**A novel Ag/Carrageenan–gelatin hybrid hydrogel nanocomposite and its biological applications: Preparation and characterization**

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**ABSTRACT**

A novel biohybrid hydrogel nanocomposite made of natural polymer carrageenan and gelatin protein were developed. The silver nanoparticles were prepared using the carrageenan polymer as reduction and capping agent. Here, the Ag/Carrageenan was combined with gelatin hydrogel using glutaraldehyde having a cross-link role in order to create the biohybrid hydrogel nanocomposite. The manufactured composite performances were anaylised by UV-visible spectroscopy, Fourier Transform infrared (FTIR) spectroscopy, Scanning Electron Microscopy (SEM), Energy dispersive X-ray (EDX) spectroscopy and Transmission Electron Microscopy (TEM) methods. The swelling behaviour of the Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite was also analyzed. The antibacterial activity was tested against human pathogens viz. *S.agalactiae 1661, S. pyogenes 1210* and *E. coli*. The bacterial cell wall damage of *S.agalactiae 1661* was analyzed by scanning electron microscopy. The cytotoxic assay was performed against the A549 lung cancer cells.

**Keywords:** Biocompatibility, Carrageenan, Polysaccharide, Capping agent, Hydrogel

## 1. Introduction

Currently, the biomaterials are involved quite often in therapeutic and diagnostic applications. Hence, the biomaterials should possess compatible interactions with components of living systems (Sanjay et al., 2018; Wu et al., 2015; Williams., 2009). Biomaterials derived from seaweed possess good biocompatibility, biodegradability, high visco-elasticity and non-toxicity characteristics that enable cell proliferation and metabolic activities (Khan, F. and Tanaka, M., 2018; Venkatesan et al., 2015; Vinod et al., 2020). They are assumed to be inert, not interfering

with biological systems and should be friendly with the human body. The natural seaweed containing polymers based are more attractive compared to different compounds because high biocompatibility, rich in water, and have promising functions in most biomedical use (i.e. tissue engineering, drug delivery and so on) (Ige et al., 2012; Venkatesan et al., 2016). There, to improve the biomaterials properties used in scaffolds for tissue engineering it was proposed a combination between design and association of natural polymers from seaweed. It can produce tissues which replicate the physical properties of implanted location (Burg et al., 2000; Katti., 2004). The seaweeds are marine micro algae, which benefits of high bioactive secondary metabolites properties favorable for development of novel pharmaceutical agents (Ishitsuka et al., 1979).

Red algae are assumed to be a reach source of numerous biologically active-metabolites comparing to other algal classes (El Gamal., 2010; Maruthupandian et al., 2015). Phycocolloids (i.e. agar agar, alginic acid and carrageenan) are the basic constituents of brown and red algal cell walls, widespread employed in many industry. Carrageenan represents a sulphated polygalactan containing 15 to 40% ester-sulphate with an average relative molecular mass over 100 kDa. It is generally produced by alternating some units of D-galactose and 3,6-anhydro-galactose (3,6-AG) that are joined by α-1,3 and β-1,4-glycosidic linkage (Necas, and Bartosikova., 2013). The carrageenan sulphated polysaccharide chains are cross-linked with functional protein molecules present in the gelatin which create a unique material. In view of their biodegradability, biocompatibility and antimicrobial properties, seaweed containing polymers are attractive for hydrogel preparation and explored in biomedical applications. The drug formulation can be improved by the gelling nature of carrageenan and also by the use of filler materials in the hydrogel (Kalsoom Khan et al., 2017). Modified hydrogel with polymeric

nanoparticles can exhibit superior biological and swelling properties, improved immune modulatory effects, antitumor and anticoagulant activities (Zhao et al., 2015). Wei Long Ng et al. 2016 developed polyelectrolyte hybrid hydrogel from chitosan-gelatin material. They showed that the prepared materials possess unique ability when used as bioprinted material for tissue engineering applications. The hydrogels together with 2 D and 3D materials can play a major role in tissue engineering. It is because the hydrogels is formed from naturally native extracellular matrices (ECM) which provide a well hydrated porous micro environment. In fact their porous and micro-environment nature of hydrogels enables a nutrient supply smoothly. Collagen, gelatin, gelatin methacrylate, alginate, fibrin, hylauronicacid, polyethylene hydrogel based on polymeric materials were recently used for rapid prototyping of hydrogel preparations. (Wei Long Ng et al. 2020). Some filler materials are embedded into hydrogel scaffold and used for cell proliferation and tissue engineering applications. They helps to improve the mechanical properties, sweeling ratio and cell stiffness. Recently, 3D printing techniques i.e. laser based-3D printing, nozzle based-3D printing and inkjet printer based-3D printing systems were noted as very attractive towards preparations of novel hydrogel materials with unique scaffold charcteritics that are relevant for a wide range of applications (Tae-Sik Jang 2017). Further, the solvent based extrusion 3D printing technique was used for preparation of different kinds of biocomposites, including gelatin-alginate bioprinted gel that typically is used for the replacement of human adipose-derived mesenchymal stem cells (ASCs) because of its fast reacting charcteristic (Krishna C. R. Kolan 2019).

Among various metal nanoparticles, silver nanoparticles may posses great properties for drug delivery (Kumar, and Yadav, 2009), wound healing (Cho et al., 2005), sensors, textile industry, cosmetics (Kumar et al., 2008). They also have superior antimicrobial performances which make

them favorable for medical science especially for food pathogens (Li et al., 2009). The bio applications of polysaccharide nanoparticles with bio hybrid hydrogel showed novel functionalities in oral drug delivery systems (Li et al., 2014). To overcome the toxicity, side effects, lower bioavailability within drug delivery system, currently the natural polymers are coated with magnetic nanoparticles and were introduced as a carrier for drug delivery (Shanmuga et al., 2015). Hydrogels are considered soft materials with origins either synthetic and/or natural used in biomedical applications (i.e. tissue engineering, regenerative medicine, and drug delivery) (Biondi et al., 2008) having comparable physical, chemical, and biological properties with natural biological tissues (Fisher et al., 2010).

