ORIGINAL ARTICLE

Influence of Algal Based Protein Nanoparticles Loading on Antibacterial Activity, *In Vitro* Drug Release and Cytotoxicity of Cephalosporine Derivative

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Abstract

Aim: In this study, algal based protein, such as phycocyanin nanoparticles loaded cephalosporine derivative cefotaxime nano drug conjugate, has been prepared for the improved antibacterial activity, *in vitro* drug release and cytotoxicity. **Materials and Methods:** Nano drug conjugate was prepared by simple coacervation technique. Method for preparation of nano drug conjugate was optimized with various parameters such as pH, ethanol to phycocyanin ratio, and crosslinking time. Well diffusion assay and turbidimetric assay were carried out to determine the antibacterial activity. Effect of nanoparticles loading on drug release was studied by continuous dialysis method, and cytotoxicity was determined by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay using Vero cell line. **Results and Discussion:** Nano drug conjugate was prepared using the optimized conditions at pH were 8.0 pH, 6:1 the ethanol to phycocyanin ratio, 10 h. Crosslinking time and the nanospheres formed were characterized using scanning electron microscopy which showed a particle size of nanosphere in the range of 70-90 nm and Fourier transform infrared spectroscopy revealed the possible functional groups of nano drug conjugate. Nano drug conjugate showed distinct drug loading, entrapment efficacy, and release profile. Antibacterial activity against human pathogenic bacterial strains revealed effective inhibition at the least concentration. Biocompatibility studies using Vero cell line adopting MTT assay was confirmed by showing maximum cell viability. **Conclusion:** This study would suggest the possible utilization of phycocyanin nanoparticles loaded cefotaxime nano drug conjugate as an antibacterial agent against pathogenic bacteria.

Key words: Antibacterial activity, biocompatibility, cefotaxime, nano drug conjugate, nanoparticles, phycocyanin

INTRODUCTION

Delivery of the drug by the various methods can have a distinct effect on its efficacy. Some drugs have an optimum concentration range within which maximum benefit is derived, and concentrations above or below this range can be toxic or produce no therapeutic benefit at all.[1] On the other hand, the very slow progress in the efficacy of the treatment of severe diseases has suggested a growing need for a multidisciplinary approach to the delivery of therapeutics to targets in tissues.[2] From this, new ideas on controlling the pharmacokinetics, pharmacodynamics, nonspecific toxicity, immunogenicity, biorecognition, and efficacy of drugs were generated. These new strategies, often called drug delivery systems (DDSs), are based on interdisciplinary approaches that combine polymer science, pharmaceutics, bioconjugate chemistry, and molecular biology.[3,4]

The nanoparticle technology used in the recent years has great significance in improving the efficacy of the drugs. The nanoparticles fit into colloidal DDSs, which offer advantages of drug targeting by modified body distribution as well as the enhancement of the cellular uptake which benefits from reduction of undesired toxic side effects of the free drugs.[5-7] With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites, thus aiding in systemic treatments. Nanoparticles (including nanospheres and nanocapsules of size 10-200 nm) are in the solid state and are either amorphous or crystalline.[8] They are

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able to adsorb and/or encapsulate a drug, thus protecting it against chemical and enzymatic degradation. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. Nanoparticles as drug carriers can be formed from both biodegradable polymers and nonbiodegradable polymers.[9]

In recent years, biological nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, in targeting particular organs/tissues, as carriers of DNA in gene therapy, and in their ability to deliver proteins, peptides, and genes through the peroral route. Biological nanoparticles are mainly developed for DDSs as an alternative to liposome technology, to overcome the problems related to the stability of these vesicles in biological fluids and during storage.[10] The nanoparticles technology used in the recent years has great significance in improving the efficacy of the drugs. The nanoparticles fit into colloidal drug delivery systems, which offer advantages of drug targeting by modified body distribution as well as the enhancement of the cellular uptake which benefits from the reduction of undesired toxic side effects of the free drugs.[11] With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites thus aiding in systemic treatments. Nanoparticles can be prepared from a variety of materials such as protein, polysaccharides, and synthetic polymers.[12] The need for developing biodegradable nanoparticles (liposome, virus-like particle, protein, etc.) as effective drug delivery devices was felt years ago.[13] The reason being in addition to the general advantages of nanoparticles, biopolymer nanoparticles in particular offer several advantages, which include the ease of their preparation from well-understood biodegradable polymers and their high stability in biological fluids and during storage.[14]

