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Evaluation of Chitosan-Alginate Nanoparticle as A Stable Antibacterial Formula in Biological Fluids

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Abstract

This research mainly focuses on the preparation of chitosan-alginate Nanoparticle by ionotropic gelation method using calcium chloride and sodium alginate to form nanocomposites of CH-ALg, examine their antibacterial activity against multidrug resistance (MDR) bacteria, and evaluate the stability of chitosan-alginate formula in different biological fluids, including simulated gastric fluid (SGF) and intestinal fluid (SIF). The average diameter of particles size prepared was measured by an Atomic force microscope (AFM) and it was 61.91 nm. Otherwise, the nature of functional groups present in CH-ALg nanoparticle was investigated by Fourier transforms infrared (FTIR) analysis. The stability of synthesized CH-ALg nanoparticle was measured by Zeta potential measurement and show high stability with a 79 mV ratio. The SEM picture shows that particles are shown to be in the form of bundles and the size of nanoparticles was in the range of (14-84nm). The antibacterial activity of CH-ALg was tested against (MDR) Gram-positive bacteria (*staphylococcus aureus*) and Gram-negative bacteria (*Salmonella typhi*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*). The results exhibited a significant impact of antibacterial action against isolates of MDR. CH-ALg nanoparticles were loaded with antibiotic doxycycline in order to strengthen the antibacterial action and drug delivery effectiveness. The synergistic effects of prepared CH-ALg loaded with DOX antibiotic at several concentrations toward MDR bacteria were also examined. The results indicate that DOX-loaded nanoparticles have a significant improvement for antibacterial activity against Gram-negative and Gram-positive compared to doxycycline alone. The formula of chitosan- alginate was stabile in various biological fluids including simulated gastric fluid (SGF) and simulated intestinal fluid (SiF)

Keywords: antibacterial, chitosan-alginate, simulated gastric fluid (SGF), simulated intestinal fluid (SIF), synergistic effect and doxycycline nanoparticle .

تقييم التوليفة النانوية من الكيتوسان الجينيت كمضاد بكتيري ثابت في السوائل الحيوية

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الخلاصة

ركز هذا البحث بشكل رئيسي على التحضير النانوي لل chitosan–alginate بطريقة ionotropic gelation باستخدام calcium chloride و sodium alginate.وتكوين chitosan–alginate nanocomposite, فحص فعاليتها المضادة للبكتريا ضد البكتريا المقاومة للعديد من المضادات الحيوية وتقييم الاستقرارية للتوليفة CH–ALg في مختلف السوائل البايولوجية والتي تتضمن simulated gastric

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حجم الدقائق النانوية المحضرة تم قياسها . simulated intestinal fluid (SIF) و fluid (SGF) بواسطة Atomic force microscope (AFM) حيث كان الحجم 61.91nm غير ذلك المجاميع الفعالة الموجودة في الكيتوسان تم التحقق منها بواسطة تحليل (FTIR). ان الاستقرارية للدقائق النانوية المصنعة تم قياسها بواسطة مقياس ال Zeta Potential حيث عرضت النتائج استقرارية عالية بقيمة 79mV . كما ان صورة ال SEM اظهرت بأنه الدقائق تظهر على شكل حزم وحجم الدقائق النانوية بمعدل يتراوح بين (14–84 نانوميتر). الفعالية المضادة للبكتريا للكيتوسان تم فحصها ضد البكتيريا المقاومة لمختلف المضادات الحيوية الموجبة لصبغة غرام (*Staphylococcus aureus*) والسالبة لصبغة غرام *Salmonella typhi,* (*Enterobacter cloacae, Pseudomonas aerogenosa*) . عرضت النتائج تاثيرا كبيرا على هذه العزلات . تم تحميل الدقائق النانوية chitosan alginate بالمضاد الحيوي doxycycline من أجل تعزيز العمل المضاد للبكتيريا وفعالية توصيل الدواء. الفعل التأزري للدقائق النانوية chitosan alginate محملة بالمضاد الحيوي doxycycline ضد البكتيريا المقاومة لعديد من المضادات الحيوية ايضا تم التحقق منه.واظهرت النتائج ان المضاد الحيوي doxycycline المحمل بواسطة الدقائق النانوية له تأثير كبير مقارنة مع المضاد لوحده ضد البكتريا السالبة والموجبة لصبغة غرام. وان التوليفة chitosan–alginate كان مستقر في مختلف السوائل البايولوجية والتي تتضمن simulated gastric fluid (SGF) و simulated . intestinal fluid (SIF)

1. Introduction

Nanotechnology has been proven to be a powerful effective antimicrobial approach in the pharmaceutical and biomedical application areas, because of the infectious diseases re-emergence of antibacterial-resistant strains, particularly Gram-negative bacteria [1]. Currently, biodegradable nanoparticles are of significant relevance because they can safely and very quickly break down into nature's raw materials and disappear into the environment when they have already served their purpose [2]. They could also be categorized as environmentally friendly, such as starch, chitosan, dextran, cellulose, and alginate [3]. Chitosan is a modified biopolymer, generated through gradual deacetylation of chitin, It is mainly composed of alternating units of (1–4) connected N-acetyl glucosamine and glucosamine [4]. The most important biological functions of chitosan would include the antivirals, antimicrobials, antioxidants, and antitumor functions [5]. Those properties are particularly appropriate for a broad range of biomedical and pharmaceutical applications, including wound healing [6], gene delivery [7], tissue engineering [8], and drug delivery [9]. Gastroenteritis is one of the main causes of illness and death worldwide [10]. The most common pathogenic agent strongly associated with acute gastroenteritis and diarrheas are *Enterobacteriaceae,* [11]. This family is the main cause of opportunistic infections (meningitis, septicemia, pneumonia, and urinary tract infections) [12]. Though several current antibiotics have been developed in recent years, none of them has improved their effectiveness against MDR bacteria [13], [14]. Chitosan has a broad spectrum of antibacterial functions with a high rate of destruction against Gram- positive and Gram-negative bacteria via the interaction of CH and its derivative products mostly with the cell wall of bacteria [15]. Doxycycline antibiotic encapsulation by chitosan-alginate nanoparticles helps to avoid adverse health effects by protecting sensitive tissues from rapid exposure to drugs while also helping to improve drug efficacy by gaining slow, sustained release directly at the infection site [16]. Most of the clinical-phase therapies fail to achieve clinical outcomes due to the lack of ability to reach the target site. A successful way to solve this deficiency is by designing controlled drug release systems that release drugs or bioactive compounds at the target sites [17]. Several studies have examined the stability of chitosan in different biological fluids, including simulated gastric fluid (SGF) and intestinal fluid (SIF), as well as their mucoadhesive properties, cellular absorption, and drug solubility [18],[19]. These studies aimed to achieve a drug delivery system for oral administration which has the ability to delay

the release of drugs in an acidic environment and facilitate them in the intestinal compartment [20]. The aim of this study was to synthesize and characterize the chitosan-alginate nanoparticle which prepared by the ionotropic gelation method. In addition, analyze their antibacterial effect against MDR bacteria which isolated from patients who suffered from diarrhea. like *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. And also evaluate the stability of chitosan-alginate formula in different biological fluids, including simulated gastric fluid (SGF) and intestinal fluid (SIF).

2. Materials and methods

Collection of samples

About 235 stools sample were collected randomly from children and adults with intestinal infections for both sexes from different Baghdad hospitals such as (medical city), Protect Children's hospital Baghdad hospital, Al-Aelweia hospital for children, during the period from September 2018 to December 2018. All specimens were obtained under controlled conditions and held within 1-2 hours in the laboratory.

Bacterial isolation and identification

Gram stain has been performed to study bacterial appearance under microscope of isolated bacteria. Then all Samples were inoculated on different culture media such as (MacConkey agar, Blood agar, SS agar, Nutrient agar, and Bile esculine agar), incubated at 37 ˚C for 24 hrs. The biochemical tests were applied on these isolates, such as (sugar fermentation, catalase, oxidase, and (IMVC) and then studied,other Biochemical tests using the API 20 E kit were tested to complete the diagnosis. The identification of isolates was also confirmed by Vitek 2 compact auto analyzer system.

Antibiotic susceptibility testing

Antibiotic susceptibility of bacterial isolates was carried out by modifying Kirby-Bauer's disk diffusion method [21] according to the Clinical Laboratory Standards Institute (CLSI) guidelines [22]. Cultures of *Enterobacteriaceae* and *Staphylococcus aureus* were performed to 0.5 McFarland standards. The streaking method was used to culture the bacterial suspension on Müller-Hinton agar using sterile swab. Each antibiotic disk was placed by a sterile forceps on the inoculated agar plates and then were incubated for 24 hrs at 37°C. The results were assigned as sensitive (S) or resistant (R) as indicated by (CLSI) 2018. The following antibiotics were tested: Cefotaime(CTX), Metronidazole(MET), Amikacin(AK), Clindamycin(DA), Trimethoprim(TMP), Rifampin(Ra),Tobramycin(TOB),ErythromycinI, Ciprofloxacin(CIP) ,Nitrofurantoin(F),Impenem (IPM), Doxycycline(DOX), Colistin(CT).