In the present paper, to further improve the state of art, we been focused to evaluate the carrageenan polymer extracted from red seaweed (*Acanthophora spicifera)* for its biological application and to create a procedure which can be used to prepare the hydrogel for wound healing study. It was employed as agent reduction in order to synthesise the silver nanoparticles, which further were incorporated into gelatin yielding a new hybrid hydogel nanocomposite. The bio-nanocomposite has been evaluated using UV-visible, FTIR, EDX, SEM and TEM methods. The swelling behaviour of Ag/Carrageenan-gelatin hybrid hydrogel has been analysed. The prepared hydrogel has been tested against the human pathogens and the anticancer activity of hydrogel has been tested against A549 lung cancer cell lines. Further the suitability of the hybrid hydogel nanocomposite in drug delivery application has been demonstrated. The encouraging results obtained in this study enable the use of AgNP synthesize from natural materials as a robust platform for wound healing study and some other critical biomedical applications.

## 2. Experimental

### 2.1. Material collection

The red seaweed *Acanthophora spicifera* was collected from near the Mandabam area, Ramanathapuram district, Tamil Nadu, India. Silver nitrate, gelatin and glutaradehyde were procured from Sigma-Aldrich, USA. The Mueller-Hinton agar, Todd-Hewitt broth, yeast extract and LB broth were procured from Hi-Media, India. The human pathogens *S.agalactiae1661* and *S. pyogenes 1210* used for antibacterial studies were obtained from Apollo Hospital, Chennai, India. The *E. coli* culture was obtained from Microbiology Laboratory, School of Biology, Madurai Kamaraj University, India. A549 cells (non-small human lung cancer) was acquired from the National Centre for Cell Sciences (Pune, India) and Dulbecco's modified Eagle's medium-DMEM, (Gibco), Fetal bovine serum 10% (FBS; Gibco) and 1% antibiotic/antimycotic solution were procured from Hi-Media, India.

### 2.2. Carrageenan extraction

The extraction of carrageenan polymer from the seaweed was carried out as previously reported (Sebaaly et al., 2012.). The seaweed was thoroughly washed with water in order to eliminate the potential impurities (i.e. salt, sand) and further rinsed with distilled water. About 50 g of the fresh seaweeds were heated in 500 ml of water. It showed an alkaline pH (8-9) (0.5 M $NaHCO_3$) during submission to water bath at $90^0$ C for 3 h. The formed mixture were filtered in order to eliminate the insoluble residues and viscous solution that may contain carrageenan. The filtered solution was precipitated by using excess alcohol. The obtained carrageenan was rinsed in ethanol, following by dry at room temperature for 24 h, further grinded into fine powder. This process yielded about 2.5 g of carrageenan powder.

### 2.3. Synthesis of carrageenan polymer capped silver nanoparticles

About 0.5 mg of the extracted carrageenan powder were homogeneuous dispersed in 15 mL water and it was incubated for 2 h at 42.5$^{o}$C. Then 15 mL of one mM $AgNO_3$ (silver nitrate) were added and then heated in a chamber at 37$^{o}$ C for 24 h in dark condition for the generation of silver nanoparticles (Ibrahee et al., 2016).

### 2.4. Synthesis of bio-adaptive Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

The gelatin stock solution were manufactured by dissolving about 1 g of gelatin granules within 10 mL of distilled water having a temperature of 50ºC, while were used a constant stirring of 600 rpm for 30 min. About 0.1 g of Ag/ carrageenan were introduced in 10 mL of distilled water which were mixed at 40ºC with constant stirring at 700 rpm for about 30 min. A 2.1 mL of cross-linker were prepared by mixing 1mL of glutaraldehyde, 1 mL of ethanol and 0.1 mL of HCl (0.01N). The gelatin and Ag/Carrageenan mixtures were blended at 200 rpm on the magnetic stirrer at 45 ºC for 20 min. with the addition of the cross-linker (Khade et al., 2014). For control experiment, a hydrogel was prepared with the addition of carrageenan, instead of Ag/Carrageenan.

### 2.5. Characterization of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

The biosynthesis of Ag nanopaticles from carrageenan was identified using an UV-visible spectral analysis having a spectrophotometer (JASCO V 630) which work within the wavelength range from 300 nm to 800 nm. Fourier transform infrared spectral (FTIR) analysis was performed under a SHIMADZU -8400S FTIR spectrophotometer to detect the biomolecules

responsible for the reduction of the Ag+ ions as well as the capping of the Ag nanoparticles synthesised by carrageenan extraction was analyzed. The main spectrum was recorded within the range of 400-4000 $cm^{-1}$. The surface morphology and elemental composition of Ag/Carrageenan-gelatin hydrogel samples were examined by scanning electron microscopy (SEM) (JEOL Model JSM - 6390LV) and Energy Dispersive X-ray (EDX) (OXFORD XMX N) methods. The particle distribution of Ag nanoparticles in the sample was examined by High Resolution Transmission Electron Microscope (HRTEM) (Jeol/JEM 2100) and the particle size were measured using a 'smart TIFF' software.

**2.6. Swelling studies**

The swelling ratio (Q) was obtained by immersing the Ag/Carrageenan hybrid hydrogel nanocomposite in distilled water and using 0.1 M NaCl at room temperature for about 5 h. At each interval of 1 h the gel was withdrawn and weighed after removing excess wetness from the surface of gel by using tissue paper (Azizi et al., 2017). The swelling ratio were determined using Eq. 1

$$\text{Swelling ratio (\%)} = \{(W_f - W_i/W_i)\} \times 100 \quad (1)$$

Here, $W_i$ =initial gel weight and $W_f$ = swollen gel weight at each interval.

**2.7. Assessment of antimicrobial activity**

The antibacterial activity of synthesized Ag Carrageenan hybrid hydrogel nanocomposite against human pathogens *S.agalactiae 1661, S. pyogenes 1210* and *E. coli*. were evaluated using a disc diffusion procedure. The Mueller- Hinton agar were amended with 5% sheep blood with and without (MHA) ([g/L]: Beef extract-3, Casein acid hydrolysate - 17.5, Starch- 1.5 and Agar- 17).

