IJPSR (2019), Volume 10, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 12 February 2019; received in revised form, 25 May 2019; accepted, 06 June 2019; published 01 November 2019

SYNTHESIS AND CYTOTOXICITY STUDY OF PLANT DERIVED NANOCOMPOSITE

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Keywords:

Musa balbisiana, Gold nanoparticles, Cisplatin, HeLa cell line

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ABSTRACT: The present study is a small attempt of synthesis of gold nanocomposites with anticancer drug cisplatin and their cytotoxicity test on HeLa cell line. There are many well-established reports on the synthesis of gold nanoparticles (AuNPs) chemically. But as an ecofriendly alternative various plant extracts also been used for the reduction and synthesis of AuNPs. AuNPs in this study have been synthesized from ethanolic bract extract of *Musa balbisiana*. Further the drug-loaded nanocomposite (AuNPCHCS) was synthesized using the synthesized AuNPs with chitosan (CH) and anticancer drug cisplatin (CS). AuNPs were tested for their cytotoxicity in a cancerous cell line, HeLa using MTT assay and found nontoxic up to the concentration of 100 µM. At the concentration of 10 µg/ml, cell viability reduced to 52% in case of nanocomposites and 55.7% in case of free drug. Results suggested that when nontoxic AuNPCH composites were loaded with CS, were able to kill the cancer cells efficiently compared to free drug. These synthesized nanoparticles are non-covalently conjugated with anticancer drug cisplatin. The synthesized nanocomposites show similar cytotoxic effect compared with the free drug, indicating an increased susceptibility of cells to cisplatin.

INTRODUCTION: Gold nanoparticles (AuNPs) have several biomedical and industrial applications mostly in the diagnosis of diseases, targeted delivery of drug, gene, protein, and in imaging due to their biocompatibility and cytotoxicity. Small size and multifunctionality are the main characteristics of AuNPs. Nanoparticles can be integrated with ligands, imaging labels, therapeutic agents and other functional groups for specific drug delivery and cellular uptake.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(11).5167-78

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(11).5167-78

Research shows that a number of anticancer drugs, *e.g.* Doxorubicin, Cisplatin, Capecitabine, *etc.* can conjugate with AuNPs and by conjugation, there is increase in the potency of the drugs. Physical and chemical methods are establishing methods for metal nanoparticle synthesis, but recent studies also show the potential of plants in the synthesis of NPs.

Therefore, the present study attempts on the conjugation of green synthesized gold nano-particles with cisplatin and their effect on cancerous (HeLa) cells.

MATERIALS AND METHODS: Gold nanoparticles are synthesized using ethanolic bracts extract of *Musa balbisiana* and details of synthesis and characterization.

HeLa (human cervical cancer) cells were maintained in Eagle's minimal essential medium (EMEM) sup- plemented with 10% and 4% fetal and bovine serum (FBS heat inactivated) and 1% antibiotic-antimycotic solution (1,000 U/mL penicillin G, 10 mg/mL streptomycin sulfate, 5mg/mL gentamycin, and 25 µg/mL amphotericin B).

Synthesis and Characterization of Cisplatin (CS) Loaded AuNP-Chitosan (CH) Nano-composites: To develop the drug conjugated NPs, first chitosan (2% wt/vol) was prepared in double-distilled water and pH was adjusted to 7.0. 3 mL of AuNP solution was centrifuged at 14,000 rpm for 20 min. Then pellet was re-dispersed in 0.5 mL of PBS. To this 200 µL of chitosan (CH) was added. To this resulting AuNPCH solution different concentration of Cisplatin (CS) was added (0.67 to 30 µg/mL) and final volume made up to 1.5 mL [1, 2, 3, 4].

UV Visible Spectroscopy: Product samples were subjected to UV-Vis spectroscopic studies [Shimedzu (UV 1601PC)], the most confirmatory tool for the detection of surface Plasmon resonance property (SPR) of AuNPs. AuNPs drug composites (AuNPCHCS) were characterized by using UV-Vis spectroscopy immediately within few minutes after addition of the drug conjugate.

FTIR: Synthesized AuNPs and AuNPCHCS nanocomposites were centrifuged at 20000 and 14000 rpm for 20 min. The pellets washed with deionized water, lyophilized and then analyzed using a spectrophotometer (Bruker Vector 26 spectrophotometer) in the range of 400-4000 cm^{-1}.

Zeta Potential and DLS Study: Synthesized (i) AuNPs and (ii) AuNPCHCS nanocomposites were evaluated for their surface charges in Zetasizer ZS90 (Model no ZEN3690 Malvern). Particle size analysis was carried out by a dynamic light scattering (DLS) based technique using a Zetasizer nano ZS90 instrument fitted with a 633 nm red laser (Model no ZEN3690, Malvern).

Transmission Electron Microscopy (TEM) Analysis: Morphological details of the synthesized (i) AuNPs and (ii) drug-loaded composite NPs (AuNPCHCS) were revealed with the transmission electron microscope (JEOL, JEM-2100 Electron Microscope, 180 kV) analysis. For TEM analysis, 10 µL of the AuNP solutions, as well as AuNP drug composites, were deposited onto carbon-coated copper grids and air-dried well, before recording the images. The average particle size of the AuNPs was calculated using Image J software. The TEM analysis was done in Sophisticated Analytical Instrument Facility (SAIF), North Eastern Hill University (NEHU), Shillong, India.

***In-vitro* Drug (Cisplatin) Release Study:** The *in-vitro* release of the drug, cisplatin (Cs) from the nanocomposites was studied in phosphate buffer saline (PBS) and acetate buffer for 24 h. For this 1.5 mL of AuNPCHCS was mixed with 6 mL of PBS and acetate buffer separately in different vials and incubated in a shaking water bath for 24 h. At different time intervals (up to 24 h), the samples were collected and centrifuged at 13000 rpm at 4ºC for 15 min [4, 5]. The supernatant was analyzed by UV-Vis spectroscopy at 301 nm to determine the amount of cisplatin that had been released. The cumulative release of the drug was calculated using the following formula,

$$\% \text{ of drug release} = CS_F / CS_I \times 100$$

Where CS_F is the concentration of the CS released in supernatant and CF_I is the concentration of the CS loaded in the nanocomposites.

Cytotoxicity Test of AuNPS:

Qualitative Test: Cells are grown on 96 well plates. After 24 h different concentration of nanocomposites was given to the cells. Treated cells are observed after 24 h for any morphological change under an Inverted light microscope.

Staining of Cells using Mac-Grunwald Giemsa Stain: Both treated and untreated cells grown on coverslips were fixed in methanol for 5 min. Methanol was discarded and the coverslips were flooded with undiluted May- Grunwald stain for 5 minutes.