Synthesis of chitosan-alginate nanoparticles

A modified method for preparation of Chitosan-alginate nanoparticle was intended in two steps based on the inotropic pre-gelation method already mentioned by Chopra *et al.,* [23]. For stock preparation 7.35 gm of calcium chloride was dissolved in 50 ml distal water, 0.0018 gm Of sodium alginate was dissolved in 50 ml and 0.060gm Of chitosan was dissolved in 50 ml HCL (0.25M). The PH was adjusted to 5 for all the stocks.

Steps for chitosan-alginate nanoparticle preparation are described below:

- The first step: 12 ml (1.2 mg/ml) was dropped from chitosan stock and then added slowly to 6 ml of calcium chloride (147 mg/ml) after that it was stirred for 30 minutes.
- The second step: 6 ml of sodium alginate (0.036 mg/ml) stock was diluted two folds in distal water and added drop-wise to the solution in the first step with regular stirring for 90 minutes.
- The third step: ultra-sonication for 90 minutes.

Preparation of chitosan–alginate loaded with doxycycline antibiotic

A modified method which was already mentioned by Chopra *et al.,* [23] to prepare doxycycline loaded with chitosan-alginate was done by the following:

- A stock solution of (0.01) gm/ml of (1Mm) doxycycline was prepared and stored at -18 °C

- 76 µl of doxycycline with a final concentration (16 µg/ml) was added slowly to 6 ml of calcium chloride (147 mg/ml) and stirred for 30 minutes
- 12 ml of chitosan (1.2 mg/ml) was added gradually to this mix and stirred for 30 minutes,
- The final step was to add 6 ml (0.036 mg/ml) of sodium alginate (which was diluted twofold with 6 ml distaled water) and stirred for 90 minutes
- Another mix was prepared in the same steps, but with different concentrations of 0.15mg/ml and 0.3mg/ml of chitosan and 16µg/ml and 8µg/ml of doxycycline
- Ultra-sonication was used for 90 minutes and stored in a refrigerator.

Characterization of prepared nanoparticle

Atomic Force Microscopy (AFM) offers 3-dimensional surface topography that is dependent on Van der Waals or even other attractive and repulsive forces [24]. In the glass slide, 5 drops of CH-Alg nanoparticle have been applied and left to dry and precipitate upon this. Analysis gained using zeta potential measurement to determine the surface charge and particle size distribution of chitosan nanoparticle. For sample preparation of the chitosan nanoparticle 1-3 drops of potassium chloride (KCl) 1Mm was added to the solution and mixed well. The FT-IR spectra of chitosan, alginate, chitosan-alginate nanoparticle, doxycycline antibiotic alone, and chitosan-alginate nanoparticle loaded with doxycycline were analyzed. About 5 drops for each of these components were dispersed on different slides then it was dried in an incubator overnight. The Scanning electron microscope (SEM) was used to study the morphology and surface shape of CH-ALg nanoparticle .

Optimized conditions for nanoparticles synthesis

The effect of Concentrations

The present study used different concentrations of chitosan, alginate, and calcium chloride in order to prepare chitosan-alginate nanoparticles with high antibacterial activity with a stabile formula. These concentrations are listed in table (1).

Table 1-Different Concentrations in CH-Alg nanoparticle Formulations

Materials / **Formulation**	Chitosan	Sodium alginate	Calcium chloride	References
Formula 1	4 ml of (0.8 mg/ml)	10 ml of (3 mg/ml)	2 ml of (3.35 mg/ml)	(Li, 2008) [25]
Formula 2	25 ml of (50 mg/ml)	117.5 ml of (0.063 mg/ml)	7.5 ml of (147 mg/ml)	(Chopra, 2012) [23]
Formula 3	12 ml of (1.2 mg/ml)	6 ml of (0.036 mg/ml)	6 ml of (147 mg/ml)	Modified method

The effect of time

In order to investigate the antibacterial activity and degradation of chitosan-alginate nanoparticle per time.1 ml of CH-Alg nanoparticle was mixed with 1 ml of bacterial suspension and incubated with shaking,10 µl was dropped and incubated on Muller Hinton agar at 37℃ for (2,4, and 6 hrs).

The effect of pH

CH-Alg nanoparticle solutions which consisted of (chitosan 1.2 mg/ml, sodium alginate 0.0036 mg/ml, and calcium chloride 18.3 mg/ml) were prepared with different pH, and the antibacterial effect of CH-ALg nanoparticles were investigated within different pH value at (5, 7and 9). The pH was adjusted using HCL (0.1 N) and NaOH (0.1N).

Antibacterial activity CH-Alg nanoparticle alone and CH-Alg nanoparticle loaded with doxycycline antibiotic

A single bacterial isolate was inoculated in a 10 ml Muller Hinton broth tube and incubated for 24 hrs. for activation, after overnight 1ml of bacterial inoculation which was adjusted to McFarland with $1.5*10^8$ CFU/ml was added to 1 ml of synthesized CH-ALg nanoparticle according to Divya *et al.*,[26],as shown in table (2)

Table 2-Chitosan and Doxycycline Antibiotic Different Final Concentrations in the Antibacterial Experiment.

Cooperation's	Chitosan concentration	DOX concentration
Tube 1	0.3 mg/ml	————
Tube 2	0.3 mg/ml	16 µg/ml
Tube 3	0.15 mg/ml	16 µg/ml
Tube 4	0.3 mg/ml	8 µg/ml
Tube 5		16 µg/ml

For negative control 1ml of bacterial suspension was applied to 1 ml of normal saline in another tube. After that, it was incubated with shaking for 24 hrs. A volume of 0.1 was withdrawn from those tubes to determine the vailable cell count and passed to Muller Hinton agar plates, spread by a loop.

In vitro stability of chitosan-alginate nanoparticle

Chitosan-alginate stability was examined *in vitro* in simulated gastric and intestinal fluids (SGF and SIF) according to the preparation method which previously described by He *et al.*, [19]. The Simulated Gastric Fluid preparation was done by mixing 2gm of NaCl , 7 ml of HCL 36–38%, and about 3.2gm of pepsin enzyme in 1L of D.W, with regulating PH to 1.2 with 1M HCl. On the other hand, for simulating intestinal fluid preparation: 6.8gm of KH_2PO_4, 10gm of Trypsin enzyme, and 5gm of bile salts were added to 1L of D.W. then, PH was regulated to 7.4 with 1 Mol of NaOH. The nanoparticle stability was examined in SGF, 3ml of CH-ALg nanoparticle solution added to 3 ml of SGF or SIF, after that incubated for overnight in water bath at 37 C˚. At different time periods (zero, 4, and 6 hrs.) Particle size and zeta potential were measured. Every simulation has been replicated three times.

3. Result and discussion

Isolation and Identification of bacterial isolate

About 235 stool samples include 20 isolates for *Salmonella typhi*, 36 isolates for *Enterobacter cloacae*, two isolates for *Staphylococcus aureus*, and 6 isolates for *Pseudomonas aeruginosa*.The total bacterial isolates according to the cultural and microscopically characteristics showed that The purple-colored bacterial isolate signifies Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis*, otherwise the bacterial isolate appeared in red, it reflects Gram-negative bacteria such as: *E.coli*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeroginosa*, and *Salmonella typhi*. The Api 20E system and VITEC2 compact system were also used. The results of bacterial identification showed that percentage of *E.coli* 36.2% whereas 28.8% belonging to *Klebsiella pneumonia*,*Pseudomonas aeroginosa* 2.6%,*Enterobacter cloacae* 15.7%, *Proteus mirabilis*12.7%, *Salmonella typhi* 1.7 %, *Enterococcus faecalis* 1.7%, and *Staphylococcus aureus* 0.4%.

Antibiotic susceptibility testing:

The results of susceptibility tests for the bacteria, which were isolated in the present research, showed various patterns of resistance to the antimicrobials as shown in Figure 1. Multi-resistant bacteria increased according to the transfer of resistance factors via mobile genetic elements, which include gene cassettes and transposons plasmids [27]. In the present study, all the 229 bacterial isolates have a 100% resistance rate to erythromycin, metronidazole, rifampin, and clindamycin. These results agree with those of Kilonzo *et al.,* [28] and Moawad *et al.,* [29] who found that (100%) of the *Enterobacteriaceae* isolates were resistant to penicillin, erythromycin, and rifampin with 98.2%, 96.4%, and 96.4% respectively.

