ORIGINAL ARTICLE

Phycocyanin Stabilized Chitosan Nanoparticles Loaded Cephalothin-Bacteriocin Nano Drug Conjugate Preparation for the Enhanced Antibacterial Activity, Controlled Drug Release and Biocompatibility

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Abstract

Aim: The aim of the present work is to evaluate improved or enhanced antibacterial activity, controlled drug release, and biocompatibility of bacteriocin - chitosan nanoparticles (NPs) -cephalothin nano drug conjugate formulation stabilized by phycocyanin. **Materials and Methods:** Ionic gelation method was carried out to synthesize phycocyanin stabilized nano drug conjugate, and the synthesized nano drug conjugate was characterized by electron microscopy for determination of particle size and shape, energy dispersive atomic X-ray spectroscopy for elemental composition and surface modification by Fourier transform infrared spectroscopy. Antibacterial activity was studied by well diffusion assay, microdillution colorimetric assay, and biofilm inhibition assay against food spoilage pathogenic bacterial strains. *In vitro* drug release of cephalothin from the nano drug, conjugate preparation was done by continuous dialysis method. Effect of temperature on the antibacterial activity of nano drug conjugate was also studied biocompatibility of nano drug conjugate was evaluated using *in vitro* 3-(4, 5-dimethyl thiazol-2-yl)-2-5-dephenyl tetrazoliumbromide (MTT) cyctotoxicty test using vero cell line. Hemocompatibility of nano drug conjugate was studied by spectrophotometric determination of plasma hemoglobin (Hb) and hemolysis of nano drug conjugate treated blood. **Results and Discussion:** Nano drug conjugate synthesized by ionic gelation method revealed electron-dense core-shell spherical particles with nano size range. An increase in antibacterial activity against the tested bacterial strains was recorded in nano drug conjugate-bacteriocin preparation treatment studied by all the assays which revealed an effective synergistic activity and the activity was not affected under the influence of temperature except 70°C. Nanoformulation brought about a steady constant release of cephalothin at increasing time. Less cytotoxic effect of nano drug conjugate against vero cell line adopting MTT assay and changes on plasma Hb and hemolysis supported the biocompatibility. Hemocompatibility of nano drug conjugate revealed no distinct changes in plasma Hb and complete absence of hemolysis. **Conclusion:** Formulation of cephalothin-bacteriocin by the phycocyanin stabilized chitosan NPs preparation showed distinct antibacterial activity, controlled release pattern, and best biocompatibility. This study would suggest the possible utilization of the synthesized nano drug conjugate against pathogenic bacterial strains.

Key words: Bacterial strains, bacteriocin, biocompatibility, cephalothin, chitosan nanoparticles, controlled release, synergistic activity

INTRODUCTION

Nanoscience and nanotechnology are new frontiers of this century. Their applications to the agriculture and food sector are relatively recent compared with their use in drug delivery and pharmaceuticals.[1,2] Smart delivery of nutrients, bioseparation of proteins, rapid sampling of biological and chemical

contaminants and nanoencapsulation of nutraceuticals are some of the emerging topics of nanotechnology for food and agriculture. Advances in technologies, such as DNA microarrays, microelectromechanical systems and microfluidics, will enable the realization of the potential of nanotechnology for food applications.[3,4]

The four major areas in food industry that will probably be significantly enhanced by nanotechnology are development of new functional materials; micro- and nanoscale processing; product development; and design of methods and instrumentation for food safety and biosecurity. The potential applications of nanotechnology in the agro-food production chain are claimed to be applicable throughout all phases of food production.[5,6] The food market demands technologies, which are essential to keep market leadership in the food processing industry to produce fresh authentic, convenient and flavored food products. Prolonging the product shelf life and freshness as well as improving the quality of food are the target.[7] Nanotechnology is a technology that has the potential to revolutionize the food industry. Detection of very small amounts of chemical contaminants, virus or bacteria in food systems is another potential use of nanotechnology.[8] Current nanotechnology applications in the agro-food production chain are focused on the development of nano-sized food ingredients and additives, delivery systems for bioactive compounds, and innovative food packaging. As a consequence of their small size, nanoparticles (NPs) show different physical and chemical properties compared to their respective conventional-sized materials, which probably results in different biological interactions.[9,10]

