THERANOSTICS

Supporting Information

Potential theranostics application of bio-synthesized silver nanoparticles (4-in-1 system)

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Running title: Multifunctional silver nanoparticles

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1. CHARACTERIZATION TECHNIQUES

The as-synthesized b-AgNPs were thoroughly characterized by several physico-chemical techniques that are described below:

1.1. UV-VIS spectroscopy

The absorption of all as-synthesized b-AgNPs was measured by UV–Vis spectroscopy (JASCO dual-beam spectrophotometer (*Model V-570*) in a quartz cuvette from 800 to 200 nm with a resolution of 1 nm.

1.2. X-ray diffraction (XRD)

The intense ash colored loose pellets of as-synthesized b-AgNPs samples were obtained by centrifugation at a 14,000 rpm at 10^0 C for 1 h in Thermo scientific, Sorvall-WX ultra 100. The glass slide was coated with this loose b-AgNPs pellet by evaporating the solvent repeatedly and submitted for XRD analysis. The structure and phase purity of the as-synthesized b-AgNPs samples were determined by X-ray diffraction (XRD) analysis using a Bruker AXS D8 Advance Powder X-ray diffractometer (using CuKαλ=1.5406 Å radiation).

1.3. Transmission electron microscopy (TEM)

Initially, the ash colored intense loose pellet of as-synthesized b-AgNPs samples were obtained by centrifugation at a 14,000 rpm at 10^0 C for 1 h and diluted with water (1:3 ratio). Samples for TEM analysis were prepared by placing a drop of this diluted loose pellet solution on carbon-coated copper grid and allowed the grid to evaporate the solvent. The morphology and shape of nanoparticles were examined on a FEI Tecnai F12 (Philips Electron Optics, Holland) instrument operated at 100 kV. Selected area electron diffraction (SAED) patterns were also taken using this instrument.

1.4. Dynamic light scattering (DLS)

Zeta potential of b-AgNPs solution was measured in zeta potential analyzer (HORIBA SZ-100) using quartz cuvette taking 20 µL of AgNPs in 1mL of deionized water.

1.5. Fourier transformed infrared spectroscopy (FTIR)

FTIR measurements were carried out to identify the possible functional groups in biomolecules (from plant extract) responsible for the synthesis of b-AgNPs. The fourier transformed infrared (FTIR) spectra were recorded using thermo Nicolet Nexus 670 spectrometer in the diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets. The ash colored intense loose pellet of as-synthesized AgNPs samples were obtained by centrifugation at a 14,000 rpm at 10^0 C for 1 h in Thermo scientific, Sorvall-WX ultra 100. The glass slide was coated with this loose AgNPs pellet along with the *Olax* leaf extract by evaporating the solvent repeatedly and submitted for FTIR analysis.

1.6. Gel electrophoresis (SDS PAGE)

In order to find out the specific *Olax Scandens* proteins responsible for the formation and stabilization of silver nanoparticles, we have carried out 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) where we have loaded the concentrated part of the supernatant of b-AgNPs after centrifugation at 10^0 C for 1h at 14 k rpm and the water extract of *Olax* leaves. The concentrated extract and supernatant of b-AgNPs were mixed with one third of 4x laemmli sample loading buffer (Rainbow) with 2-mercaptoethanol (Sigma) and were heated at 100 ºC for 5 mins before loading and 50 µL of this mixture was loaded in 12% SDS-PAGE. A standard protein molecular weight marker (BIORAD) was loaded in separate well. Finally, the gel was stained with silver nitrate according to standard protocol and scanned with a photo scanner *HP*.

1.7. Inductively coupled plasma optical emission spectrometry (ICP-OES)

An inductively coupled plasma optical emission spectrometer (ICP-OES, IRIS intrepid II XDL, Thermo Jarrel Ash) was used to determine the concentrations of silver ions in the

aqueous solutions. Initially, a series of standard $AgNO_3$ solution with concentration range of 5-50 ppm was prepared for silver standard curve. Then we have calculated the concentration of silver ion in b-AgNPs from ICP-OES analysis using this standard curve.

2. EXPERIMENTAL PROCEDURES

2.1. Antibacterial activity of b-AgNPs towards E.coli

Sterile LB (Luria bertani) broth media was used for the determination of bacterial growth inhibition at 37ºC in shaking condition at 180 rpm. After pre-inoculation of a single colony of *E.coli* into 1 mL of sterile LB -broth, pre-culture (around 0.6 OD at 600 nm contains 10^7 CFU/ mL) was inoculated into 20 ml of each sterile LB- broth medium at 1:100 dilutions. At regular intervals the bacterial growth inhibition was checked by multimode spectrophotometer (Varioskan) at 600 nm up to 7 hours. Silver nanoparticles with media as a blank, culture with media as a negative control, culture plus ampicillin (100 µg/µl) with media as a positive control and culture with different concentration of silver nanoparticles (1.5-30 µM) with media were taken for experimental groups.

Spread plate technique was used to measure the antibacterial activity of b-AgNPs at concentration range 3-30 µM by counting of CFU (colony forming units). First b-AgNPs was mixed in sterile hot 30 ml LB-agar (Agar- 1.5%) medium. Then medium was poured in Petri-plates. After solidification of media, 2.5 µl of pre-culture of *E.coli* (0.6 OD at 600 nm of culture grown by inoculation of a single colony into 1 ml of LB-broth medium contains 10^7 CFU/ mL) was used to spread onto the upper surface area of solidified LB-agar media containing AgNPs. Then plates were incubated at 37°C with inverted position for 16 hours. As a positive control without any antimicrobial drug and a negative control with ampicillin (100 µg/ mL) LB-agar medium were used.

Agar diffusion technique also used to evaluate antimicrobial activity of Gram negative *E.coli* against Ag-NPs. Here 100 µl pre-culture (OD around 0.6 at 600 nm contains 10^7 CFU/ mL) was taken to inoculate into sterilized semisolid LB-agar medium (agar-1.2%) at 37°C to 42°C.Then the medium was poured into the Petri-dish. b-AgNPs solution was poured into the well in the centre of the plate. After diffusion of antibacterial drug, plates are incubated at 37°C with the inverted condition for 16 hours. Ampicillin (100 µg/ ml) containing plates were used for distinguishing the inhibition zone of different classes.