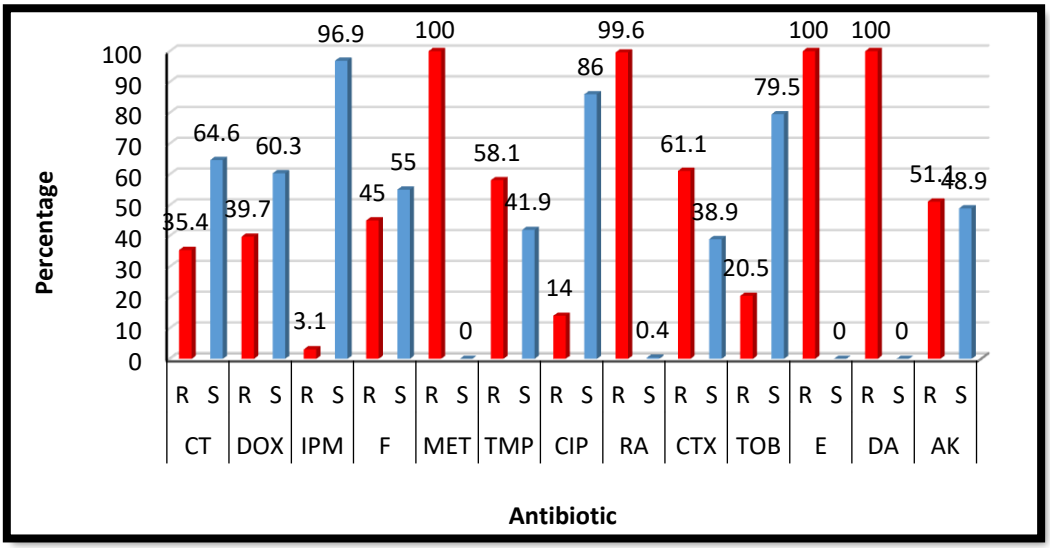

Figure 1- Percentage result of antibiotics susceptibility test for all bacterial isolate. AK = Amikacin , DA = Clindamycin , E = Erythromycin , TOB = Tobramycin , CTX = Cefotaxime , RA = Refampin , CIP = Ciprofloxacin , TMP = Trimethoprim , MET = Metronidazole , F = Nitrofurantoin , IPM = Imipenem , DOX = Doxycycline , CT = Colistin**.** *R: Resistance, S: Sensitive

Preparation of chitosan-alginate nanoparticle

As a principle, the simple gelling properties of both chitosan and alginate were used to prepare polyelectrolyte complexes of polycations and polyanions [30]. Based on an ionotropic gelation method, the preparation of CH-ALg nanoparticle systems involves mixing the two aqueous phases at room temperature [31]. CH-ALG polyionic combination is produced through ionic interactions between the chitosan amine group and the alginate carboxylic group by ionic gelation [32].

Optimized conditions for nanoparticle synthesis

The effect of concentration

Chitosan-alginate nanoparticle were prepared in different concentrations of chitosan (4 ml of 0.8 mg/ml, 25 ml of 50 mg/ml, 12 ml of 1.2 mg/ml). Sodium alginate (10 ml of 3 mg/ml, 117.5 ml of 0.063 mg/ml, 6 ml of 0.036 mg/ml). Moreover, calcium chloride (2 ml of 3.35 mg/ml, 7.5 ml of 147 mg/ml, 6 ml of 147 mg/ml. The results showed that formula 3 with higher $CaCl_2$ (6 ml of 147 mg/ml) and lower chitosan (12 ml of 1.2 mg/ml) and sodium alginate (6 ml of 0.036 mg/ml) concentrations were more stable. In addition, it had more antibacterial activity in comparison with the other formula, which estimated by Li *et al.,* [25] and Chopra *et al.,* [23] as shown in Figure 2.

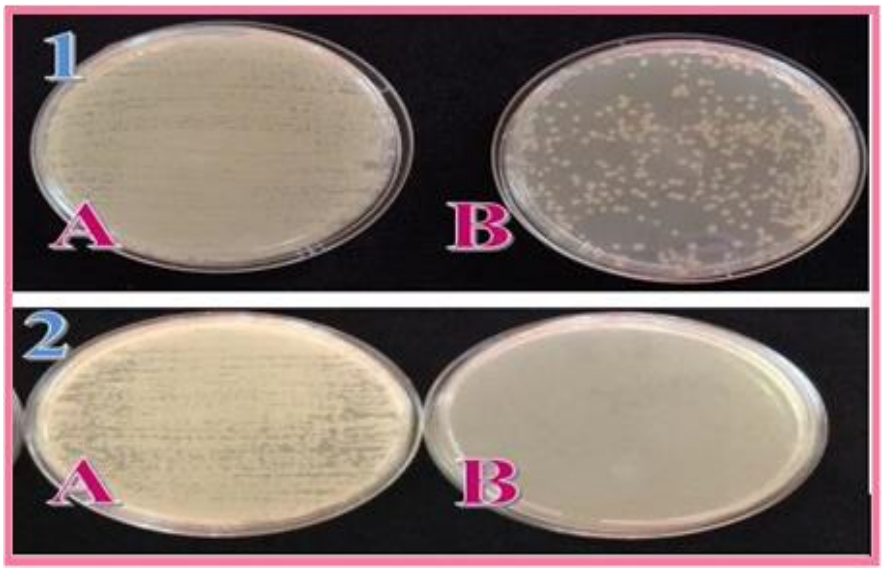

Figure 2-The Differences in Antibacterial Activity between CH- ALg nanoparticle Formula: (1) Formula two, (2) Formula three, (A): Negative Control, (B): CH-ALg nanoparticle

In addition, also the results showed slight differences in particle size which was analysed by AFM where the average diameter of formula three was 61.91 nm while another formula was 68.77 nm as shown in table (3):

Table 3-AFM Results for Chitosan-Aginate nanoparticle Formulations

Formulation	AFM results	
Formula 3	Avg. Diameter:61.91 nm	<=10% Diameter:25.00 nm
	<=50% Diameter:60.00 nm	<=90% Diameter:90.00 nm
Formula 2	Avg. Diameter:68.77 nm	<=10% Diameter:60.00 nm
	<=50% Diameter:65.00 nm	<=90% Diameter:80.00 nm

This improvement in mean particle size was related to the decrease of alginate concentration and subsequent reduction in sodium alginate chains and number of the carboxylate, ether, and hydroxyl groups that are jointed to the calcium cation. The rise in alginate concentration causes more functional groups to assemble around the Ca crosslinking agent and thus additional layers of alginate chains may join the Ca cations; in concluding, the size of nanoparticle increases with the rise in alginate concentration [33]. Suppositions, with the increasment of Ca ion concentration, lower polymer chains are involved with higher contents of Ca cations so, the size of nanoparticle has to be substantially smaller [34]. In addition, the use of low molecular weight chitosan in formula 3 led to the formation of smaller particles for the majority of ALG/CS mass ratios, resulting in fewer aggregates and the solution was cloudy in comparison with formula 2 solution which had high viscosity as shown in Figure 3. This could be dependent on the potential of low molecular weight CH to diffuse in the alginate gel matrix to produce smaller and more homogeneous particles, Polymers of high molecular weight or viscosity, on the other hand, bind to the surface of such matrices, forming an external membrane, resulting in increased particle size [35], [36].

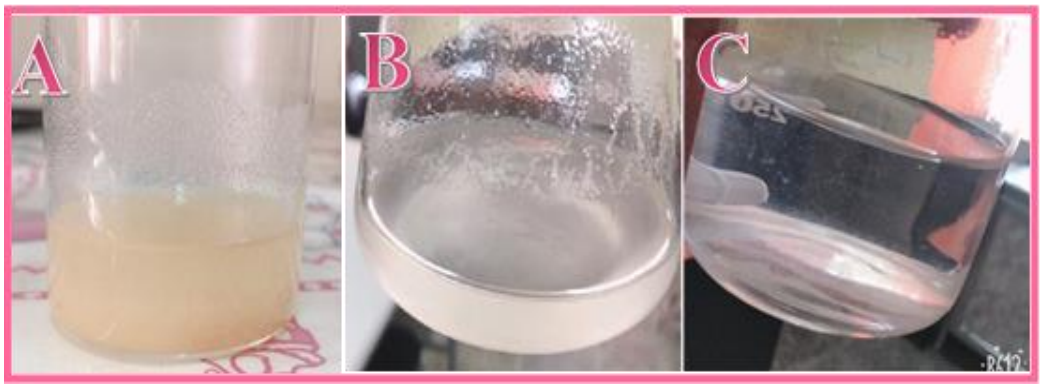

Figure 3-Solutions of (A): Chitosan Stock in Formula 2 (B): CH-ALg nanoparticles in Formula 2, (C): CH-ALg nanoparticle in Formula 3.

The effect of time

The antibacterial activity of CH-Alg nanoparticle was investigated for (2, 4, and 6 hours) as shown in Figure 4. The results showed that the number of vital bacterial cells decreased with an increased reaction time. There is reasonable concordance between these results and the previous results by Divya *et al.*,[26].

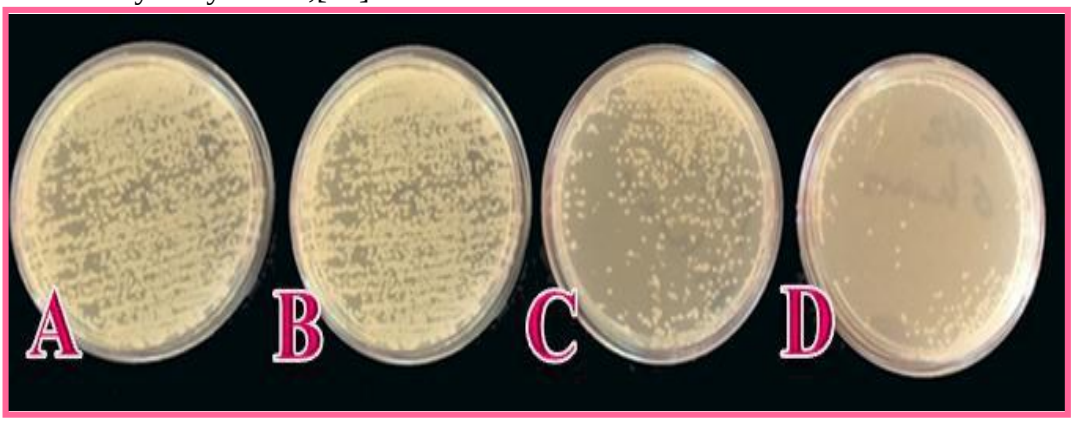

Figure 4-The Antibacterial Activity of CH-ALg nanoparticle against S.typhi During (A): Negative control, (B): 2 hours, (C): 4 hours, and (D): 6 hours.