The selected standard strains were inoculated in a 5 ml of sterile Todd-Hewitt broth with 1% yeast extract and LB broth, respectively, which were incubated to 37°C during the overnight. The produced cultures were swabbed with sterile cotton buds on Mueller-Hinton agar medium with 5% sheep blood for *S. agalactiae 1661, S. pyogenes 1210* and without blood for *E. coli*. The Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite, Ag nanopaticless, carrageenan-gelatin hydrogel were placed separately on the pathogen spread medium. The amphotericin B (drug) loaded hydrogel and tetracycline (antibiotic) was used as a positive test refernce while sterile distilled water were considered as negative reference. The experimental plates were incubated for 24 h at 37 °C. Following the incubation period the studied zone of clearance were evaluated by a meter ruler. At least three experiments were performed to obtain reproducible results.

**2.8. Examination of bacterial cell wall damage by SEM analysis**

1 mL of Todd-Hewit broth with 1% yeast extract amended medium was taken in two eppendorf tubes. *S. agalactiae* fresh 1-5 colonies were inoculated at 37 °C within an incubation chamber. Subsequently, the Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite was added to one tube (test) and the other was kept for control experiment. After 24 hours incubation it was detached from the medium carefully which were than rinsed for the bacterial cells by sterile phosphate buffer saline. The bacterial cells were fixed into the glass slide and allowed to air dry and then frozen by liquid nitrogen followed by plasma coating. The bacterial cell wall damage were analysed under a scanning electron microscopic images (JEOL Model JSM - 6390LV) and the results were compared with treated (test) and untreated bacterial cells (control).

**2.9. MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

Non-small lung Cancer Cells A459 were keept within Dulbecco's Modified Eagle (DME) Medium. Cell suspensions were distributed in specifc 96 well plates that later was preserved at 37 $^{o}$C in wet atmosphere (5% carbon dioxide) for 24 h. After 24 h the cell suspensions were washed with trypsin and 10 µL of MTT dye were incorporated, coumpound that were preserved within a $CO_2$ incubator for 2 to 24 h. A549 cancer cells was cultivated in DME medium. The medium used contains 10% foetal bovine serum (FBS) enriched by 50 units/mL of penicillin and 50 µg /mL of streptomycin. Here, the examination were conducted under a MTT to detect the toxicity accordingly to standard procedures. Briefly, the cells was cultivated within a 96-well plate during overnight. The samples were incubated for 48 h with Ag/Carrageenan hybrid hydrogel nanocomposite (dissolved in sterile hot water) in escalating concentrations of (5, 10, 25, 50, 75 and 100 µg/ml) and the produced cells which were wahed with phosphate buffered saline was preserved by using 100 µL of 0.5 mg/mL MTT (Sigma-Aldrich, USA) at 37°C. As successive incubation for 30 min allowed to form the dark blue crystals of formazan (MTT metabolites) which was dissolved using 100 µL of DMSO and later preserved at 37 °C for a duration of 30 min. Normally, the process of cell proliferation is considred effective when the mitochondrial succinate-tetrazoliumreductase system transforms 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a compound having a blue colored formazan. The sample spectrophotometric absorbance were evaluated under a microplate (ELISA) reader. It is well known that the wavelength which validate the absorbance performnaces of the formazan product being 570 nm. In order to obtain reproducible results, there were replicate the experiments at least three times using the same protocol which was followed until the completion

of the experiment. The growth rate of inhibition of A549 cells by Ag/Carrageenan-gelatin hydrogel nanocomposite were determined by applying (Eq.2)

The rate of inhibition (%) = (1- mean OD value of treated group/mean OD value of control group) x 100. (2)

## 3. Results and Discussion

### 3.1. Nature of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

The Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite were manufactured using a simple two-step routine described in the experimental section. The prepared hybrid hydrogel nanocomposite appeared in a gel form initially with pale yellow in colour and finally turned from transparent yellow to brown colour which may be due to the plasmonic effects of Ag nanoparticles present in the hydrogel (Marques et al., 2019). Fig. 1 shows the prepared Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite patterns in comparison to that of carrageenan-gelatin hydrogel (control) one.

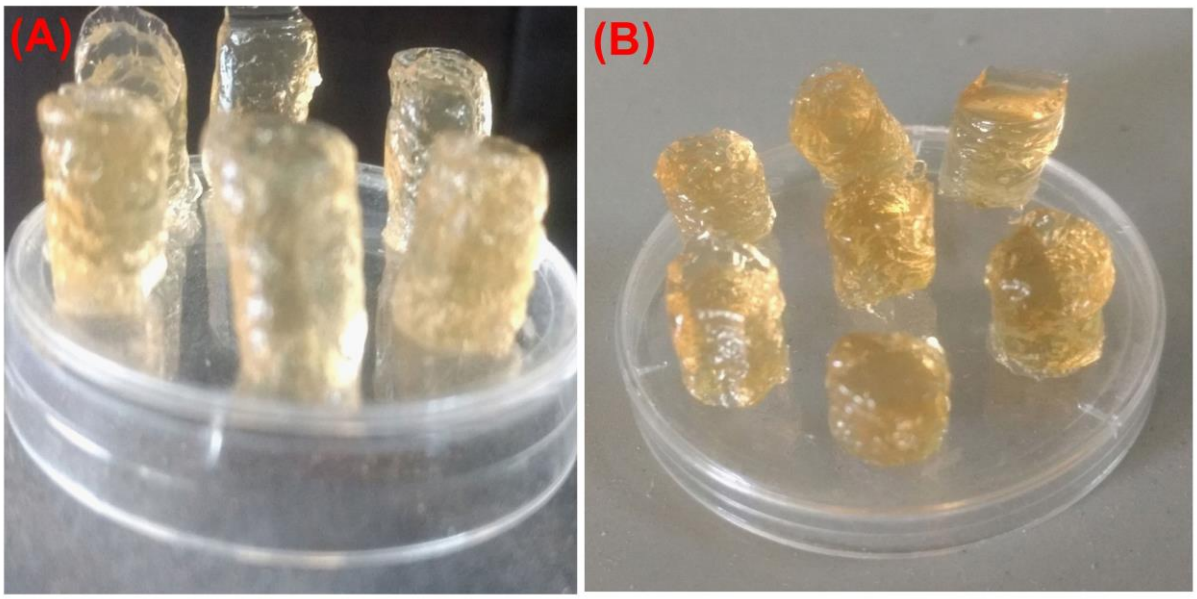

**Fig. 1.** As-prepared (A) Carrageenan-gelatin hybrid hydrogel and (B) Ag/Carrageenan–gelatin hybrid hydrogel nanocomposite