Nanoparticles made of biodegradable polymers such as proteins and polysaccharides can act as efficient drug delivery vehicles for sustained, controlled and targeted release, aiming to improve the therapeutic effects and also to reduce the side effects of the formulated drugs. Among the different naturally occurring proteins, very few are used for the synthesis of protein nanoparticles such as albumins and gelatine.[15] In this study, an attempt has been made to synthesize phycocyanin, naturally occurring pigment-protein based protein nanoparticles and the synthesized nanoparticles were loaded with cefotaxime, a cephalosporine derivative for the improved antibacterial activity and controlled drug release. Phycocyanin is a pigment-protein complex produced by cyanobacteria known to have distinct biological activities. It is a biodegradable, biocompatible, photosensitive, and poor immunogenic protein molecule. These unique properties will be created premier place for it in the field of nanotechnology as drug delivery agent. Phycocyanin nanoparticles loaded cefotaxime nano drug conjugate has been prepared by simple coacervation method in this study will be helpful to develop effective biocompatible nanoparticles formulated antibacterial agent against severe bacterial infection. Once the formulation passes through the various clinical trials, it will be used for filing a product patent application and the product will be recommended as an effective and safer antibacterial agent.

MATERIALS AND METHODS

Reagents and chemicals

All of the chemicals were analytical grade and used as purchased without further purification (99.99% purity). Phycocyanin was derived from Sigma. Cefotaxime, Mueller-Hinton broth and agar, Roswell Park Memorial Institute media, fetal bovine serum (FBS), trypsin, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide were purchased from HiMedia, Mumbai, India.

Phycocyanin nanoparticles synthesis

Simple coacervation method was used for preparation of protein nanoparticles was also utilized in phycocyanin nanoparticles synthesis.[16] In this method, 1 ml of phycocyanin (0.25 %) was suspended in 50 ml of tris HCL buffer (5 mg/lit.) followed by the addition of ethanol and 0.1 ml of cross linker (25% glutaraldehyde), and the reaction mixture was kept under stirring. The reaction was continued at room temperature. Tween 20 was added at final concentration of 0.1% to stabilize the preparation. The suspension was centrifuged on 10,000 rpm for 10 min. The collected pellet was lyophilized and used for further studies.

Optimization of process parameters for the synthesis of phycocyanin-cefotaxime nano drug conjugate

Phycocyanin nanoparticles-cefotaxime synthesis was carried out at by optimizing various parameters such as pH, ethanol to phycocyanin concentration, and crosslinking time as described in our earlier studies. In all the optimization process, minimum inhibition concentration of cefotaxime was used.

Characterization

Field emission scanning electron microscopy (FESEM)

Size and shape of the BSA nanoparticles and drug loaded nano conjugate was studied by SEM using Carl Zeiss supra 55 (Germany) at 65,000 magnification. Specimen preparation and examination was done by standard condition.

Further characterization of nano drug conjugate pelletized with KBr was done by Fourier transform infrared spectroscopy (FTIR) using Bruker Optik GmbH Tensor 27 (scanning in the range of 400-4000/cm).

In vitro drug release study

In vitro drug release profile was studied by dialysis bag method. Known quantity of freeze dried form of nano drug conjugate suspended in saline was transferred to the dialysis bag (HiMedia) and dialyzed against saline under stirring at 37°C and 75 rpm. At every 1 h interval, dialysate was collected for quantification of cefotaxime and release percentage against time was calculated.

Antibacterial activity

Acinetobacter baumannii and *Staphylococcus aureus* were selected in this study. Both the tested bacterial strains were obtained from Madurai Medical College Hospital, Madurai, Tamil Nadu, India. Both the strains were maintained on Tryptic soy agar slant. Inocula for the antibacterial activity were prepared in Tryptic soy broth under optimum condition.