The effect of PH

The results of the present study showed that the antibacterial activity of CH-ALg nanoparticle was higher in pH 5 than in pH 7 and 9 as shown in Figure 5; This may be clarified by the positive charge in the chitosan polymer is increased by a low environmental pH, which promotes binding to the bacterial cell wall [37]. In addition, a study done by Younes *et al.*, [38] had been reported that a lower degree of chitosan acetylation and a lower pH are favorable to the antibacterial activity of chitosan.

Figure 5-The Effect of Different pH Values on the Antibacterial Activity of CH-ALg nanoparticle. (A): Negative control, (B): pH 9, (C): pH 5, (D): pH 7.

Characterization of prepared chitosan-alginate nanoparticle Atomic force microscope (AFM)

AFM is ideal for quantitatively measuring the nanometer scale surface roughness and for visualizing the surface nano texture on many types of material surfaces including polymer nanocomposite AFM makes it easier to examine the mechanical characteristic of nano for each particle and molecule with better Physiological considerations [39]. The (AFM) gives a 2D and 3D image of the nanoparticles surface at an atomic level as shown in; Figure 6

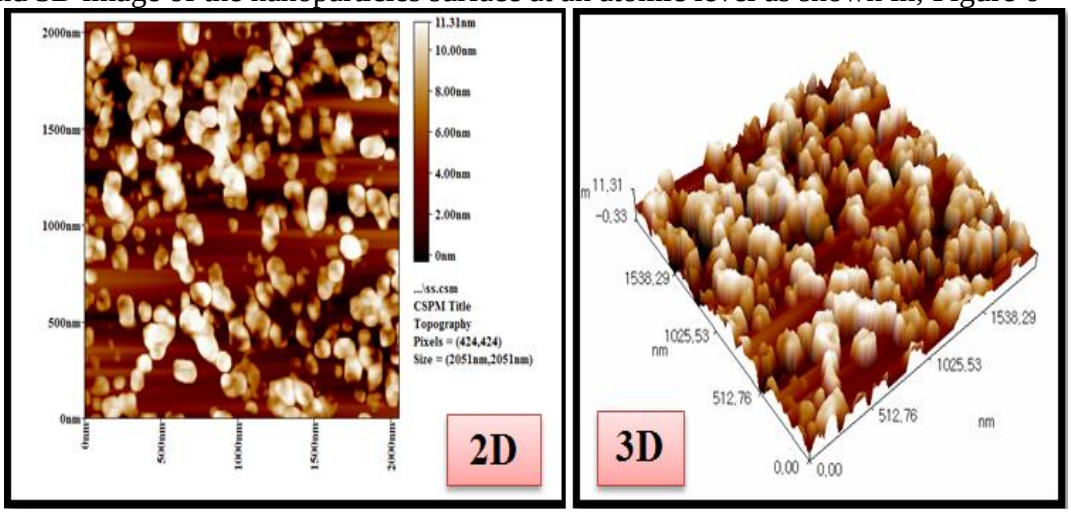

Figure 6-Atomic Force Microscopy of CH-Alg nanoparticle illustrate 2D And 3D Topological of Chitosan Nanoparticles.

The average particle size of CH-ALg nanoparticle was 61.91 nm which agrees with Körpe *et al.*,[40] because he found that size of particles was homogeneous and 60±20 nm.as shown in table (4)

Table 4- Chitosan Average Size Analysis by (AFM)

Avg. Diameter:61.91 nm	<=10% Diameter:25.00 nm
<=50% Diameter:60.00 nm	<=90% Diameter:90.00 nm

Fourier transforms infrared (FTIR) characterization

The results of mixed oppositely charged polysaccharides (chitosan and alginate), observed changes in some bands placement and disappearance or appearance of new peaks in comparison to single alginate or chitosan, and this is associated with possible electrostatic interaction between mixed polymers as mentioned in previous research [41]. A broad band around 3500-3100 cm-1, indicating enhanced hydrogen bonding compared to that of chitosan or sodium alginate alone [42]. The amide-I peak of chitosan shifted from 1627 cm-1 to 1731 cm-1 as shown in Figure 8, on the other hand, as shown in Figure 7, the absorption of OH bands for sodium alginate at 3261 cm-1 had been disappeared when it interacted with chitosan that suggested an electrostatic interaction between alginate and chitosan [43]. The Potential interactions between CH-alginate nanoparticle and Doxycycline were evaluated by comparing the FTIR spectra of CH-ALg nanoparticle, DOX, and a mixture of CH-ALg nanoparticle and DOX as shown in Figure 9. DOX had a peak at 1558 cm-1 which agrees with Zehtabi and his colleagues [44] study, and a peak at 1458 cm-1 which represent -CH2 bending [45]. Whereas the peak at 1396 cm-1 can be assigned to hydroxyl group [46]. Peaks at 1622 cm-1, 1677 cm-1 and 3461 cm-1 in infrared spectra of DOX are attributed to C-C stretching, C-O group and primary O-H group respectively [47]. The peak at 1699 cm-1 is due to presence of carbonyl group (C= O stretching) and the peak at 1643 cm-1 has represented the amide I band due to carbonyl absorption [48]. The mixture results of CH-ALg nanoparticle loaded with

doxycycline antibiotic FTIR spectra showed that the characteristic peaks of doxycycline at 1458 cm-1 and 1396 cm-1 were observed in the mixture FTIR spectra, which indicate the assurance of the interaction between CH-ALG nanoparticle and DOX antibiotic. But the peak at 1558 cm-1 was shifted to 1560 cm-1 and the peak at 3461 cm-1 shifted to 3263 cm-1, these results also indicate the interactions between CH-ALG nanoparticle and DOX antibiotic [16].

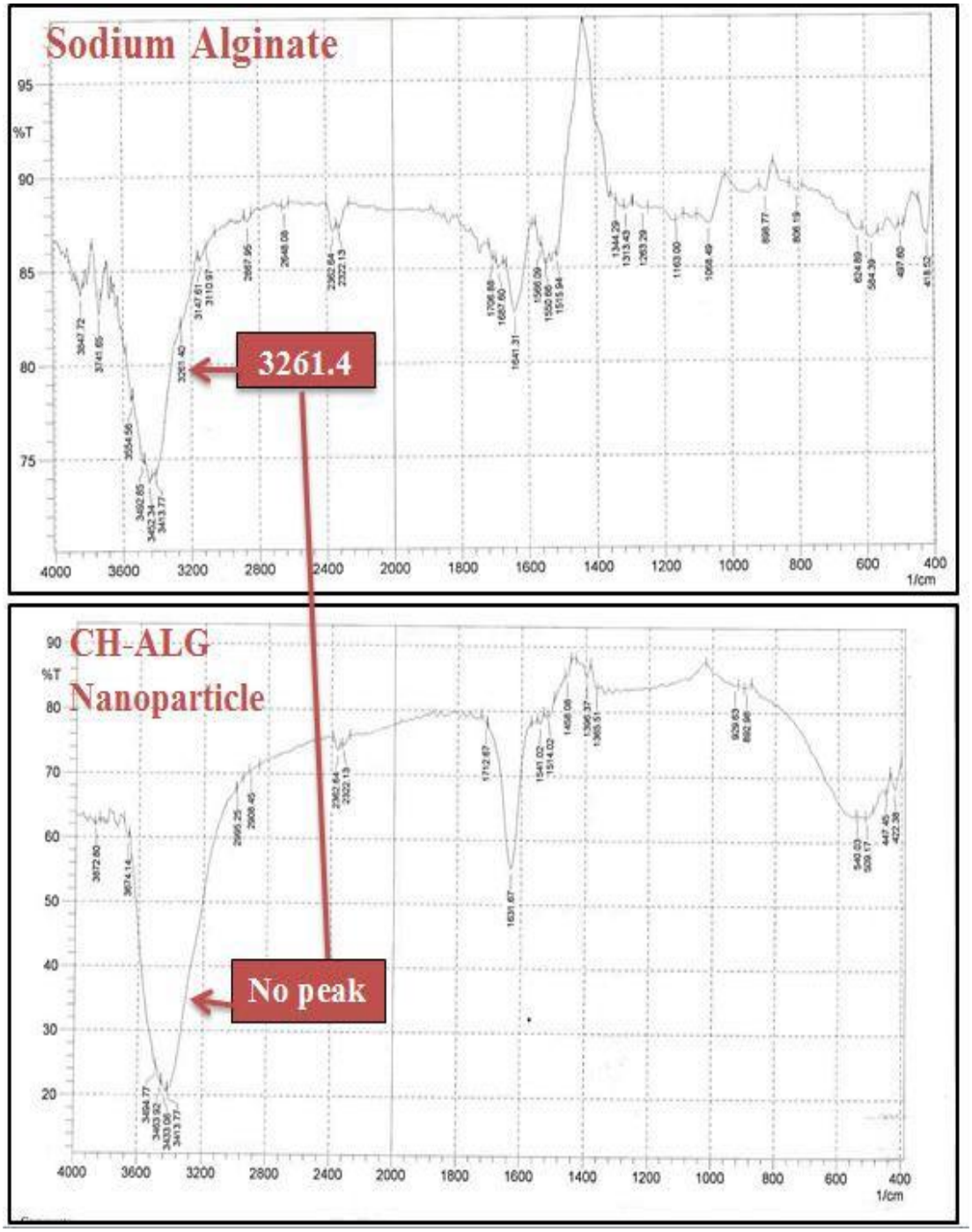

Figure 7-The Fourier Transforms Infrared (FT-IR) Spectroscopy Measurement of Sodium Alginate and CH-ALg Nanoparticle.