### 3.2. Analysis of UV-visible absorption

UV spectrophotometric analysis allows to detect Ag nanoparticles presence on the hybrid. The absorption spectrum of Ag/Carrageenan solution (Fig. 2) indicated details of surface plasmon resonance peak around at 429 nm endorsing the presence of Ag nanoparticles in the carrageenan matrix (Rhim, and Wang., 2014; Pandey et al., 2013). In contrast, the carrageenan solution shows no visible peak in the range between 300 - 750 nm.

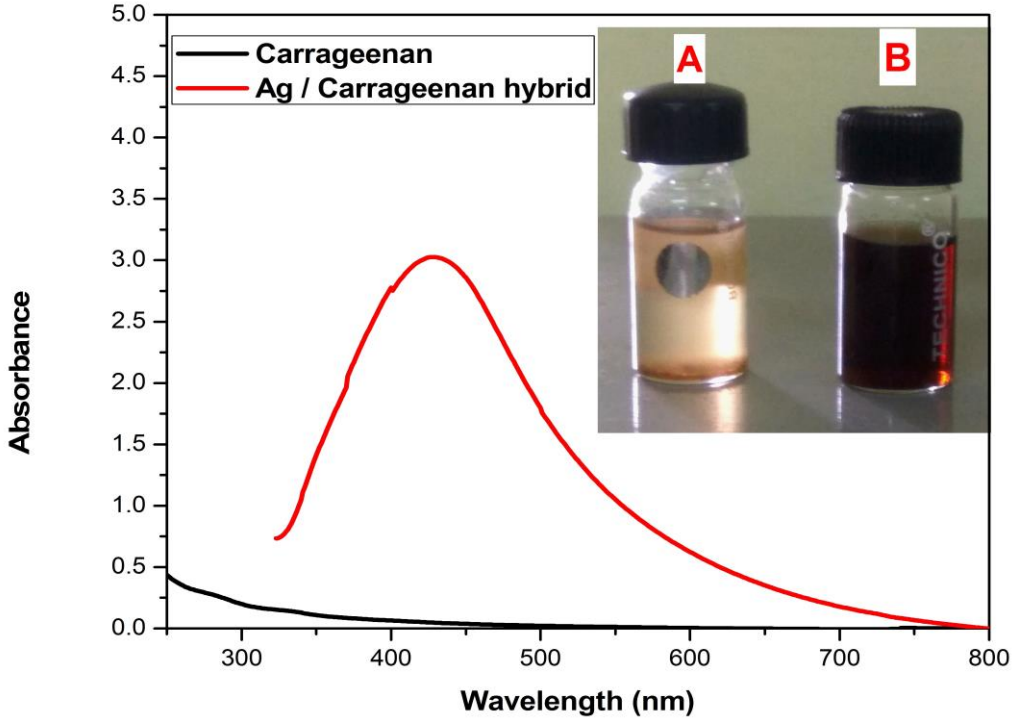


**Fig. 2.** Absorption spectra of Carrageenan (control) and Ag/Carrageenan hybrid. Inset shows dispersions of (A) Carrageenan and (B) Ag/Carrageenan hybrid.

### 3.3. SEM observation of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

The SEM image of the hybrid hydrogel nanocomposite has been shown in Fig. 3A. The arrow marks indicate the presence of spherical Ag nanoparticles (white spots) dispersed over the carrageenan-gelatin matrix which appears to be a spongy, highly interconnected porous network. The cross-linking between the carrageenan and gelatin has triggreded the formation of a porous hydrogel and the interconnected pores can be expected to be beneficial for drug delivery application (Varghese et al., 2014). The EDX spectrum in Fig. 3B shows a typically strong signal for Ag at 3keV confirming the incorporation of Ag nanoparticles in the hydrogel matrix.

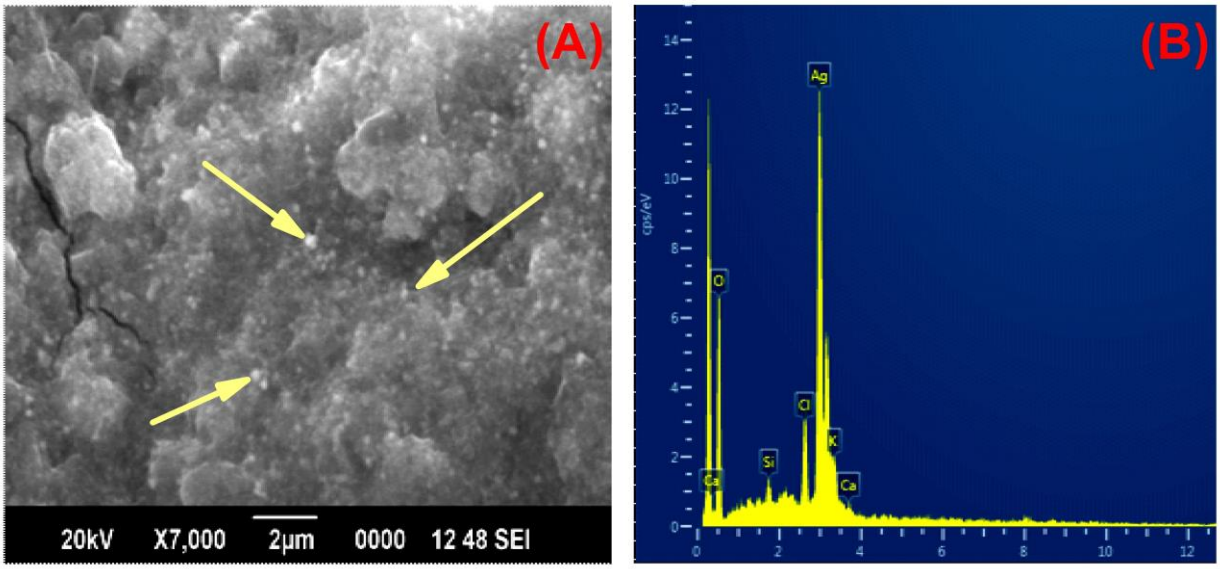


**Fig. 3.** (A) SEM morphology and (B) EDX spectrum of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite.

### 3.4. TEM studies

The Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite has been subjected to TEM characterization to get insight about Ag nanoparticles geometry and its size distribution profile as shown in Fig. 4A and 4B, respectively. The results indicate that the Ag nanoparticles in the