Interaction of silver nanoparticles with *E.coli* was analyzed by SEM. To analyze the interaction of silver nanoparticles with *E.coli* was grown through a log phase (OD around 0.6 at 600 nm contains 10^7 CFU/ mL) in LB culture medium. Then, silver nanoparticles of 30 µM and *Olax* of 100µg/ mL had been added to bacterial solution and leaving the bacteria to grow for 30 min. The cells were collected by centrifugation (3000 rpm, 5 min, 4 ◦C), washed and then re suspended with a PBS buffer solution. Then the bacterial cells were fixed by exposure to a 2.5% glutaraldehyde solution in PBS for 30 min. Then again the fixed cells have been stained with 2% OsO_4 for 45 minutes. After that, the stained *E.coli* cells have been undergoing dehydration by using a series of 50, 60, 80, 90 and 100% ethanol/PBS solutions. Then, these samples were analyzed by using SEM (Hitachi S-3000 N, Japan).

2.2. Oxidative Stress: In-vitro GSH Oxidation: Ellman's Assay

The concentration of thiols in GSH was quantified by the Ellman's assay. b-AgNPs pellets (225 µL) in different concentrations in 50 mM bicarbonate buffer (pH 8.6) was added into 225 µL of GSH (0.8 mM in the bicarbonate buffer) to initiate oxidation. GSH solution without b-AgNPs was used as a negative control and GSH (0.4 mM) oxidization by H_2O_2 (1 mM) was used as a positive control. The b-AgNPs mixtures were transferred into a 24-well plate covered with alumina foil to prevent illumination, placed in a shaker with a speed of 150 rpm at room temperature for 2 h. After incubation, 785 µL of 0.05 M Tris-HCl and 15 µL

of DTNB (Ellman's reagent, 5,50-dithio-bis-(2-nitrobenzoic acid), Sigma-Aldrich) were added into the mixtures to yield a yellow product. A 250 μL aliquot of filtered solutions (0.22 μM PVDF, Merck Millipore) from each sample was then placed in a 96- well plate and absorbance was measured by at 570 nm using a microplate reader (Varioskan Flash). The loss of GSH was calculated by the following formula: loss of GSH % = (absorbance of negative control - absorbance of sample)/absorbance of negative control×100.

2.3. Cell viability test using MTT reagent

The MTT assay (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide), a colorimetric assay has been used for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. This assay has been used to determine the cell viability in presence of any cytotoxic potential medicinal agents/ nanomaterials. Briefly, we have plated 10,000 cells (counted by haemocytometer) of different cancer cells and normal cells (A549, B16 and H9C2, HUVEC) in a 96 well plate for 24 h and then the cells were incubated with b-AgNPs in dose dependent condition for another 24 h. 1 mL of MTT stock solution (concentration 5 mg/ mL) was diluted to 10 mL solution using DMEM/ EBM for HUVEC media and 100 μL of this MTT solution was added to each well by replacing the media and allowed to incubate for 4 h. After 4 h, the media in each well was replaced by 100 μL of DMSO-Methanol mixture (1:1 volume ratio) for solubilizing the violet crystal and kept the mixture on the shaker for homogeneous mixture. Finally, the absorbance of the mixture was measured at 570 nm using a microplate reader (Varioskan Flash). All the experiments were carried out in triplicate and the results are expressed as normalized viability= $\{1/Abs_{\lambda=570}$ (untreated $cells_{blank})\}\times\{Abs_{\lambda=570}$ (treated cells-blank)$\}$.

2.4. Experimental procedures for AAS and UV to check the release of Ag+ ions in acidic and basic pH

The as synthesized b-AgNPs and c-AgNPs were centrifuged at 14,000 r.p.m and the as collected pellets have incubated with acidic (pH-5.0) and neutral buffer solutions (pH-7.4) for 24 hours [10 ppm of b-Ag and c-Ag has been incubated with 10 mL of pH-5.0 and pH-7.4 buffers]. After I day incubation the solutions have been centrifuged at 25,000 rpm to eliminate all the AgNPs and to keep only the silver ions. The supernatant is submitted for AAS to determine the concentration of silver ions.

Also, the b-Ag and c-Ag incubated with acidic and neutral buffers for 24 hours is monitored in the UV-Visible spectroscopy.

3. RESULTS AND DISCUSSION

3.1. Characterization of c-AgNPs and comparisons of size, shape & charge with b-AgNPs

We have performed various characterization techniques (UV-Visible, TEM, DLS, Zeta potential) of chemically synthesized silver nanoparticles (c-AgNPs). The results are provided in the supporting information (SI-Fig. 4.a-c). It is already well established that for a particular nanoparticles, biological activity changes with its size, shape and charge. In this present communication, there is no significant difference in size, shape & charge between chemically synthesized (c-AgNPs) and biologically synthesized nanoparticles (b-AgNPs). However, we have found that b-AgNPs show better anti-bacterial, anti-cancer and imaging properties

SI.Table-1: Comparison between the size, shape & charge between b-AgNPs & c-AgNPs