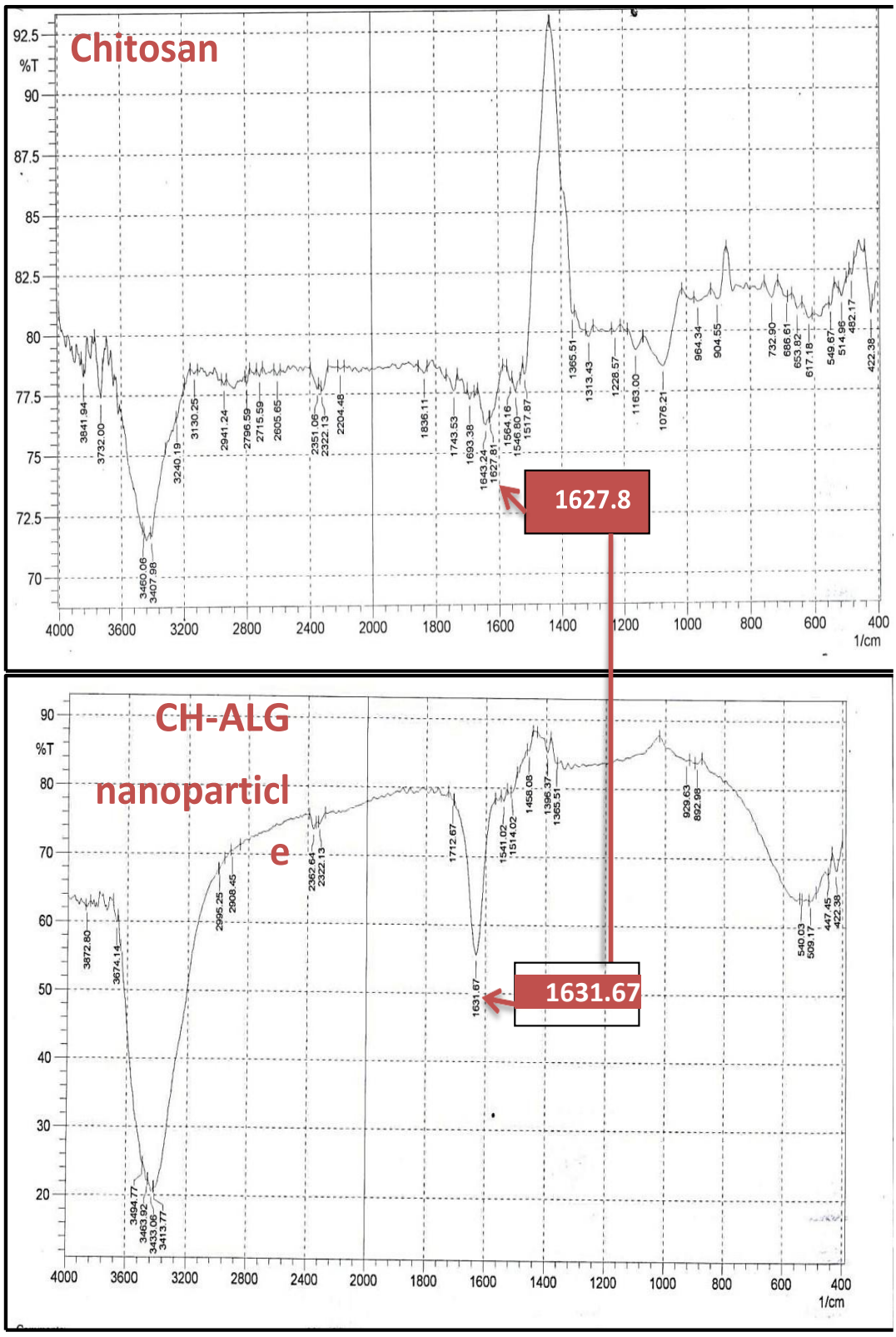

Figure 8-The Fourier Transforms Infrared (FT-IR) Spectroscopy Measurement of Chitosan and CH-ALg Nanoparticle.

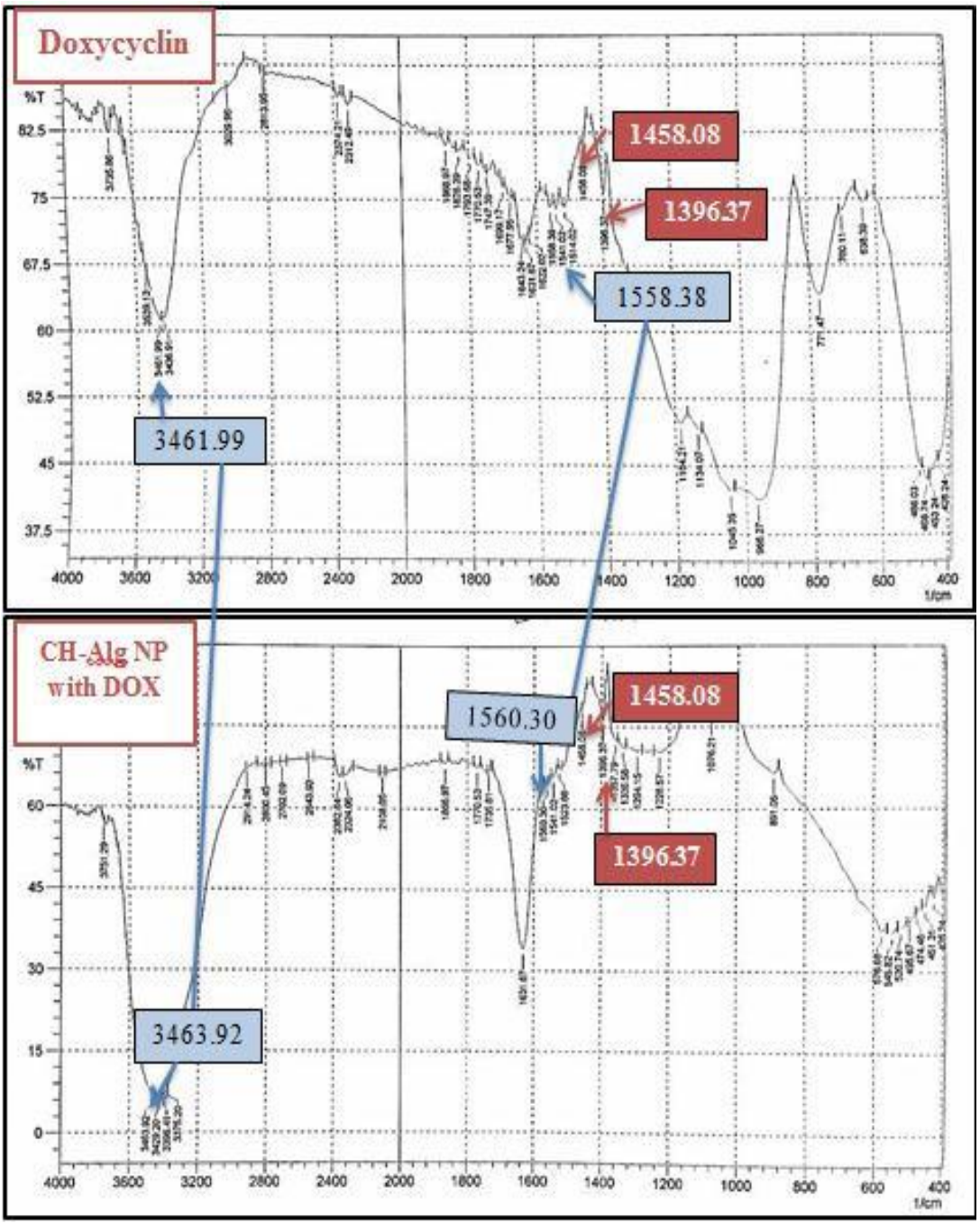

Figure 9-The Fourier transforms Infrared (FT-IR) Spectroscopy Measurement of Doxycycline and CH-ALG nanoparticle loaded with Doxycycline Antibiotic.