Antibacterial activity of the tested bacterial strains was studied by well diffusion assay. Inocula of the respective bacterial culture thus prepared were uniformly spread with sterile cotton swabs on sterile Mueller-Hinton Agar Media (HiMedia, India). The wells were made using cork borer and aliquots of silver nanoparticles (aliquots of 25, 50, and 75 µg/ml were prepared from concentrated nanoparticles) was loaded into the wells. The plates were incubated at 37°C for 24 h. After the incubation period, the plates were observed for zone of inhibition. Three replicates were maintained.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was determined by a turbidimetric method.[17] Bacterial inocula prepared in Tryptic soy broth was incubated with different concentration of nano drug conjugate, incubated under shaking condition (150 rpm/min) at 37°C for 20 h for the determination of MIC and MBC. Triplicates were maintained in each treatment.

Cytotoxicity study

Cytotoxicity of nano drug conjugate was carried out by MTT assay using Vero cell line.[18,19] The cells maintained in minimal essential media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C incubated with different concentration of nano drug conjugate and percentage of viability was determined.

RESULTS AND DISCUSSION

Nanomaterials derived from proteins as protein nanoparticles are biodegradable, nonantigenic, metabolizable and can also be easily amenable for surface modification, and covalent attachment of drugs and ligands 19. Because of the defined primary structure of proteins, the protein-based nanoparticles may suggest various possibilities for surface alteration and covalent drug attachment.[20] In this study, phycocyanin - a natural pigment protein complex was utilized for the nano drug conjugate preparation based on cefotaxime-an antibacterial agent under optimum condition. The process conditions for the synthesis of the nano drug conjugate was optimized based on the factors such as pH, crosslinking time, ethanol-phycocyanin ratio. Table 1 shows the process parameters. pH is the major parameter which influences the protein nanoparticles synthesis. With increase in pH, the mean diameter of the nanoparticles decreased gradually and a significant increase in the yield percentage was also observed (P = 5%). Among the different pH, controlled sized particles were obtained at pH 8 and the yield percentage was also high (89.0%). However, gradual decrease in yield and increased size of particles were observed at increasing pH. Ethanol concentration in the coacervation process is critical as it acts as the desolvating agent. The intermittent addition of desolvating agent improves the reproducibility of the protein nanoparticles preparation. It is noted from the optimization process is that the volume of ethanol added is key to the yield of controlled size nanoparticles. 6:1 ratio of ethanol to phycocyanin revealed maximum yield of controlled size particles. However, increased ratio of ethanol-phycocyanin ratio showed larger size particles. The crosslinking of the particles by the glutaraldehyde is a critical factor in the synthesis of protein nanoparticles. The time for crosslinking influences the yield and particle size. Crosslinking plays a major role in the stability and drug release. In this investigation, the crosslinking time was varied between 6 h and 14 h. The yield percentage of about 90.0% was obtained at a crosslinking time of 10 h with the controlled size particles. The previous studies on optimization of preparation process of protein nanoparticles loaded cytotoxic and antibacterial drugs were supported our present findings.

Table 1: Parameters selection for nano drug conjugate preparation

pH	7.0	7.5	8.0	8.5	9.0
Yield percentage (%)	53.0	65.0	89.0[a]	78.0	67.0
Ethanol/ phycocyanin ratio	3:1	4:1	5:1	6:1	7:1
Yield percentage	30.0	41.2	73.4	87.0[a]	54.1
Crosslinking time (hour)	6.0	8.0	10.0	12.0	14.0
Yield percentage	70.0	81.2	90.0[a]	76.0	60.0

[a]Column carries alphabet is statistically significant at 5% level by DMRT

Characterization of both free and drug loaded phycocyanin nano formulation was carried out by scanning and transmission electron microscopy which used to determine morphology (size and shape) and conformational features. Electron microscopy studies of nano drug conjugate revealed spherical, smooth monodispersive particles with size of 120 nm [Figure 1a and b]. Characterization of the synthesized nano drug conjugate was also carried out by FTIR. FTIR analysis helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanoparticles.[21] The profiles of FTIR spectroscopy of the free phycocyanin and nano drug conjugate was depicted in Figures 2 and 3 which showed changes in absorption peaks at specific wave lengths indicating loading of antibiotic with the phycocyanin nanoparticles.