The aim of the present investigation is to formulate phycocyanin stabilized cephalothin, bacteriocin complex with biocompatible chitosan NPs and the formulated drug preparation was evaluated for the antibacterial activity, controlled release and cytotoxicity. Functionalization of NPs is necessary for their stability, functionality, and biocompatibility. Functionalization is essential to preserve the properties of nano drug conjugates In other words, the biological molecule should be stable and able to retain its biorecognition properties and NPs should be able to retain their unique properties. In this study, phycocyanin is used for stabilization of nano drug conjugate. 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. Cephalothin is the cephalosporin derivative active against Gram-positive and -negative bacteria. Howeer, the continuous and high rate of usage causes various adverse side effects which progressively leads to severe health complications. Moreover, the poor solubility of the drug may causes less bioavailability and delivery to the target. Due to the unique biological effects based on the structure, size, and biocompatibility of the chitosan NPs was selected in the present work. Phycocyanin stabilized cephalothin-bacteriocin nano drug conjugate prepared in the present investigation will helpful to develop effective antibacterial agent against pathogenic bacteria.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents used in the study were analytical grade with high purity and the required agents were prepared in double distilled water. Culture medium and other reagents used for antibacterial and cytotoxicity studies were obtained from HI Media, India. Cephalothin and nisin – (bacteriocin) were purchased from Sigma.

Preparation of phycocyanin stabilized chitosan NP incorporated nisin-cephalothin drug conjugate

Ionic gelation method was carried out to prepare nano drug conjugate-bacteriocin complex using 1 mL phycocyann obtained from *Spirullina maxima* with final concentration of 10 µg/mL. The reaction mixture consists of 25 mg chitosan, 25 mg nisin, 10 mg cephalothin and 1 mL phycocyanin suspended in 1% acetic acid in 100 mL distilled water. The preparation was kept under stirring followed by drop-wise addition of sodium tripolyphosphate at room temperature for 3 h. The slurry thus obtained was centrifuged at 10,000 rpm for 10 min. The collected pellet was dried at 48°C overnight and used for further studies.

Characterization of nano drug conjugate

The nano drug conjugate thus obtained was characterized by scanning electron microscope (SEM) equipped with energy dispersive atomic X-ray spectroscopy analysis (EDAX) (performed by SUPRA 55-CARL ZEISS, Germany). Fourier transform infrared radiation (FTIR) was studied using the dried samples (pelletized with potassium bromide Kbr). Transmission electron microscope (TEM) was also used to study particle morphology of the nano drug conjugate. The sample was dispersed in ethanol and the solution was sonicated for 20 min. A few drops of the solution was dropped on a copper grid at room temperature. Then, the sample was analyzed by TEM (performed by HITACHI H9500 300kV TEM).

Antibacterial activity

Bacterial strains

Klebsiella pneumoniae, Salmonella typhi, Escherichia coli, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris, Enterobacter cloacae, Serratia marcescens were obtained from Madurai Medical

College, Department of Microbiology, Chennai. Transport and maintenance was carried out by standard methods. All the strains were stored on nutrients agar slant at 4°C.

Well diffusion assay

Antibacterial activity was carried out by well-diffusion assay using the above mentioned bacterial strains causing foodborne diseases. Respective bacterial strains were grown in Luria-Bertani (LB) broth 37°C for 12-18 h to reach mid-log phase. After the incubation period, respective bacterial cultures were uniformly spread with sterile cotton swab on sterile molten Mueller-Hinton agar plate. Wells were made using sterile gel puncher (8 mm) and loaded with 10, 25, 50 and 100 µg/mL concentration of free nisin previously reconstituted. The seeded plates were incubated at 37°C for 12-24 h. Zone of inhibition was recorded after the incubation.

Microdilution colorimetric assay

Modified method of microdilution calorimetric assay using the chromogenic reagent 3-(4, 5-dimethyl thiazol-2-yl)-2-5-dephenyl tetrazoliumbromide (MTT) was used to study antibacterial activity.[11] Respective bacterial strains were grown in LB medium (yeast extract 5 g/peptone 10 g/L, sodium chloride 5 g/L, and pH 7.0) overnight at 28°C. 10 µl of different concentration of nisin 10, 25, 50, 75 and 100 µg/ml reconstituted in acetonitrile and prepared bacterial suspension (90 µl) containing 1×10^6 CFU/mL were added into each well of the 96 well microplate. Each well of the negative control contained 90 µl of inoculum 1×10^6 CFU/mL and 10 µl of acetonitrile. Cephalothin was used on positive control. The microtiter plates were incubated in the dark at 28°C for 24 h. 10 µl of MTT (5 mg/mL in 0.2 mol/L, pH 7.2, phosphate buffer saline) was added to each well and the plates were incubated another 4 h. The minimum inhibitory concentration value was defined as the lowest sample concentration that inhibited visible growth of the test bacterium, as indicated by MTT staining. Only living microorganisms can convert MTT to formaldehyde and a blue color appeared in the well.

