AgNPs (10,000µgmL^{-1}) were loaded into the corresponding wells. Gentamicin, nystatin and clotrimazole were used as control. Zones of growth inhibition were determined by measuring the diameter (mm) of bacterial clearance after 24 h of incubation at 37°C and 28°C for bacteria and fungi, respectively. For determination of minimum inhibitory concentration (MIC), micro dilution method was used which is described elsewhere with slight modification.[36] Briefly organisms at concentration of 10^5 to 10^6 CFU mL^{-1} in a 96-well microplates were exposed to serial dilutions of the AgNPs ranging from 10,000 µgmL^{-1} to 50µgmL^{-1} for 24 h at appropriate temperature under aerobic conditions. After 24 h of incubation MIC values were determined visually as the lowest concentration where no turbidity in the wells was observed. All assays were performed in triplicate.

In vitro time dependent killing assay

To determine the microbial killing time of AgNPs, the overnight grown cultures were resuspended in LB or PDB to get the concentration of 10^5 to 10^6 CFU mL^{-1}. AgNPs at the concentration of 10mg mL^{-1} was added in microbial culture in 96-well plates. Medium without AgNPs were used as positive control. Microbes were harvested at the indicated time points and serially diluted in 2 mL centrifuge tube, 10^{-4} dilution was spreaded on the LBA and PDA plates and incubated for 24 h. After incubation the number of colony forming units (CFUs) was determined and compared with the control. All samples were plated in triplicate and values were averaged from three independent trials.

Change in cellular morphology after AgNPs treatment

To determine the effect of AgNPs on cell surface morphology of microorganism, *S. aureus, E. coli* and *C. albicans* were grown overnight and resuspended in LB or PDB to achieve cell concentrations of 10^5 to 10^6 CFU $mL^{-1.}$ Cells were treated with AgNPs (10 mg mL^{-1}) for 30 min. After that, control and treated microbial cells were harvested and washed with phosphate-buffered saline (PBS, pH 7.4). After washing, the cells were fixed with 2.5% glutaraldehyde overnight and the fixed samples were then dehydrated for 30 min in a graded series of ethanol (50-100%). Changes in the microbial cellular morphology were observed by scanning electron microscopy (SEM) (JEOL JSM-6390LV, Tokyo, Japan).

In vitro biofilm inhibition assay: Tissue culture plate method (TCP)

To determine the efficacy of AgNPs in biofilm inhibition, TCP method was carried out as described elsewhere with a slight modification.[52] Briefly, individual wells of sterile, polystyrene

96-well-flat bottom tissue culture plates were filled with 10^5 to 10^6CFU mL^{-1} microbial culture in 100 µL of tryptic soy broth (TSB). To the culture, 50µl of AgNPs at MIC range of 250 µg mL^{-1} to62.5 µg mL^{-1} were added. The tissue culture plates were incubated for 24 h at 37°C (28°C for fungus). Then the content of each well was gently removed. The wells were washed thrice with phosphate buffer saline (PBS pH 7.4) to remove free-floating planktonic bacteria. Biofilms formed by adherent organisms in plate were fixed by keeping the plate at 60°C for 1 hand stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. After drying, 100% ethanol was added to the wells and the optical densities (OD) of stained adherent bacteria were determined with a microplate reader (Thermo Scientific Multiskan GO) at 570 nm. The biofilm inhibition was calculated based on the solubility of the retained dye in wells by using the formula-

% Biofilm inhibition= $[(OD_{control}- OD_{test}) / OD_{control}] \times 100$ where control OD is the absorbance which is obtained without addition of AgNPs. Experiment was carried in triplicate.

Effect of AgNPs on amplification of target DNA sequence using PCR

In this work, we mechanistically investigated the effects of AgNPs (1000 µgmL^{-1}- 62.5 µgmL^{-1}) on *in vitro* DNA replication on DNA fragments (1500 bp) using PCR. Briefly, PCR amplification of 16S rDNA was performed using genomic DNA isolated from *E. coli* (MTCC 40) with primer pairs (16F 5'-ACATAGGATCCAGCGAACGCTGGCGGCAG-3' and 16R 5'-ATCTGAAGCTTTTAGCAGGTTCCCCTACGGCTA-3'). The 16S rDNAPCR consisted of an initial denaturation at 94°C for 5 min, followed by 18 cycles or 35 cycle of 40 sec at 94°C, 30 sec at 55°C and 1.6 minat72°C, and a final extension of 10 min at 72°C. To visualize the PCR product gel electrophoresiswas conducted.

Cytotoxicity studies of AgNPs on Human Embryonic kidney cells (HEK 293)

The human embryonic kidney cells(HEK 293) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin solution, 0.05 % L-glutamine, 1% non-essential amino acid and 0.6 % 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). To determine the cytotoxicity of the AgNPs, 1×10^4HEK293 cells were grown in each well of a 96-well plate at 37°Cand 5% CO_2 for 24 h before addition of three different concentrations of AgNPs (250 μgmL^{-1}, 125 μgmL^{-1} and 62.5 μgmL^{-1}). To determine the morphology and cell viability, after 24 h incubation with the AgNPs, cells were analysed by inverted microscope Zeiss, Model AXIOVERT A1) and treated with MTT solution for 4 h at 37°C in a cell culture incubator (Eppendorf Galaxy 170). In metabolically active cells, MTT was reduced to an insoluble dark purple formazan by mitochondrial dehydrogenases.The formazan crystals were dissolved in denaturing buffer (SDS, HCl and isopropanol) and the absorbance was read at 570 nm in 96 well plate reader (Thermo Scientific Multiskan GO). The cell viability was calculated based on the production of formazan in treated and untreated wells.

Results and discussion

Preparation of AgNPs

'Green' synthesis of silver nanoparticles has drawn copious attention of the researchers because of its easy preparation method. Earlier studies have demonstrated that the leaf extract of *S. cumini* is rich in polyphenolic compounds such as gallic acid, narigin, quercetin, caffeic acid, rutin, catechins, etc. Polyphenolics consist of many classes like anthocyanins, isoflavonoids, flavonols and flavones, which are well known for their metal chelating and metal reducing property that convert metal ions into nanoparticles.[53] However, the exact mechanism of reduction

and stabilization of silver nanoparticles (AgNPs) using plant extract remains largely unknown. It might be that the polyphenolic compounds which are present in plant extract acting as a reducing and stabilizing agent. The putative mechanism for the formation and stabilization of AgNPs using *S. cumini* leaf extract is schematically presented in scheme 1. Polyphenols have been shown to form complexes with silver ions resulting in the release of electrons. [54] This reduces the metal ions which can be visualized by a change in colour from transparent to dark brown/ black.

Characterization of AgNPs

UV-visible spectra showed the characteristic plasmon peak of silver at around 442 nm (Fig.1a). The absorbances were recorded for AgNPs with a final concentration of 10 mg mL^{-1}. Generation of nanoparticles was monitored from 0 to 72 h and increase in absorbance was observed during this period (Fig.1a inset). The absorbance spectra of plant extract alone and 1mM $AgNO_3$ was recorded (Fig. 1b). After 72 h the colour changes was also monitored Fig. 1b inset).The stability of the prepared solution was determined by measuring its absorption spectrum at an interval of 24 h for 90 days. A minor change in the absorbance was observed during the storage period of 90 days (Fig ESI S2). This indicated that the prepared nanoparticles were stable for a significant time.

The elemental composition of EDX data revealed the presence of silver in the formed AgNPs Fig.2b. Apart from the silver ions there are other signals of carbon, oxygen and sulphur which could be the residual presence of plant extracts. Lattice parameters of the prepared nanoparticles were studied by XRD technique. The pattern shows the formation of face centered cubic (fcc) silver, which could be confirmed by the peak positions at 2θ values 38.3, 44.6, 64.2 and 74.1 (JCPDS 89-3722). These correspond to the characteristic Bragg's reflection planes

(111), (200), (220) and (311). Average crystallite size of AgNPs is also presented in Fig.2b. This could depict that AgNPs were formed within a narrow size spectrum.

This was further supported by TEM analysis Fig. 2c, where maximum particle density was observed within 10-15 nm. Moreover, no sign of agglomeration was indicated by the TEM images. This affirms the stability of the prepared nanoparticles, which was initially observed by UV-visible spectroscopy. Silver nanoparticles with smaller size and uniform distribution are found to be efficient for many interesting biological activities.