hybrid hydrogel nanocomposite are mostly spherical and polydispersed. The particle size profile shows that Ag nanoparticles vary between 2 to 28 nm and have a nominal diameter of about 12 nm. The SAED pattern (Fig. 4C) corresponds to a polycrystalline Ag with a face-centred cubic structure (Jyoti et al., 2016).

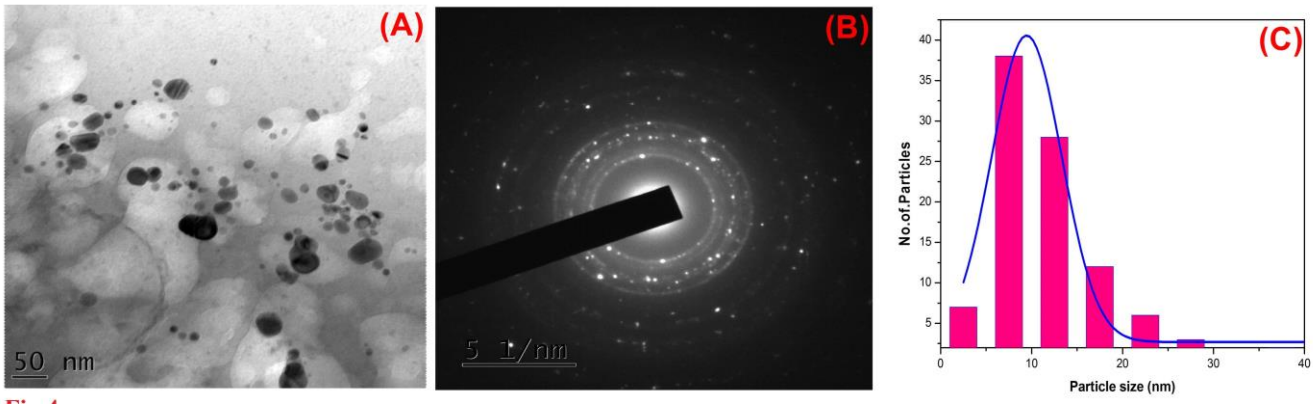


**Fig. 4. (A) TEM morphology (B) SAED pattern details and (C) Particle size historgram of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite.**

### 3.5. FTIR analysis of Ag/ Carrageenan hybrid hydrogel nanocomposite

Fig. 5 shows the FT-IR spectrum of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite in comparison to that of carrageenan-gelatin hydrogel. The two spectra are almost similar with superimposing bands at 2360, 1450, 1400, 1194, 1123, 1101, 753, 656 and 601 $cm^{-1}$. The 2360 $cm^{-1}$ band coresponds to interlayer of C-H stretching within *k*-carrageenan (Elsupikhe et al., 2015). The weak band detected at about 1450 $cm^{-1}$ coresponds to sulfate stretching vibration. We suspect that the one at 1194 $cm^{-1}$ is caused by the C=O in the ester sulfate group. Than, we noted a strong band formed at about 1400 $cm^{-1}$ which may linked to wagging vibration of proline side chains in gelatin and its presence implies the formation of type I gel (Hossan et al., 2015). The

doublet bands formed at 1123 and 1101 $cm^{-1}$, respectively, may corresponds to the glycosidic linkage.

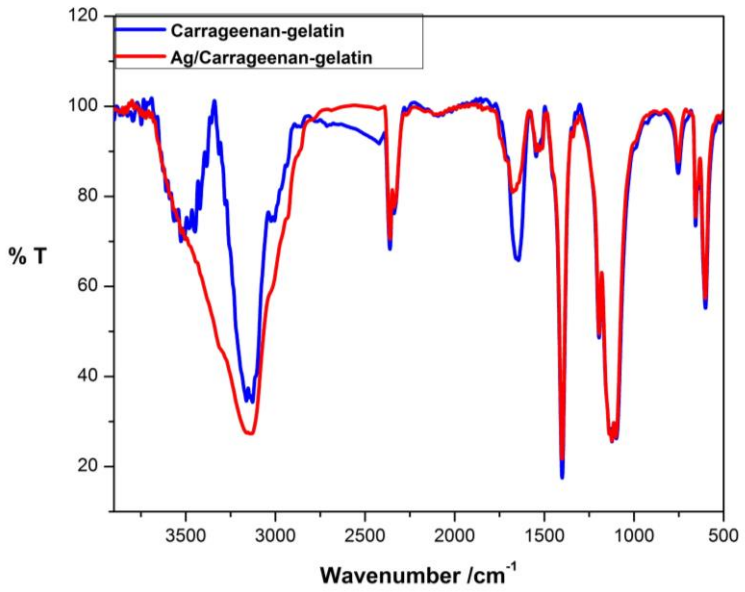


**Fig. 5**. FTIR spectra of carrageenan-gelatin hydrogel and Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

The three vibrational bands developed over 600-753 $cm^{-1}$ range are specific to the 3,6- anhydro-d-galactose in *k*-carrageenan. This characteristic C = O stretching at 1644 $cm^{-1}$ assosiated to amide I and N-H deformation at 1545 $cm^{-1}$ associated to amide II, in the FTIR spectrum of carrageenan-gelatin hydrogel, arising out of their cross-linking during gel formation were found to have considerably loosened to 1676 and 1526 $cm^{-1}$, respectively, on the spectrum of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite. The characteristic absorptions due to –OH stretching of *k*-Carrageenan and the N-H bending in gelatin can be distinctly observed at about 3526 and 3127-3162 $cm^{-1}$ in the spectrum of Carrageenan-gelatin hydrogel. It is notable that in the spectrum of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite, no such separate bands are observed and instead a large absorption variation in range between 2800-3600 $cm^{-1}$ has

appeared suggesting strong interaction between the –OH group with Ag nanoparticles. Thus the FTIR data enebled to confirm the cross-linking between *k*-carrageenan and gelatin for hydrogel formation and also the interaction between Ag nanoparticles and the hydrogel. It worth mentioning here that *k*-Carrageenan aids in the preparation of Ag nanoparticles which can act as a reducing process and capping agent, respectively.