Biofilm inhibition assay

Free nisin, free cephalothin, and nisin-cephalothin incorporated drug conjugate NPs were dissolved in deionized water at different concentration as 10, 25, 50, 75 and 100 µg/ml in sterile screw cap vials, gently shaken well to obtain complete homogenous mixture and used for biofilm inhibition assay. Biofilm inhibition carried out in 96 well plates and nitrocellulose membrane adopting modified method of biofilm inhibition spectrophotometric assay.[12] 100 µl of cell suspension of different bacterial culture was added to 96 well titer plate and 50 µg/ml of cephalothin, bacteriocin, chitosan NPs and nano drug conjugate was added separately, incubated 37°C for 3 days. After the incubation, the liquid suspension was removed, and 100 µl of 1% w/v aqueous solution of crystal violet was added. The following staining at room temperature for 30 min the dye was removed

and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 min. The reaction mixture was read spectrophotometrically at 570 nm.

Evaluation of temperature mediated stress on the antibacterial activity of nano drug conjugate

Temperature-mediated influence of nano drug conjugate on the antibacterial activity was studied by incubating the known quantity of nano drug conjugate (0.05 mg/ml) dispersed in phosphate buffered saline (pH 7.6) at 30, 40, 50, 60 and 70°C separately for 1 h in water bath. After the incubation period, 50 µl of the suspension from each heat treatment was used for well diffusion, microdilution, and colorimetric assays as described earlier.

Cytotoxicity assay

Cytotoxicity of nano drug conjugate was determined by inhibition of cell growth of Vero cell line using a tetrazolium dye (MTT) assay and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the untreated ones. Vero cell line was obtained from National Centre for Cell Sciences, Pune, India. RPMI1640 was used as the source of cell growth medium and a humidified atmosphere (d 5% CO_2) was maintained for cell culture. Cells harvested in a logarithmic growth phase were seeded on 96 wells at a cellular density of 5×10^3 cells/ml followed by the addition of 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml concentrations of nano drug conjugate, incubated for 24 h at 5% CO_2 incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 µl/well (5 mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4 h incubation, 1 ml of dimethyl sulfoxide was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability was determined graphically. The effect of the nano drug conjugate on the proliferation of vero cells was expressed as the % cell viability.

In vitro hemolysis assay

Hemocompatibility of nano drug conjugate was studied by spectrophotometric determination of plasma hemoglobin (Hb) and hemolysis of nano drug conjugate treated blood. An aliquot containing 0.1 ml of nano drug conjugate was treated with 0.9 ml of blood and incubated for 3 h at 37°C under shaking. After the incubation, plasma was collected by centrifuging the samples at 4500 rpm for 10 min. The concentration of Hb in the plasma was quantified by spectrophotometry; plasma Hb concentration directly correlates the percentage of lysed blood cells. Plasma Hb concentration was quantified spectrophotometrically.

Here in our experiment, Triton-X (1%) treated blood as the positive control, saline treated blood as the negative control. Plasma Hb concentration (mg/mL) was calculated based on the following equation:

$$\text{Plasma Hb} = \frac{2 \times A_{415} - A_{380} + A_{450} \times 1000 \times \text{Dilution factor}}{E \times 1.655}$$

Where, A_{415}, A_{380} and A_{450} are the absorbance values at 415, 380 and 450 nm. A_{415} is the soret band based absorption of Hb. A_{380} and A_{450} are correction factors applied for uroporphyrin absorption falling in the same wavelength range. E is molar absorptivity value of oxyhemoglobin at 415 nm which is 79.46 and 1.655 is the correction factor accounting the turbidity of plasma sample.

$$\% \text{ Hemolysis} = \frac{\text{Plasma Hb value of sample}}{\text{Total Hb value of blood} \times 100}$$