The loading efficiency and the entrapment efficiency of cefotaxime were determined by the spectrophotometric analysis of the nano drug conjugate suspension. The concentration of the drug was done by correlating the absorbance of the supernatant after the centrifugation with the standard absorbance concentration ratio. The drug loading and entrapment efficiency was in the range of 85% and 87%, respectively. *In vitro* drug release of the drug was studied by continuous dialysis method. The sample was taken at regular intervals and analyzed spectrometrically. The release percentage was calculated using the initial drug concentration and the release at specified time [Figure 4]. The drug release was calculated for 24 h. There was a steady release of drug in the early hours, and a total release of about 99.0% was observed during 24 h.

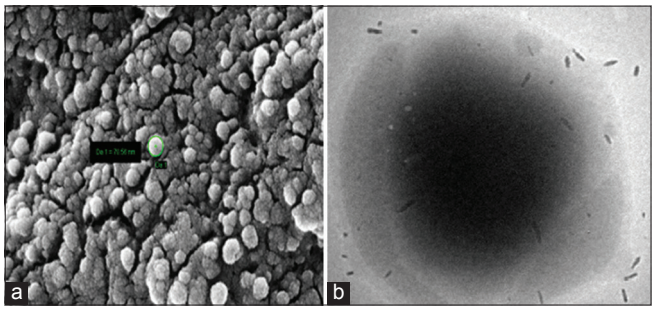

Figure 1: (a) Scanning electron microscopy image of nano drug conjugate (b) transmission electron microscopy image of nano drug conjugate

Antibacterial activity of nano drug conjugate was studied against *A. baumannii* and *S. aureus* by well diffusion assay [Table 2]. In both the tested strains, distinct variation in antibacterial activity of nano drug conjugate has been observed. Zone of inhibition of nano drug conjugate against both the tested strains have been increased with minimum 6 mm to maximum 11 mm [Figures 5 and 6]. MIC and MLC of the nano drug conjugate against both the tested bacterial strains was studied by broth dilution method. It can be seen that nano drug conjugate showed lesser MIC and MLC values than free cefotaxime [Table 3]. A study by Karthick *et al*.[22] supported, our present findings by showing improved antibacterial activity of BSA nanoparticles loaded levofloxacin against *Pseudomonas aeruginosa* studied by determination of well diffusion assay and tube broth assay.

Biocompatibility of the nano drug conjugate was assessed by determination of cytotoxicity against Vero cell line adopting MTT assay. Vero is the most commonly used cell line used for studying efficacy testing. The Vero cell lineage is continuous