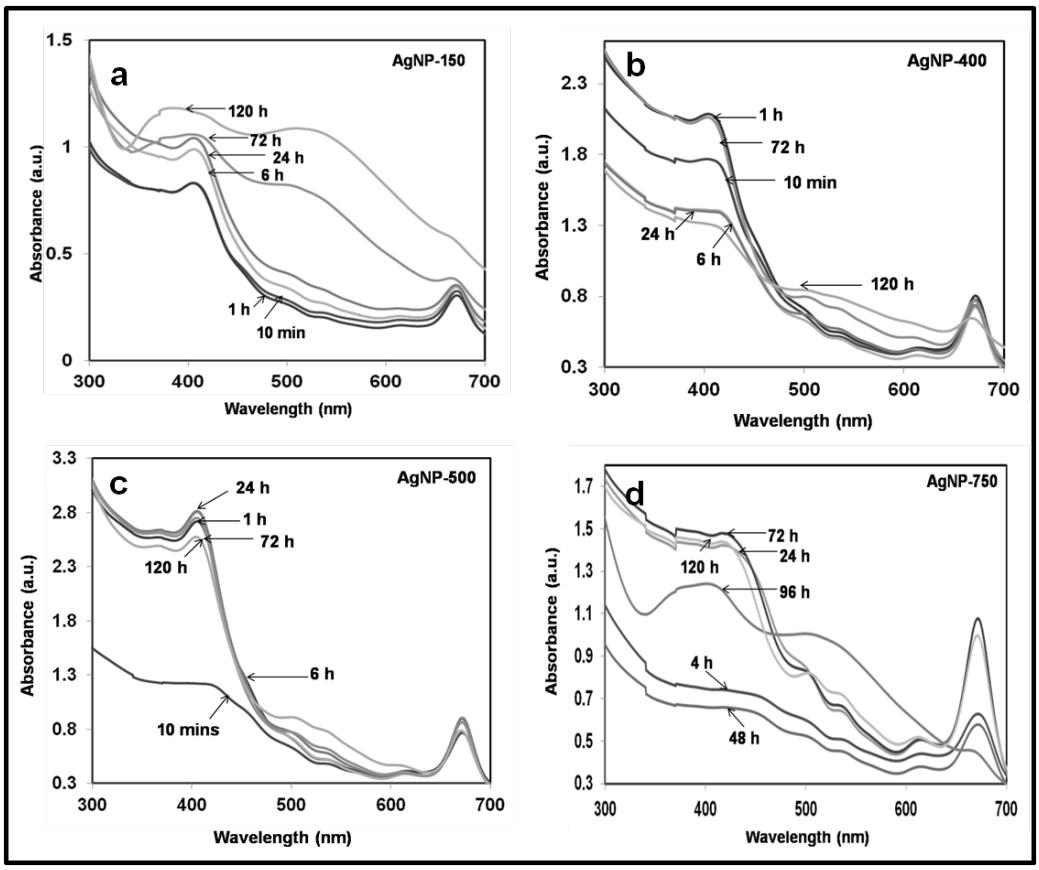
Parameters	b-AgNPs	c-AgNPs
SPR (λ_{max})	402 nm	385 nm
TEM (size)	10-55 nm	5-50 nm
Shape	Spherical	Spherical
DLS (size)	55-100 nm	20-80 nm
DLS (charge)	– 15.2 ± 0.5 mV	- 20.6 ± 1.2 mV

presented in the SI-Table-1.

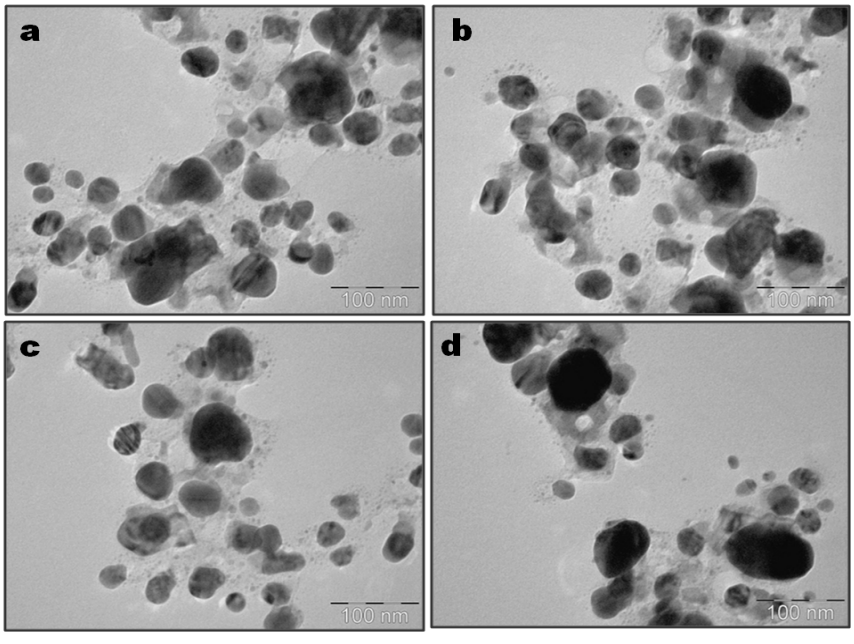
3.2. Fourier transformed infrared (FTIR) spectroscopy

The bio-synthesis of silver nanoparticles has been carried out using *Olax scandens* leaf extract that contains some phytochemicals which are responsible for synthesis and stabilization of the nanomaterials. In order to find out the exact role of phytochemicals present in the leaf extract, FTIR spectroscopy has been carried out for both *Olax leaf* extract and b-AgNPs-500 that are presented in SI.Fig.5.a-b. The major stretching frequencies appeared at $\nu = 1512.18$ cm^{-1} and $\nu = 1258.79$ cm^{-1} due to the presence as amide II and amide III in proteins of *Olax scandens,* have been almost disappeared for b-AgNPs-500 (SI.Fig.5.b) confirming the role of proteins for the synthesis and stablization of b-AgNPs-500 (SI.Fig.5.b). These results are supported by our previous literature.[22-23] The role of proteins for the synthesis and stablization of b-AgNPs-500 has been further confirmed by SDS gel electrophoresis The other significant IR spectrum mainly at $\nu = 1738.01$ cm^{-1} in curve-e shifts to 1737.53 cm^{-1} in curve-b which may be due to the participation of C=O stretching modes of aldehydes or ketones in curve-e which arises from C=O stretching modes of acids. Another peak at $\nu = 3384.31$ cm^{-1} in curve-e is due to the O-H stretching modes present in the several polyphenols like otacosanol, β-sitosterol and glucosides of β-sitosterol in the Olax leaf extract. [23]Thus, several proteins and polyphenols are mainly responsible for the generation of b-AgNPs. The remaining IR peaks in curve –a and -b remains almost unchanged.

