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Chitosan nanoparticles loaded antibiotics as drug delivery biomaterial

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ABSTRACT

Herein we describe the preparation, characterization and utilization of chitosan nanoparticles for the intracellular delivery of the poorly cell-penetrating antibiotic e.g. Ciprofloxacin, Chlortetracycline hydrochloride and Gentamycin sulfate to improve their treatment of bacterial infections. Chitosan nanoparticles were prepared via the ionic gelation of chitosan with tri polyphosphate anions. Several parameters were studied to optimize the particle size of chitosan nanoparticles, here we select the concentration of chitosan and the concentrations of sodium tri poly phosphate (TPP) as optimizing parameters and the other factors stay constant such as pH of solution and ultrasonication time. Chitosan nanoparticles formed characterized by using FT-IR and transmission electron microscope (TEM). Results show that chitosan nanoparticles and its loaded antibiotics kill and inhibits the growth of gram (+) and gram (-) bacteria tested due to nanoparticles structures, and the antibacterial activity increased with increasing the anti biotic content.

INTRODUCTION

The purpose of this study was to explore the possible improvement of antimicrobial treatment by its incorporation into chitosan-based nanoparticles. Limited cellular penetration reduces the effectiveness of many antimicrobial treatments; and overcome the side effects of selected antibiotecs such as Ciprofloxacin, Chlortetracycline hydrochloride and Gentamycin sulfate to improve their treatment of bacterial infections(Zaki and Hafez, 2012). Chitosan is a copolymer composed of N-acetyl glucosamine and glucosamine units (Chattopadhyay and Inamdar, 2013).It has been used as antibacterial and anti-fungal substance (Benhabiles *et al.,* 2012; Lahmer *et al.,* 2012; Cruz-Romero *et al.,* 2013; Limam *et al.,* 2013). There are several mechanisms that explain the antibacterial activity of chitosan, the most common one assume that chitosan binds to the negatively charged bacterial surface disrupting the cell membrane and altering its permeability. This allows materials to leak out of the bacterial cells resulting in cell death (Abou-Zeid *et al.,* 2011).

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Chitosan has antibacterial activity only in an acidic medium because of its poor solubility above pH 6.5.There are several factors affect the antibacterial activity of chitosan such as chitin type, degree of polymerization, molecular weight solvent some physciochemical properties and pH of the solution (Li *et al.,* 2002, Dash *et al.,* 2011; Jimtaisong and Saewan, 2014; El-Sherbiny and El-Baz, 2015). Chitosan exhibits higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria, this is due to their different cell walls. In Gram-positive bacterium, its cell wall is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow foreign molecules to come into the cell without difficulty. ButIn Gram-negative bacterium, the cell wall of which is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules (Jia and Xu, 2001; No *et al.,* 2002; Abou-Zeid *et al.,* 2011; Lu *et al.,* 2014). Chitosan is a mucoadhesive polymer that is able to open tight junctions and allow the paracellular transport of molecules across mucosal delivery of vaccines (Van der Lubben *et al.,* 2001; Sawaengsak *et al.,* 2014; Del Guidice and Baudner, 2015).

Chitosan nanoparticles exhibit higher antibacterial activity than chitosan based on the special character of the nanoparticles. The negatively charged surface of the bacterial cell is the target site of the polycation. Therefore, the polycationic chitosan nanoparticles with higher surface charge density interact with the bacteria to a greater degree than chitosan itself. Chitosan nano-particles provide higher affinity with bacteria cells for a quantum-size effect, due to the larger surface area of the chitosan nanoparticles, which could be tightly adsorbed onto the surface of the bacteria cells to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacteria cells(Avadi *et al.,* 2004, Abdel-Fattah *et al.,* 2014).

Chitosan nanoparticles has been used as drug carriers and gene carrier to enhance their transfer efficiency in the cells as reported in several studies(Chopra *et al.,* 2014, Csaba and Alonso, 2014, Garrait *et al.,* 2014, Liu *et al.,* 2014, Lu *et al.,* 2014, Nascimento *et al.,* 2014, Ragelle *et al.,* 2014).