Antimicrobial assays and Minimum Inhibitory concentration

Synthesized AgNPs were tested for their antimicrobial activity. AgNPs exhibited significant antibacterial activity against Gram-negative bacteria (*K. pneumoniae*, *P. aeruginosa* and *E. coli*), Gram-positive bacteria (*S. aureus*, *B. subtilis*, *M. smegmatis*) and fungus (*T. rubrum, Aspergillussp*, *F. oxysporum* and *C. albicans*) grown in MHA and PDA medium. The antimicrobial activity expressed in terms of zone of inhibition was quite visible on MHA and PDA plates (Fig. 3a-f) and corresponding Table 1 shows the measurement of zone of inhibition of AgNPs, methanolic leaf extract of *S.cumini* and $AgNO_3$ alone. Mean MIC values for the studied microbes were found to range between 250 and 62.5µgmL^{-1} .Some researchers have been reported that $AgNO_3$ do not contain any antifungal activity.[55] But some authors have reported a high antifungal activity of $AgNO_3$.[56] However, the antifungal activity of silver nitrate observed in this study might be attributed to strain differences and their differential susceptibility.

In vitro time dependent killing assay

From the antimicrobial assay it is clear that AgNPs have prominent activity against microorganisms. Again AgNPs showed significant inhibitory activity toward *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, *M. smegmatis C. albicans* and *F. oxysporrum* in a time dependent manner. As shown in Fig. 4 a-f 'green' AgNPs significantly inhibited the growth of the aforementioned strains. After 30 min of exposure to AgNPs at 10,000µg mL^{-1}, more than 75% of the microbes were killed, whereas after 2 h of incubation more than 95% killing was observed, relative to the control.

Change in cellular morphology after AgNPs treatment

SEM images of three representative microbial strains (*E. coli, S. aureus* and *C. albicans*) were taken to observe changes in the surface morphology of the microbial cells after treatments with AgNPs (10,000µg mL^{-1}) for 30 min. Native *E. coli, S. aureus and C. albicans* cells showed normal morphologies without any treatment (Fig.5 control) and the cell wall of the treated microorganisms is showing some abnormalities (Fig. 5 AgNPs).Thus, membrane damage may be one mechanism for inactivation of microorganism treated with AgNPs.

Effect of AgNPs on amplification of target DNA sequence using PCR

It has been reported that AgNPs interacts with phosphorous and sulphur containing macromolecules like DNA. This plausibility may be even complemented by their probable interaction with the polymerase enzyme used during polymerase chain reaction (PCR). However, there is limited information on whether or how AgNPs brings changes in genetic materials. In this work, we mechanistically investigated the effects of AgNPs on *in vitro* DNA replication fidelity using (1500 bp) DNA fragments in a PCR. The results showed that the replication fidelity of small gene (1500 bp) was compromised by AgNPs in a dose-dependent manner (Fig. 6a). The AgNPs concentrations (µg mL^{-1}) used in the wells, lane 1 and 6: 1000; lane 2 and 7:

500; lane 3 and 8: 250; lane 4 and 9: 125; lane 5 , 10: 62.5 and control reaction (C) without AgNPs at 18 and 35 cycle of PCR. As shown in Fig.7a that AgNPs at the concentration of 1000µg $mL^{-1}$and 500µg mL^{-1}compromised the reaction at 18 and 35 cycle of PCR and concentration from 250-62.5 µg mL^{-1} showing the positive effect on PCR efficiency and Fig. 7b shows that addition of bovine serum albumin (BSA) to PCR reactions reversed the effect of AgNPs, whereas BSA itself had no effect on PCR (Fig. 7b; lane 1-3). Fig. ESI S1 shows that plant extract has no effect on PCR efficiency.

Action of AgNPs on biofilm formation

Among the microbes tested here, *P. aeruginosa*, *S. aureus* and *C. albicans* have been studied in detail with respect to their ability to form biofilms. In this study, we tested the effect of AgNPs on biofilm formation under *in vitro* conditions by monitoring the binding of the crystal violet to adherent cells, which directly reflects the effective ability of the biofilm inhibition. Treatment of AgNPs for 24 h resulted in a decrease of biofilm formation with increasing AgNPs concentration (Fig. 7a-c). More than 90% biofilm inhibition was observed at 250 µg mL^{-1}concentrations and 85% of the biofilm inhibition was observed at 125 µg mL^{-1}. Also the lowest MIC value (62.5 µg mL^{-1}) showed the prominent antibiofilm activity. In addition the controls DMSO and water did not have significant biofilm inhibition activity, whereas 1mM $AgNO_3$ has the biofilm inhibition activity which may be due its antimicrobial activity. These data indicate that AgNPs impede biofilm formation of *P. aeruginosa, S. aureus* and *C. albicans.*

Cytotoxicity studies of AgNPs on Human Embryonic kidney cells (HEK 293)

Despite of effective antimicrobial activity and wide biological applications, the use of AgNPs as therapeutic agent is limited due to their cytotoxicity against mammalian cells. In this

study, we tested the cytotoxicity of green AgNPs against human embryonic kidney cells by the cell morphology and MTT assay. MTT assay relies on the fact that metabolically active cells reduce MTT to purple formazan. Cells treated with different concentrations of AgNPs for 24 h were observed for their morphological changes using inverted microscope. As shown in Fig. 8a-d, the cells did not show any visible morphological changes on treatment with AgNPs at different concentrations. However 1mM $AgNO_3$ showed significant cytotoxicity towards the cells (Fig. 8e). Absorbance of MTT at570 nm was directly proportional to the number of viable cells. Treatment with 250µg mL^{-1} AgNPs, which is maximum in MIC range resulted in approximately 20% reduction in cell viability (Fig. 9). However concentrations below 250µgmL^{-1}AgNPs exerted no significant cytotoxic effect on these cells (Fig. 9).No significant cytotoxicity was found in cells treated with water, DMSO or plant extract.

Conclusion

AgNPs synthesized using methanolic leaf extract of *S. cumini* is an economical, efficient and environment-friendly process. The 'green' AgNPs showed effective antibacterial and antifungal behaviour against a broad spectrum of microbes tested in this study. Further synthesized AgNPs have the potential to inhibit biofilm formation in a variety of micro-organisms. The nanoparticles have no cytotoxic effect on HEK cells at the MIC range. Thus the prepared AgNPs could be of promising application in biomedical field as an efficient antimicrobial and antibiofilm agent.

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Figure legends

Fig. 1. UV-Vis of AgNPs: (a) Absorption spectra of time dependent formation of AgNPs. Kinetics of AgNPs formation is shown in inset **(b)**. The UV-visible spectrum of AgNPs, plant extract and aqueous solution of $AgNO_3$ are recorded after a period of 72 h incubation1 mM $AgNO_3$ in aqueous (blue mark); Plant extract (red mark) and AgNPs (black mark). Color changes of 1 mM aqueous solution of $AgNO_3$ (blue line marked), plant extract (red lined marked) and 72 h incubated reaction mixture of $AgNO_3$ plant extract (black lined marked)(Inset)

Fig. 2 Characterization of AgNPs: EDX spectrum of the green synthesized AgNPs (a), X-ray powder diffraction pattern of the green synthesized silver nanoparticles(b), representative TEM micrographs of phytosynthesized silver nanoparticles(c), histogram frequency for the particle size distribution derived by counting over multiple images

Fig. 3 Antimicrobial activity: Antimicrobial activity of (1) plant extract,(2) silver nitrate, (3)AgNPs and (4) antibiotics against (a)*Escherichiacoli*, (b) *Bacillus subtilis*, (c)*Staphylococcus aureus*, (d)*Mycobacterium smegmatis*, (e) *Candida albicans* and(f) *Fusariumoxysporum*

Fig.4 Time dependent Effect of AgNPs on microbial survivability:effect of AgNPs (10 mg mL^{-1}) on the microbial survival using CFU assay. (a) *Escherichiacoli*, (b) *Pseudomonas aeruginosa*, (c) *Staphylococcus aureus*, (d) *Bacillus subtilis*, (e) *Mycobacterium smegmatis*, (f) *Candida albicans*and (g) *Fusariumoxysporum*

Fig.5Scanning electron microscopy images of microbial cells upon treatment with AgNPs: SEM micrograph of microbial cells (a) *Escherichiacoli*(b) *Staphylococcus aureus* and (c) *Candida albicans*without treatment of AgNPs (control) andafter treatment(AgNPs) Solid arrows show morphological deformation of treated cells.