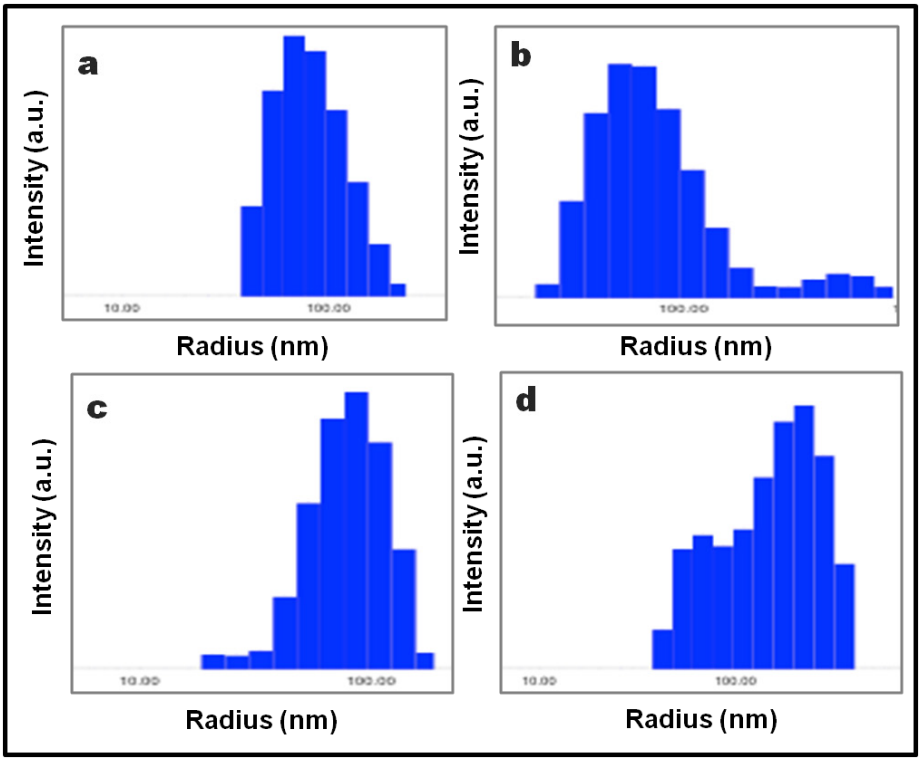
SUPPORTING FIGURES



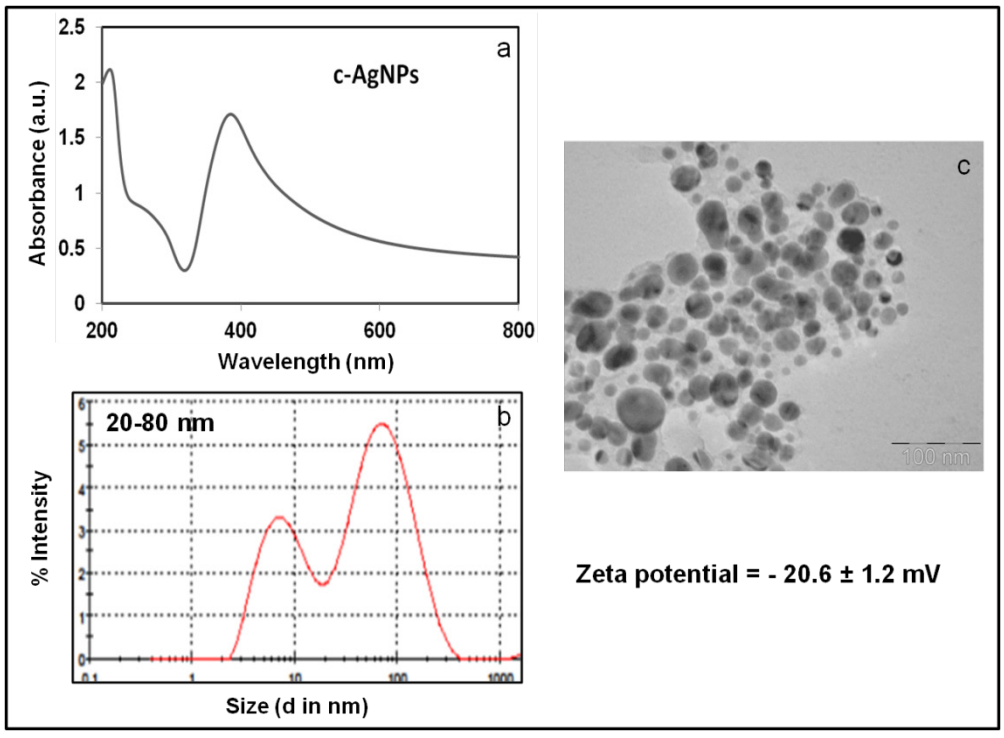
SI.Fig.1.a-d: Time dependent UV-VIS study of (a) b-AgNPs-150; (b) b-AgNPs-400; (c) b-AgNPs-500 and (d) b-AgNPs-750.(using 150;400;500 and 750 µL of *Olax* extract)



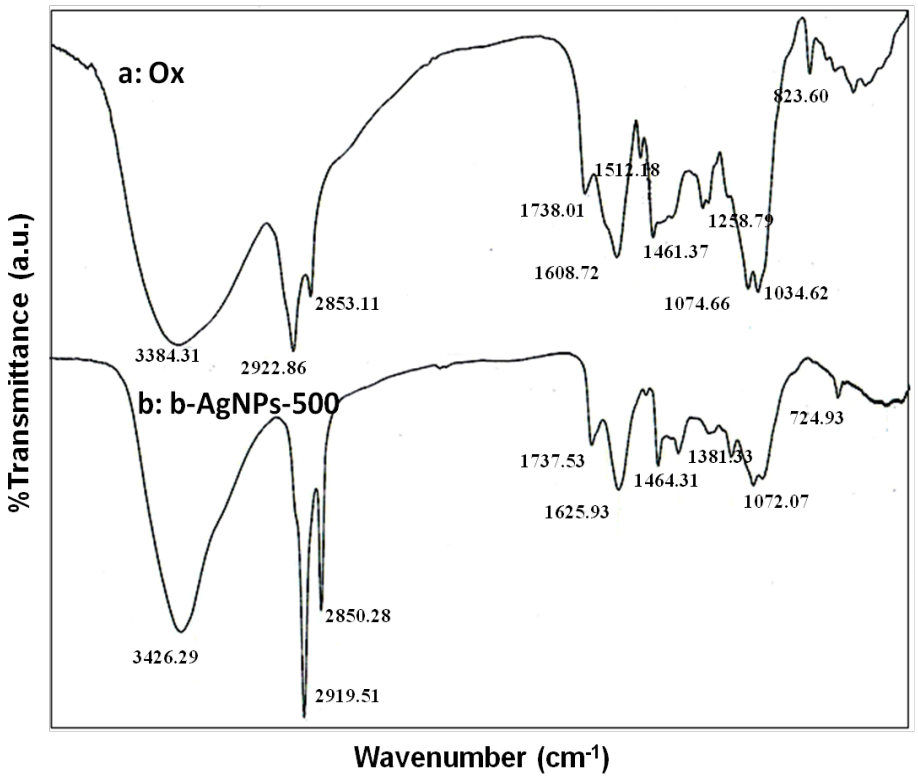
SI.Fig.2.a-d: Size and shape of b-AgNPs synthesized from different volumes of *Olax* extract from TEM. (a-d) TEM images of b-AgNPs-150, b-AgNPs-400, b-AgNPs-500 and b-AgNPs-750 at 24 hours. The images show mainly small and large spherical b-AgNPs.