Chitosan nanoparticles were used as drug delivery carriers due to it offers many advantages. First, chitosan is safe material as it has biocompatible and biodegradable properties. Second, it is water-soluble polymers which is an ideal property for drug delivery carriers, therefore, simple and mild preparation methods can be applied. This renders chitosan nanoparticle are suitable for a broad category of drugs including macromolecules and labile drugs. Third, chitosan is available in a wide range of molecular weights and is easily chemically modified by coupling with ligands providing flexibility in formulation development. Forth, chitosan provides absorption promoting effect that prolongs the contact time between substrate and cell membrane. In addition, their nano-sized facilitates the drug uptake through the cell membrane. Together, the absorption enhancing effect and nano-sized particles exhibited ability to improve drug bioavailability. Fifth, chitosan nanoparticles offer versatile routes of administration, especially non-invasive routes, i.e. per oral, nasal, and ocular mucosa, which are preferable routes administration. Furthermore, chitosan nanoparticles demonstrated to be good adjuvant for vaccine delivery (Kim *et al.,* 2004; Liu *et al.,* 2014; Lu *et al.,* 2014; Nascimento *et al.,* 2014; Ragelle *et al.,* 2014).

An antibiotic is a drug that kills or inhibit the growth of bacteria, antibiotics are classified into four categories: antibacterial, antiviral, antifungal, and antineoplastic. Antibiotics work by a number of different actions including inhibition or regulation of cell wall synthesis, nucleic acid metabolism, and protein synthesis. The main classes of antibiotics are β-Lactams, Macrolides, Fluoroquinolones (Ciprofloxacin), Tetracyclines (tetra Chlortetracycline) and Aminoglycosides (gentamycin) (Chopra *et al.,* 1997; Walsh, 2003; Walsh, 2003; Taubes, 2008).

The present work is aimed to prepare chitosan nanoparticles by using ionic gelation method and characterize it by using FT-IR and TEM to be used as polyloaddrug delivery of some antibiotics names, Ciprofloxacin antibiotic as ciprofloxacin HCL, Chlortetracycline hydrochloride and Gentamycin sulfate. Then evaluate their antibacterial activities.

EXPERIMENTAL

Materials

Chitosan (Alfa Aesar Company, Medium molecular weight, viscosity 1860 cps, degree of deacetylation 79.0%), penta sodium tri poly phosphate (TPP).Sodium hydroxide (Modern Lab chemicals, Egypt), Methyl alcohol, ethyl alcohol and acetic acid (Sisco Research Laboratories, India) and all other chemicals used are analytical grade and were used without further purification Antibiotics used are: ciprofloxacin HCL, Chlortetracycline hydrochloride and Gentamycin sulfate (Memphis pharm and chemical industry).

Methods

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared based on the modified ionotropic gelation with slight modification (Qi *et al.,* 2004; Du *et al.,* 2009; Lu *et al.,* 2009). Briefly, Chitosan was dissolved in 1% (v/v) acetic acid and leaving it under stirring for 24 h. The pH was adjusted to pH 5.5 with 0.01N NaOH. TPP was dissolved separately in deionized water to final concentration of 0.1 mg/ml. Then, the TPP solution was added to the chitosan solution drop wise at different TPP:chitosan ratios under vigorous magnetic stirring at room temperature. The resulting suspension was then left under ultrasonication for 45 min**.**

Preparation of Antibiotic-loaded chitosan nanoparticles:

Different concentration of the antibiotic dissolved in distilled water was added to nano-chitosan solution in the same molar ratio with stirring for 20 min. and The resulting suspension was then left under ultrasoincation for 45 min. then finally stirring for another 20 min., to obtain a final antibiotic concentration (0.05, 0.1, 0.15, 0.2, 0.5% gm/ml)(Jain and Banerjee, 2008, Du *et al.,* 2009).

Characterization of chitosan nanoparticle and its loaded

The FTIR spectra of the samples were recorded by using an FT- IR spectrophotometer (Nexus 670, Nicolet, USA) in the region of 4000-400cm-1 with spectra resolution of 4 cm^{-1}.

Shape and size of chitosan Nanoparticle was investigated using JEOL, TEM-Speciments for TEM measurements were prepared by placing a drop of colloidal solution on 400 mesh copper grid coated by an amorphous carbon film and evaporating the solvent in air at room temperature. The average diameter of chitosan nanoparticle was determined from diameter of 100 nanoparticle found in several arbitrarily chosen are in enlarged microphotographs.