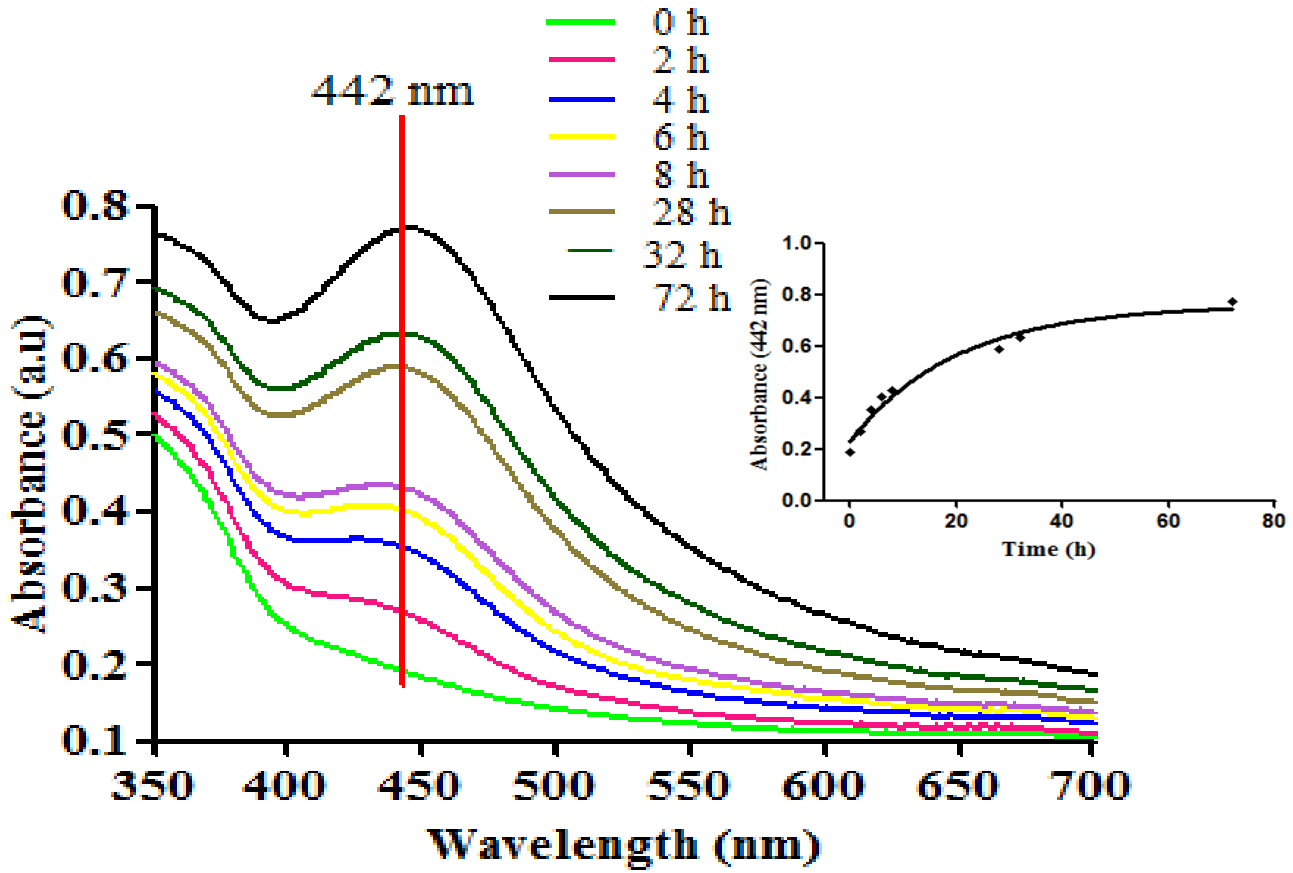
Fig. 6 Effect of AgNPs on PCR efficiency (16srRNA gene) and role of BSA on removal of AgNP effect: (a)AgNPs inhibit the PCR amplification of a small gene (16s rRNA gene). M, marker; (Thermo Scientific ; Gene 1kb plus ladder; Cat No. #SM1333)C, control; lane 1 was 1000 µg mL^{-1}; lane 2 was 500 µg mL^{-1}; lane 3 was 250 µg mL^{-1}; lane 4 was 125 µg mL^{-1}; lane 5 was 62.5 µg mL^{-1}; AgNPs (18 cycles) lane 6 was 1000 µg mL^{-1}; lane 7 was 500 µg mL^{-1}; lane 8 was 250 µg mL^{-1}; lane 9 was 125 µg mL^{-1}; lane 10 was 62.5 mL^{-1}AgNPs (35 cycles.).(b) BSA reverses the effect of AgNPs on PCR (35 cycle). M, marker (Thermo Scientific ; Gene 1kb plus ladder; Cat No. #SM1333); C, control. The concentration of BSA in lane 1 was 8 µg mL^{-1}; lane 2 was 4 µg mL^{-1}; lane 3 was 2 µg mL^{-1}. The concentration of AgNPs in lane 4,5,6 was 500 µg mL^{-1}; lane 7,8,9, was 1000 µg mL^{-1}. The concentration of BSA in lane 4 and 7 was 8 µg mL^{-1}; lane 5 and 8 was 4 µg mL^{-1}; lane 6 and 9 was 2 µg mL^{-1}.

Fig. 7 Biofilm inhibition using crystal violet (CV) assay. Inhibition of biofilm formation of (a)*Staphylococcus aureus,*(b) *Pseudomonas aeruginosa*and(c) *Candida albicans* Experiments were performed in triplicates; mean ± SD are shown.

Fig. 8 Cytotoxicity of AgNPs on cell morphology of Human Embryonic Kidney cells (HEK 293). (a) Cells without treatment, Treated with (b) 250 µg mL^{-1} AgNPs, (c) 125 µg mL^{-1}AgNPs, (d)62.5 µg mL^{-1} AgNPsand (e) with 1mM $AgNO_3$

Fig. 9 Cytotoxicity of AgNPs concentration on Cell viability of Human Embryonic Kidney cells (HEK 293). Cell viability of Human Embryonic Kidney cells (HEK 293) upon treatment with MIC range of AgNPs.

Fig. 1 (a)



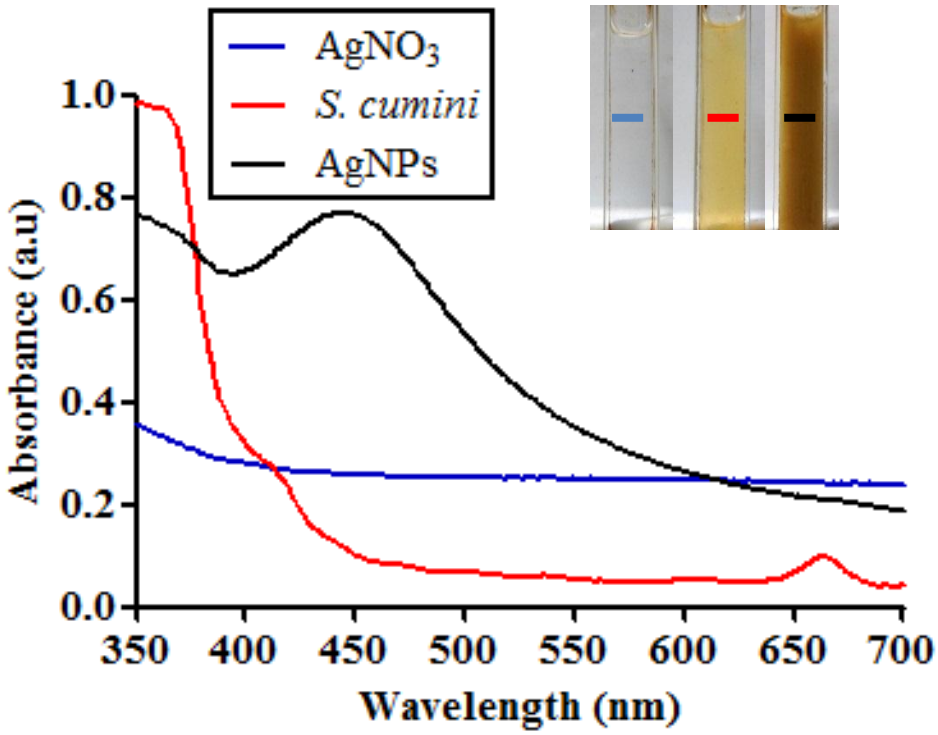
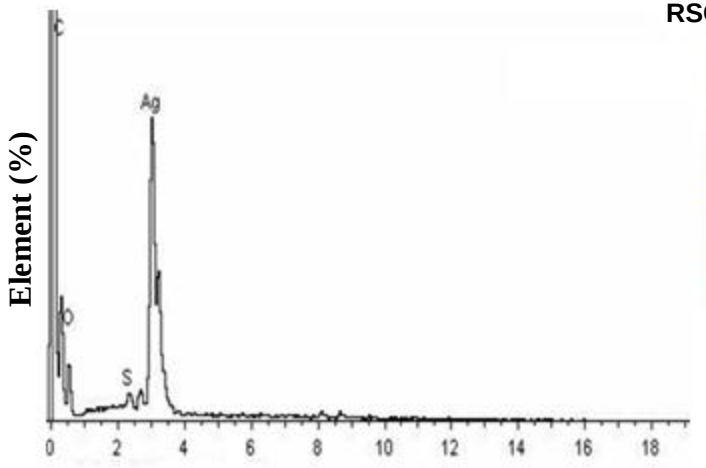