After 5 min the May-Grunwald stain was discarded and coverslips were flooded with Giemsa stock diluted to 1:10 ratio with distilled water for 15 min. The stained cells were washed with tap water and air-dried. After drying coverslips were mounted on glass slide (cell side down) using DPX.

Quantitative Test–MTT Assay (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium): To examine the cytotoxicity of AuNPs, monocultures of the HeLa cell line was incubated with increasing concentrations of AuNPs for 24 h and the cell viability was investigated by 3-(4,5-dimethylthiazol- 2-yl)- 2, 5- diphenyltetrazolium bromide (MTT) dye conversion assay.

For MTT assay, HeLa (1×10^4) cells were seeded in a 96-well plate (Nun clone). After 24 h of growth, the medium was replaced with the serum-free medium (plain medium) that contained varying concentrations of nanocomposites (0.6-33.3 µg/ml) as well as free drug cisplatin (CS) (0.6-33.3 µg/ml) differently.

After 24 h of treatment, media were removed and cells were washed with phosphate-buffered saline (PBS). This was followed by addition of 100 µL of MTT (0.5 mg/mL) prepared in serum-free medium to each well and incubation for 4 h at 37 ºC.

After incubation, the medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals.

The concentration of formazan was determined by measuring its absorbance at 550 nm using a multiwell plate reader (Bio Red PR4100 model).

For each of the samples, the assay was carried out in triplicates.

SEM Analysis of the Untreated and Treated Cells: Cells (2×10^4) were seeded in 6 well plates, grown for 24 h. After that medium was removed, the cells were washed with Phosphate buffered saline (PBS; 0.01M, pH 7.4), and serum-free medium containing composite nanoparticles was added to the cells. For control sample, serum-free medium without composite nanoparticles was added to the cells. The cells were incubated for 24 h and then washed with PBS several times. Next the cells were trypsinized, centrifuged and fixed in glutaraldehyde solution (2%) for 4 h. The cells were dehydrated in graded ethanol solutions and finally suspended in absolute ethanol.

TEM Analysis of Untreated and Treated Cells: Cells (2×10^4) were seeded in 6 well plates, grown for 24 h. After that medium was removed, the cells were washed with Phosphate buffered saline (PBS; 0.01M, pH 7.4), and serum-free medium containing composite nanoparticles was added to the cells. For control sample, serum-free medium without composite nanoparticles was added to the cells. The cells were incubated for 24 h. After 24 h treatment cells were primarily fixed with 2.5-3% of Karnovsky's Fixative, followed by washing 3 changes of 15 min each with 0.1 M Sodium Cacodylate buffer at 4 ºC. After the primary fixation of the sample the post-fixation which includes washing, dehydration, clearing, infiltration, embedding, ultramicrotomy, and staining was done according to the standard protocol followed by Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong, Meghalaya, India.

DNA Fragmentation Study: The cells (2×10^4) were grown on 6 well plates, treated with or without samples and incubated for 24 h in a CO_2 incubator for DNA fragmentation study. Cells were then harvested, washed with ice-cold PBS (pH 7.2) and centrifuged at 1000 rpm for 10 min at 4 ºC. The resulting cell pellet was dispersed in 30 µL of lysis buffer (10 mM EDTA; 50 nM Tris HCl, pH 7.8, and 1% SDS) by gentle vortexing. 4 µL of Proteinase-K (10 µg/µl) was then added to the mixture followed by incubation at 45 ºC for 1-2 h. Then 2 µL of RNase (10 µg/µL) was added to the cell lysate, which is further incubated for 1 h at 57 ºC. After incubation, cell lysate was mixed with 4 µL of 6X DNA loading dye and subjected to run at 2% agarose gel electrophoresis. The gel was stained with ethidium bromide (0.5 µg/ml) and visualized under gel documentation system [6].

RESULTS AND DISCUSSION:

Synthesis and Characterization of the Drug loaded Composite Nanoparticles (AuNPCHCS):

UV-Visible Spectroscopy of the Synthesized AuNPCHCS: AuNPs synthesized from *M. balbisiana* bract extracts were further taken for the synthesis of nanocomposites with chitosan (CH) and anticancer drug cisplatin (CS). UV-Visible spectra of only AuNPs showed a peak at 530 nm, which was shifted to 532 nm after addition of CH. Again with the addition of CS, the peak broadens and further shifted to 534 nm. This is possibly due to formation of the composite NPs (AuNPCHCS) which brings Au NPs closer *i.e.*, induce agglomeration due to electrostatic interaction with the chitosan, causing red shifting of SPR signal. This confirms the formation of cisplatin loaded composite NPs (AuNPCHCS) **Fig. 1A**. One of the important parameter of an efficient drug carrier which needs to validate before drug delivery is its stability in physiological environment. Thus, the present study probed the stability of the AuNPs and AuNPCHCS by monitoring its UV-Vis characteristics which were recorded after 1 month that shows no significant difference **Fig. 1B**.

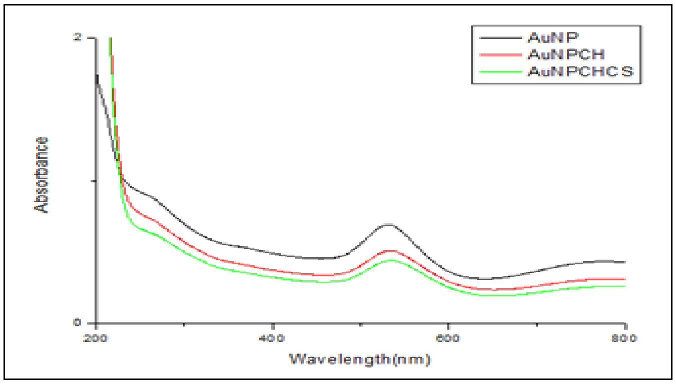

FIG. 1A: UV-VIS SPECTRA OF THE COMPOSITES, (ONLY AuNP, AuNP WITH CHITOSAN (CH), AuNPCH WITH CISPLATIN (CS)

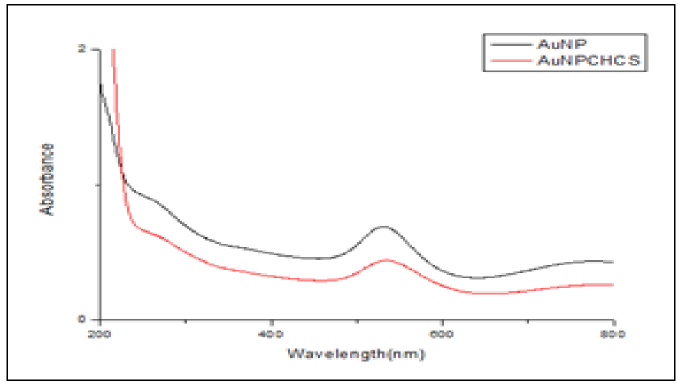

FIG. 1B: UV-VIS SPECTRA OF THE COMPOSITES, (ONLY AuNP, AuNPCH WITH CISPLATIN (CS)