Evaluation of Antibacterial Activity *in vitro*

Materials

Two bacterial strains from Bacterial Lab, Botany Department, the Faculty of women for Art, Science & Education, Ain Shams University, Cairo, Egypt were employed. They include *Staphylococcus aureus (S. aureus)* as Gram-positive (G +ve)

bacteria and *Escherichia coli (E. coli)* as gram-negative (G−ve) bacteria. *S. aureus* and *E. coli* were selected as test cells because they are the most frequent bacteria in the wound infection and represent Gram positive and Gram negative bacteria, respectively. Fresh inoculants for antibacterial assessment were prepared on nutrient broth at 37 °C for 24 hours.

Test Method

The antibacterial spectrum of chitosan, nanochitosan and their loaded antibiotic samples were determined against the test bacteria by disk diffusion method on an agar plate(Abou-Zeid *et al.*, 2011). Briefly,1 cm diameter blended film samples were cut and put into 10 ml of nutrient agar, to which 10 µl of microbe culture was inoculated, after the solidification. The plates were incubated at 37 ^{o}C for 24 hrs, after which the diameter of inhibition zone were measured and recorded.

RESULT AND DISCUSSION

Preparation and Characterization of chitosan nanoparticles

The preparation of chitosan nanoparticles is based on an ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate at room temperature represented figure 1. The interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution and ultra sonication time.

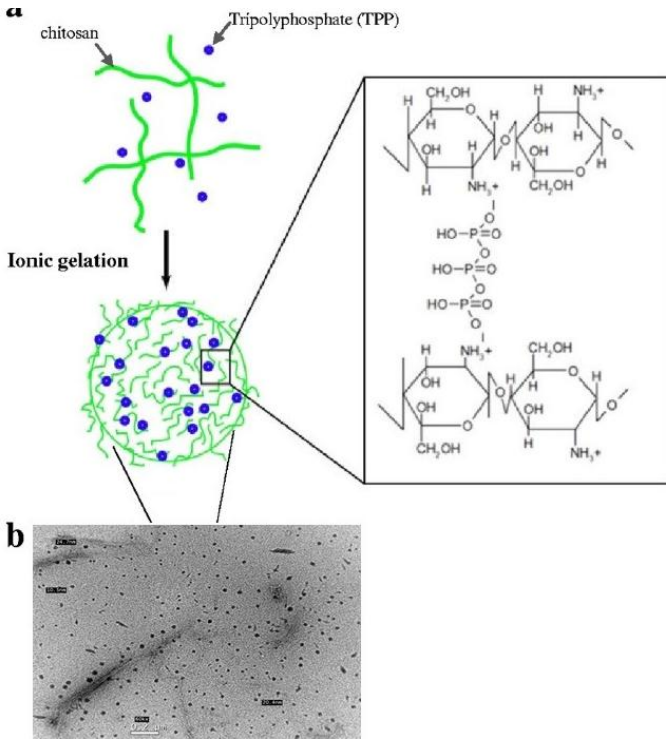
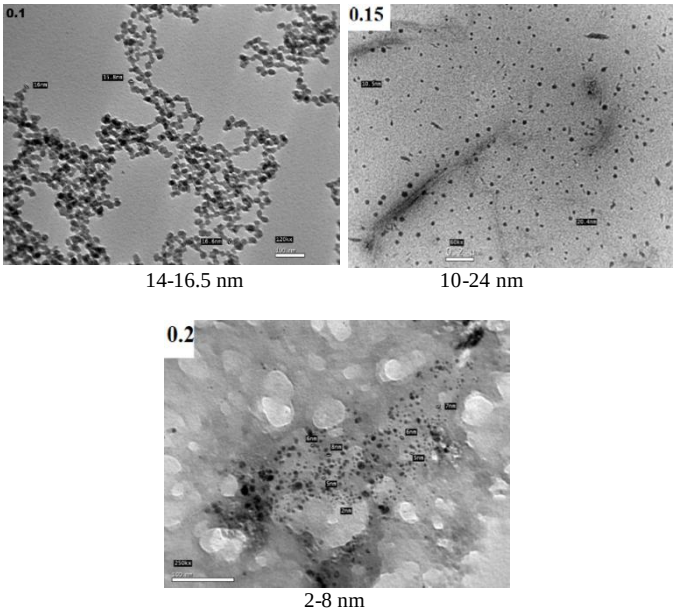


Fig. 1: Formation of the chitosan-tripolyphosphate complex byionotropic gelation. (a) Schematic illustration of the chitosan-TPPcomplex and (b) TEM image.of chitosan nanoparticles with 25 nm diameter.