Fig. 2 (a)



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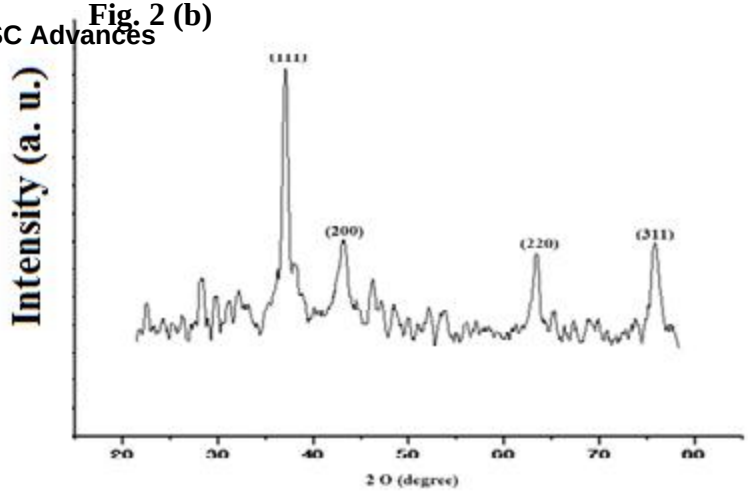

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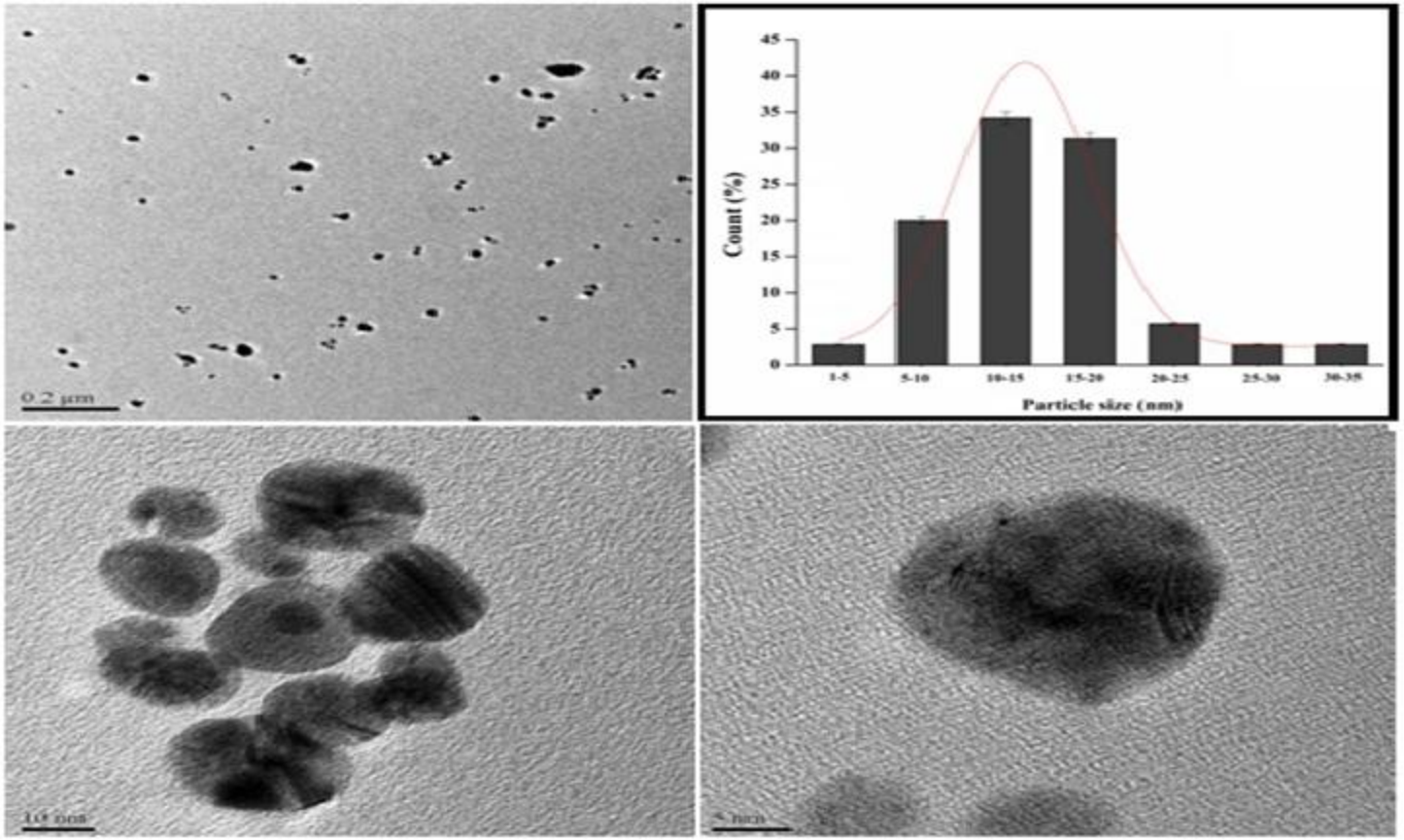
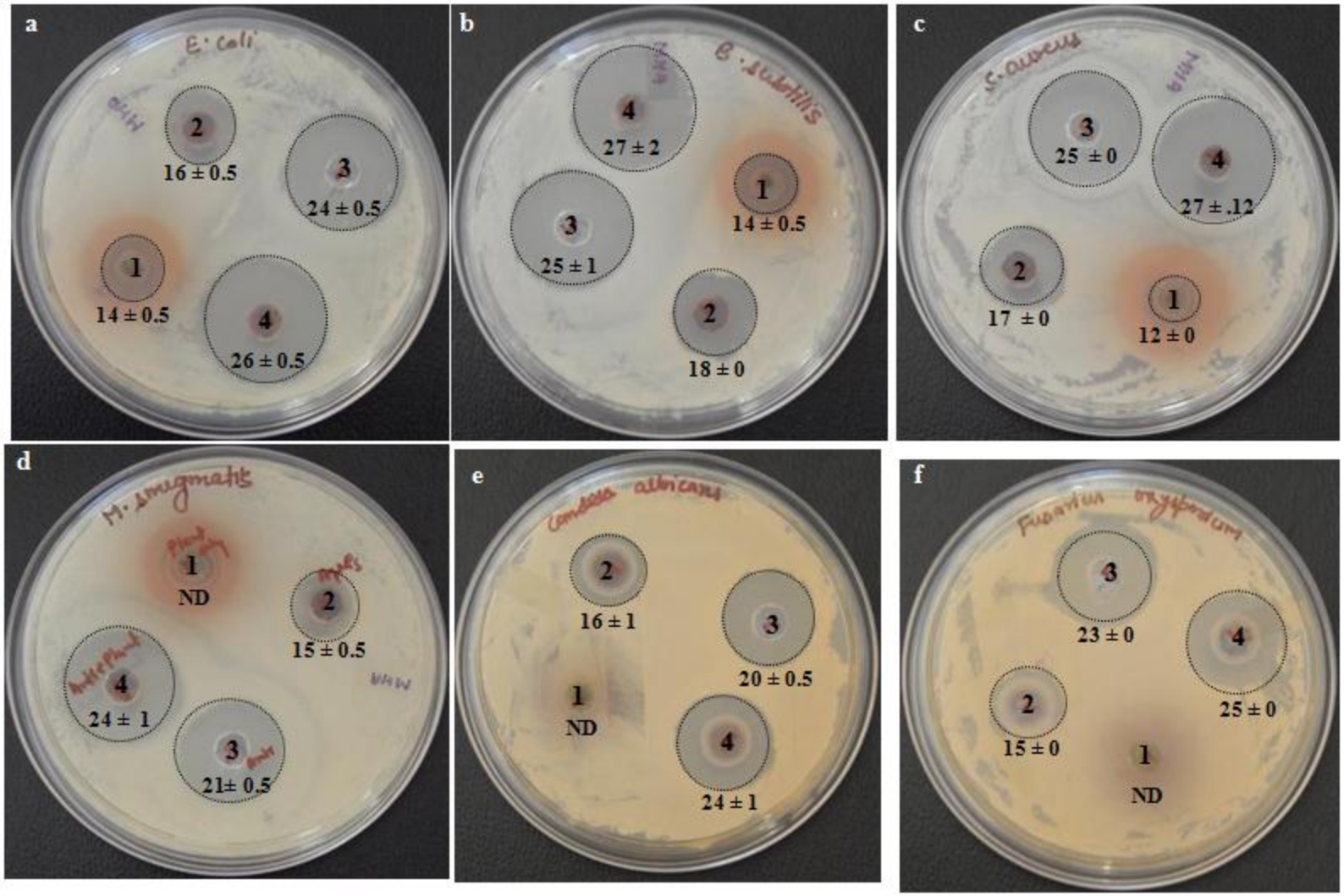
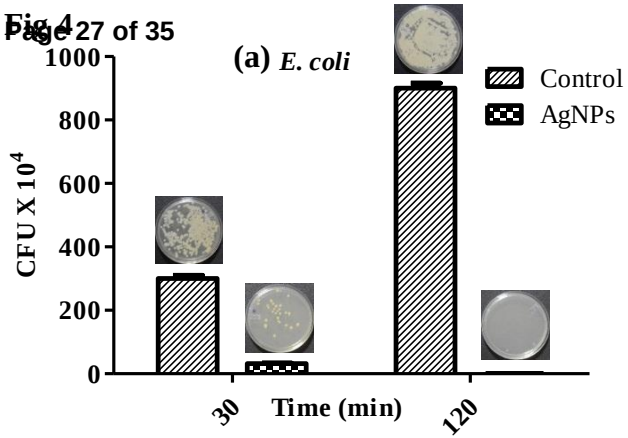
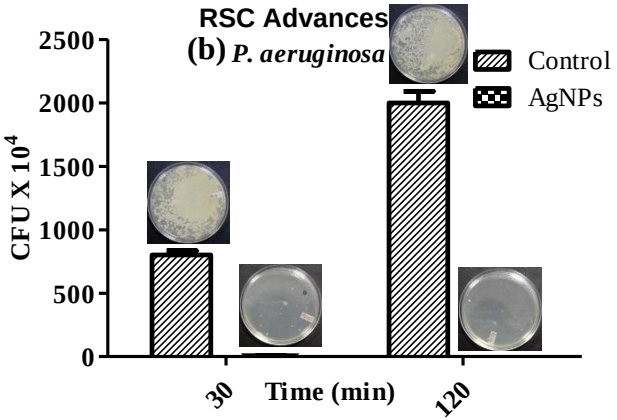
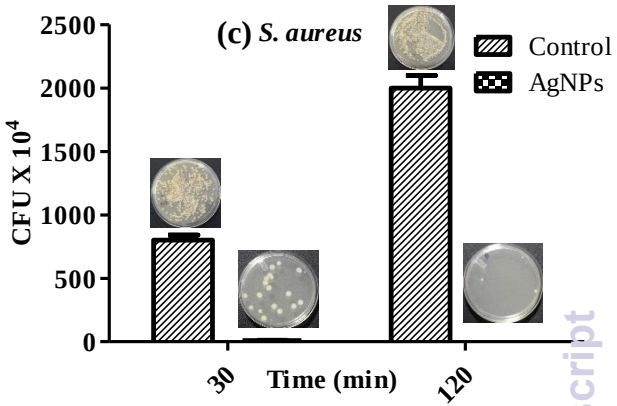
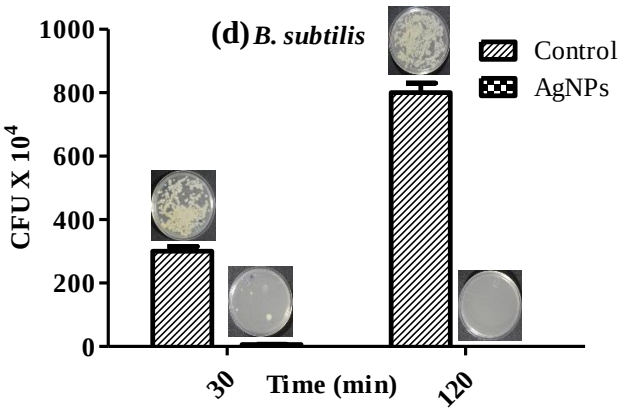