RESULTS AND DISCUSSION

NPs (including nanospheres and nanocapsules of size 10-200 nm) are in the solid state and are either amorphous or crystalline. They are 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. NPs as drug carriers can be formed from both biodegradable polymers and non-biodegradable polymers.[13] In recent years, biodegradable polymeric NPs, particularly Chitosan NPs 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.[14] In the present study, chitosan NPs loaded cephalothin was prepared to determine antibacterial activity, controlled drug release, and cytotoxicity
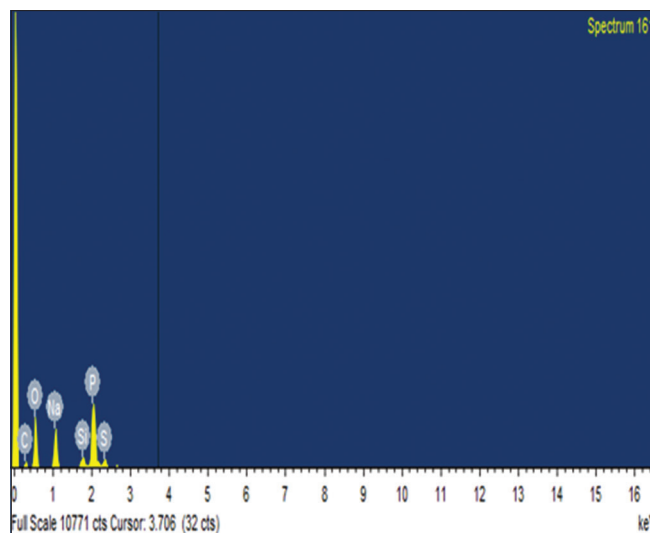
Preparation of phycocyanin stabilised chitosan NPs incorporated cephalothin-bacteriocin drug conjugate

Size, surface charge, and hydrophobicity/hydrophilicity are parameters that affect the body distribution and interactions with the biological environment. The NP characterization by Conventional light microscopy is not suitable because of its resolution limited to about 1 μm. Instead, techniques such as SEM and TEM are nowadays, studied with 5000-30,000-fold magnifications and provide visual and descriptive information about the NPs population. In a SEM setup, the nanoparticulate sample, coated to be conductive (e.g., platinum), is scanned in a high vacuum chamber with a focused electron beam. SEM measurement on phycocyanin stabilized nano drug conjugate revealed spherical particles with the size of 53-55 nm [Figure 1]. Such size distribution analysis of nano drug

conjugate confirms that the particles are well dispersed. SEM equipped with EDAX analysis shows quantitative detection and localization of elements in the nano specimens. The EDX images illustrated the presence of elements in the nanosuspension [Figure 2]. TEM analysis of the nano drug conjugate recorded electron dense core-shell particles with the size range 53-55 nm [Figure 3]. Further characterization was carried out by FTIR analysis which 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 nano drug conjugate. Figure 4a-c depicted FTIR spectra of phycocyanin, chitosan NPs and phycocyanin stabilized chitosan nanoparticles- bacteriocin stabilized nano drug conjugate which reveals specific changes in absorption peaks. In Figure 4c, the main absorption peaks at 3904.1/cm (OH), 3770.2/cm (OH) and 2165.0/cm (C≡C), 3433.5/cm (OH), 2928.3/cm (CH), 2361.3/cm (H–C=O, C≡N), 1737.3/cm (C=O) and a new absorption peaks at 1406.7/cm (C-C), 1127.7/cm (C-N), 896.1/cm (CH) and 532/cm (C-Cl)

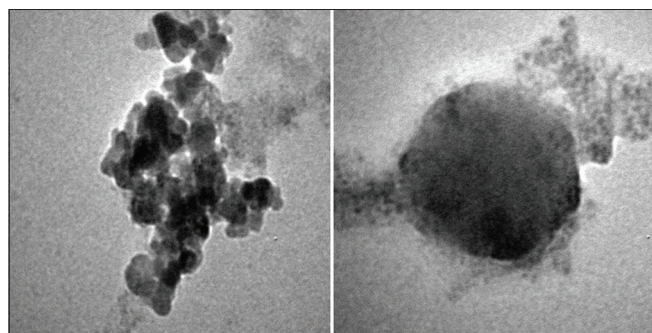**Figure 1:** Scanning electron microscopic image of nano drug conjugate

Figure 2: Energy dispersive atomic X-ray spectra of nano drug conjugate

which illustrates the specific interaction of functional groups which confirms the phycocyanin stabilized the nano drug conjugate.