There are many factor have effect on the size of chitosan nanoparticles: chitosan concentration, sodium tri poly phosphate concentration, pH of solution and ultrasonication time; herein we kept the pH of solution and ultrasoincation time constant at pH 5.5 and 45 min. respectively based on previous study(Xu and Du, 2003)and changing the concentration of both chitosan and TPP.

Effect of chitosan concentration on nanoparticle size

By studying the chitosan concentration effect (0.1, 0.15, 0.2) g/mlat constant other parameter such as concentration of sodium tri polyphosphate (0.1) g/l and pH 5.5 and ultrasonication time 45min., It was observed that with increase in the concentration of chitosan the appearance of the solution changed from clear viscous liquid to opalescent fluid and then precipitated the solution became opalescent indicating the formation of nanochitosan with smallest nano size ranged (2-8) nm. From figure2 is obvious that by increasing the chitosan concentration from 0.1 to 0.2 g/mL at a constant TPP concentration (0.1 g/mL), the size of nanoparticles decrease, nano size of particles are more favorable at the chitosan concentration of 0. 2 g/ml (2-8nm) than 0.15(10-24 nm) and 0.1 mg/mL.



14-16.5 nm 10-24 nm

2-8 nm

Fig. 2: TEM image of nano chitosan prepared at different concentration of chitosan and its nano size range.

Effect of concentration of sodium tri poly phosphate

The effect of concentration of sodium tri poly phosphate (0.05, 0.1, 0.15) g/ml was studied at constant other parameters:concentration of chitosan at (0.2) g/l, at pH(5.5), sonication time 45 minutes at room temperature. Sodium tri poly phosphate (TPP) a major ingredient for cross linking has a pronounced effect on the properties of chitosan dispersion, the concentration of TPP was increased gradually, the solution became opalescent indicating the formation of nanochitosan. It was observed from figure 3 with increase in concentration of TPP, the particle size of chitosan nanoparticle increased, Concentration of TPP above 0.10 g resulted precipitation. The

precipitation at excessively higher concentration of TPP may be attributed to the aggregation of chitosan molecules due to excessive cross linking through TPP bridging. As shown at figure 3, obvious tha tby increasing the chitosan concentration from 0.05 to 0.15 g/mL at a constant chitosan concentration (0.2 g/mL), the size of chitosan nanoparticles changed from 19 nm at (0.05) to 12 nm at (0.15) but the more favorable size 23 nm at 0.1 of STTP which have a good distribution for a nanoparticle all over the solution.

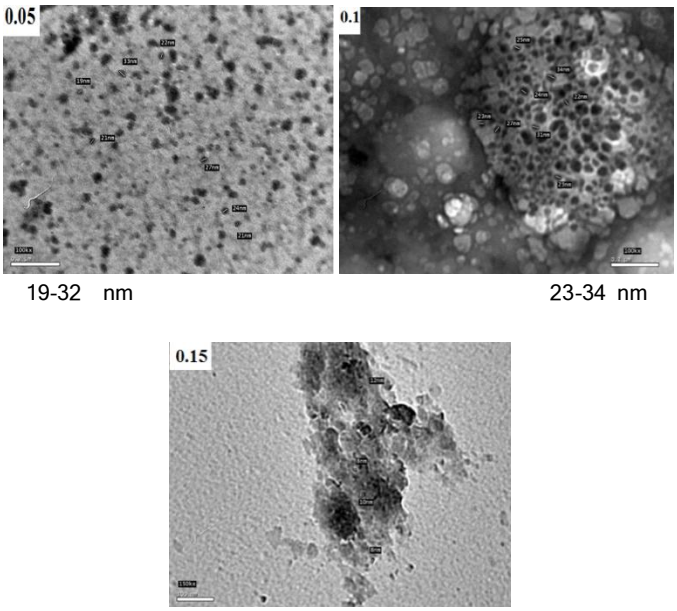


Fig. 3: TEM images of nano chitosan prepared at different concentration of TTP and its nano size range.