### 3.6. Swelling behavior of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite

To investigate the swelling behaviour of hybrid hydrogel nanocomposite, the prepared hybrid hydrogel nanocomposite was immersed in water (pH 7) and maintained until it reached an equilibrium. The physical transformation results of the material upon swelling are shown in Fig. 6.

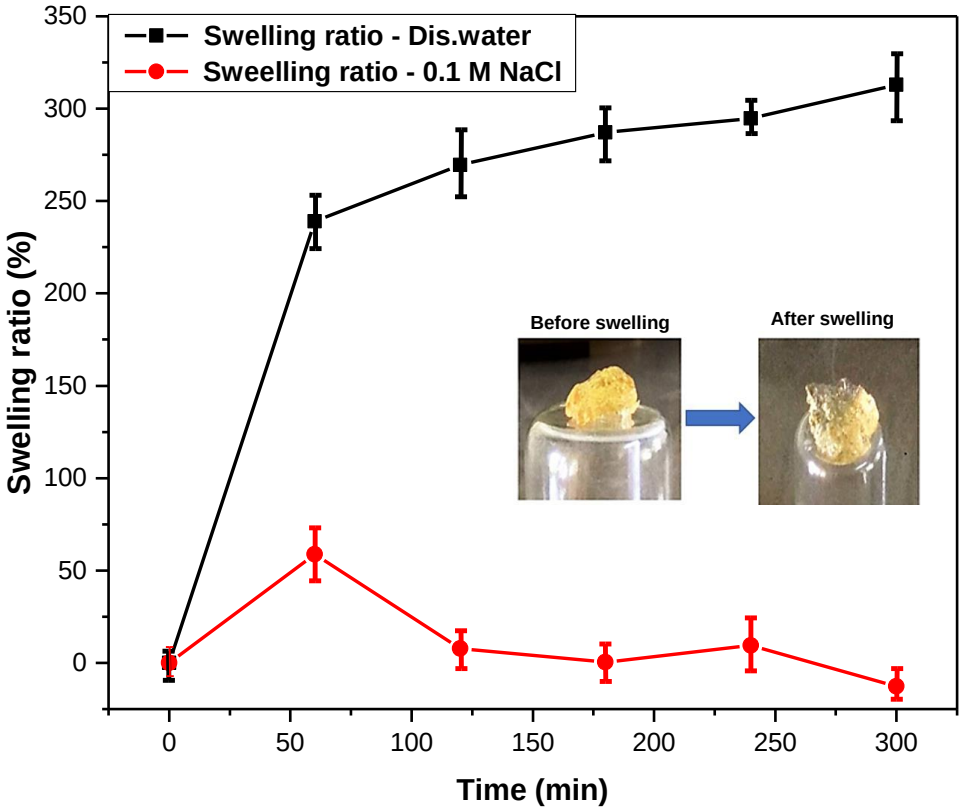

**Fig. 6.** Swelling behaviour of Ag/Carrageenan-gelatin hybird hydrogel nanocomposite with distilled water and 0.1M NaCl. Inset shows the hydrogel nanocomposite before and after swelling.

The swelling ratio was obtained by measuring the change in the mass of the gel to the original gel mass at an interval of 1 h in pure water and as well as in 0.1 M NaCl electrolyte solution. The results suggest that the hydrogel shows a nearly 250% water absorption in the first 1 h of immersion and further shows a moderate increase upto about 300 % after a standing time of 5 h. In 0.1 M NaCl, the hydrogel nanocomposite shows a tendency to swell during the initial 1 h period of time followed by slow de-swelling for the remaining 4 h period of standing.

### 3.7. Antimicrobial activity

The antimicrobial study of four samples (AmB Drug loaded hydrogel, Ag/Carrageena-gelatin hybrid hydrogel nanocomposite, Carrageenan-gelatin hydrogel, Ag nanoparticles) were tested against human pathogens *S.agalactiae 1661*, *S. pyogenes 1210* and *E. coli*. Among the four samples tested, Ag/Carrageenan hybrid hydrogel nanocomposite showed better antibacterial activity followed by drug loaded hybrid hydrogel and antibiotic one. Table 1 presents details of comparison for the antimicrobial activities of all four samples. Among the three bacteria tested against four different samples we have noted that *E. coli* has a higher sensitivity (12.8%), followed by *S. Agalactiae 1661* (11.6%) and *S. pyogenes 1210* (10.6%) when compared to tetracycline (21%, 15% and 11%) as a reference control (Fig. 7). The antimicrobial activity results enabled to sustain that the prepared hybrid hydrogel nanocomposite is suitable for biomedical applications.