Zeta potential and dynamic light Scattering (DLS)

Zeta characterization method was used to characterize the stability and dispersion of CH-Alg nanoparticle. The results of Zeta-potential measurement presented a positive charge for zeta value of 79.12 mV reflecting the stability of the colloidal suspensions as shown in Figure 10. It is an additional benefit when the surfaces of NANOPARTICLE have a positive charge especially through using nanoparticles in drug delivery because they can easily be transferred in the cell membrane through negative channels [49]. Although several studies by Caetano *et al.*, [36] Katuwavila *et al.*, [49] and Azizi *et al.*, [50] which had found the zeta potential value of CH nanoparticles was about 30 ± 5 mV which considered suitable and stable, whereas another study by Agarwal *et al.*, [51], it was found the zeta value was about 50 mV. The

present study found a very high zeta value, which indicates it has high stability according to this explanation: when the zeta potential value increases, the particles repel each other with more force, the tendency of aggregation decreases, and the particles form a more stable profile [52]. Dynamic light scattering technique analyses the fluctuation of the intensity of the scattered light due to the movement of particles in the diluted nanoparticles sample. The results of DLS analysis showed that CH-ALG nanoparticle size was 542.27 nm that was larger than nanoscale and the sizes, which were acquired from AFM because DLS measures the "hydrodynamic diameter". Accordingly, the real diameter of these particles can be considerably smaller than this [53]. On the other hand, the polydispersity was 0.244, which is in agreement with Mohammadbaghban *et al.*, [54] study that found the PDI was 0.210± 0.087. A low PDI value is considered ideal, as it indicates higher particle homogeneity in the formulation.

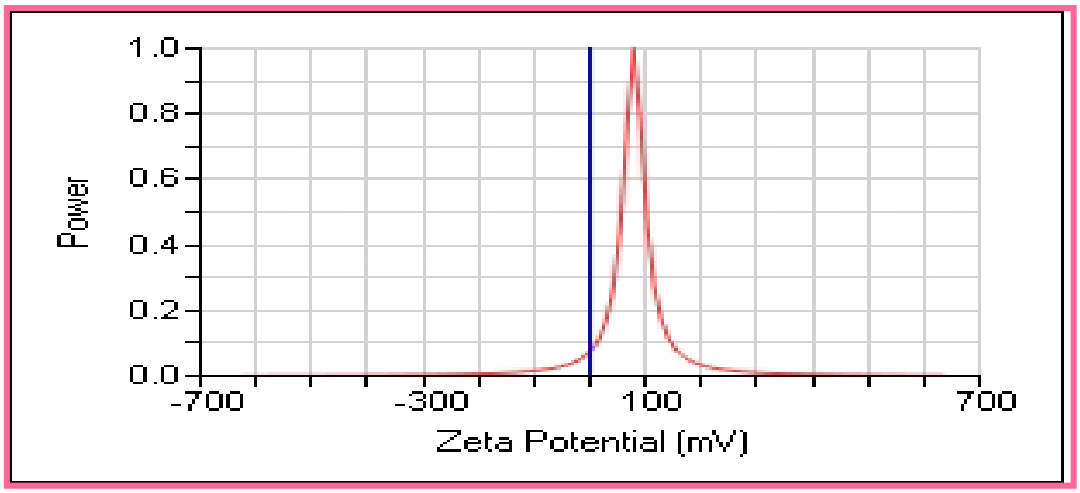

Figure 10- The Zeta Potential Measurement of CH-ALg Nanoparticle

Scanning electron microscope analysis of chitosan-alginate nanoparticle

The morphology and size distribution of CH-ALg nanoparticle synthesized as shown in Figure 11. The SEM picture shows that particles are shown to be in The form of bundles and threads with the morphology of a leaf and this result is confirmed by Govindan *et al.*, [55] who found the same bundles shape.The average particles size and distribution are determined randomly on the (SEM). The main particles size of the prepared composite nanoparticles is about (14 to 86 nm) which is confined to the nano range.

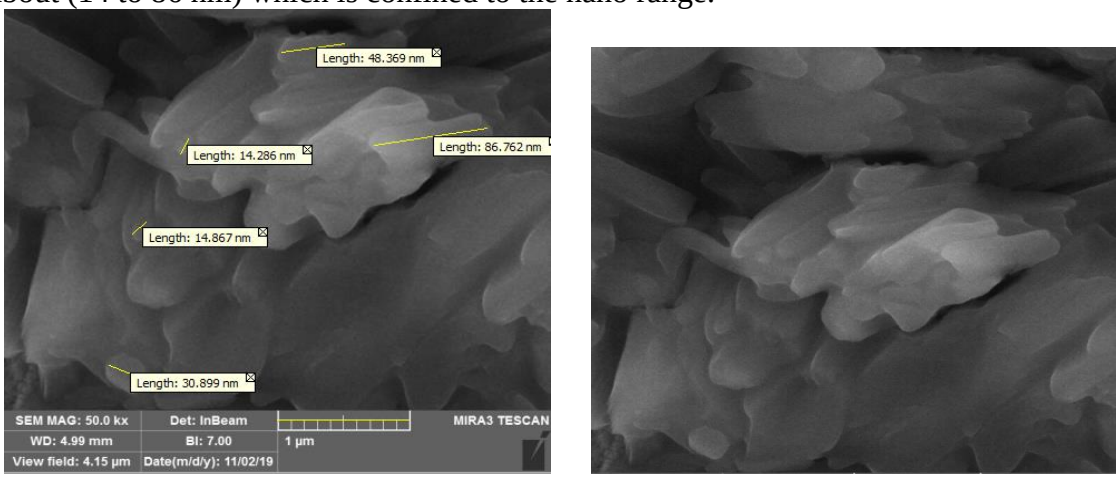

Figure 11- SEM image of CH-ALg nanoparticle

The antibacterial activity of chitosan-alginate nanoparticle

Chitosan-alginate nanoparticle antimicrobial effect was evaluated against studied bacterial pathogens which included MDR Gram-negative bacteria (*Salmonella typhi*), (*Pseudomonas aeruginosa*), (*Enterobacter cloacae*), and Gram-positive bacteria (*Staphylococcus aureus*) bacteria that selected according to the susceptibility testing because it exhibit highly resistance ratio toward many types of antibiotics .as shown in Figure 12 and Table 5 CH-Alg nanoparticle had been shown bactericidal action against *S.typhi* and *P.aeruginosa* while it showed bacteriostatic action against *Staphylococcus aureus* and *E.cloacae* with 4.7×10 and 4.3×10 CFU/ml respectively because of the selected MDR isolate was differentiated in resistance toward 13 different types of antibiotic which were used in the present study, for example, *E.cloacae* was highly resistant toward 10 different types of antibiotics. While *Pseudomonas aeruginosa* and *S.typhi* showed intermediate resistance about 7 or 8 antibiotics out of 13 which were used in present study. On the other hand The agar plate of negative control contained 10^8 CFU/ml while, the agar plate of doxycycline antibiotic with a final concentration of 16 µg/ml according to CLSI MIC resistance rate was contained more than 3×10^3 CFU/ml. Chitosan's nanoparticle antibacterial properties are dependent on the electrostatic interaction of NH_2+ groups of chitosan and phosphoryl group of lipids on the bacterial cell membrane [56]. Chitosan possesses a better antibacterial effect toward Gram positive than Gram negative bacteria, due to their cell walls diversity. In Gram-positive, the cell wall consists entirely of peptidoglycogen [57]. Peptidoglycan layer is composed of networks having pores that allow foreign molecules to reach the cell easily. However, in Gram-negative bacterium, cell wall consists of a thin peptidoglycogen membrane and an outer membrane consisting of lipopolysaccharide, lipoprotein, and phospholipids. The outer membrane is a strong barrier to foreign molecules because of its bilayer structure [58].