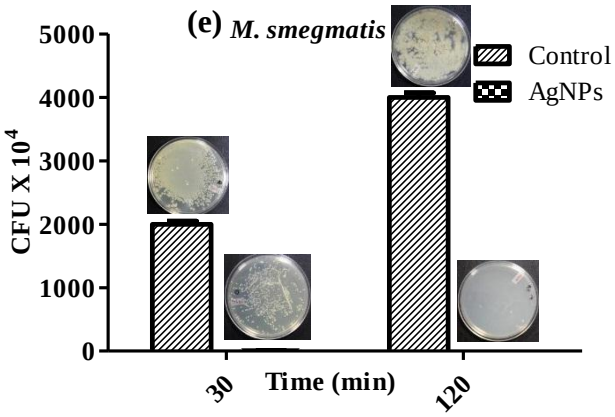


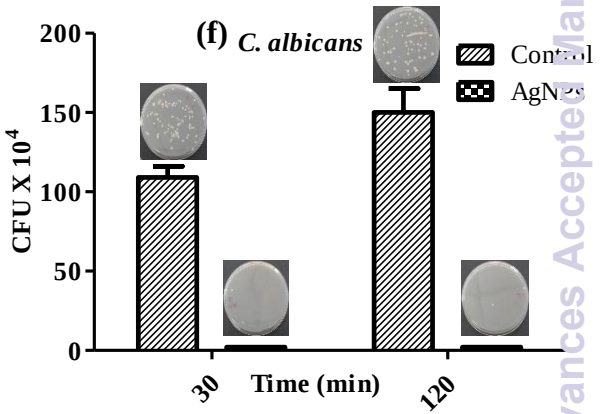












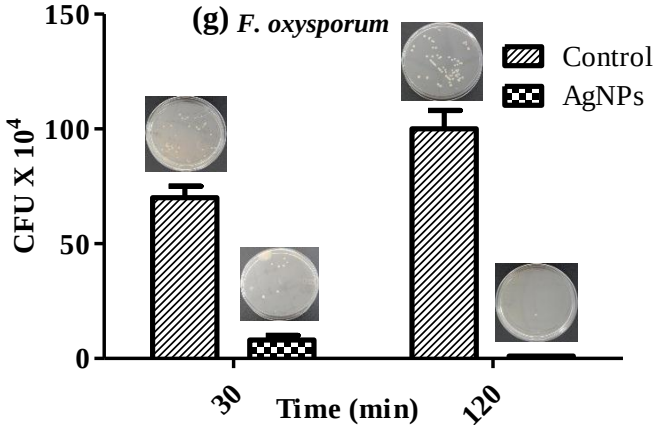


Fig. 5

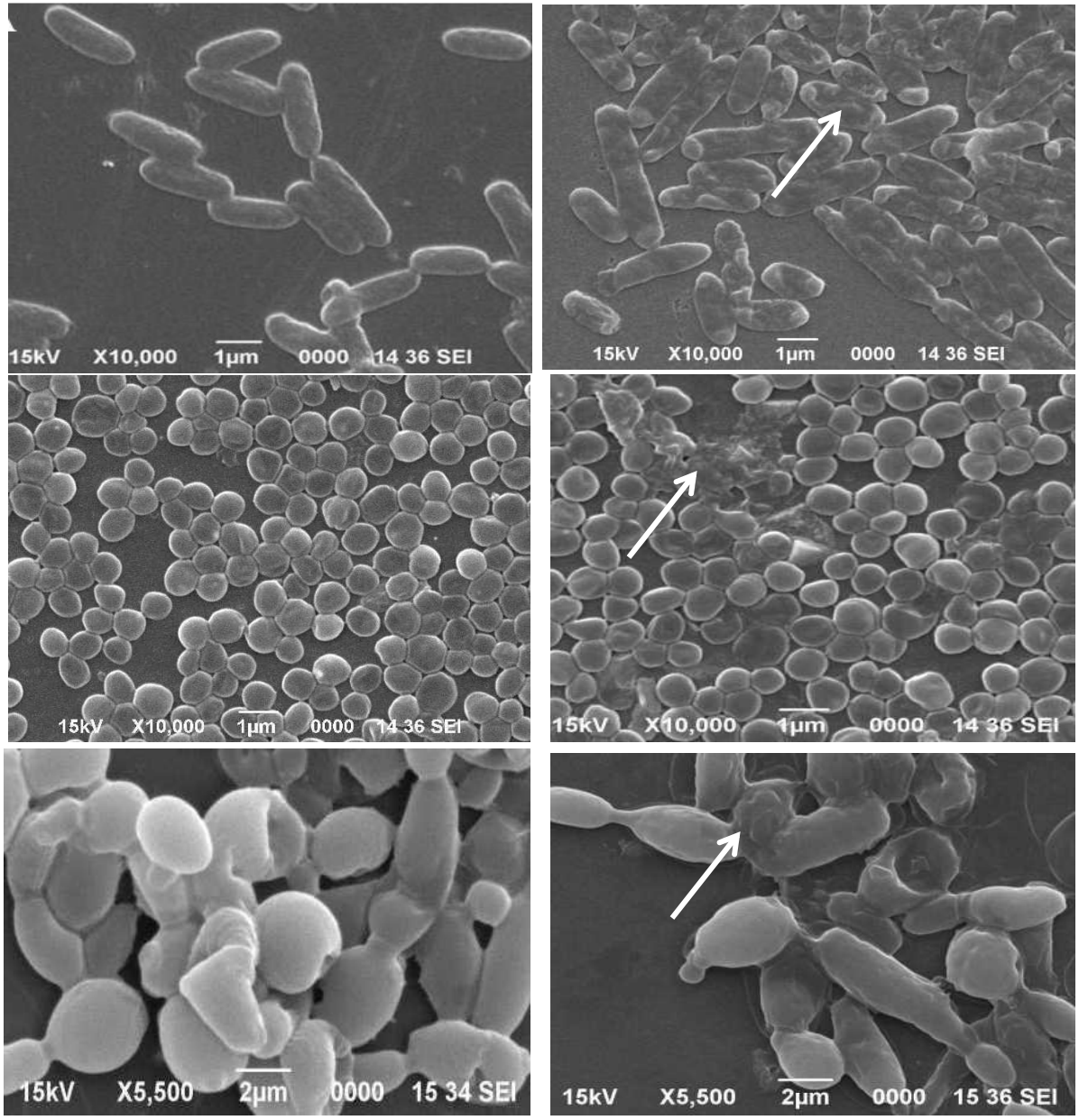


Fig. 6 (a)