**Table 1** Comparison of antimicrobial activity (mm) of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite for the three pathogens

| Pathogen | Drug coated hydrogel | Tetra cycline | Ag/carrageenan-gelatin hybrid hydrogel nano nanocomposite | Carrageenan-gelatin hybrid hydrogel | Ag nanoparticles | $H_20$ |
|---|---|---|---|---|---|---|
| *E.coli* | 14 | 21 | 19 | 21 | 3 | |
| *S.agalactiae1661* | 15 | 15 | 21 | 4 | 6 | -- |
| *S.pyogenes 1210* | 10 | 11 | 18 | 10 | 4 | -- |

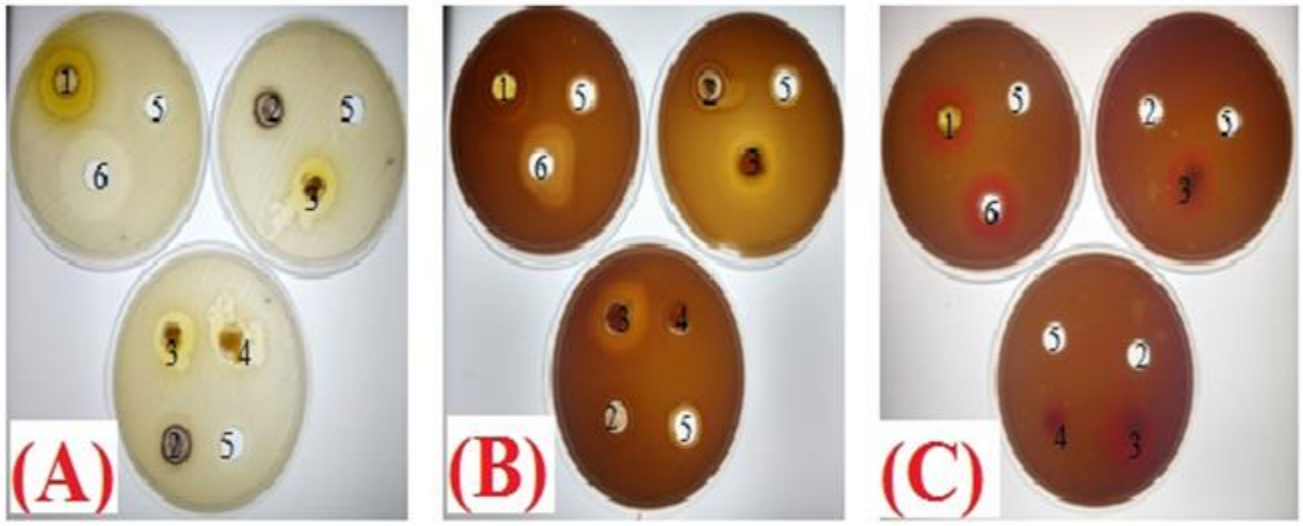


**Fig. 7.** Antimicrobial activity of Ag/Carrageenan hybird hydrogel nanocomposite against a) *E.coli*, b) *S.agalactiae 1661* and c) *S. Pyogenes 1210* human pathogens.

1. AmB Drug loaded hydrogel, 2. Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite, 3. Carrageenan-gelatin hydrogel, 4. Ag nanoparticles, 5. $H_2O$, 6. Tetracycline

### 3.9. Effect of Ag/Carrageenan hybrid hydrogel nanocomposite on the cell wall of *S.agalactiae 1661*

It has been well known that antimicrobial agents target the cell wall. The bacterial membrane can be very important for the bacterial cell survival and for the strength of antimicrobial agents which is very protective for the host. Hence, it was noted that the antimicrobial agents is responsible for both degradation of inner and outer bacterial membranes (Epand et al., 2016). Based on the antimicrobial activity results, we have tried to further explore the effect of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite on human pathogen *S. agalactiae* 1661**.** The *S. agalactiae* bacterial cell wall has been treated with Ag/Carrageenan-gelatin hybrid hydrogel and the effect of cell damage has been clearly observed by SEM images. The SEM images clearly showed the damage of pathogenic bacteria cell wall when was used Ag/Carrageenan hybrid hydrogel nanocomposite (Fig. 8B). Also we can note that the untreated bacterial cells showed no changes in the cell wall or damage (Fig. 8A) (Ng et al., 2014). The bacterial cell wall damage was observed only to be cause by Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite treated cell which clearly endorses that hydrogel has superior activity against bacterial cell wall protein. It is considered that the electrostatic interaction that occurs between the hydrogel and bacteria cell wall possibly leads to cell wall disruption and as a result bacterial cells could be dying. Fig. 8B indicates the damaged cellular envelope of *S. agalactiae* bacterial cell wall.

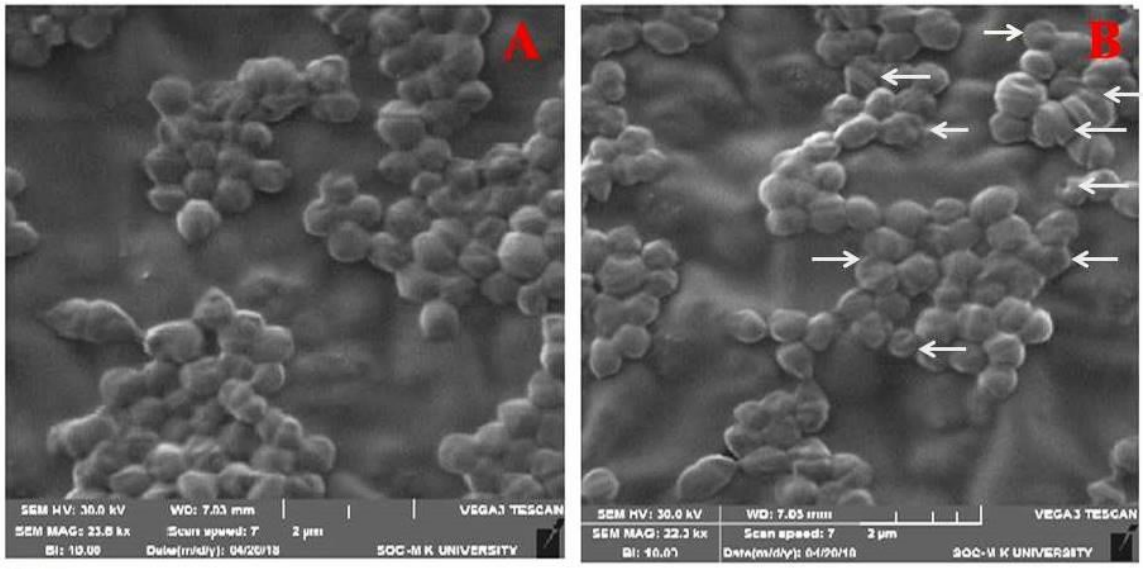


**Fig. 8.** SEM images showing the effect of Ag/Carrageenan hybrid hydrogel nanocomposite on bacterial cell wall (*S.agalactiae 1661)* damage.