In the present study, the AuNPs and the nanoparticle-based composite (AuNPCHCS) were studied using UV-Vis spectroscopy. A shift in the absorption spectrum of AuNPs observed after addition of CH, which was indicative of agglomeration of AuNPs and CH. Initially AuNPs have absorption maxima at 530 nm which changes to 532 nm on addition of CH, which further changes to 534 nm on addition of CS **Fig. 1A**. Though the changes in the absorbance wavelength were indicative of interactions of the AuNPs with the added molecules, additionally the small changes in the SPR spectra also suggest that there were no major alterations of the agglomerated nano-structures present in the medium. It was also reported that AuNPs are known to absorb a specific light band with absorption peak ranging from 520 nm to 540 nm due to surface plasmon resonance or SPR [7].

The position of the maximum peak and the width of the absorption band depend on the morphology of the particles (size, shape, and uniformity), coagulation among the particles and the dielectric environment [8, 9, 10, 11]. The stability of the thiol stabilized AuNPs and doxorubicin conjugates was reported up to 3 months at room temperature [7]. In the present study also both the synthesized AuNPs and AuNPCHCS show stability up to one month at room temperature and there was no significant difference in the spectra of the two **Fig. 1B**.

In another study, the nuclear delivery of doxorubicin from dextran-coated AuNPs, the citrate synthesized AuNPs had aminated for better stability for various biological applications [12]. Here also, the redshift was observed in the UV-Vis spectra of AuNPs with the addition of molecules.

FTIR Analysis of the AuNPCHCS: As mention in the earlier publication, the FTIR of AuNPs from *M. balbisiana* showed peaks at 1047 cm^{-1} (amine), 1399 cm^{-1} (aromatic amines), 1639 cm^{-1} (C=C of benzene), 2939 cm^{-1} (alkane) and 3414 cm^{-1} (hydroxyl). Addition of CH, to these AuNPs shows peaks at 1058 cm^{-1}, 1643 cm^{-1}, 2940 cm^{-1} and 3420 cm^{-1}. Again addition of CS showed peaks at 1079 cm^{-1}, 1653 cm^{-1}, 2953 cm^{-1} and 3479 cm^{-1}. This peaks shifting possibly due to the interaction of CH and CS with AuNPs and formation of cisplatin loaded composite NPs (AuNPCHCS) **Fig. 2**.

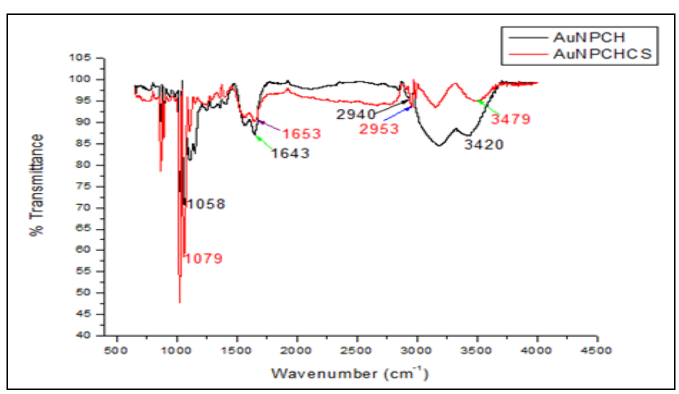

FIG. 2: FTIR ANALYSIS OF AuNPCH AND AuNPCHCS

The FTIR analysis of the nanoconjugate shows after binding the drug to the AuNPs, the band, observed at the range of 3200-3700 cm^{-1}, which were due to the presence of surface-bound –N and –OH groups. The peak shifting from 2980 to 2978 cm^{-1} represented the stretching vibrations of C-H bonds from the rings[2]. In the present study, the FTIR peaks of AuNPs (*M. balbisiana)* found at 1047cm^{-1} (amine), 1399 cm^{-1} (aromatic amines), 1639 cm^{-1} (C=C of benzene or N-H stretching of primary amine and amide bands), 2939 cm^{-1} (alkane) and 3414 cm^{-1} (hydroxyl). Addition of chitosan (CH) to these AuNPs shows peaks at 1058 cm^{-1}, 1643 cm^{-1}, 2940 cm^{-1} and 3420 cm^{-1}. As mentioned earlier, chitosan (CH) is also reached in primary amines and hydroxyl group [13]. Again addition of cisplatin (CS) shows peaks at 1079 cm^{-1}, 1403 cm^{-1}, 1653 cm^{-1}, 2953 cm^{-1} and 3479 cm^{-1} **Fig. 2**. This peaks shifting showed the interaction of CH and CS with AuNPs and formation of nanocomposites. It was observed that the stretching in the amine and the amide rich regions, as well as the hydroxyl regions, was involved in the synthesis and agglomeration of the nanocomposites. Therefore, it can be suggested that the flavonoids and phenolic groups present in the plant extracts might be responsible for the synthesis as well as agglomeration of both AuNPs and AuNPCHCS.

ZETA Potential and DLS Study:

Zeta Potential of the Synthesized AuNPS and AuNPCHCS: Zeta potential of the AuNPs synthesized from *M. balbisiana* was found to be -30 mV **Fig. 3A** as mentioned earlier in this chapter, while zeta potential of AuNPCHCS was found to be 23.1 mV **Fig. 3B**. The changes in charge of the particles confirmation the interaction of the AuNPs with CH and CS.