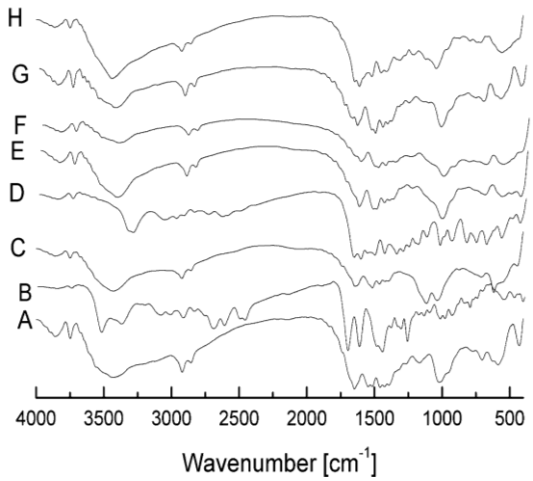


Fig. 4: charts for original and loaded samples A (chitosan), B (ciprofloxacin HCL), C (Gentamycin sulfate) and D (Chlortetracycline HCl), E (CS NPs), F (CS NPs-Cipro), G (CS NPs-Genta) and H (CS NPs-Chloro)

FT-IR Analysis

Infrared analysis used to detect ciprofloxacin, Gentamycin sulfate and Chlortetracyclinein chitosan nanoparticles. As expected, chitosan and chitosan nanoparticles shows identical peak absorption as shown in Figure 4 In addition, figure 4 B, C and D shows typical absorption peaks for ciprofloxacin HCL, Gentamycin sulfate and Chlortetracycline HCl as shown (Agyare *et al.*, 2008; Trapani *et al.*, 2010; Tiyaboonchai, 2013). Chitosan nanoparticles loaded with ciprofloxacin HCL, Gentamycin sulfate and Chlortetracycline HCl showed different infrared spectra: for ciprofloxacin HCL (Lopez-Gresa *et al.*, 2002), Gentamycin sulfate (Sivakumar *et al.*, 2002; Leypold *et al.*, 2003; Sivakumar and Rao, 2003) and Chlortetracycline HCl (Friess and Schlapp, 2002)in addition to chitosan nanoparticles itself with proof that these antibiotics successfully loaded on chitosan nanoparticles as shown in figure 1 F, G and H.

Antibacterial Activity of chitosan nanoparticles and its loaded antibiotic

The zntibacterial properties of chitosan nanoparticles and its loaded antibiotic are evaluated against *Staphylococcus aureus (S. aureus)* as Gram-positive bacteria and *Escherichia coli (E. coli) as* gram-negative bacteria.

Figure 5 shows that the antibacterial activity of chitosan nanoparticles loaded with ciproflaxin, tetra chlorocycline and gentamycin sulfate are increased with increasing absorbed dose for both gram-positive and gram-negative bacterium. The inhibition zone of chitosan nanoparticles loaded Ciprofloxacin HCl of *S. aureus as* gram positive bacteria was enhanced from 6.5 mm to 22mm and against *E. coli* as gram negative bacteria from 5.5 mm to 21 which increase with increasing the concentration of antibiotic as shown in figures 5 and 6.

The inhibition zone of chitosan nanoparticles loaded with tetra chlorocyclin of *S. aureus as* gram positive bacteria was enhanced from 0 mm to 10 mm and 0 mm to 7 mm for *E. coli* as gram negative bacteria which increased with increasing the concentration of tetra chlorocycline.

