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Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, *O*-carboxymethyl and *N*,*O*-carboxymethyl chitosan nanoparticles

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ABSTRACT

Chitosan (CS) is a naturally occurring biopolymer. It has important biological properties such as biocompatibility, antifungal and antibacterial activity, wound healing ability, anticancerous property, anticholesteremic properties, and immunoenhancing effect. Recently, CS nanoparticles have been used for biomedical applications. However, due to the limited solubility of CS in water its water-soluble derivatives are preferred for the above said applications. In this work, the nanoparticles of CS and its water-soluble derivatives such as *O*-carboxymethyl chitosan (*O*-CMC) and *N*,*O*-carboxymethyl chitosan (*N*,*O*-CMC) was synthesized and characterized. In addition, cytotoxicity and antibacterial activity of the prepared nanoparticles was also evaluated for biomedical applications.

1. Introduction

Chitin is the second most abundant polymer in nature after cellulose. The refuses of seafood industries, which are rich in chitin, and the shells of crustaceans are the raw materials for the production of this polymer. It is a linear homopolysaccharide composed of 2-acetamide-2-deoxy-D-glucopyranose units linked by β (1–4) bonds. Chitosan is the derivative of chitin, a copolymer of 2-amino-2-deoxy-D-glucopyranose and 2-acetamide-2-deoxy-D-glucopyranose units. It is present in the cell walls of some fungi, but the partial deacetylation of chitin is the main route used for its preparation in industry as well as in research laboratories (Fernando & Sérgio, 2004). Chitin and chitosan are biocompatible, biodegradable and nontoxic polymers. Because of these properties, it has many applications such as biomaterials for tissue engineering, in wound healing, as excipients for drug delivery (Elzatahry & Mohy, 2008; Kashappa & Hyun, 2005; Kofuji, Ito, Murata, & Kawashima, 2001; Thanou, Verhoef, & Junginger, 2001) and also in gene delivery (Gerrit, 2001; Huang, Khor, & Lim, 2004; Muzzarelli, 1988; Richardson, Kolbe, & Duncan, 1999). CS nanoparticles used for the delivery of polypeptides such as insulin, tetanus toxoid, and diphtheria toxoids are widely explored (Calvo, Remunan-Lopez, Vila-Jato, & Alonso, 1997, 1997a; Janes & Alonso, 2003; Vander et al., 2003; Xu & Du, 2003).

Chitosan is soluble only under acidic conditions, which limits some of its applications. The limited solubility of chitosan in water can be overcome by chemical modification (Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Reis, & Mano, 2005). Thus chemical modifications of chitin/chitosan are generally preferred to improve the polymer processability as well as to modify some of its properties such as solubility, antimicrobial activity and the ability to interact with other substances (Jayakumar & Tamura, 2006; Jayakumar, Nagahama, Furuike, & Tamura, 2007a; Singh et al., 2008). An important chemical modification method is carboxymethylation. Polymers with carboxylate functionality have been found to elicit a broad range of biological activities. Polymers carrying –NH–CH_2–COOH (as in *N*-carboxymethyl, *N*,*O*-carboxymethyl and *N*-succinyl chitosan) group have good chelating ability due to the presence of additional functional groups. For carboxymethyl chitin and chitosan, their original structure based on *N*-acetyl glucosamine enhances their performances and make them more biocompatible, hydrophilic, biodegradable and amenable to various physical forms than synthetic polymers (Muzzarelli, 1988). The important carboxymethyl derivatives are *O*-carboxymethyl chitosan (*O*-CMC), *N*,*O*-carboxymethyl chitosan (*N*,*O*-CMC), *N*-carboxymethyl chitosan (*N*-CMC) and *N*-succinyl chitosan. Nanoparticles of these carboxymethylated derivatives have been synthesized through simple chemical reactions for use in various applications such as in drug delivery (Shi, Du, Yang, Zhang, &

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Sun, 2005). In the present work, we are described and reporting about the synthesis, characterization, cytotoxicity and antibacterial studies of CS, *O*-CMC and *N,O*-CMC nanoparticles in detail.

2. Experimental

2.1. Materials

CS (Molecular weight 100–150 kDa, DA-80) and *O-O*MC (DDA 61.8% & DS-0.54) was purchased from Koyo chemical Co Ltd., Japan, sodium tripolyphosphate (TPP) and calcium chloride ($CaCl_2$) was purchased from Sigma Aldrich, isopropyl alcohol was purchased from Qualigens, chloroacetic acid from Nice chemicals, breast cancer cell lines (MCF-7) for cytotoxicity studies was purchased from NCCS Pune and ATCC strains of *staphylococcus aureus* (*S. aureus*) for antibacterial study was obtained from AIMS microbiology lab.