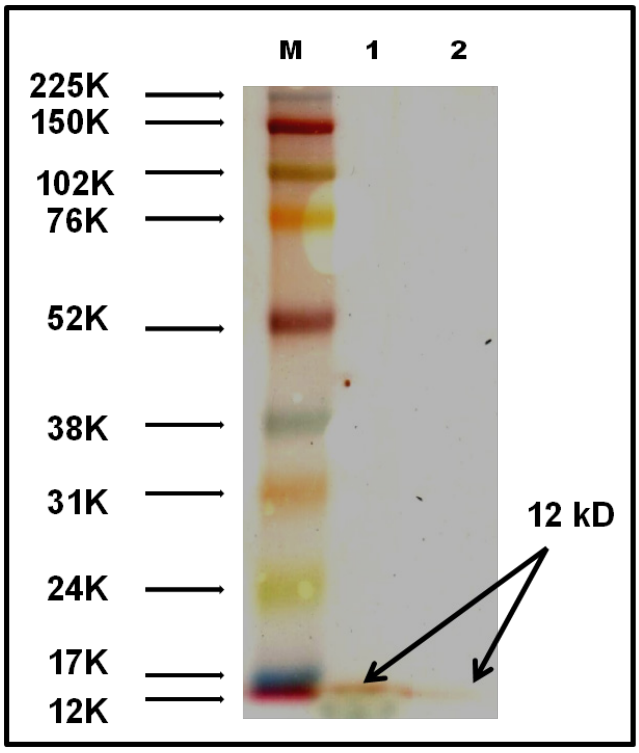
SI.Fig.3 (a-d): Size distribution pattern of different sets of b-AgNPs by DLS. (a) DLS size distribution patterns of b-AgNP-150, (b) DLS size distribution patterns of b-AgNP-400 (c) DLS size distribution patterns of b-AgNP-500 and (d) DLS size distribution patterns of b-AgNP-750.



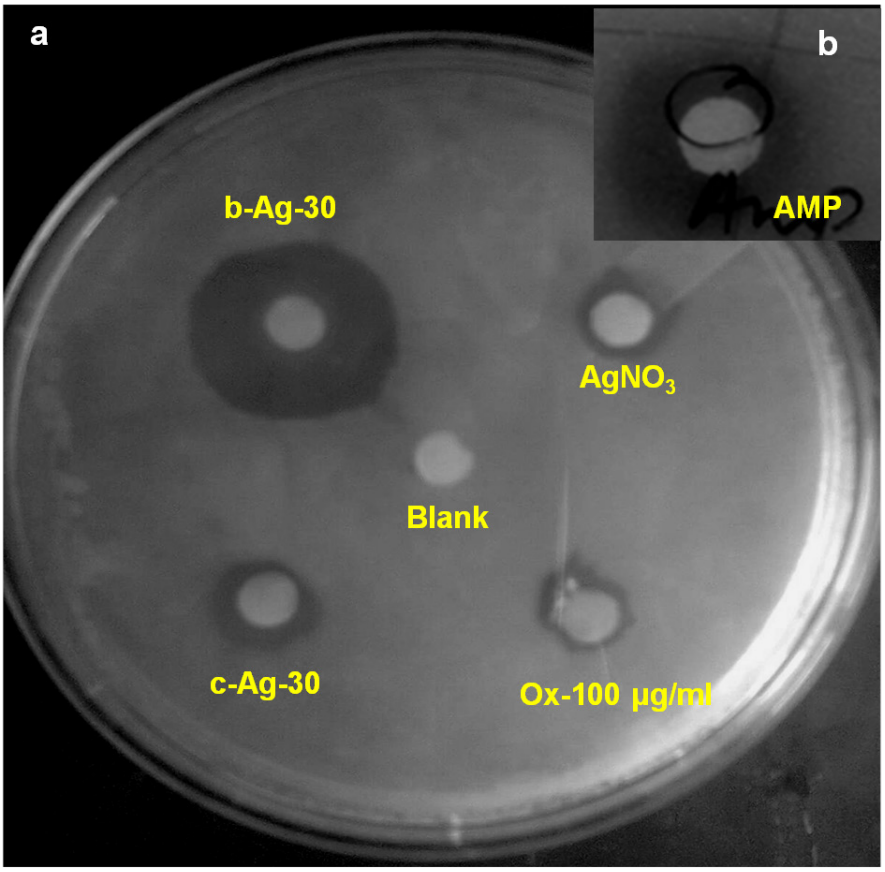
SI.Fig.4 (a-c): (a) UV-Visible spectra, (b)Size distribution by DLS and (c) TEM images of c-AgNPs.