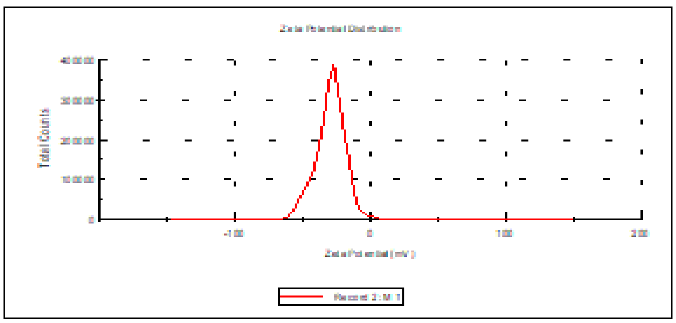

FIG. 3A: ZETA POTENTIAL OF AuNPs SYNTHESIZED FROM *MUSA BALBISIANA* (-30)

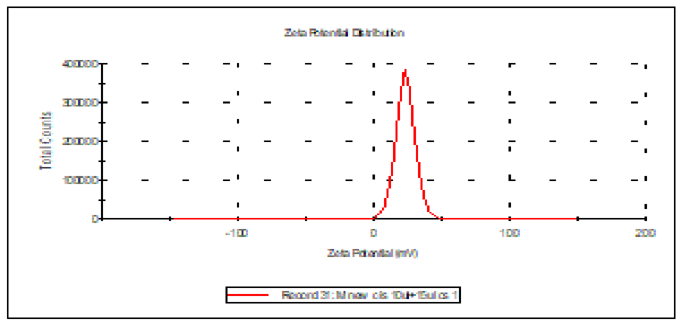

FIG. 3B: ZETA POTENTIAL OF AuNPCHCS (23.1)

DLS (Dynamic Light Scattering) based Particle Size Distribution Curves:

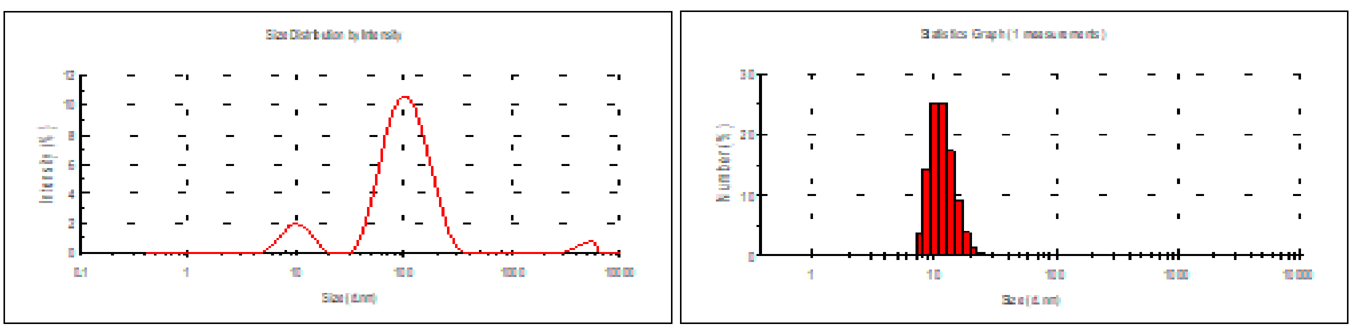

FIG. 4A: DLS SPECTRA OF AuNP SYNTHESIZED FROM *M. BALBISIANA*

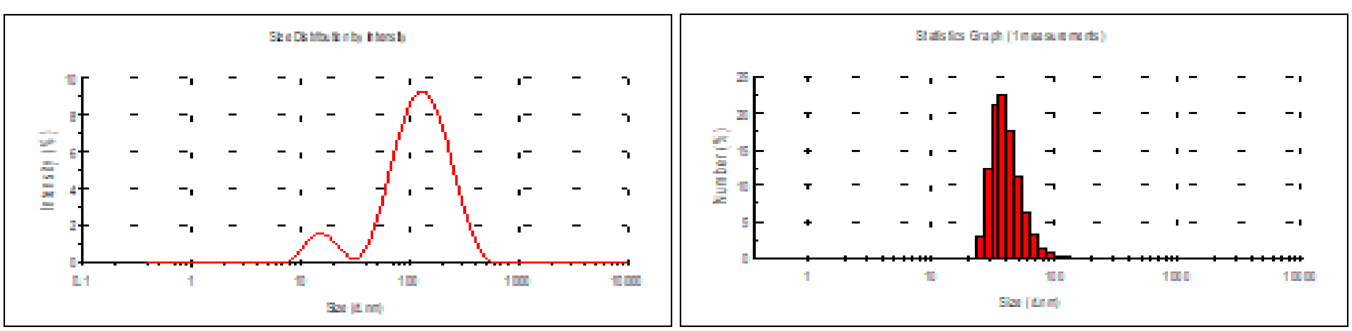

FIG. 4B: DLS SPECTRA OF DRUG LOADED COMPOSITE NANOPARTICLES (AuNPCHCS)

From DLS spectra the average particle size of the AuNPs synthesized from *M. balbisiana* was found to be 63.59 nm while the AuNPCHCS was 97 nm **Fig. 4 A** and **B**.

Zeta potential of the AuNPs synthesized from *M. balbisiana* was found to be -30, while zeta potential of AuNPCHCS was found to be 23.1. In a study of loading and releasing of anticancer drug doxorubicin CM (Carboxymethyl)-Chitosan stabilized AuNP, the zeta potential value of CM (Carboxymethyl)-Chitosan reduce AuNPs was reported to be -21.6 where doxorubin loaded AuNPs was -10.6 mV. Doxorubicin is a positively charged drug and Zeta value towards positive side indicates the successful interaction between the drug and the AuNPs [14]. Similarly, cisplatin was also a positively charged drug, and the changes in charge of the particles confirm the interaction of the AuNPs with CS in the present study **Fig. 3 A, B**.

DLS based study was also conducted to analyze the particle diameter. The DLS based diameter of the AuNPs found to be 63.59 nm and composite to be 97 nm. Similar literature had reported the average hydrodynamic diameter of the synthesized AuNPs as 19 nm, which changes to 116 nm on addition of lysozyme and further to 195nm on addition of doxorubicin [4].

Jang *et al*., (2013) had also analyzed the size of the AuNPs and reported that the DLS spectra shifted to higher wavelength indicating increase in the overall size [12]. These findings are found to be similar to the present study. Here, the DLS based diameter of the AuNPs found to be 63.59 nm and composite to be 97 nm **Fig. 4 A, B**.

Transmission Electron Microscopy (TEM) Analysis of the AuNPCHCS: It's to be deemed that the addition of the chitosan with as-synthesized Au NPs brings closer, *i.e.*, induce agglomeration of Au NPs due to electrostatic interaction with the chitosan. The assembly of the Au NPs employed in encapsulation of a well known anti-cancer drug cisplatin. TEM images of the nanocomposites showed the agglomeration of AuNPs in presence of CH and CS. The nanocomposites rearranged in the nanoscale and the diameter obtained around 82 nm.