Antibacterial activity

Well diffusion assay

All the tested bacteria were susceptible to nano drug conjugate treatment [Figure 5]. Among the different bacterial species, maximum zone of inhibition was recorded in *P. aeruginosa* (26.5 mm) followed by *S. typhi* (24.0 mm). Minimum zone of inhibition was recorded in *S. aureus* (13.0).
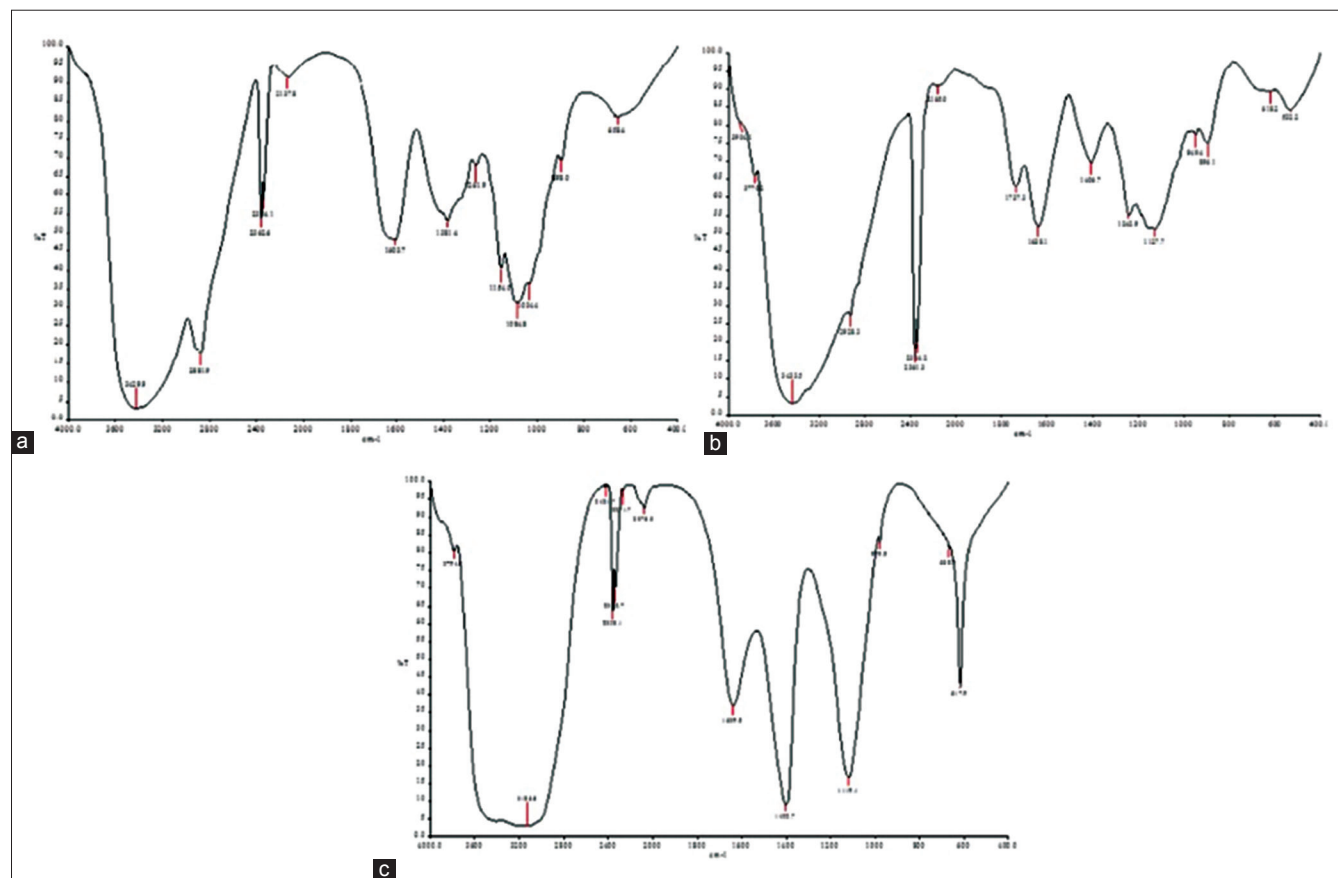

Figure 3: Transmission electron microscope image of nano drug conjugate

Microdilution colorimetric assay

Antibacterial activity was also evaluated by growth inhibition assay using MTT microdilution colorimetric microplate assay. All the tested bacterial strains were susceptible to nano drug conjugate as concentration-dependent manner. In all the tested bacterial strains, maximum growth inhibition was recorded in high concentration of nano drug conjugate [Figure 6a and b].