2.2. Preparation of CS nanoparticles

CS nanoparticles were obtained by ionic cross-linking of CS with TPP, which was described in literatures (Quan & Tao, 2007; Quan, Tao, Colette, & Paul, 2005; Li feng et al., 2004; Devika & Varsha, 2006; Shi et al., 2005). About 0.1% CS solution was prepared in 1% acetic acid and 1% TPP solution was prepared in water. Nanoparticles were spontaneously obtained by the addition of 1 ml of TPP aqueous solution into 10 ml CS solution under constant stirring at room temperature for 30 min. The prepared nanoparticles were separated by centrifugation at 10,000 rpm for 1 h, and then purified, dispersed in water and lyophilized.

2.3. Preparation of O-CMC nanoparticles

*O-*CMC nanoparticles were prepared by cross-linking of *O*-CMC solution with calcium chloride solution as described in literature (Shi et al., 2005). About 0.5% *O*-CMC solution was prepared in distilled water. To 5 ml of this solution, 1 ml of 1.5% $CaCl_2$ solution was added under constant stirring. The resulting nanoparticles were purified by centrifugation for 30 min at 10,000 rpm and lyophilized.

2.4. Preparation of N,O-CMC nanoparticles

N,O-CMC was prepared from CS based on the reported literature (Chen et al., 2004) using monochloroacetic acid as the carboxymethylating agent, in alkaline medium. *N,O*-CMC nanoparticles were synthesized from *N,O*-CMC by the ionic cross-linking with TPP as described in literature. Nanoparticles were spontaneously obtained by the addition of 1 ml of 0.25% TPP aqueous solution to 10 ml of 0.1% *N,O*-CMC solution under constant stirring at room temperature for 30 min. The prepared nanoparticles were separated by centrifugation for 1 h at 10,000 rpm and lyophilized.

2.5. Cell culture

Breast cancer cells (MCF-7) were used in this study. The cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in CO_2 incubator with 5% CO_2. After reaching confluency, the cells were detached from the flask with Trypsin–EDTA. The cell suspension was centrifuged at 3000 rpm for 3 min and then re-suspended in the growth medium for further studies.

2.6. Cytotoxicity studies

For cytotoxicity experiments, MCF-7 cells were seeded on a 96 well plate with a density of 10,000 cells/cm^2. MTT [3-(4,5-Dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium] assay was used to evaluate cytotoxicity of the prepared nanoparticles and this is a colorimetric test based the selective ability of viable cells to reduce the tetrazolium component of MTT into purple colored formazan crystals. Three different concentrations of the nanoparticles (0.4 mg/ml, 0.8 mg/ml and 1 mg/ml) were prepared by dilution with the media. After reaching 90% confluency, the cells were washed with PBS buffer and different concentration of the nanoparticles (100 μl) were added and incubated along with tissue cultured wells without any nanoparticle as negative control and wells treated with Triton X-100 as positive control for a period of 24 h. About 5 mg of MTT (Sigma) was dissolved in 1 ml of PBS and filter sterilized. About 10 μl of the MTT solution was further diluted to 100 μl with 90 μl of serum–free phenol red free minimum essential medium. The cells were incubated with 100 μl of the above solution for 4 h to form formazan crystals by mitochondrial dehydrogenases. About 100 μl of the solubilization solution (10% Triton X-100, 0.1 N HCl and isopropanol) was added in each well and incubated at room temperature for 1 h to dissolve the formazan crystals. The optical density of the solution was measured at a wavelength of 570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment.

2.7. Antibacterial studies

Antibacterial studies of the prepared nanoparticles of CS, *O*-CMC and *N,O*-CMC were done with ATCC strains of *S. aureus* (ATCC 25923) using minimum inhibitory concentration method (MIC). The least concentration of the sample, which can be used (MIC) for these studies, for CS and *N,O*-CMC 5 ml of 1 mg/ml sample and for *O*-CMC it was 5 ml of 5 mg/ml sample. In this study, the overnight culture of *S. aureus* was taken and turbidity was adjusted to 0.5 McFarland standards. From this 10 μl of this culture was incubated in a nutrient broth (10 ml) along with three different concentrations (5 ml, 8 ml and 10 ml of 1 mg/ml) of each of these three nanoparticles and kept in a water bath shaker at 37 °C for over night. After overnight incubation these samples were serially diluted with 1 mM magnesium sulphate solution up to the 6th dilution and plated on a Nutrient Agar (NA), *S. aureus* without any nanoparticle plated as control. Plates were kept in an incubator at 37 °C for 12 h. After 12 h incubation these plates were observed for colony counting. Based on the number of colonies of control and samples we calculated the antibacterial activity of these nanoparticles.