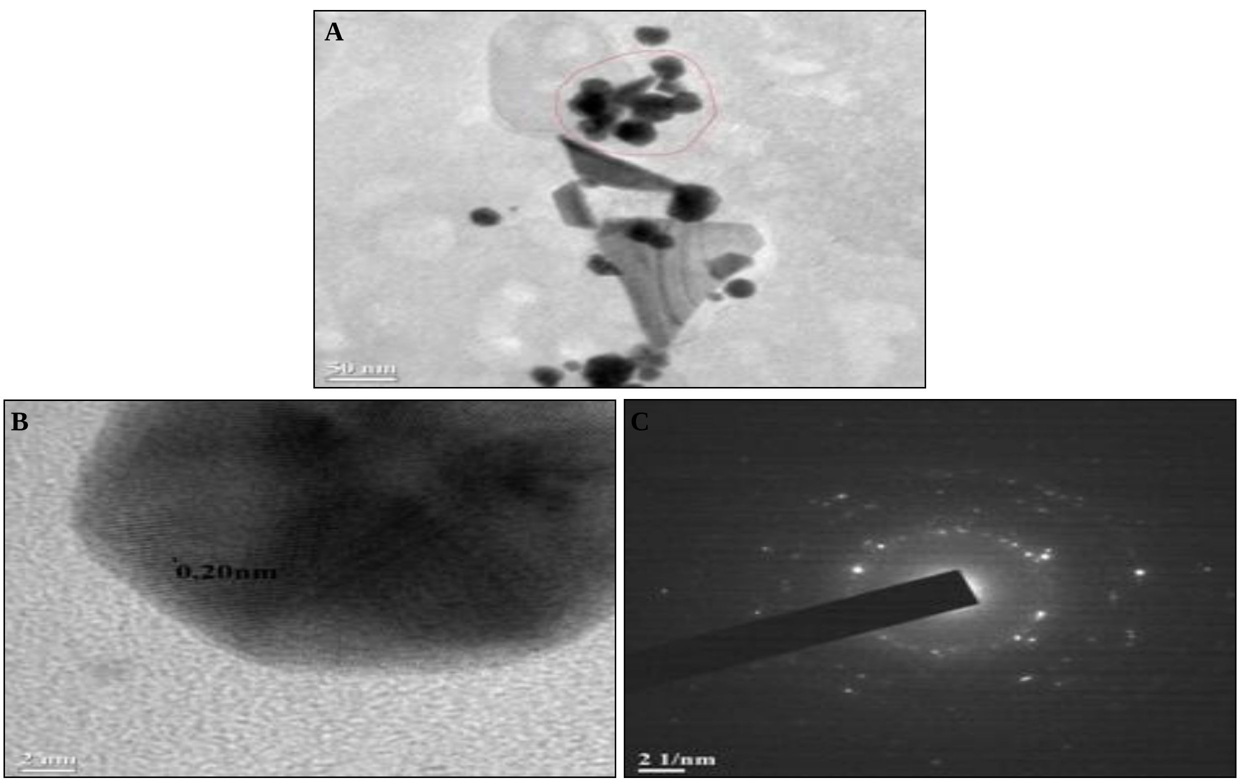

FIG. 5A: TEM IMAGE OF AuNPCHCS, WHICH IS SHOWING ASSEMBLY OF AuNPs AFTER INTERACTION WITH DRUG. B. HRTEM OF AuNP, C. SADE PATTERN

In another study the effect of AuNP conjugated with cisplatin, doxorubicin and capecitabine on hepatocellular carcinoma derived cancer cells, the cell viability was found to be lower in presence of the anticancer drugs delivered by AuNPs compared to the drug alone [2], in which synthesis of the AuNPs was done using L-aspartate as reducing and capping agent. Further, the aspartate nanostructures were functionalized with the desired drugs. The TEM analysis of the structures involving the AuNPs was rearranged in the presence of the drug and maintains a nanometric size [2, 4]. This study was also found to be similar to the present study. In the present findings AuNPCHCS structures observed were bigger than the only AuNPs. AuNPs sizes from TEM images were calculated to be around 8 nm, while AuNPCHCS was found to be around 82 nm **Fig. 5 A, B, C**.

***In-vitro* Drug Release Study:** The *in-vitro* release of cisplatin from the composite NPs was studied in physiological conditions such as phosphate buffer saline (PBS) (pH 7.4) and acetate buffer (pH 5.6), at 37 ºC for 24 h was studied. Drug release percentage at 24 h was found to be 12.05% in PBS and 21.3% in acetate buffer, respectively. All of the experiments were performed in triplicates.

One of the important parameters for good nanocomposites for drug delivery is the ability to release drugs efficiently in a controllable manner at the target site. In the present study, *in-vitro* **Drug** release trend of the nanocomposites was studied at physiological and acidic conditions such as PBS (pH 7.4) and acetate buffer (pH 5.6) at 37 ºC. Drug released percentage at 24 h was found to be 12.05% in PBS and 21.3% in acetate buffer (pH 5.6) respectively **Fig. 6**.

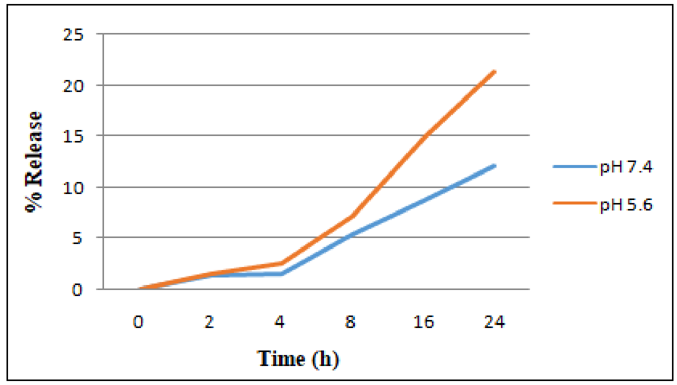

FIG. 6: *IN-VITRO* DRUG RELEASE STUDY

It is well documented that cancer cells have an extracellular acidic environment [15,] and most of anticancer drugs such as doxorubicin, cisplatin, *etc*. at Free State are not effectively taken up at acidic conditions [16]. Since many cancer cells have low intracellular pH [17], drug should be able to work at that low pH within the cells. In the present study, it was observed that the drug release from the nanocomposites in the acidic environment was comparatively more than in physiological condition. At physiological conditions, drug-loaded nanocomposites showed stability as it is necessary for the transportation of the drug with minimal loss [4, 5, 14].

Cytotoxicity Tests of Synthesised AuNPS and AuNPCHCS:

Qualitative Test:

Observation under Inverted Light Microscopy: Both treated and untreated cells were observed under an inverted light microscope.