Biofilm inhibition

Biofilms are universal, complex, interdependent communities of surface-associated microorganisms. The organisms are enclosed in an exopolysaccharide matrix occurring on any surface, particularly aquatic and industrial water systems as well as medical devices. As such, biofilms are highly relevant for public health.[14] Biofilm, likely the predominant mode of device related microbial infection exhibit resistance to antimicrobial agents They can serve as hides for disease and are often associated with high-level antimicrobial resistance of the associated organisms. Biofilms create an environment that enhances antimicrobial resistance.[15] In the present work, biofilm inhibition was observed in all the tested bacterial strains which studied by microtiter plate and nitrocellulose membrane assays [Table 1]. Nano drug conjugate exhibited maximum biofilm inhibition than the free drug and free chitosan NPs. Biofilm inhibition was not recorded in bacteriocin treatment.
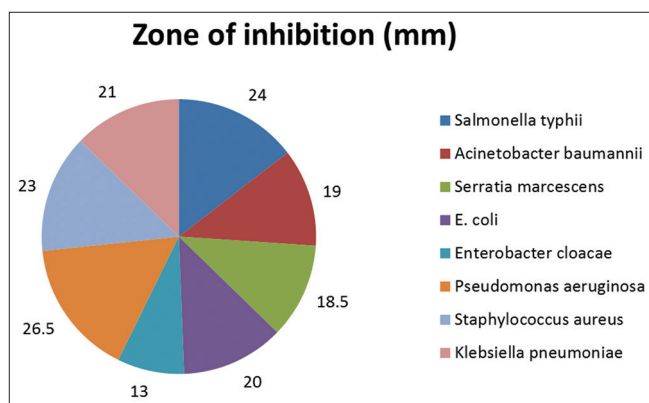

Figure 4: Fourier transform infrared spectra of nano drug conjugate, (a) Free chitosan nanoparticles, (b) Phycocyanin (C) Nano drug conjugate

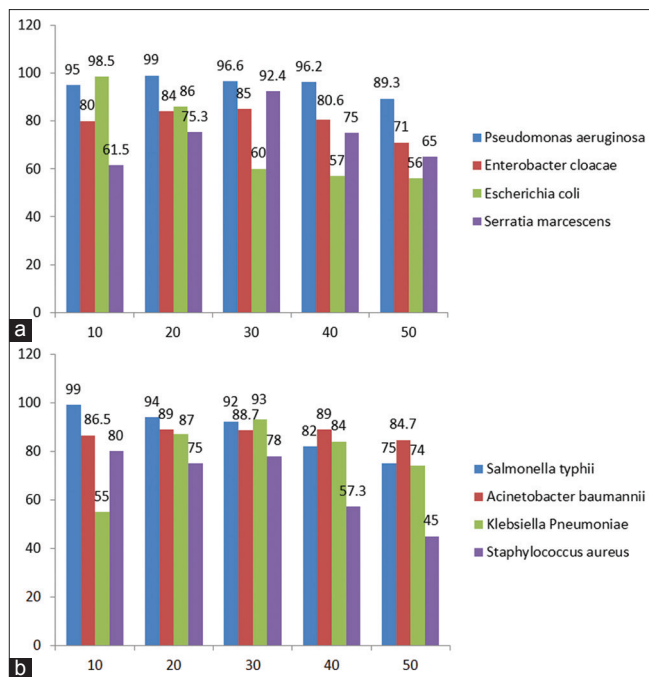## Effect of temperature on the antibacterial activity

Temperature-mediated influence on the antibacterial activity studied by all the assays of nano drug conjugate revealed that the drug conjugate retained the activity in all tested temperature except 70°C [Figures 7-9]. The results of all the tested assays showed that significant thermostability of the prepared nano drug conjugate than the free cephalothin, bacteriocin, free NPs.

Controlled drug release profile

Dialysis bag method was used to study the *in-vitro* release of cephalothin from the nano drug conjugate. In general, there was a steady state of release was observed with respect to increasing time. Release was observed from 5th h and gradual

Figure 5: Zone of inhibition (mm) of nano drug conjugate against pathogenic bacteria
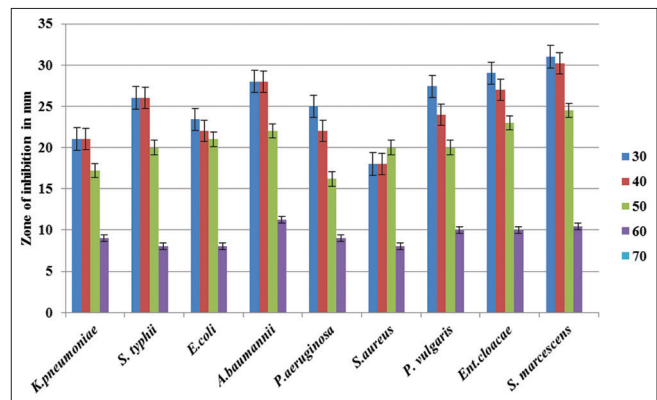
Figure 6: (a) Growth inhibition (%) of nano drug conjugate against tested bacterial strains, (b) growth inhibition (%) of nano drug conjugate against tested bacterial strains
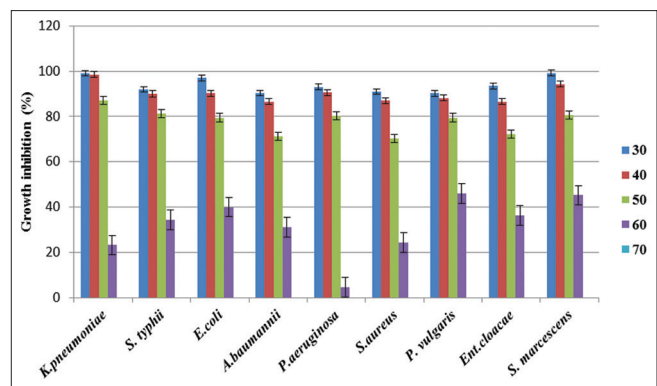
increase in release profile was recorded. Maximum release of 99.6% was noticed at the 18th h [Figure 10].