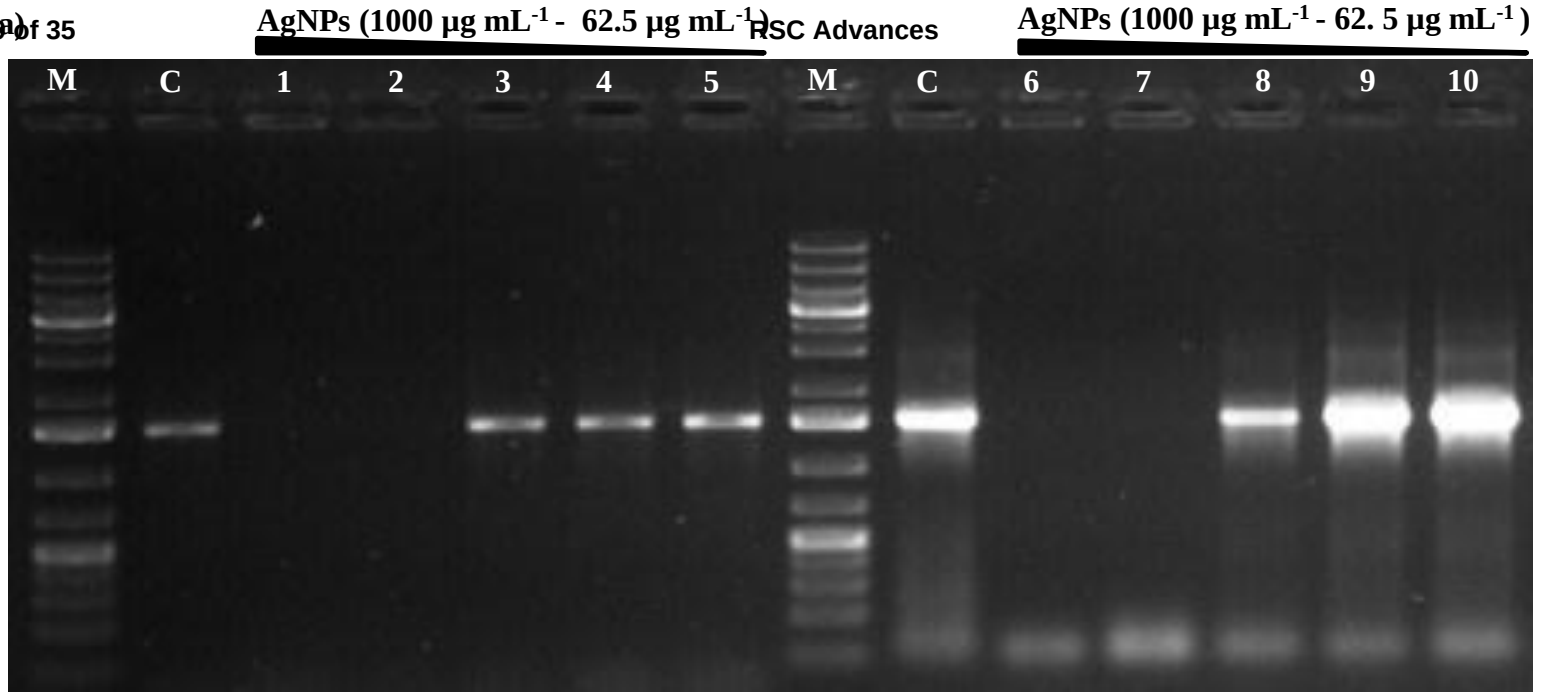
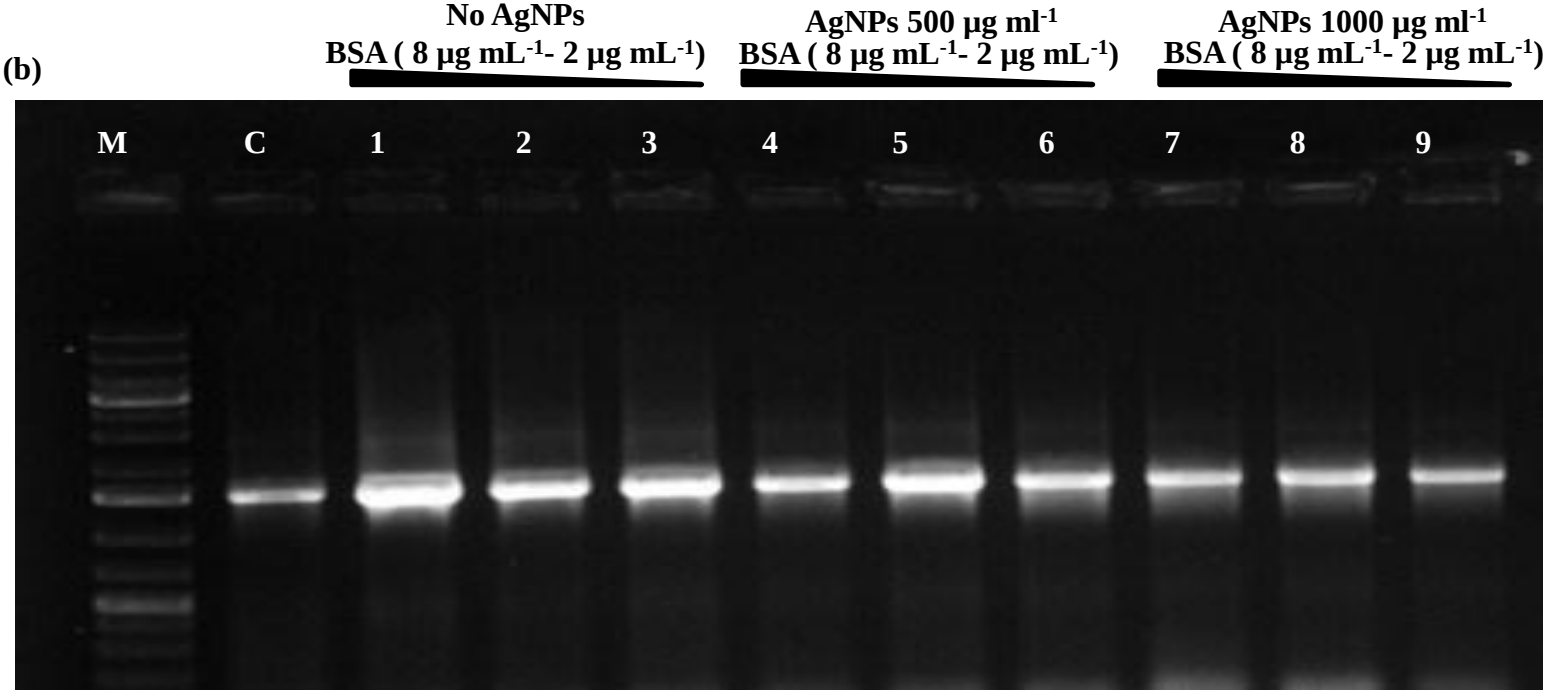
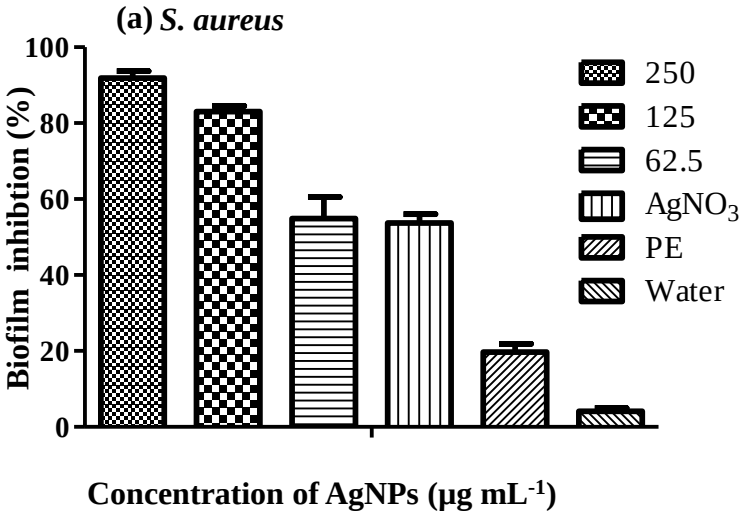
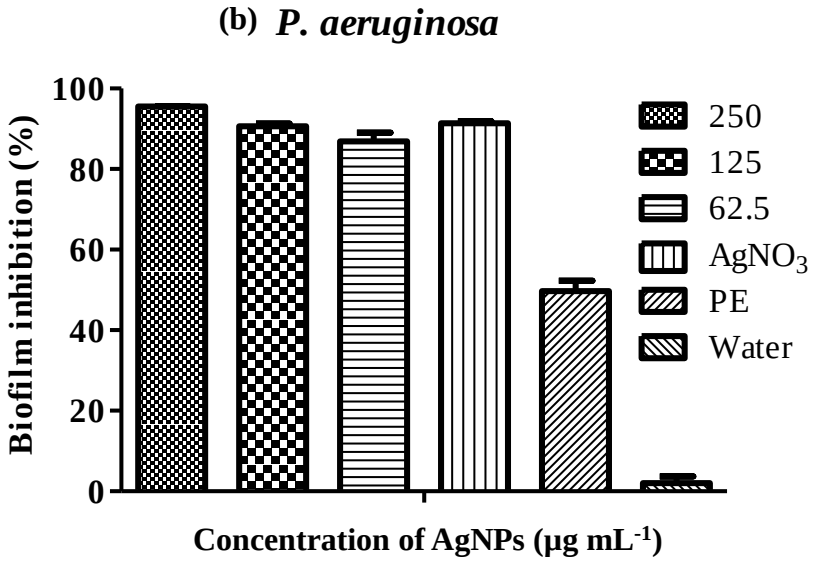
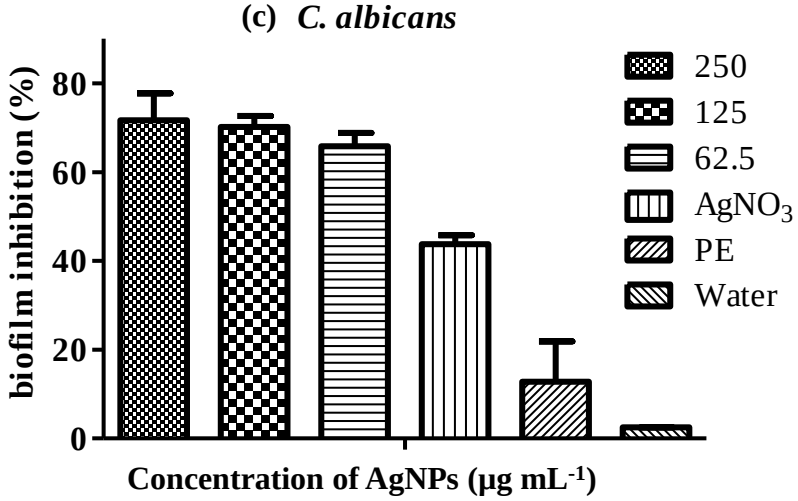