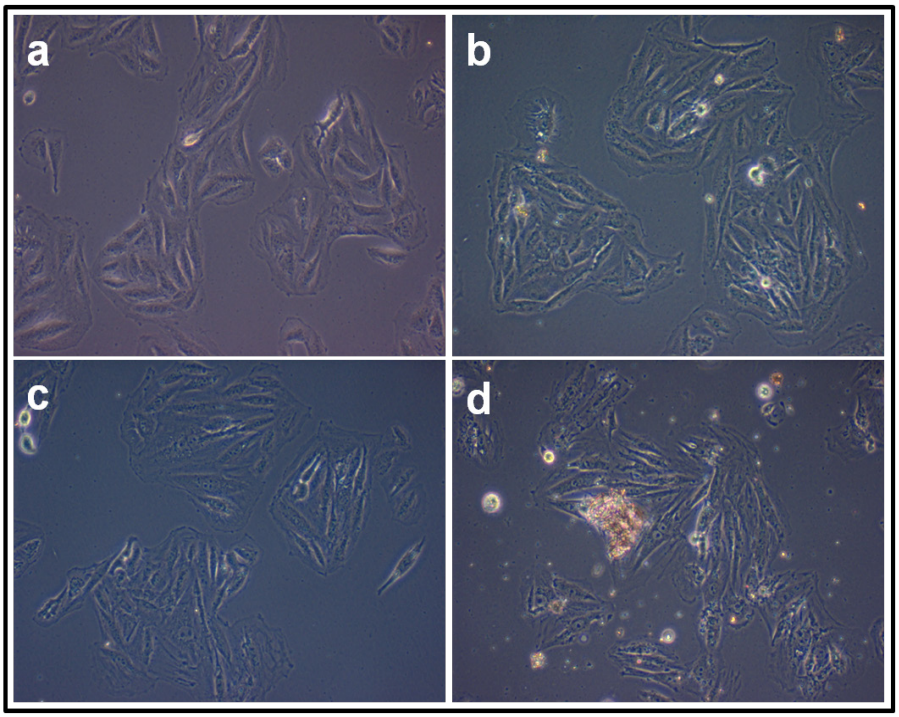
SI.Fig.5 (a-b): FTIR spectrum of Olax and b-AgNPs-500 shows the amide II and amide III from proteins and C=O from aldehydes are mainly responsible for the reduction of $AgNO_3$ for the formation of b-AgNPs.



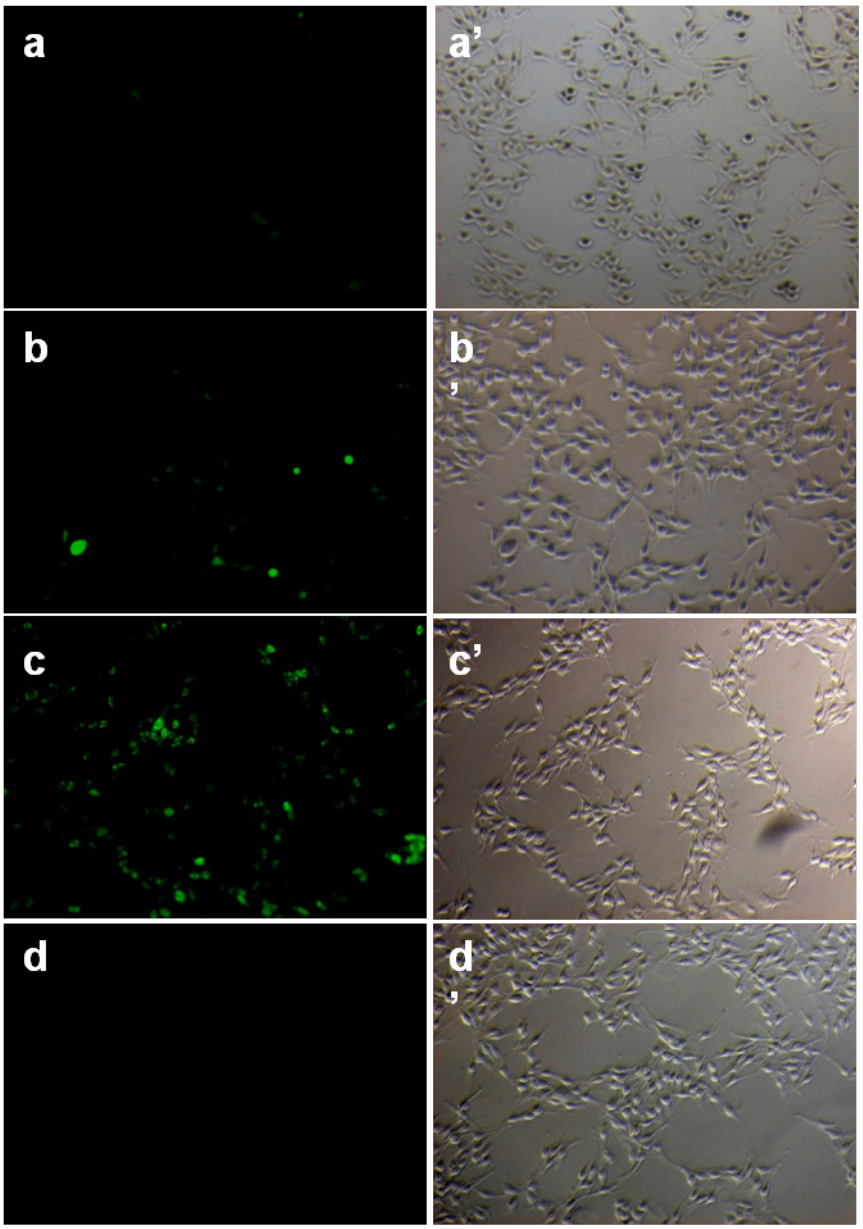
SI.Fig.6: SDS PAGE profile of *Olax* leaf extract and supernatant of b-AgNPs-500 with silver nitrate staining. M indicates the standard protein marker. Lane-1 and -2 indicate the *Olax* extract and supernatant of b-AgNPs-500 obtained after centrifugation. The silver nitrate lane staining after gel electrophoresis shows the presence of low (12-17 kDa) molecular weight proteins in only *Olax* extract in lane-1 and disappearance of 12-17 kDa proteins in *Olax* extract associated with b-AgNPs in lane-2. The results confirm the role of low molecular weight proteins (12-17 kDa) for the formation and stabilization of b-AgNPs.