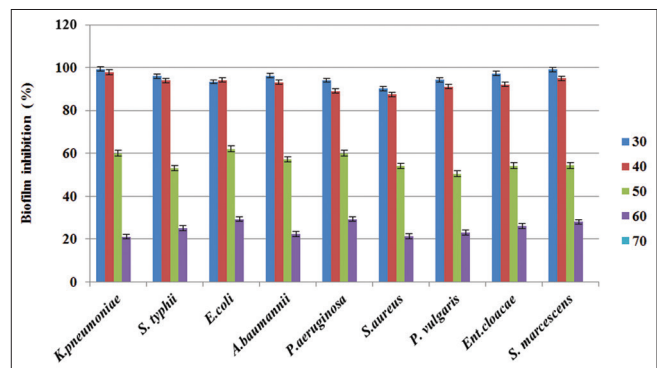## *In vitro* cytotoxicity and hemocompatibility

The MTT assay was performed to assess the viability of cells exposed to nano drug conjugate. A serial 10-fold dilution of nano drug conjugate was prepared. Vero cells grown in 96 well

Figure 7: Zone of inhibition (mm) of nano drug conjugate against pathogenic bacteria tested at different temperature

Figure 8: Growth inhibition of nano drug conjugate against pathogenic bacteria tested at different temperature by microdilution colorimetric assay
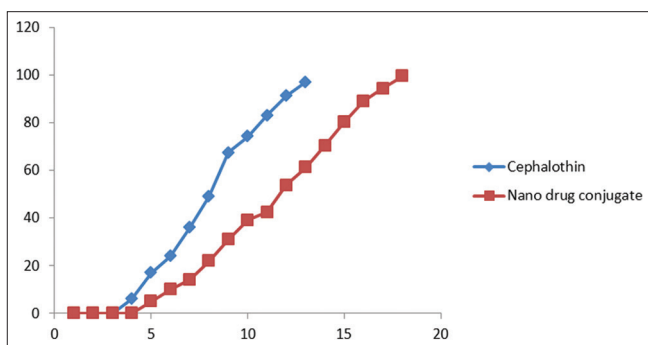
Figure 9: Biofilm inhibition (%) of nano drug conjugate against pathogenic bacteria by microtitre plate assay

Tested bacteria	Treatment	Biofilm inhibition (%)	
		Microtitre plate assay	**Membrane assay**
Klebsiella pneumoniae	Cephalothin	70.0	71.2
Salmonella typhii		69.2	71.0
Escherichia coli		76.4	80.2
Acinetobacter baumannii		81.2	84.0
Pseudomonas aeruginosa		65.5	70.0
Staphylococcus aureus		34.3	4.2
Proteus vulgaris		66.2	72.0
Enterobacter cloacae		76.2	77.2
Serratia marcescens		63.2	69.0
Klebsiella pneumoniae	Chitosan NPs	10.0	10.4
Salmonella typhii		6.5	8.5
Escherichia coli		9.0	9.5
Acinetobacter baumannii		5.2	5.7
Pseudomonas aeruginosa		6.1	6.6
Staphylococcus aureus		0.0	0.0
Proteus vulgaris		6.4	7.2
Enterobacter cloacae		23.2	29.0
Serratia marcescens		31.2	33.4
Klebsiella pneumoniae	Nano drug conjugate	98.0[a]	99.3[a]
Salmonella typhii		91.2[a]	96.0[a]
Escherichia coli		90.2[a]	93.4[a]
Acinetobacter baumannii		94.2[a]	96.2[a]
Pseudomonas aeruginosa		90.2[a]	94.0[a]
Staphylococcus aureus		84.0[a]	90.2[a]
Proteus vulgaris		90.2[a]	94.3[a]
Enterobacter cloacae		92.2[a]	97.2[a]
Serratia marcescens		96.2[a]	99.0[a]

Table 1: Effect of nano drug conjugate on biofilm inhibition (%) of the tested bacterial strains