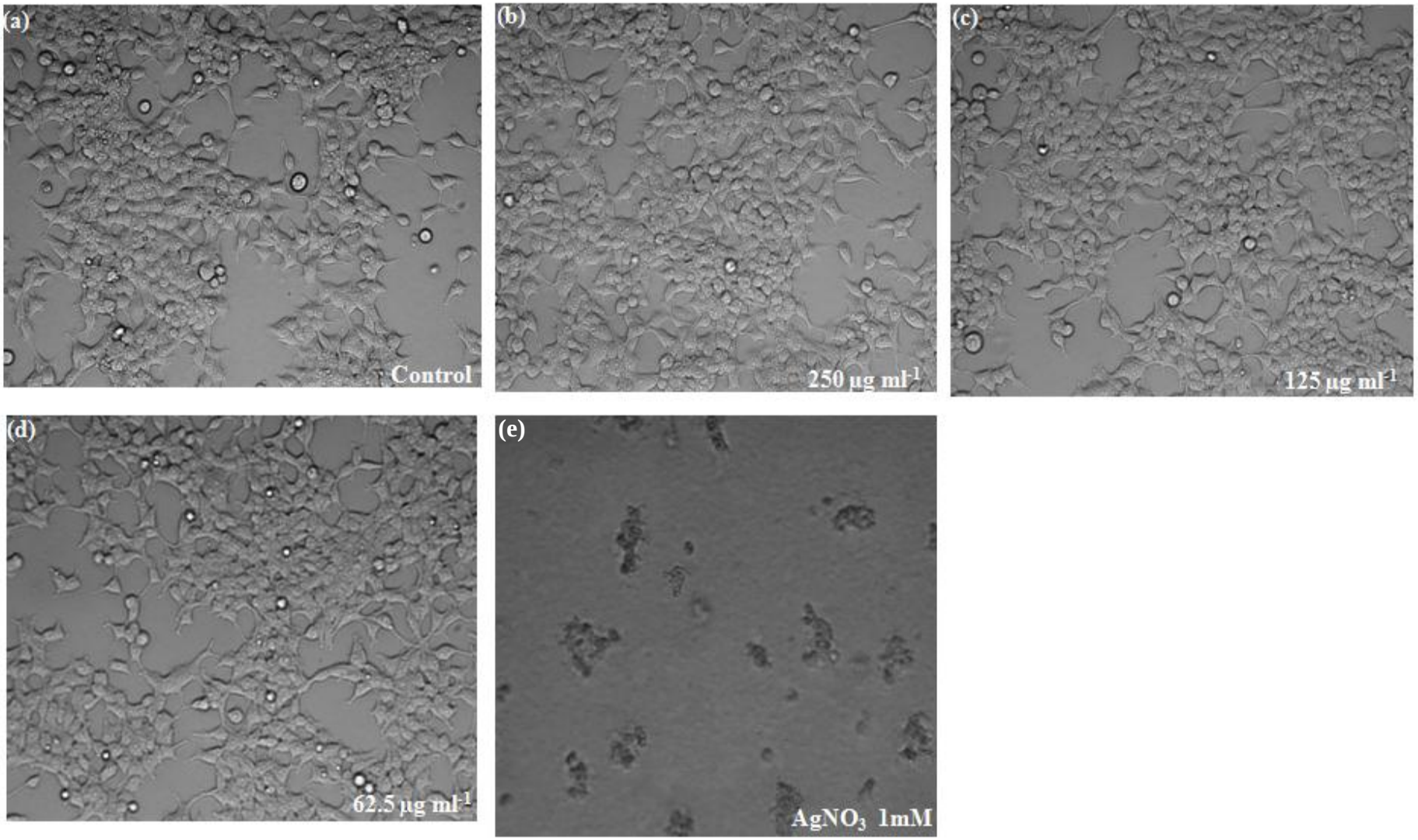
Fig. 6 (b)