2.8. Characterizations

FT-IR spectra of CS, *O*-CMC and *N,O*-CMC and their corresponding nanoparticles were recorded on Perkin Elmer Spectrum RX1 Fourier transform infrared spectrophotometer using KBr method. The mean size and size distribution for the prepared nanoparticles were determined by dynamic light scattering (DLS-ZP /Particle Sizer Nicomp™ 380 ZLS) measurements. Size of the particles further confirmed by SEM (JEOLJSM-6490LA) and AFM (JEOL JSPM-5200). Surface charge and thereby the stability of the three nanoparticles system prepared was obtained by zeta potential measurements (DLS- ZP /Particle Sizer Nicomp™ 380 ZLS). MTT assay for cytotoxicity studies was done using a micro plate reader (BioTek Power Wave XS).

3. Results and discussions

3.1. Preparation of CS, **O**-CMC and **N,O**-CMC nanoparticles

Nanoparticles of CS, *O*-CMC and *N,O*-CMC were synthesized by chemical cross-linking with TPP and $CaCl_2$. CS nanoparticles were

obtained as a result of the ionic cross-linking between the positively charged protonated amine of chitosan and negatively charged phosphate groups of TPP. By adjusting the concentration of the precursors and stirring speed, the degree of cross-linking and thus the particle size can be controlled. *N,O*-CMC nanoparticles were formed on the same mechanism, but here the protonation of amino groups took place at the unsubstituted amine since most of them are carboxymethylated. The *O*-CMC nanoparticles were obtained by reaction between carboxyl groups of *O*-CMC with Ca^{2+} ions of cross-linker.

3.2. Particles size analysis

Using dynamic light scattering (DLS) measurements, the size distributions for the three different nanoparticles were obtained. Fig. 1 shows the particle size distribution for CS, *O*-CMC and *N,O*-CMC nanoparticles. The average diameters for the three nanoparticles were found to be 40–50, 90–100 and 80–85 nm, respectively.

3.3. SEM and AFM analysis

The size of the prepared nanoparticles were again confirmed by SEM and AFM. Fig. 2 represents the SEM images of CS, *O*-CMC and *N,O*-CMC which showed a size of 120–140, 140–155 and 110–130 nm, respectively. Fig. 3 represents the AFM images of CS, *O*-CMC and *N,O*-CMC which showed a size of 160–170, 80–90 and 110–130 nm, respectively.

3.4. Zetapotential measurements

Surface charge and thereby the stability of the prepared nanoparticle systems were determined by zeta potential measurements. Zetapotential values for the CS, *O*-CMC and *N,O*-CMC nanoparticle systems were found to be +54.2 mV, −35.12 mV and +46.74 mV, respectively. All these values lie in the stable range, indicating that these nanoparticle systems prepared were stable. These results also indicated that CS and *N,O*-CMC nanoparticles possess positive surface charge and *O*-CMC nanoparticles possess negative surface charge.

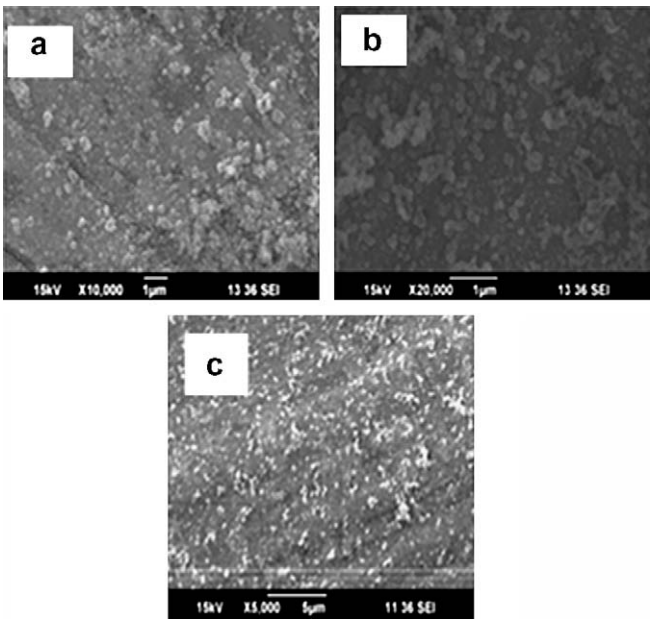

Fig. 2. SEM image of (a) CS, (b) *O*-CMC and (c) *N,O*-CMC nanoparticles.