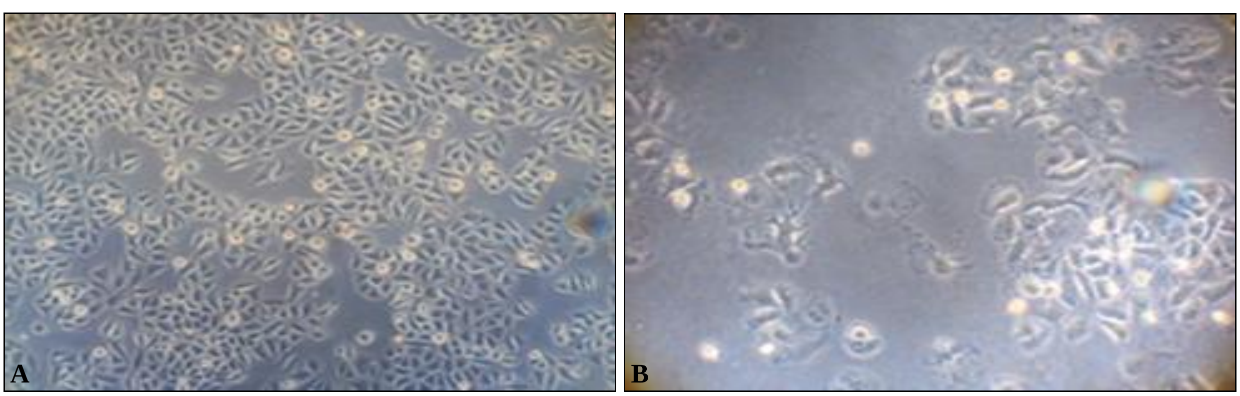

FIG. 7: HeLa CELL MORPHOLOGY AFTER 24 h OF CULTURE, (A) WITHOUT TREATMENT, (20X), (B) WITH AuNPCHCS (10 µg/mL) (20X)

Fig. 7A shows the monolayer of HeLa cells without treatment and **Fig. 7B** shows the same cells treated with 10 µg/mL concentration of drug-loaded composites nanoparticles (AuNPCHCS).

It was observed the monolayer was not intact, and also the cell morphology was distorted after treatment of 24 h.

Mac-Grunwald Giemsa Staining of Cells: Both treated and untreated cells were stained with Mac-Grunwald Giemsa staining.

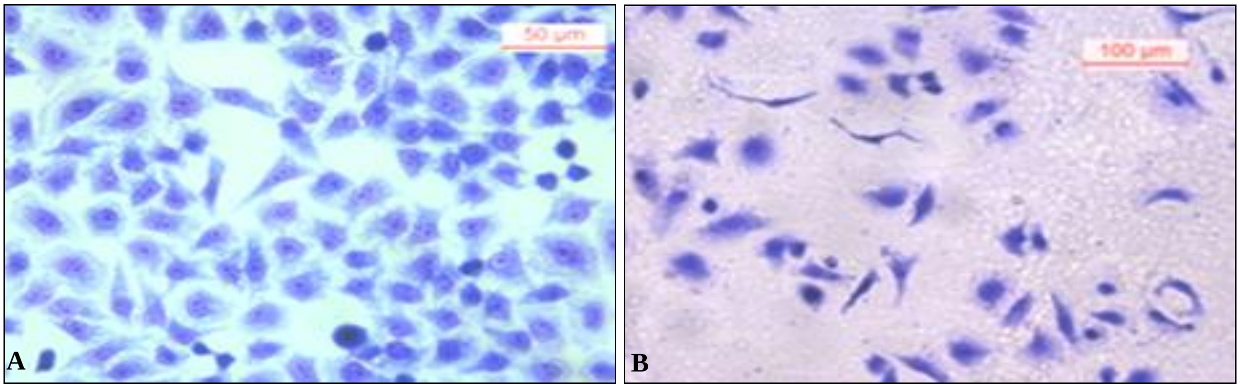

FIG. 8: MAC- GRUNWALD GIEMSA STAINING OF HeLa CELL, (A) WITHOUT TREATMENT (40X), (B) With AuNPCHCS (10 µg/mL) (40X)

In the staining photographs, **Fig. 8A** showed a healthy HeLa cell monolayer with prominent nuclei. The cytoplasm is stained blue and nuclei were dark blue. But **Fig. 8B** treated (10 µg/mL). HeLa cells showed no differentiation between cytoplasm and nuclei. Also, cell morphology was distorted which confirms the cytotoxic activity of the drug-loaded composites nanoparticles (AuNPCHCS).

Similar studies on the assessment of *in-vivo* toxicity of AuNPs on BALB/C mice include the study of toxicity by H and E staining (Haematoxylin-eosine staining) of the major organs [18]. The staining showed significant differences in the treated and untreated organs. Similarly, in the present study also Mac- Grunwald Giemsa staining of cells showed the differences in the nano-composites treated and untreated cells (HeLa).

Quantitative Test–MTT Assay:

Cytotoxicity Tests of Synthesized Gold Nanoparticles (AuNPS): Synthesized gold nanoparticles from bract extracts of *M. balbisiana* were tested for their cytotoxicity in a cancerous cell line, HeLa up to a concentration of 100 µM using MTT assay [(3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium)].

AuNPs synthesized from all the plant source showed high cell viabilities. AuNPs from *M. balbisiana* showed 92.9% in HeLa and was further taken for the nanocomposites synthesis. Cells retain their normal morphology suggesting that the AuNPs did not cause any cytotoxic effect on the cells and therefore can be used for further drug delivery assays. In the case of AuNPs synthesized from *M. balbisiana* it was found that at 100 µM concentration, the cell viability was recorded to be 92.9% **Fig. 9**.

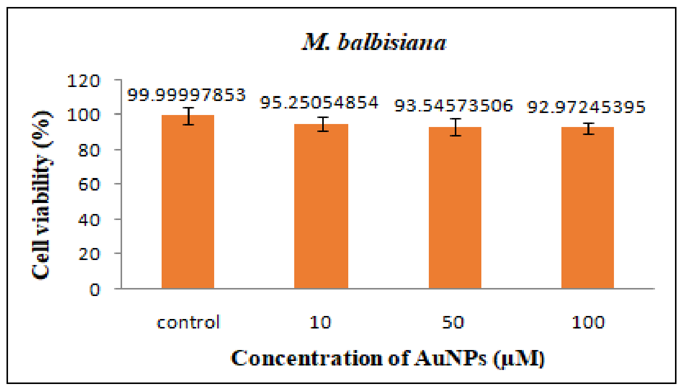

FIG. 9: MTT ASSAY OF AuNPs SYNTHESIZED FROM *M. BALBISIANA.* Results are shown with average ± SD of three individual experiments

Cell proliferation and death are essential for any cytotoxicity study and multiple procedures, are available for such study. Pharmaceutical and biomedical research largely focuses on the effects of drugs, cytotoxic agents and biologically active compounds that effect cytogenesis. MTT assay [(3-(4, 5-dimethylthiazol- 2- yl)-2, 5-diphenyl-tetrazolium)] is one of the commonly used cytotoxicity assays to evaluate the cell cytotoxicity. Synthesized gold nanoparticles and plant extracts were tested for their cytotoxicity in a cancerous cell line, HeLa, up to a concentration of 100 µM using MTT assay.