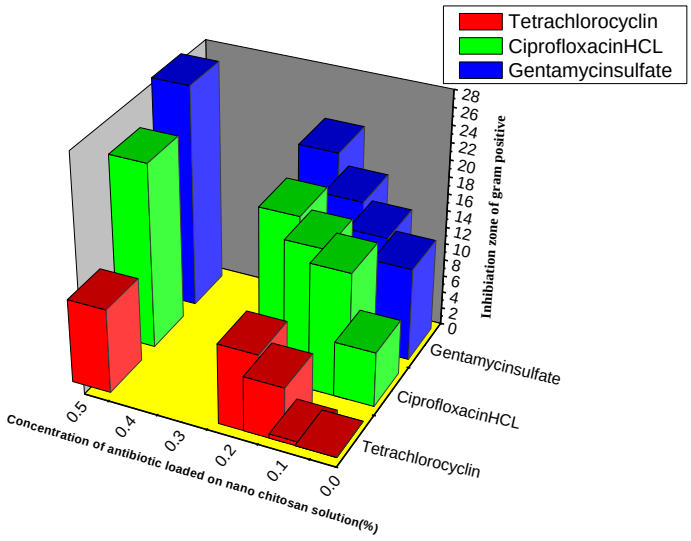


Fig. 5: Effect of Concentration of Antibiotic Loaded on Chitosan Nanoparticles against Inhibition Zone of S. aureus as Gram Positive bacteria.

As we mentioned before that Chitosan exhibits higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria, this is due to their different cell walls. But both figures 5 and 6 shows that chitosan nanoparticles shows higher antibacterial towards both Gram-positive and Gram-negative bacteria by similar inhibition zone that is due to chitosan nanoparticles are polycation with high surface charge density which interact with bacteria and tightly absorbed onto the surface of bacterial membrane to disrupt the membrane of both Gram-positive bacteria than Gram-negative bacteria, thus kill bacterial cells by the same ratio which overall due to nanoparticle structure (Avadi *et al.,* 2004; Abou-Zeid *et al.,* 2011).

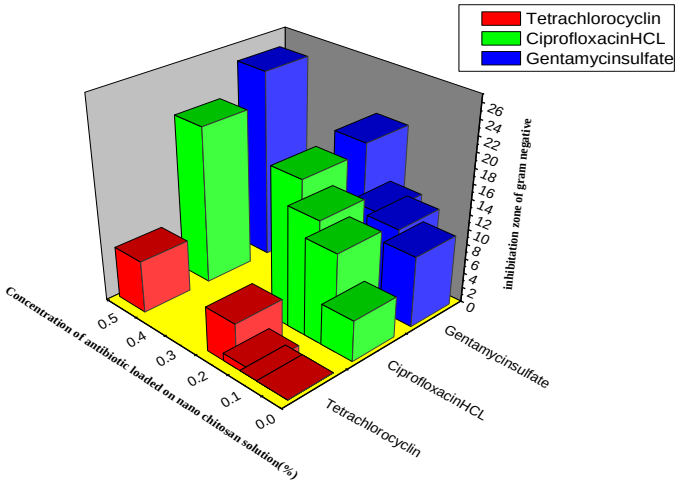


Fig. 6: Effect of Concentration of Antibiotic Loaded on Chitosan Nanoparticles against Inhibition Zone of *E. coli* as gram negative.

Also antibacterial activity of chitosan nanoparticles loaded Gentamycin sulphate increaded from 10 mm to 26 mm for *S. aureus as* gram positive bacteria and increased from 8.5 mm to 24 mm for E. coli as gram negative bacteria which increased with increasing the concentration of Gentamycin sulphate. Also antibacterial activity increased in the order chitosan nanoparticles loaded with Gentamycin sulphate then ciprofloxacin then tetra cycline at the same concentration which conclude that chitosan nanoparticles loaded Gentamycin sulphate is the best antibacterial material followed by loaded with ciprofloxacin followed by loaded by tetra cycline.

CONCLUSION

- Chitosan nanoparticles were prepared by using tripoly phosphate and characterized by using FT-IR and TEM.
- pH and ultrasonication time were kept constant at 5.5 and 45 min. respectively based on previous studies.
- The optimum conditions of preparation of chitosan nanoparticles are: chitosan concentration is 0.2 g/ml, TPP concentration is 0.1 g/ml, ultrasonication time is 45 min. and pH is 5.5.
- Chitosan nanoparticles were loaded with selected antibiotics (ciprofloxacin HCL, Chlortetracycline hydrochloride and Gentamycin sulfate) to magnify their benefits in biomedical applications.
- Antibacterial activity of chitosan nanoparticles loaded with antibiotics increase with increasing the antibiotic concentration.
- Chitosan nanoparticles exhibits higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria.
- Order of inhibition for three antbiotics are: Gentamycin sulfate > ciprofloxacin HCL > Chlortetracycline hydrochloride

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