3.5. FT-IR studies

Fig. 4 shows the FT-IR spectrum of CS, *O*-CMC and *N,O*-CMC and their corresponding nanoparticles. In the infrared spectrum of chitosan, a characteristic peak at 3428 cm^{-1} appeared and this can be attributed to the –NH2 and –OH groups stretching vibration, and a peak at 1648 cm^{-1} for the amide I, but in the chitosan nanoparticles a peak disappeared at 1648 cm^{-1} and a new peak

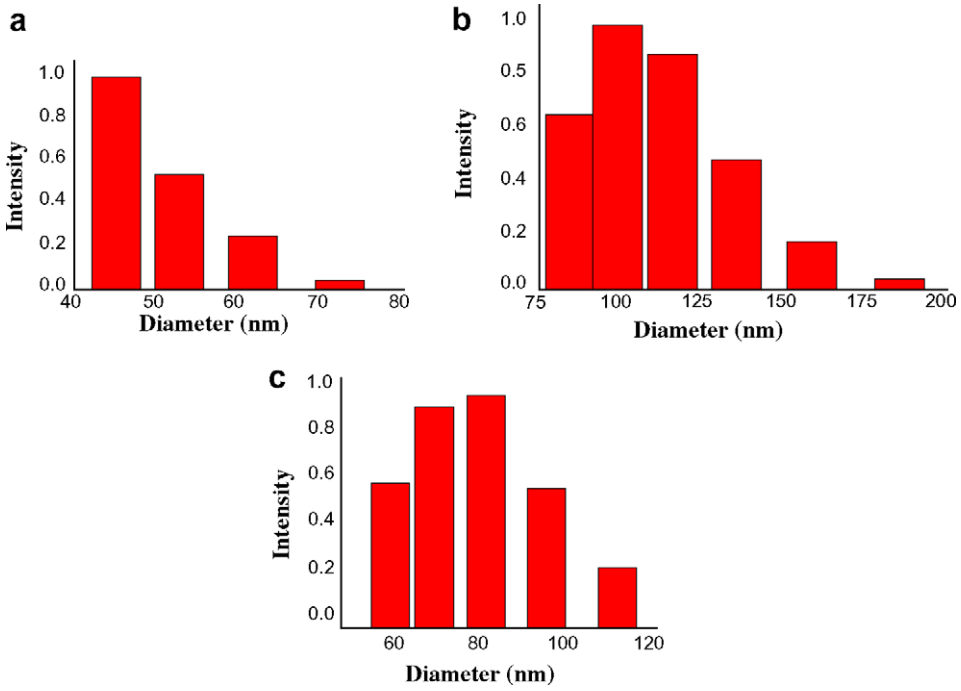

Fig. 1. Particle size distribution of (a) CS (b) *O*-CMC and (c) *N,O*-CMC nanoparticles.