Further, the significance of the experiment was studied by statistical analysis. The values of all experiments were expressed as mean ± standard deviation (SD). Cells retain their normal morphology suggesting that the AuNPs did not cause any cytotoxic effect on the cells and therefore can be used for further nanocomposites synthesis for drug delivery assays. For the synthesized AuNPs the cell viability was recorded to be 92.9% **Fig. 9**.

Up to a concentration of 100µM of biosynthesized AuNPs (from coconut water), cell viabilities observed were 87% in HeLa and 85% in MCF-7 cancer cell lines. Both cell lines retained their original cellular morphology, suggesting that the AuNPs did not induce any cytotoxic effect [19]. They also studied the cytotoxicity of AuNPs synthesized from *Bacopa monnieri* leaf extract, by MTT assay and found AuNPs were biocompatible when tested on HeLa and MCF-7 cell lines [19].

The synthesis and cytotoxicity of bioconjugated AuNPs with BSA (bovine serum albumin) towards cancer cell line (Glioma cell line- a *N*-nitroso-methyl urea induced cell line of rat brain), the biosynthesised AuNPs (using *Helminthosporium solani*) and their conjugation with doxorubicin on HEK293 cells (Human embryonic kidney 293 cells) was done using MTT assay [20, 21] were reported. Similar studies by Aryal *et al.*, (2009) had also shown the use of MTT based cell viability assay for the cytotoxicity study of doxorubicin conjugated AuNPs [7].

Cytotoxicity Tests of Synthesized AuNPCHCS: In HeLa cell line, the composite of AuNP with chitosan (CH) showed cell viability similar to control (without treatment). Therefore, it can be suggested that the AuNPCH was nontoxic in nature **Fig. 10A**. With the addition of the anticancer drug cisplatin (CS) to the AuNPCH, there was observed a gradual decrease in the cell viabilities. At the 10 µg/ml concentration of the nanocomposites-AuNPCHCS cell viability was found to be 52%.

Fig. 10B shows the cell viability of HeLa at varying concentrations, of nanocomposites and free drugs. It was observed that when the cells were incubated with 0.67 µg/ml of composite, 84% cells were viable. At the same concentration, in case of free drug, 88.4% cells were viable. At the concentration of 10 µg/ml, cell viability reduced to 52% in case of nanocomposites and 55.7% in case of free drug (CS). These findings suggested that when nontoxic AuNPCH nanocomposites were loaded with drug (CS), were able to kill the cancer cells efficiently compared to free drug **Fig. 10B**.

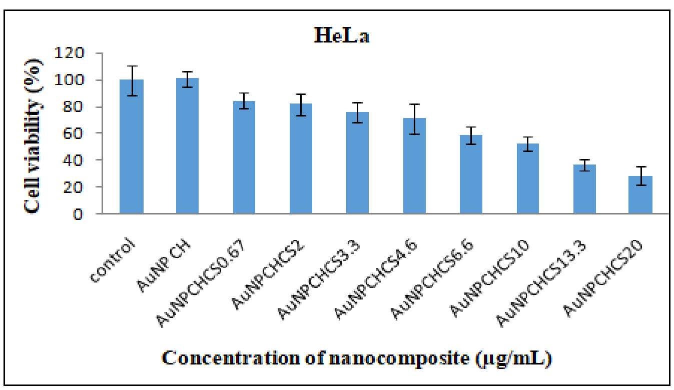

FIG. 10A: CYTOTOXICITY TEST OF THE AuNPCHCS ON THE HeLa CELLS. Results are shown with average ± SD of three individual experiments

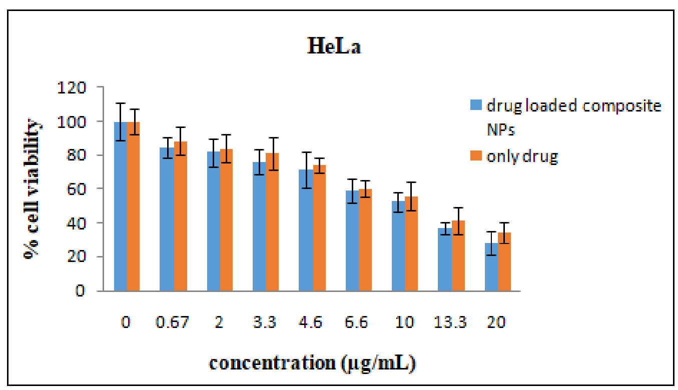

FIG: 10B: CYTOTOXICITY TEST OF THE AuNPCHCS AND ONLY DRUG ON THE HeLa CELLS. Results are shown with average ± SD of three individual experiments

Further, the significance of the experiment was studied by statistical analysis. One way ANOVA was done but no significant difference in the treatment was found. But it was important to be noted that nanocarriers exhibit similar cytotoxic effect as the free drug which is essential, as in many cases it gets compromised after loading with nanoparticles or nanoparticle mediate carriers. Thus, the nanocomposites synthesized in the present study as the same or with more suitable modifications may offer the possibility of its use in passive targeting by exploring the EPR effect, or in photothermal therapy as were nontoxic to cells [20, 21].

SEM Analysis of Treated and Untreated Cells: SEM image of HeLa cells treated with the nanocarriers shows the cell rupture and the formation of apoptotic bodies on the cell surface while control cells show smooth surface of the healthy cell **Fig. 11A, B**. Similar results of cytoplasm bebling on cell surface were reported on studies of the effect of anticancer drug doxorubicin loaded AuNPs functionalized with lysozyme nanocarriers on HeLa cells [4]. Cell bebling are the characteristics of the apoptotic cells [4]. Also studies on the effect of *Cephalotaxus griffithii* extract on HeLa cell line, and cytotoxicity showed similar cell blebbing of the cells [6]. Similar studies on studies the fluorescent Au nanocluster with chitosan (CH) for the delivery of suicidal gene in the HeLa cells. SEM results obtained showed apoptotic body on treated cells [1].