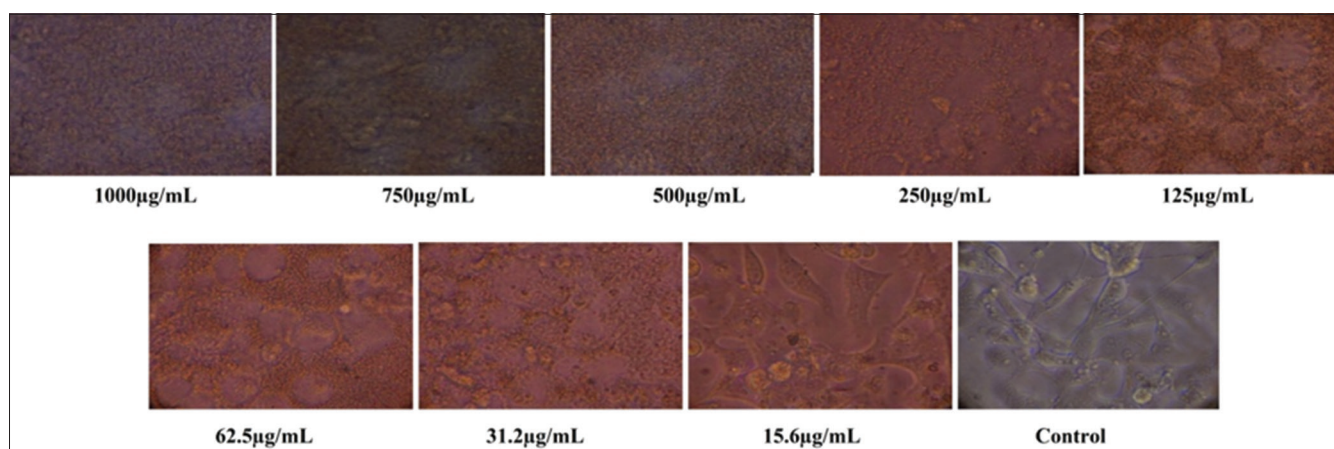[a]Column carries alphabet statistically significant at 5% level by DMRT. DMRT: Duncan's multiple range test

Figure 10: Controlled drug release (%) of nano drug conjugate

Figure 11: Effect of nano drug conjugate on the viability (%) vero cell line

plates were incubated and the viability in respective dilution was determined by MTT assay which reveals that all concentration

Figure 12: Microscopic examination of vero cell lines treated with nano drug conjugate

Table 2: Effect of nano drug conjugate on plasma Hb (g/L) and hemolysis (%)

Treatment	Plasma Hb (g/L)	Hemolysis (%)
Control	0.0	0.0
Nano drug conjugate	0.0	0.0
Triton-X (positive control)	4.0[a]	65.0[a]
Saline (negative control)	0.0	0.0

[a]Column carries alphabet statistically significant at 5% level by DMRT. DMRT: Duncan's multiple range test

if diluted NPs reduced the viability than the control. Effective high cytotoxic effect was recorded at 1000 µg/ml [Figure 11] followed by 500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg/ml and the percentage of viability at respective concentration was 9.4%, 12.8%, 14.5%, 17.0%, 20.5%, 29.9%, 49.5%, and 72.6%, respectively. Morphological characteristics of nano drug conjugate treated cells using an inverted microscope revealed that changes in the cell morphology were observed at 1000 and 500 µg/mL when compared to the cells treated at least concentrations [Figure 12]. It can be seen that cells treated with high concentration showed some changes in morphological structure whereas cytopathic effect was not recorded in least concentration. Hemocompatibility of nano drug conjugate showed no distinct effect on plasma Hb level and complete absence of blood lysis [Table 2].

CONCLUSION

Phycocyanin stabilized chitosan NPs - bacteriocin – cephalothin nano drug conjugate was prepared, characterized in the present study. Enhanced antibacterial activity, *in vitro* controlled drug release profile and biocompatibility was studied. Nano drug conjugate with spherical particles size and stabilization with phycocyanin which may improve antibacterial activity, controlled drug release profile and biocompatibility. Against vero cell line by exhibiting less cytotoxicity and no distinct changes in morphology. Nano

drug conjugate exhibited no changes in plasma Hb and complete absence of hemolysis which further confirmed the biocompatibility. These distinct properties would suggest the possible utilization of nano drug conjugate in agro –food system as the delivery agent of bioactive compounds and food packaging. Further study using animal model will helpful for mass production, formulation and clinical evaluation of nano drug conjugate which will be commanded as an effective antibacterial agent against pathogenic bacterial strains.

ACKNOWLEDGMENT

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