The antimicrobial activity of hybrid hydrogel mainly depends on the charges of carrageenan polymer, porosity of hydrogel and the permeability of microbial membranes (Li et al., 2014). Further, the bacterial cell-wall arrangement and its composition is considered a primordial factor to elucidate the functioning of the bacterial cell wall, process being responsible for drug modes control. Besides, it can enable the development of novel - generation of therapeutics (Romaniuk,. and Cegelski., 2015).

### 3.10. Cytotoxicity assay

The *in-vitro* cytotoxicity of Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite was analyzed by MTT assay with the Non-small lung Cancer Cells A459 (Ramalingam et al., 2016). The results are compared with control (Carrageenan/ gelatin hybrid hydrogel) and the standard drug (Doxorubicin). Therefore, in Fig. 9 was presented for compartion the results of three

combinations: the cell survival of A459 lung cancer cell with Ag/Carrageenan-gelatin hybrid hydrogel nanocomposite, the control carrageenan-gelatin hybrid hydrogel and standard drug Doxorubicin. There, the IC50 values of Ag/Carrageenan hybrid hydrogel nanocomposite is noted to be 74.68 ug/ml. The maximum cell death is observed at the highest concentration of Ag/Carrgeenan-gelatin hybrid hydrogel nanocomposite that was 100 µg/ml. The observed results indicate that the prepared hybrid hydrogel nanocomposite has good cytotoxicity against the lung cancer cells and the newly material formed is suitable for drug delivery and anticancer drugs.

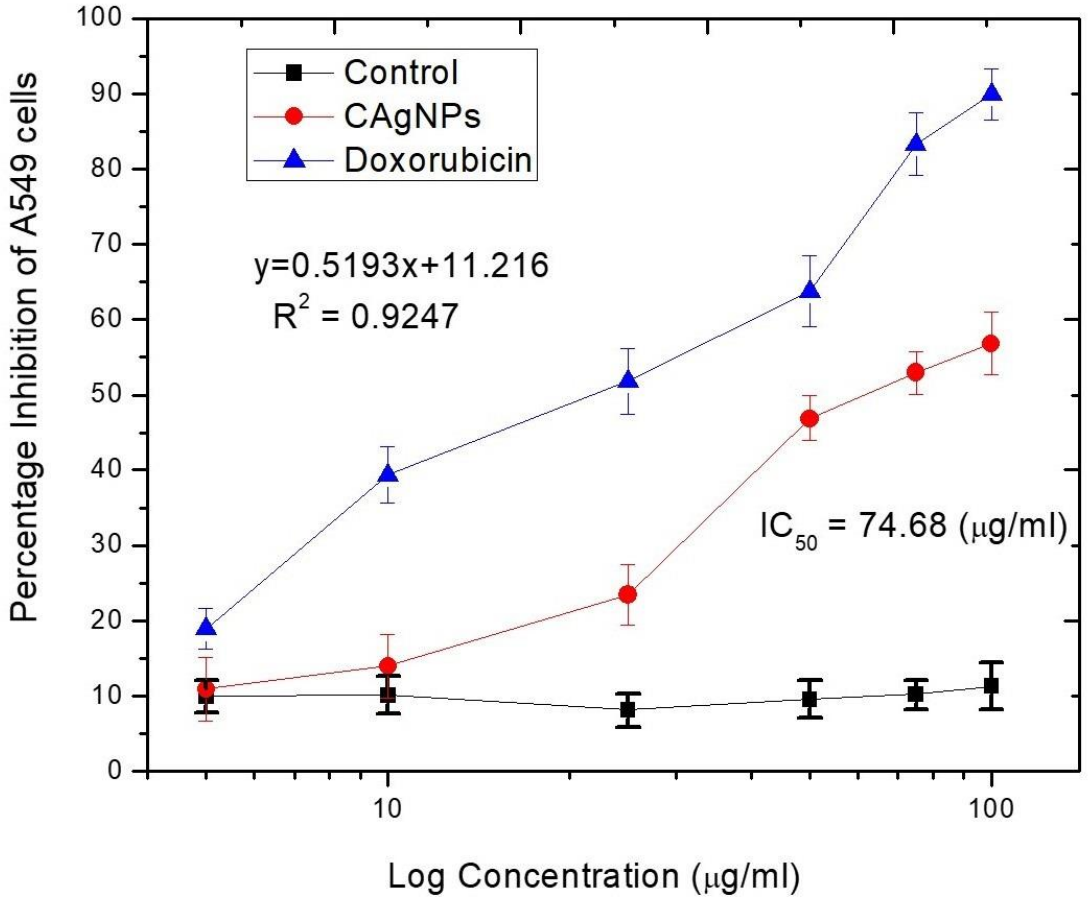


**Fig. 9**. Cytotoxic assay of prepared hybrid hydrogel nanocomposite with A549 lung cancer cells and its comaprision with standard doxorubicin drug.

## 4. Conclusions

A novel Ag/Carrageenan–gelatin hybrid hydrogel nanocomposite was prepared from red seaweed *Acanthophora spicifera*. The carrageenan polymer capped Ag nanoparticles were incorporated into gelatin hydrogel. The formation of the hybrid hydrogel nanocomposite was confirmed by using UV-visible, FTIR and EDX spectroscopy, SEM and TEM measurements. The hydrogel nanocomposite showed a maximum of water absorption of about 300 % for a standing time of 5 h. The prepared hybrid hydrogel reveald good antimicrobial activity against the human pathogens viz. *S.agalactiae 1661, S. pyogenes 1210* and *E. coli*. Further, the hydrogel nanocomposite indicated good activity against *S. agalactiae* 1661 bacterial cell wall protein. It was found that the IC50 values of hybrid hydrogel nanocomposites is 74.68 µg/mL which is comparable with the standard drug doxorubicin. The present study is considered a very promising because it reveals for the first time evidnce about the application of the new Ag/Carrageenan-gelatin hybrid hydrogel in antimicrobial, drug delivery and anticancer applications.

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