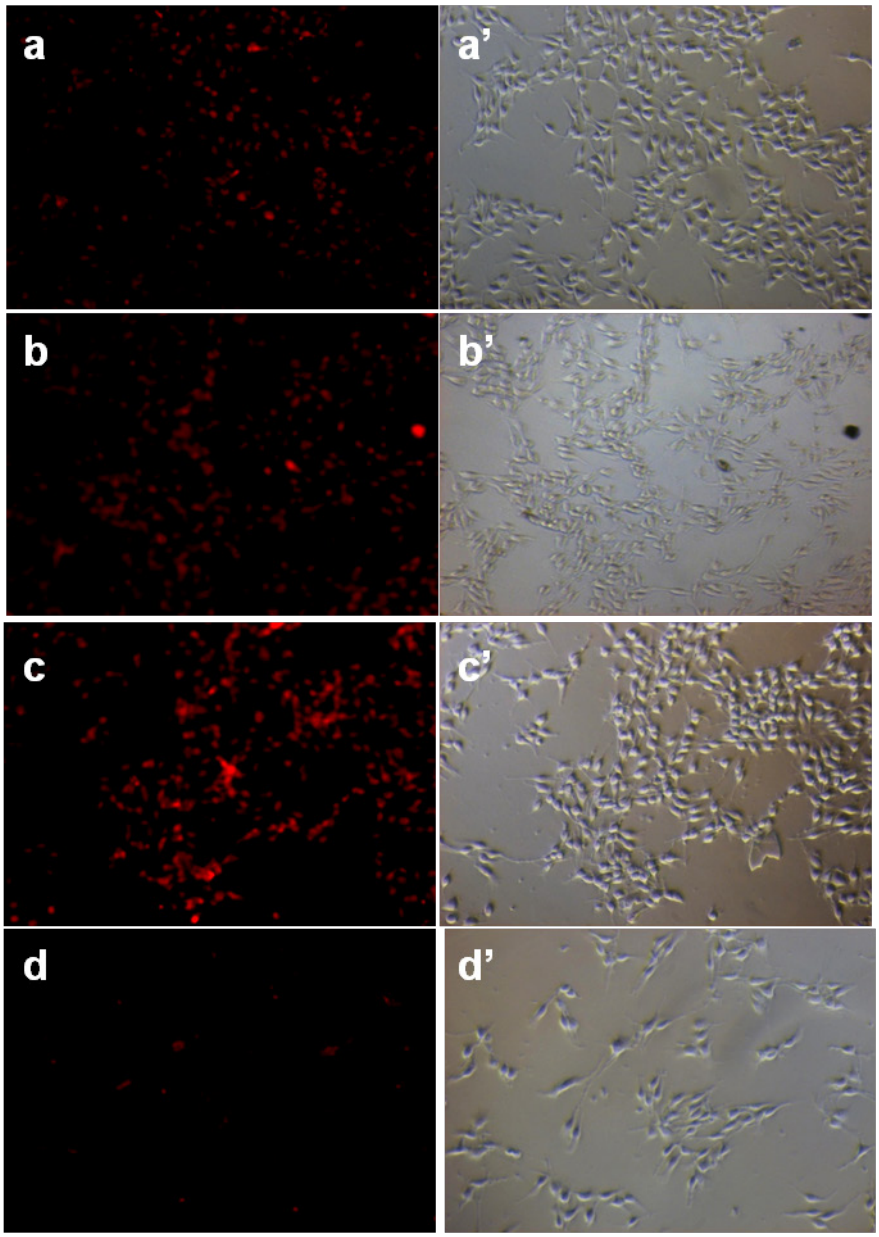
SI.Fig.7: Zone of inhibition experiment (after 24 h): Zone of inhibition of E.coli treated with Olax-100 µg/ml, $AgNO_3$ (30 µM), b-AgNPs-30 µM and c-AgNPs-30 µM (b) in the inset showing inhibition zone of Ampicillin (100µg/ml) after 24 hours.



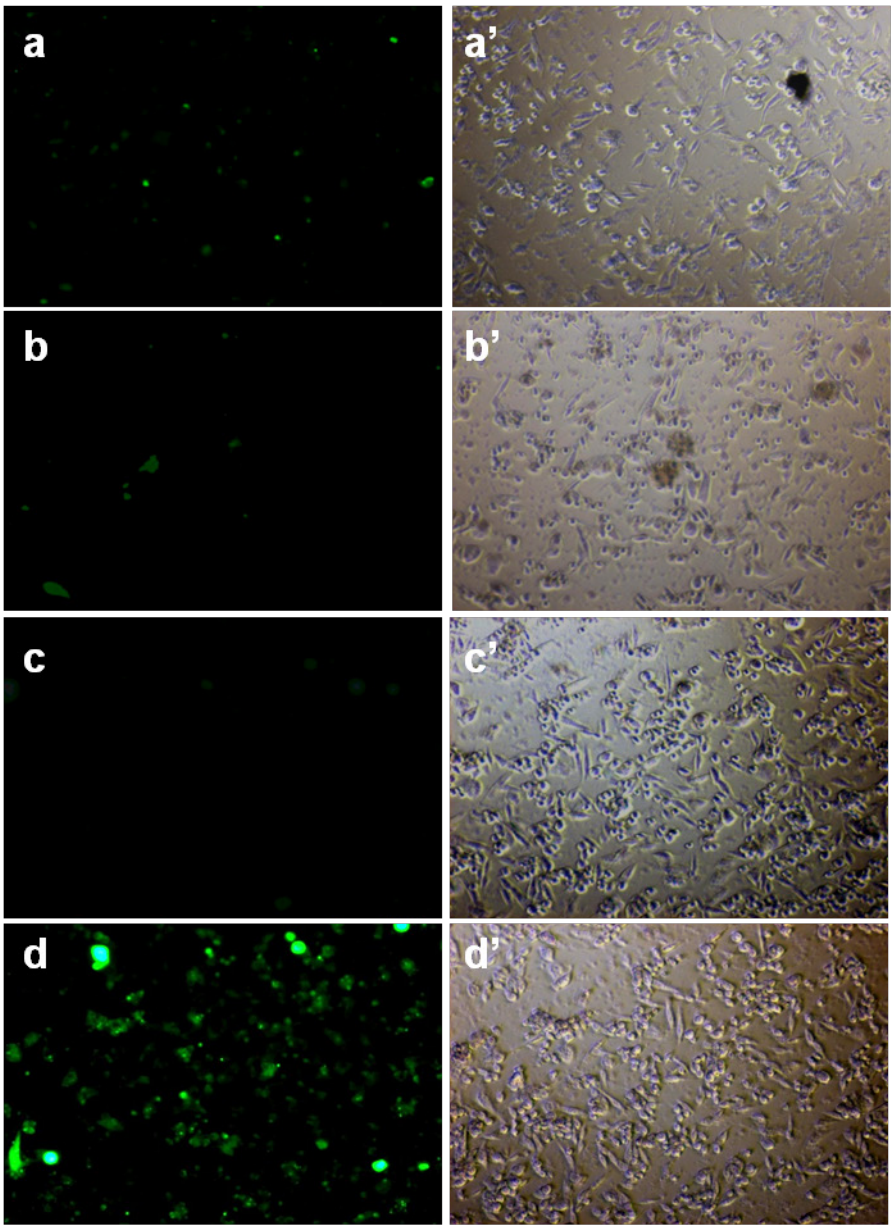
SI.Fig.8 (a-d): Microscopic images of H9C2 celllines. (a) Cells untreated, (b) cells treated with 100 µg/ml of *Olax*, (c) cells treated with 18 µM of b-AgNPs, (d) cells treated with 30 µM of b-AgNPs. Cell images show that there is negligible amount of toxicity towards these treatments.