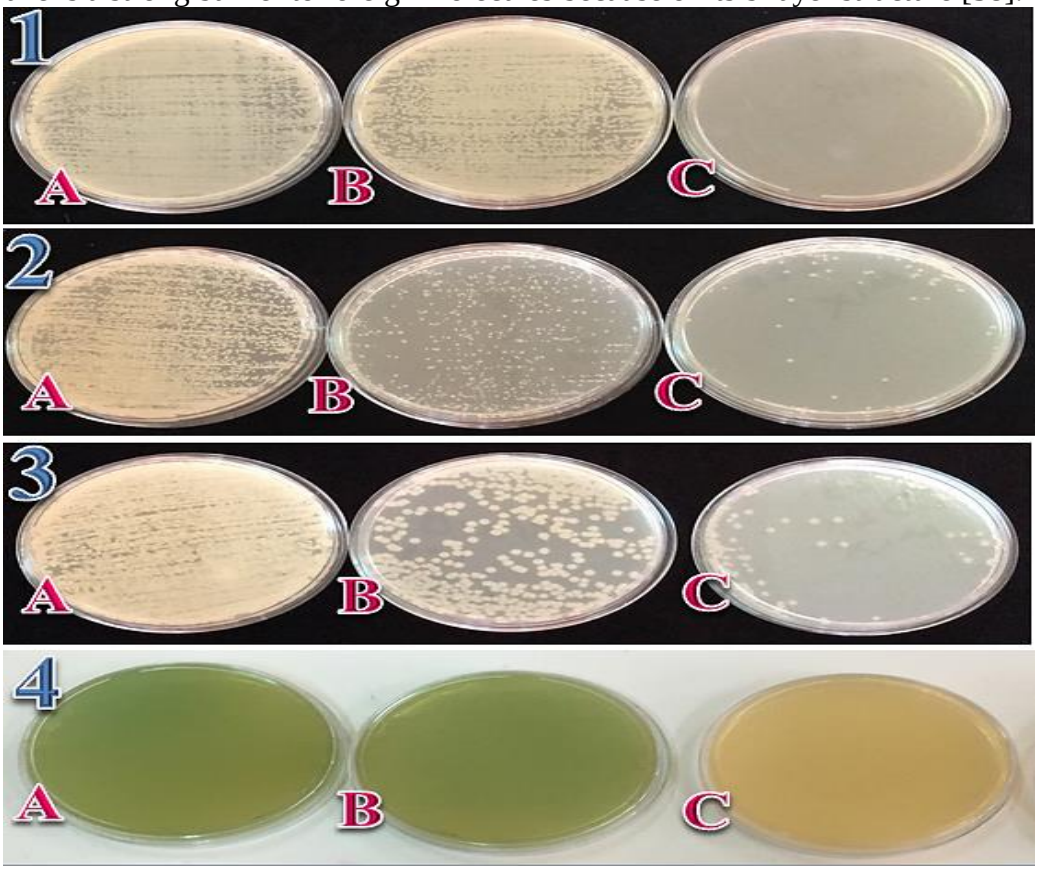

Figure 12-The Antibacterial Activity of CH-ALg nanoparticles and doxycycline antibiotic against 1: *Salmonella typhi, 2*: *Staphylococcus aureus*, 3: *Enterobacter cloacae*, 4:

Pseudomonas aeruginosa, A: Negative Control, B: Doxycycline Antibiotic, C: CH-ALg nanoparticle

Table 5-The Results of Bacterial Viable Cell Count in the Antibacterial Activity Experiment of CH-ALg NANOPARTICLEs.

Bacterial isolates	Negative control	Doxycycline	Chitosan- alginate nanoparticle
S.typhi	10^8	More than 3×10^3	Zero
P.aeruginosa	10^8	More than 3×10^3	Zero
E.cloacae	10^8	More than 3×10^3	4.7×10
S.aureus	10^8	More than 3×10^3	4.3×10

The synergistic effect of chitosan alginate nanoparticle loaded with doxycycline antibiotic

Contributing to the antimicrobial activity of DOX on a broad range particular pathogens, it is reportedly one of the most antibacterial drugs for the treatment of infectious diseases globally [59]; since it used and loaded with CH-ALg NANOPARTICLEs to strengthen drug delivery and treatment effectiveness. Different concentrations were used for chitosan in the synergistic effect (0.3 mg/ml) and (0.15mg/ml) while doxycycline (16 µg/ml) and (8 µg/ml).the results showed that the negative control plate contained 10^8 CFU/ml and more than 3×10^3 CFU/ml in the plate of doxycycline alone, while in CH-ALg NANOPARTICLE no colony appeared in *S.typhi* and *P.aeroginusa* as shown in Figures 13 and 14. In addition 4.7×10 CFU/ml appeared in *E.cloacae* respectively as shown in Figure 15, while the growth disappeared in the plates of doxycycline loaded by CH-ALg NANOPARTICLE and this suggests the occurrence of synergistic effects between prepared and nanoparticles antibiotics against Gram-positive and Gram-negative bacteria compared to DOX alone. These results agreed with Yadav *et al.*, [60] study who found an Increased inhibition potential of DOX antibiotic-loaded CH-ALg microspheres, especially in comparison to native DOX, that confirms the microspheres' antimicrobial potency. The use of these DOX-loaded nanoparticles to treat infections caused by enteric bacteria is also promising [61].

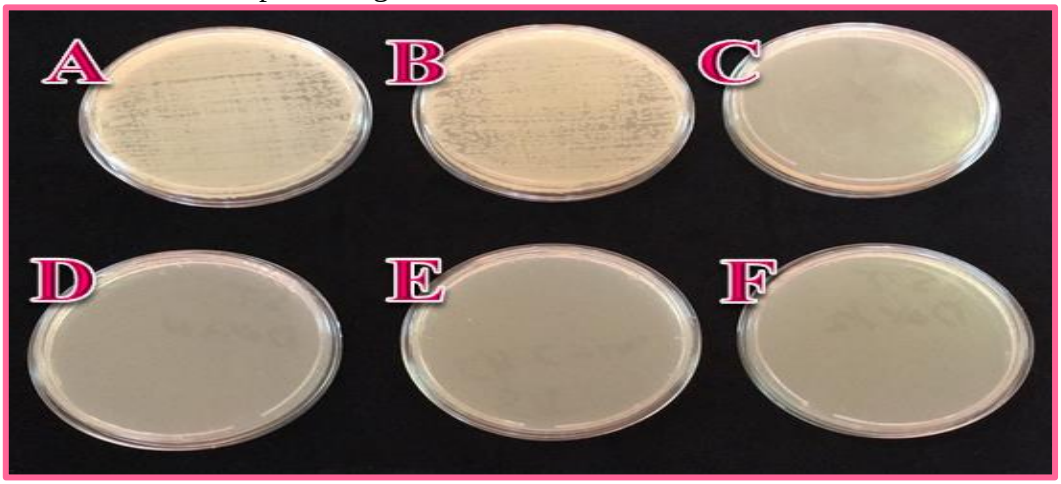

Figure 13-The Antibacterial Activity of CH-ALG NANOPARTICLE Loaded with Doxycycline against *Salmonella typhi* (A): Negative Control, (B): Doxycycline alone, (C): CH-ALg nanoparticle, (D): CH-ALg nanoparticle 0.3mg/ml loaded Doxycycline 16µg/ml, E: Doxycycline 8µg/ml Chitosan 0.3mg/ml,(F): Doxycycline 16 µg/ml Chitosan 0.15/mg ml.

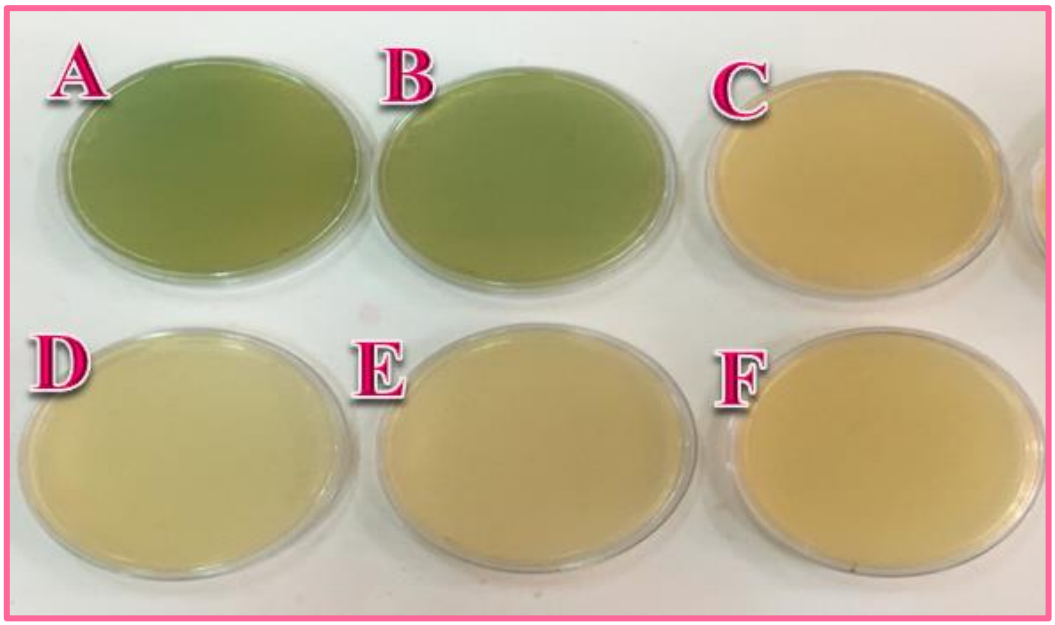

Figure 14-The Antibacterial Activity of CH-ALg nanoparticle Loaded with Doxycycline against *Pseudomonas aeroginusa* (A): Negative Control, (B): Doxycycline alone, (C): CH-ALg nanoparticle, (D): Doxycycline 16µg/ml loaded CH-ALg nanoparticle 0.3mg/ml, E: Doxycycline 8µg/ml Chitosan 0.3mg/ml,(F): Doxycycline 16 µg/ml Chitosan 0.15/mg ml.