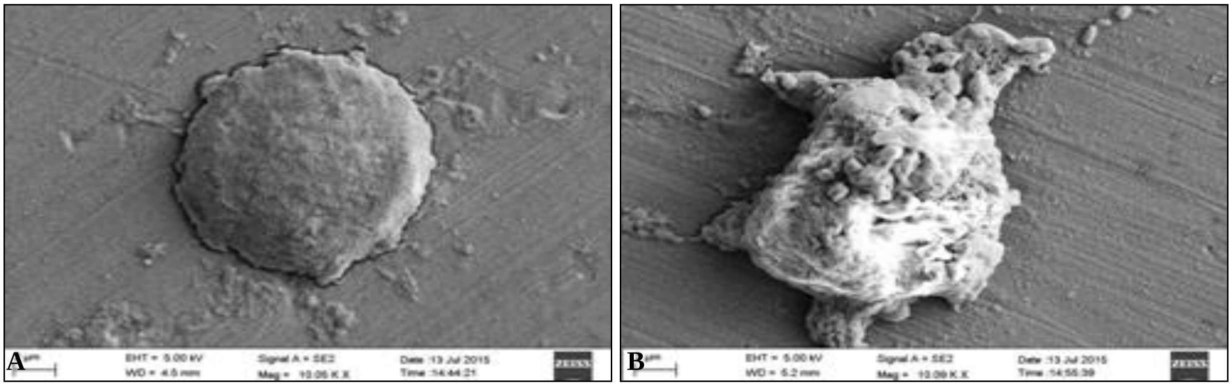

FIG. 11: SEM IMAGES OF HeLa CELLS (A) CONTROL, (B) TREATED WITH AuNPCHCS

TEM Analysis of Treated and Untreated Cells: TEM image **Fig. 12 A, B** of the treated HeLa cells show nuclear membrane blebbling and distortion of the cell shape while the control cell shows normal cell membrane and nuclear membrane structure. MC3T3 cells treated with AuNPs show doge dependent cytotoxicity and firm plasma membrane at low concentrations suggesting no toxicity [22]. Studies on cellular uptake of nanoparticles by TEM shows the internalization of the particles by endocytosis, absorption [23].

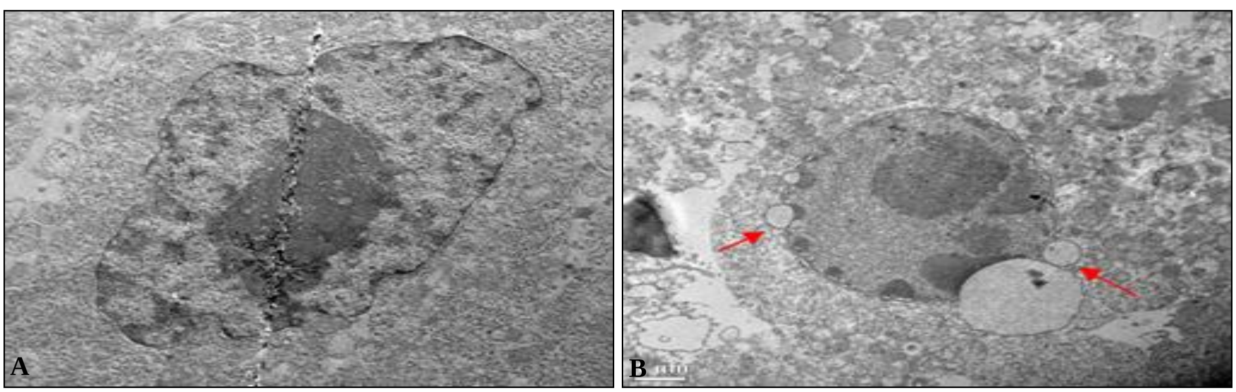

FIG. 12: TEM IMAGES OF HeLa CELL NUCLEUS (A) CONTROL, (B) TREATED WITH AuNPCHCS

DNA Fragmentation Study: DNA fragmentation study was done to observe the fragmentation or laddering pattern in the treated cells. Apoptotic cell death is characterized by nuclear fragmentation. In case of HeLa untreated cells (control), there was observed a prominent band without any sharing. But in case of the treated cells (IC_{50}, 10 µg/ml) in the lane, there observed a shearing **Fig. 13**. This could be due to nuclear damage because of the treatment. Moirangthem *et al.*, (2012) studied the effect of *Cephalotaxus griffithii* extract on HeLa cell line and cytotoxicity also studied by DNA fragmentation assay [6]. The control cells showed no fragmentation, while treated cell DNA was found to be fragmented. Similar results were also observed in the present study. Treated HeLa cells DNA showed shearing. The DNA fragmentation was a hallmark of late apoptosis. Therefore, from the present study it can be concluded that the nanocomposites developed are able to kill cancer as well as primary cells in apoptotic way.

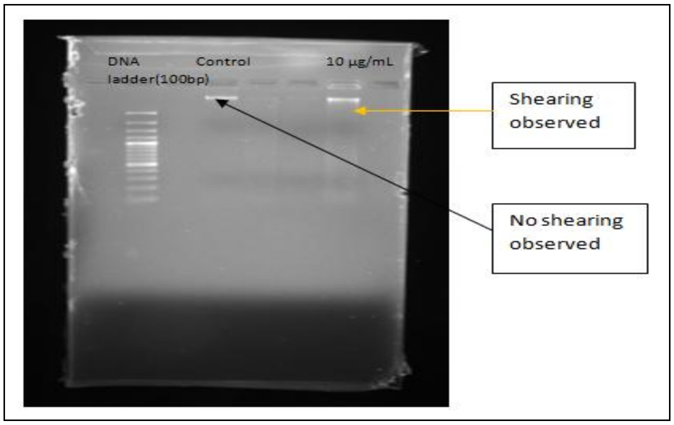

FIG. 13: DNA FRAGMENTATION ASSAY. HeLa CELLS WERE TREATED WITH 10 μg/mL OF AuNPCHCS

CONCLUSION: In summary, though the activity of the nanocomposites was not much different from the single drug, it is important to be noted that composite exhibited similar cytotoxic effect as a free drug, which is essential as in many cases it got compromised after loading with composite NPs. Thus, this composite NPs mediated delivery offers the likelihood of passive targeting by exploring the EPR effect. Moreover, the composite NPs are comprised of AuNPs, which has unique advantage such as biocompatibility. With more extensive research, it can be used as contrast agent in TEM, which will help in probing the endocytosis process, or it can also be suitably used in photothermal processes.

ACKNOWLEDGEMENT: The authors are thankful to Sophisticated Analytical Instrument Facility (SAIF), North Eastern Hill University (NEHU), Shillong, Meghalaya, Central Instrumental Facility (CIF) of Institute of Advanced Studies in Science and Technology (IASST), Boragaon, Assam, Department of Microbiology, College of Veterinary Science, AAU, Khanapara, Guwahati, and Centre for Nanotechnology, IIT Guwahati for their valuable technical help.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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How to cite this article:
Baishya B, Kalita MC and Barman NN: Synthesis and cytotoxicity study of plant derived nanocomposite. Int J Pharm Sci & Res 2019; 10(11): 5167-78. doi: 10.13040/IJPSR.0975-8232.10(11).5167-78.