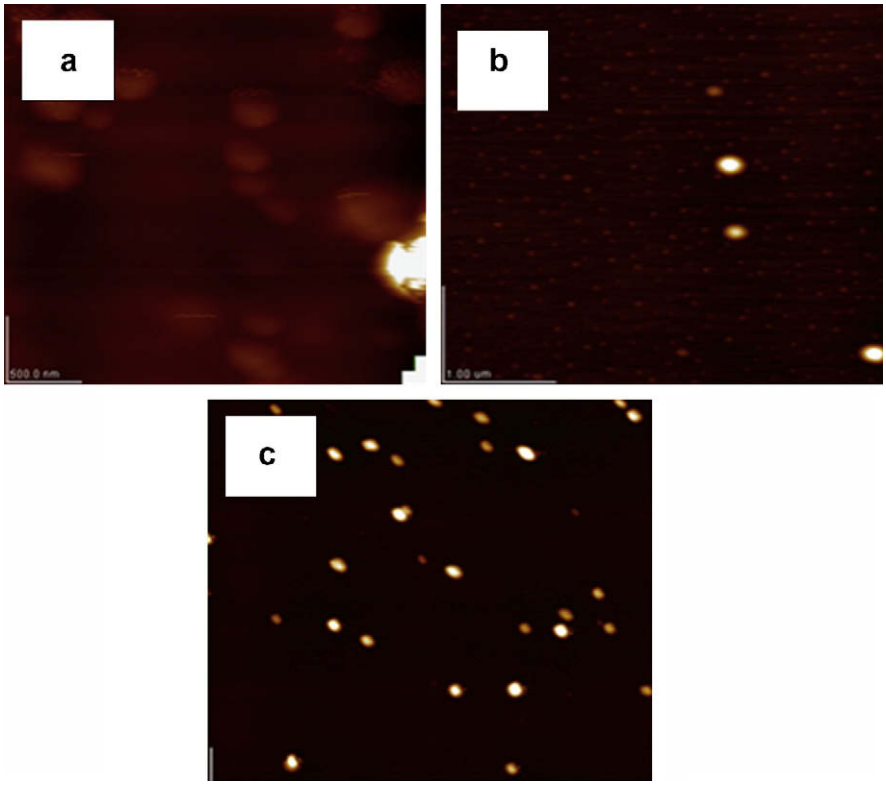

Fig. 3. AFM image of (a) CS, (b) *O*-CMC and (c) *N,O*-CMC nanoparticles.

appeared at 1641 cm^{-1}. The disappearance of the peak at 1648 cm^{-1} and the appearance of the new peak at 1641 cm^{-1} indicating that the linkage between the phosphate groups of TPP and ammonium ions of chitosan (Devika & Varsha, 2006). In the *N,O*-CMC spectrum, a peak at 1630 cm^{-1} is appeared and this can be attributed to the amine stretching, where as in the nanoparticle this peak disappeared and a new peak appeared at 1637 cm^{-1}.The disappearance of peak at 1630 cm^{-1} and the appearance of a new peak at 1637 cm^{-1} in the nanoparticle indicated the linkage between the phosphate groups of TPP and ammonium ions of *N,O*-CMC. In *O*-CMC, a strong peak at 1320 cm^{-1} appears and this can be attributed to the C–O stretching of *O*-CMC and this peak become less intense in *O*-CMC nanoparticle, this also indicate the cross-linking reaction between carboxyl groups of *O*-CMC and Ca^{2+} ions of cross-linker.

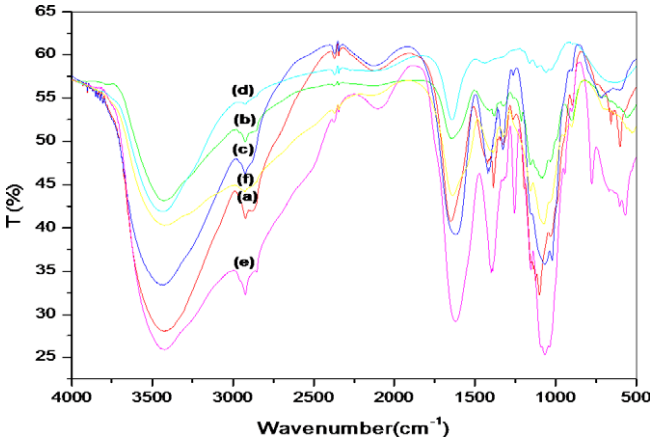

Fig. 4. FT-IR spectra of (a) CS control, (b) CS nanoparticles (c) *O*-CMC control (d) *O*-CMC nanoparticles (e) *N,O*-CMC control and (f) *N,O*-CMC nanoparticles.

3.6. Cytotoxicity studies

Cytotoxicity of the three different nanoparticles (CS, *O*-CMC and *N,O*-CMC) prepared was determined by MTT asaay, which is a colorimetric test based the selective ability of viable cells to reduce the tetrazolium component of MTT into purple colored formazan crystals. MTT assay was done for the three different concentrations viz; 0.4 mg/ml, 0.8 mg/ml and 1 mg/ml. Triton X-100 was taken as the positive control for cytotoxicity. Here in our study there is no significant difference in cytotoxicity in any of the concentrations of the three different nanoparticles studied (Fig. 5). Similarly, we also compared the level of toxicity of nanoparticles with the negative control, which is the normal tissue cultured wells. It is evident from the result that compared to the negative control almost 98% cells are viable in all the different concentrations of CS, *O*-CMC and *N,O*-CMC nanoparticles. These results indicated that the prepared nanoparticles are less toxic to breast cancer cells.