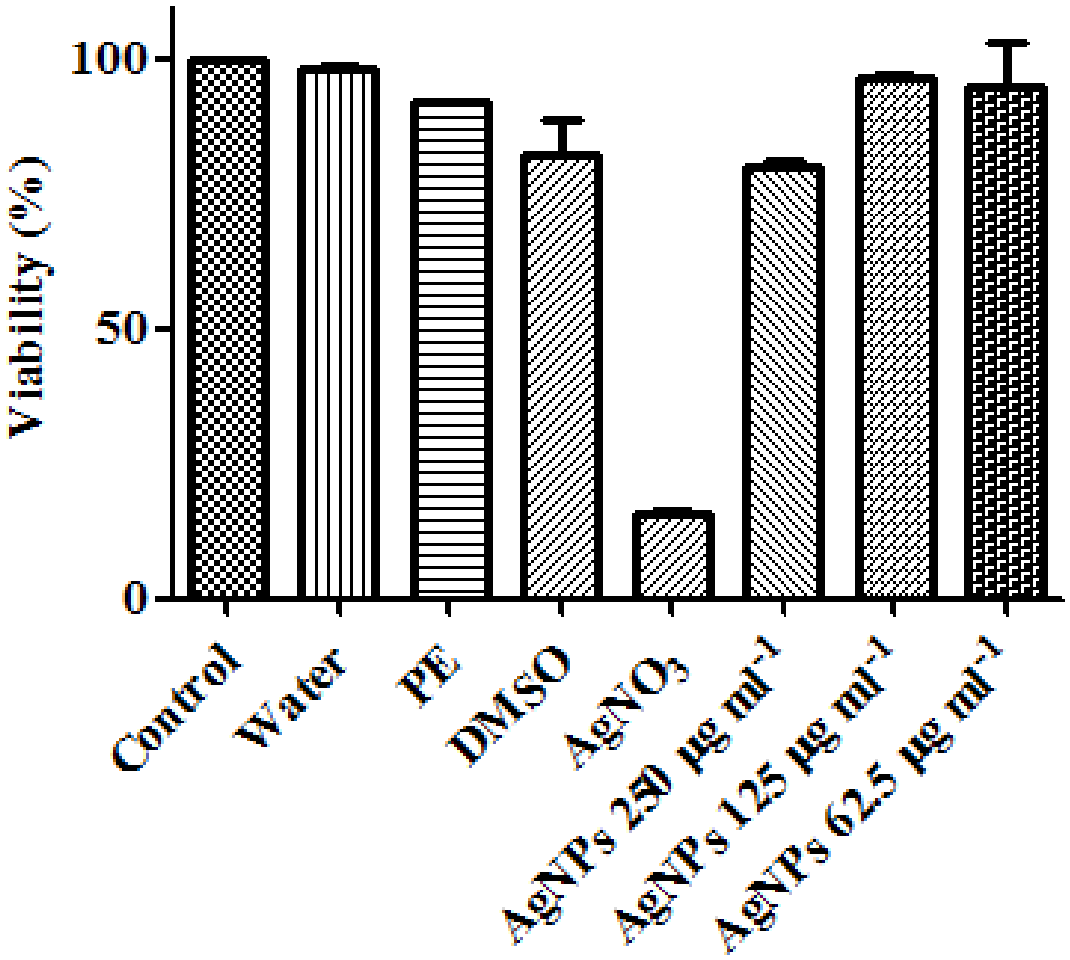


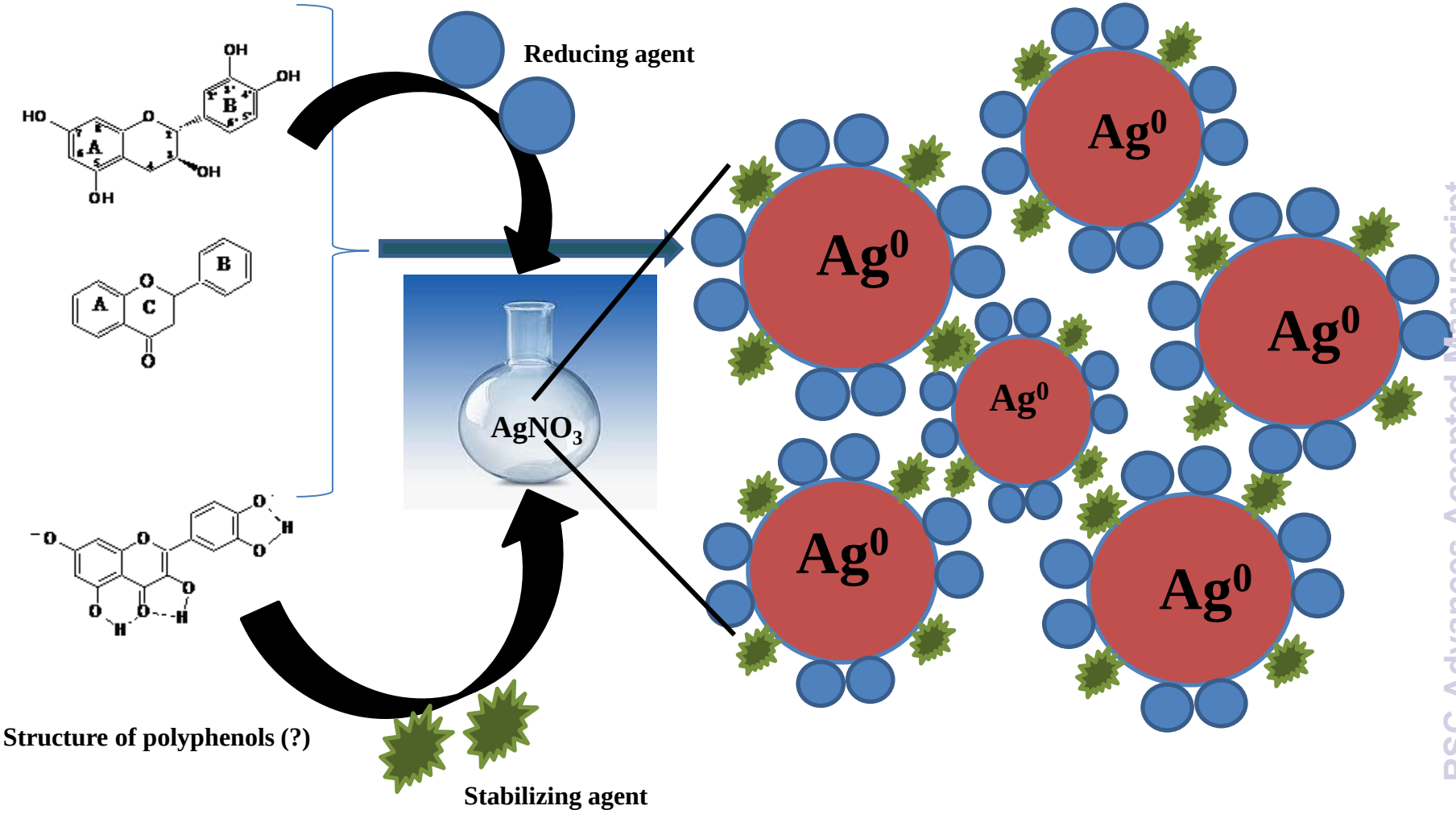












Scheme I: The proposed mechanism of reduction and stabilization of silver nanoparticles (AgNPs) using *S. cumini* leaf extract.

Table 1. Anti-microbial activity of methanol extract (ME) of *Syzygium cumini*, $AgNO_3$ (1mM), AgNPs (10,000 µg mL^{-1}) and standard antibiotics.

Table 1. Anti microbial activity of methanol extract (ME) of *Syzygium cumini*, $AgNO_3$ (1mM), AgNPs (10,000 µg mL^{-1}) and standard antibiotics by agar well diffusion assay

Pathogenic microorganism	Zone of inhibition (mm) *			
	S. cumini	$AgNO_3$	AgNPs	Antibiotics**
Escherichia coli MTCC 40	14 ± 0.5	16 ± 0.5	24 ± 0.5	26 ± 0.5
Pseudomonas aeruginosa MTCC **1688**	13 ± 0.5	16 ± 0.5	21 ± 0.5	23 ± .10
Bacillus subtilis MTCC 121	14 ± 0.5	18 ± 0	25 ± 1	27 ± 2
Staphylococcus aureus MTCC 3160	12	17	25	27 ± .12
Mycobacterium smegmatis MTCC 14468	ND	15 ± 0.5	21 ± 0.5	24 ± 1
Candida albicans MTCC 183	ND	16 ± 1	20 ± 0.5	21 ± 1
Fusarium oxysporium MTCC 284	ND	15	23	25
Trichophyton rubrum **(sk)**	ND	ND	17 ± 1	22 ± 0.5
*Aspergillus niger***(1)**	ND	ND	18 ± 0.5	22 ± 0.5
*Aspergillus versicolor***(2)**	ND	ND	10 ± 0.5	22 ± 0.5

(ND): Not detectable. Values are given as mean ± SEM (n =3). *The diameter of well is 6 mm

****Gentamicin is used for bacterial strain, Nystatin and Clotrimazole is used for fungal strain as control.**