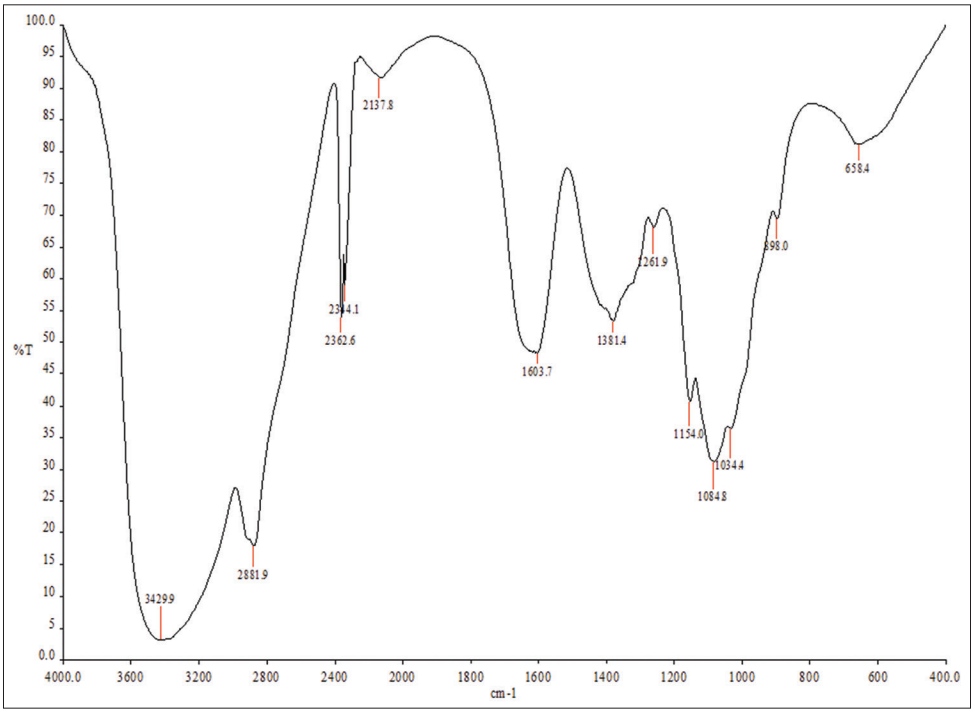

Figure 2: Fourier transform infrared spectroscopy spectra of phycocyanin Nps

Figure 3: Fourier transform infrared spectroscopy spectra of nano drug conjugate

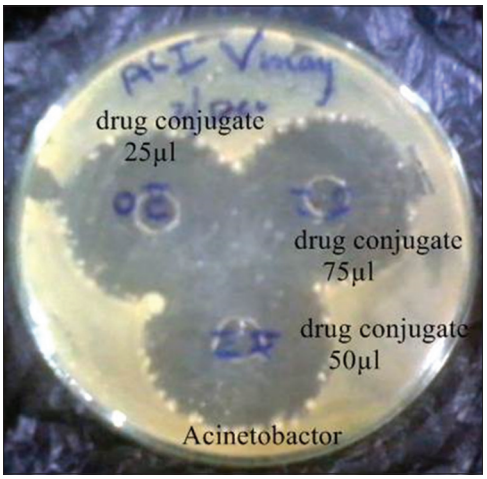

Figure 4: Zone of inhibition of nano drug conjugate against *Acinetobacter baumannii*

Figure 5: Zone of inhibition of nano drug conjugate against *Staphylococcus aureus*

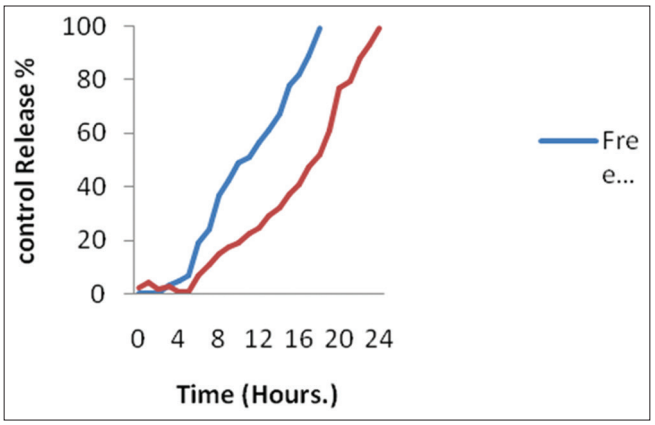

Figure 6: *In vitro* drug release profile of nano drug conjugate

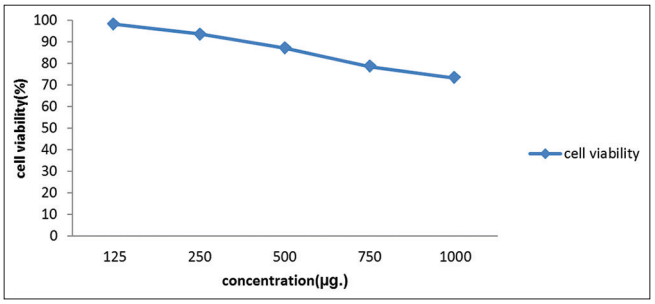

Figure 7: Cell viability (%) of nano drug conjugate treated Vero cell line

and aneuploid. A continuous cell lineage can be replicated through many cycles of division and not become senescent/ Figure 7 shows that the cell viability was not affected at all the tested concentration except 1000 and 750 µg. A significant effect (P = 5%) on the cell viability was not observed in the least concentration. Fluorescent microscopic examination of nano drug conjugate treated cells showed that less or absence