3.7. Antibacterial studies

Antibacterial activities of the CS, *O*-CMC and *N,O*-CMC nanoparticles prepared were determined by MIC method with *S. aureus* strain as explained earlier. Three different concentrations of each of these three nanoparticles were incubated in a nutrient broth (10 ml) containing *S. aureus* along with a control (without any nanoparticle) and kept in a water bath shaker for overnight. After overnight shaking, these samples were serially diluted with 1 mM $MgSO_4$ solution up to the 6th dilution and plated on a nutrient agar and again kept in a bacterial incubator for overnight. After overnight incubation these plates were observed for colony counting. Based on the number of colonies of sample and control we can calculate antibacterial activity for the three concentrations of each of the three nanoparticles.

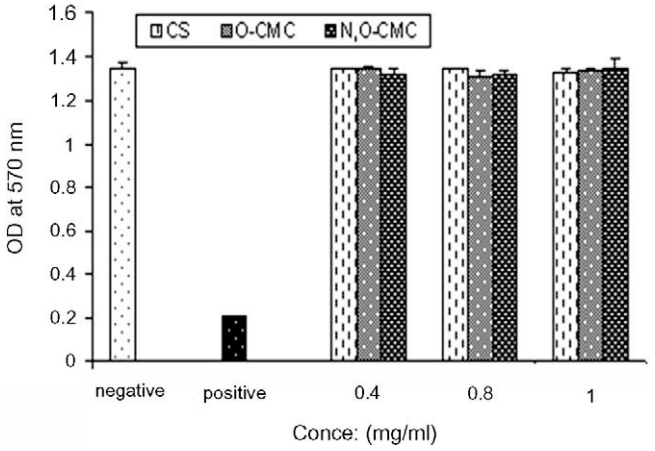

Fig. 5. MTT assay of the prepared nanoparticles.

Fig. 6 shows the antibacterial activity (which is expressed in terms of decrease in number of colonies) of CS, *O*-CMC and *N,O*-CMC nanoparticles with different concentrations. As the volume of the nanoparticles increases, number of colonies was also found to be decreasing. This antibacterial effect was higher in *N,O*-CMC and there was no colony found for the last concentration. For CS and *O*-CMC this effect is less compared to *N,O*-CMC. This effect is due to the higher degree of substitution of carboxymethyl groups in *N,O*-CMC compared to *O*-CMC, increased the antibacterial activity (Sun, Du, Fan, Chen, & Yang, 2006).

4. Conclusions

Nanoparticles of CS, *O*-CMC and *N,O*-CMC were synthesized by simple ionic cross-linking using TPP and $CaCl_2$.The prepared nanoparticles were characterized by DLS, SEM, AFM and FT-IR spectroscopy. Cytotoxicity of the prepared nanoparticles was also determined by MTT assay. MTT assay showed that the prepared nanoparticles showed less toxicity to breast cancer cells (almost 98% viability was found for breast cancer cells-MCF-7, which was treated with the nanoparticle samples). Antibacterial activities of these CS, *O*-CMC and *N,O*-CMC nanoparticles were also studied with ATCC strains of *S. aureus* by MIC method using three different concentrations of each of these three nanoparticles. These studies revealed that CS nanoparticles showed less antibacterial activity compared to *O*-CMC and *N, O*-CMC nanoparticles. This antibacterial effect was increased with increase in concentration, *N,O*-CMC nanoparticles showed maximum antibacterial activity out of the three and no colonies were found for the last concentration with respect to the control, for *O*-CMC and CS nanoparticles, the less number of colonies were found with respect to the control for the last concentration.

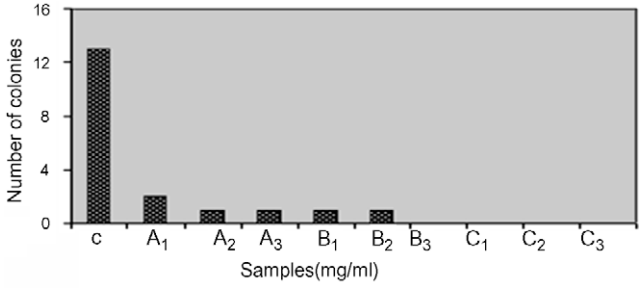

Fig. 6. Antibacterial activity of the prepared nanoparticles towards *Staphylococcus aureus* strain. [C-*S. aureus* (control), A_1, A_2, A_3– 5, 8 and 10 ml of CS (1 mg/ml), B_1, B_2, B_3–5, 8 and 10 ml of *O*-CMC (5 mg/ml) and C_1, C_2, C_3–5, 8 and 10 ml of *N,O*-CMC (1 mg/ml)].

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