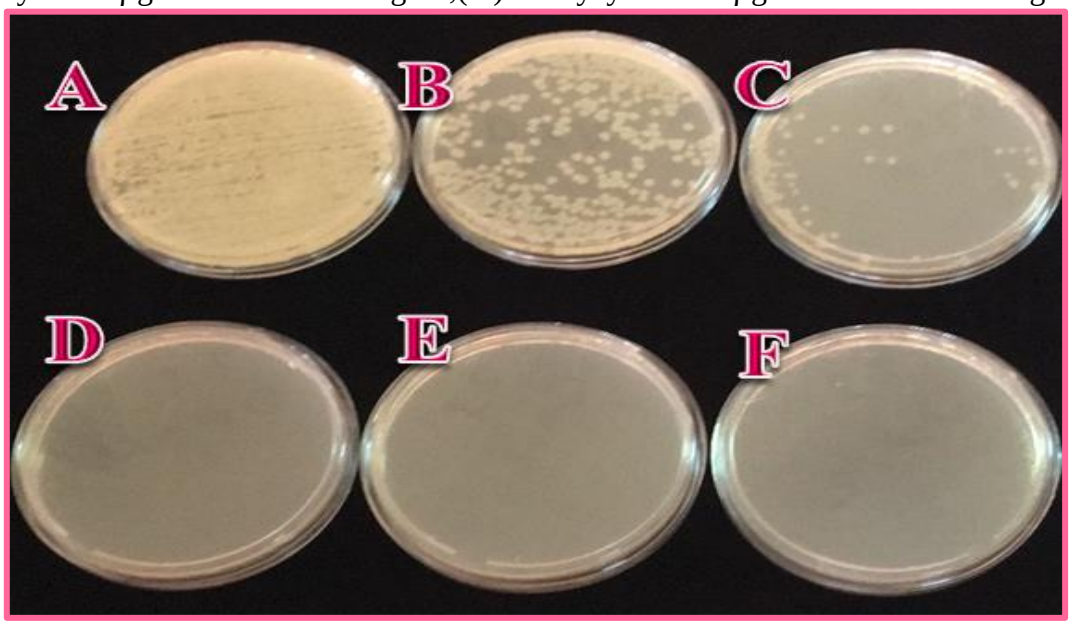

Figure 15-The Antibacterial Activity of CH-ALg nanoparticle Loaded with Doxycycline against *Enterobacter cloacae* (A): Negative Control, (B): Doxycycline alone, (C): CH-ALg nanoparticle, (D): Doxycycline 16µg/ml loaded CH-ALg nanoparticle 0.3mg/ml, I: Doxycycline 8µg/ml Chitosan 0.3mg/ml,(F): Doxycycline 16 µg/ml Chitosan 0.15/mg ml.

In Vitro Behavior of CH-Alg nanoparticle in SGF and SIF Buffers

The effect of both gastro and intestine conditions on CH-Alg nanoparticle was investigated. At SGF (low Ph) buffer the size of CH-Alg nanoparticle decrease gradually with time as shown in Figure 16. This can be explained by the presence of chitosan in the nanostructure. The high number of NH_2 groups in chitosan was protonated and subsequent increase the interaction with Alginate that lead to make nanostructure more stable in gastric enzymes. At PH 1.2 the protonation of the amine group rises and leads to an increase in the interaction between polysaccharide and chitosan. Moreover, the structure of chitosan became

an expanded form that aid to the strong affinity for the phospholipid leading to compact nanostructure more and more with reducing the degree of releasing bioactive material. Whereas the size of nanostructure was observably increased and subsequently decreased in SIF buffer at PH 7.3 as shown in Figure 17. This can be described by decreasing the connection between phospholipid and chitosan. Media tend to enter into the particles lead to increase their diameter due to the weak electrostatic force between chitosan and polysaccharide because the cationic group in chitosan is reduced. These effects are similar to the previous ones reported by Chopra *et al.*, [23] which investigated the presence of chitosan as a coating agent to the nanoliposomes could improve their stability in SGF than in SIF buffer.

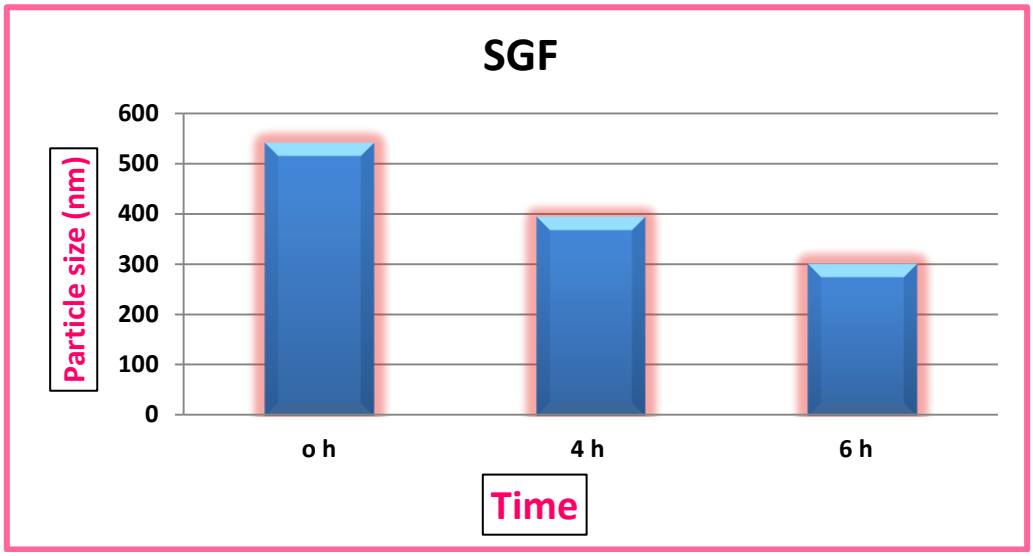

Figure 16- Particle Size of CH-Alg NANOPARTICLE Incubated in SGF at 37 °C

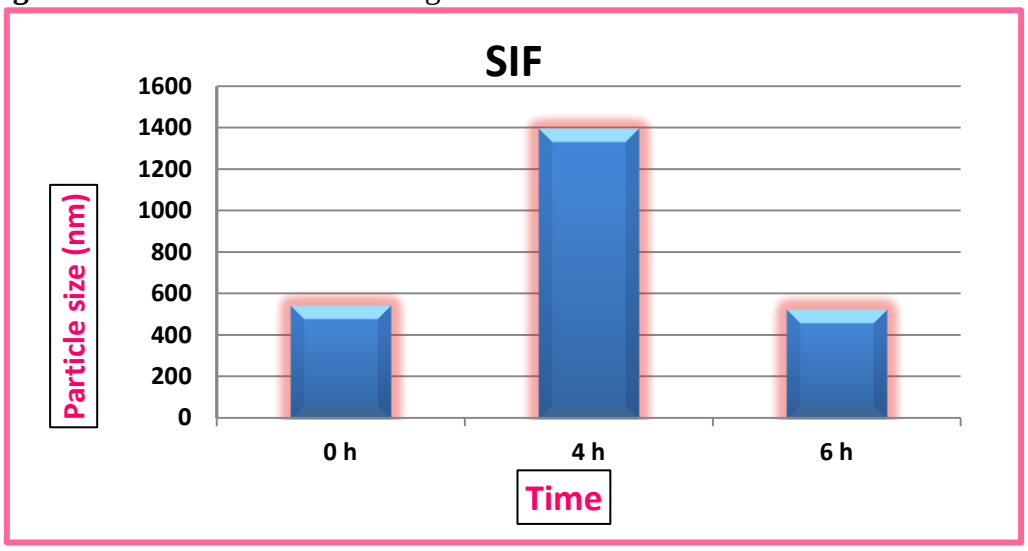

Figure 17-Particle Size of CH-Alg NANOPARTICLE Incubated in SIF at 37 °C

Conclusions

Chitosan alginate nanoparticle was synthesized by ionotropic gelation method. The prepared CH-ALg nanoparticles was characterized by AFM, and the results indicated the synthesized chitosan alginate nanoparticles were in the range of nanometer with an average diameter size of 61.91 nm. The FTIR spectrum indicated the involvement of functional activities of chitosan when it interacts with sodium alginate and calcium chloride. FTIR analysis also was done for doxycycline antibiotic and CH-ALG nanoparticle loaded with doxycycline and the results showed that some doxycycline characteristic peaks were also

existed in the mixture of CH-ALG nanoparticle loaded with doxycycline. Whereas other peaks were shifted which indicate the incidence of interaction between CH-ALG nanoparticle and DOX antibiotic. The stability of synthesized CH-ALg nanoparticles was measured by Zeta potential measurement and show high stability with a 79 mV ratio. The SEM picture shows that particles are shown to be in the form of bundles and the size of nanoparticles was in the range of (14-84nm). The optimized condition for chitosan synthesis showed that chitosan has more antibacterial activity in an acidic environment with PH 5 rather than in alkaline environments. The susceptibility test of antibiotic for collected bacteria indicates it can be resistant to different antibiotics that are commonly applied in the treatment of diarrhea and intestinal infection. The prepared nanoparticles exhibit significant antibacterial action toward the selected MDR bacterial isolates of the present study. CH-ALg nanoparticles were loaded with DOX antibiotic and the result proved that DOX-loaded nanoparticles have great inhibition effectiveness in comparison to doxycycline alone against G-ve and G+ve bacteria. The CH-ALg nanoparticles was stable in different biological fluids such as SGF and SIF.

ETHICAL CLEARANCE This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific
Research, Iraq.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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