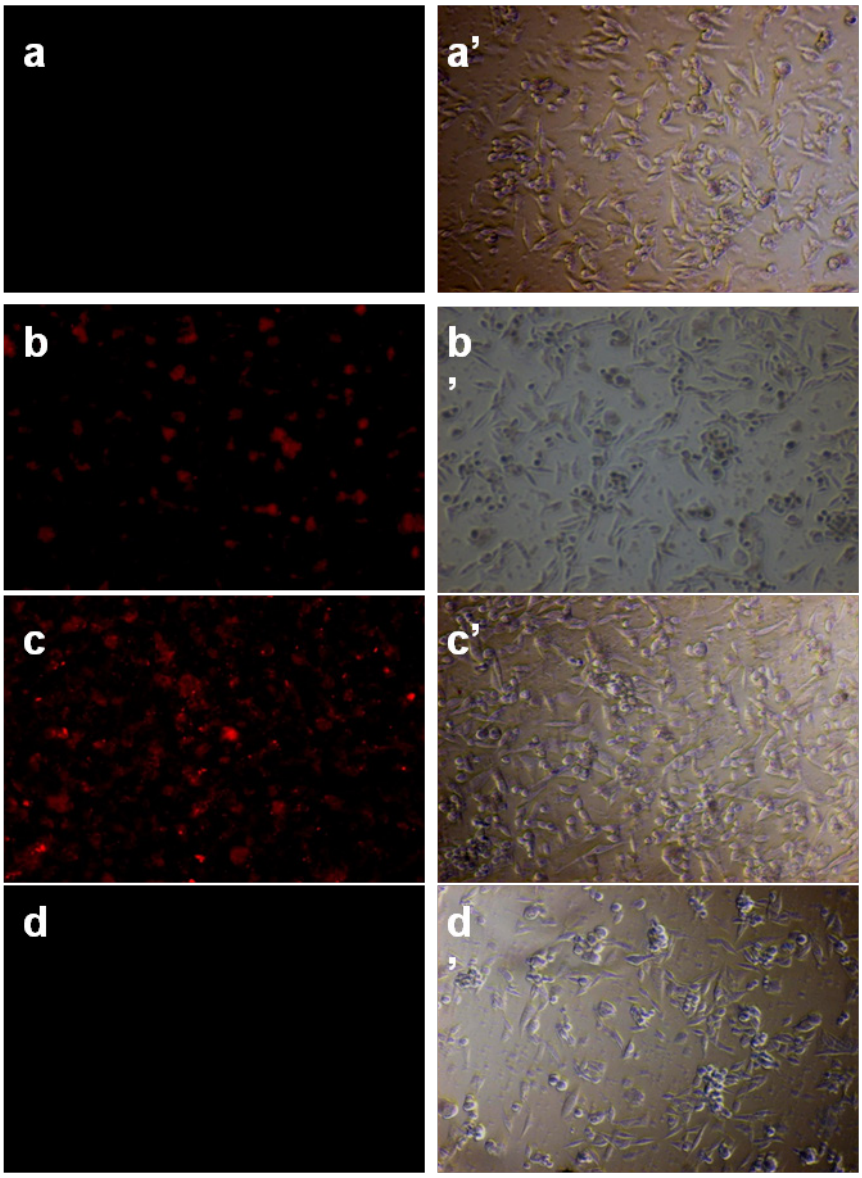
SI.Fig.9. Formation of hydrogen peroxide inside B16 cancer cell measured by fluorescence microscopy: (a) untreated or negative control, (b) Olax extract (100 µg/ ml), (c) b-AgNPs at 30 µM and (d) c-AgNPs at 30 µM, respectively. Images of a'-d' correspond to bright-filed phase images.



SI.Fig.10. Formation of Superoxide radicals inside B16 cancer cell measured by fluorescence microscopy: (a) untreated or negative control, (b) Olax extract (100 µg/ ml), (c) b-AgNPs at 30 µM and (d) c-AgNPs at 30 µM, respectively. Images of a'-d' correspond to bright-filed phase images.



SI.Fig.11. Formation of hydrogen peroxide inside CHO cancer cell measured by fluorescence microscopy: (a) untreated or negative control, (b) Olax extract (100 µg/ ml), (c) b-AgNPs at 30 µM and (d) c-AgNPs at 30 µM, respectively. Images of a'-d' correspond to bright-filed phase images.



SI.Fig.12. Fluorescence and the corresponding phase images of untreated CHO cells and cells treated with Olax, b-AgNPs c-AgNPs, observed by an Olympus Fluorescence Microscope. Fluoresence images of CHO cells treated with (a) untreated or control, (b) Olax (100 µg/ ml) leaf extract ,(c) b-AgNPs (at 30 µM) and (d) c-AgNPs (at 30 µM). Images of a‘, b‘, c‘ & d‘ correspond to phase images.