Table 2: Zone of inhibition (mm) of nano drug conjugate against tested pathogenic bacteria

Treatment	Tested bacterial strain	Zone of inhibition (mm)
Free phycocyanin	*Acinetobacter baumannii*	0.0
Free cefotaxime (MIC concentration)		16.0
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (25 µg/ml)		21.0[a]
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (50 µg/ml)		22.0[a]
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (75 µg/ml)		22.0[a]
Free phycocyanin	*Staphylococcus aureus*	0.0
Free cefotaxime (MIC concentration)		11.0
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (25 µg/ml)		15.0[a]
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (50 µg/ml)		16.5[a]
Phycocyanin nanoparticles loaded cefotaxime loaded nano drug conjugate (75 µg/ml)		16.5[a]

MIC: Minimum inhibitory concentration, *S. aureus: Staphylococcus aureus*. [a]Column carries alphabet is statistically significant at 5% level by DMRT

Table 3: MIC and MBC of nano drug conjugate against tested bacterial strains

Treatment	Tested pathogenic bacteria	MIC (µg)	MBC (µg)
Free phycocyanin	*A. baumannii*	-	-
Free cefotaxime		45.0	53.2
Phycocyanin nanoparticles loaded cefotaxime nano drug conjugate		21.2[a]	26.0[a]
Free phycocyanin	*S. aureus*	-	-
Free cefotaxime		54.0	59.5
Phycocyanin nanoparticles loaded cefotaxime nano drug conjugate		24.3[a]	26.0[a]

[a]Column carries alphabet is statistically significant at 5% level by DMRT. *S. aureus: Staphylococcus aureus, A. baumannii: Acinetobacter baumannii*, MBC: Minimum bactericidal concentration, MIC: Minimum inhibitory concentration

of morphological changes at less concentration. It can be seen that high concentration revealed some morphological changes and least cell density [Figure 8].

CONCLUSION

This study clearly shows that the effective inhibition of human pathogenic bacteria, distinct drug loading, entrapment efficacy, controlled drug release, and best biocompatibility of phycocyanin nanoparticles loaded cefotaxime nano drug conjugate which synthesized by simple coacervation method under optimum condition which would suggest the nano drug conjugate as an effective and safer antibacterial agent against pathogenic bacterial strains.

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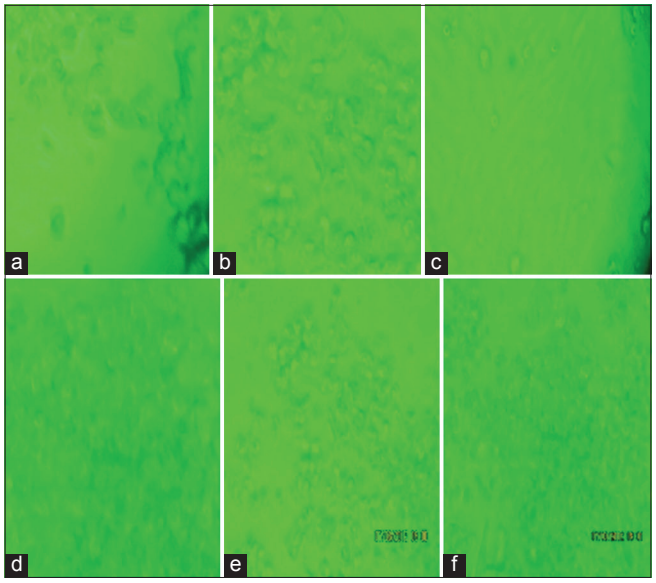

Figure 8: Microscopic images of Vero cell line treated with nano drug conjugate. (a) 1000 (b) 750 (c) 500 (d) 250 (e) 100 (f) control

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