Republic of Iraq
Ministry of Higher Education and Scientific Research
Al-Nahrain University
College of science

Effect of Carboxymethyl Nanochitosan Loaded with Streptomycin on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A Thesis

Submitted to the council of College of Science/Al-Nahrain University as a partial fulfilment of the requirements for the Degree of Master of Science in Biotechnology

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April. 2017 Rajab.1438

بسم الله الرحمن الرحيم

﴿قَـٰلُوا۟ سُبْحَـٰنَكَ لَا عِلْمَ لَنَـآ إِلَّا مَا عَلَّمْتَنَآ إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ﴾

صدق الله العظيم

((البقرة 32))

Acknowledgement

In the name of Allah, the Most Gracious and the Most Merciful Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis.

Special appreciation goes to my supervisors; ***Dr. Ali shihab*** *for his supervision, constant support, and invaluable help of constructive comments and suggestions throughout the experimental and thesis works have contributed to the success of this research.*

My thanks are due to the Dean of College of Science as well as to the head and staff of the Department of Biotechnology.

I would like to extend my thanks to Dr. Shehla Mahdi Saleh and Dr. Mohammad Mahmoud for their help.

Last but not least, it would not have been possible to write this thesis without the help and support of the kind people around me, my deepest gratitude goes to my family and for their endless love, prayers and encouragement.

To those who indirectly contributed in this research, your kindness means a lot to me. Thank you very much.

MUNA

Supervisor Certification

I, certify that this dissertation entitled **"Effect of Carboxymethyl Nanochitosan Loaded with Streptomycin on *Staphylococcus aureus* and *Pseudomonas aeruginosa*"** was prepared by the student **"Muna Mohammad Khairy Najeeb"** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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Summary

This study aimed to synthesise carboxymethylnanochitosn as nanochitosan derivative from nanochitosan synthesized from soluble chitosan (low molecular weight) and encapsulate streptomycin then characterize and bioassay them on pathogenic gram positive and gram negative bacteria and Rhabdomyosarcoma cell- line. Ionic gelation method was used to synthesize nanchitosan from soluble and insoluble chitosan (high molecular weight). Dissolved chitosan was found to be superior to non-soluble in the preparation of nanochitosan. The concentration of (2 mg/100 of 2% glacial acetic acid) chitosan was selected as the highest concentration to obtain the highest yield of nanochitosan with a constant ratio of Tripolyphosphate. Carboxymethylnanochitosan was synthesized from nanochitosan in an acid-base method with a yield of 420 mg / 3g nanochitosan. It has been appointed a maximum absorbance (λ max) for streptomycin, carboxymethylnanochitosan and carboxymethylnanochitosan encapsulate streptomycin will be 275, 265 and 270 nm respectively. It has been appointed the standard curves for streptomycin, carboxymethylnanochitosan and carboxymethylnanochitosan encapsulate streptomycin compounds and calculated the absorbency values (α) which were 0.002, 0.004 and 0.006 respectively. The loading efficiency of carboxymethylnanochitosan with streptomycin was 90%. FTIR spectroscopy of carboxymethylnanochitosan and carboxymethylnanochitosan with streptomycin showed that, absorption peaks at the same frequencies 3000 -3800 cm^{-1} with the deep in stretching of the absorption percent for carboxymethylnanochitosan with streptomycin. Scanning electron Microscope (SEM) three-dimensional morphological structures of chitosan, nanochitosan, carboxymethylnanochitosan and carboxymethylnanochitosan with streptomycin showed different forms. It was found the aggregation of

carboxymethylnanochitosan spheres and entrapped the streptomycin within carboxymethylnanochitosan. Results of Atomic force spectroscopy (AFM) three dimensions images illustrated that the concentration 2mg/100 ml of 2% glacial acetic acid of soluble chitosan gave 54.64 nm average diameter and 90% of nanoparticles with diameter 80.00 nm. Insoluble chitosan at concentration 2mg/100 ml of 2%glacial acetic acid gave average diameter 105.52 nm and 90% of nanoparticles with diameter 170 nm. According to the process of carboxymethylnanochitosan preparation, it was obtained nanoparticles size with average diameter 35nm and 90% of the 56nm diameter. After loading carboxymethylnanochitosan on streptomycin, the average size of the nanoparticles was increased to reach 37nm and 90% of particles diameter increased to 62nm. These results confirmed the loading process of carboxymethylnanochitosan with streptomycin. Results of particle size distribution showed the median particle size of carboxymethylnanochitosan was 770 nm and 526 nm for carboxymethylnanochitosan with streptomycin. Nanochitosan prepared from insoluble chitosan showed no inhibition zone on *Staphylococcus aureus* and *Pseudomonas aeruginosa* whereas, from soluble chitosan (2 and 20 mg), nanochitosan gave inhibition zone against *Pseudomonas aeruginosa* only in comparison with the results of sensitivity to streptomycin. It was found that carboxymethylnanochitosan was not affected on the response of isolated. *Staphylococcus aureus* was become sensitive against streptomycin starting from concentration 100 µg/mL while; *Pseudomonas aeruginosa* became sensitivity from concentration 300 µg/mL and was more resistant than *Staphylococcus aureus.* The results of sensitivity carboxymethylnanochitosan with streptomycin were similar to streptomycin for each isolate. *In vitro* anticancer test efficacy of streptomycin, carboxymethylnanochitosan and carboxymethylnanochitosan with streptomycin against Rhabdomyosarcoma cell line was evaluated. It was found that 50 µg/ml of

carboxymethylnanochitosan with streptomycin more effective than 100µg/ml of streptomycin and growth inhibition was 37.5%, 38.5% respectively after 24 hrs of incubation.

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List of Abbreviation

Short name	Complete name
AC	Absorbance of Control
AFM	Atomic Force Microscope
AS	Absorbance of Sample
CMNC	Carboxymethyl nanochitosan
CMNC-S	Carboxymethyl nanochitosan-streptomycin
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared spectroscopy
G+ve	Gram positive
G-ve	Gram negative
KDa	Kilo Dalton
M.H.A	Muller Hinton agar
MSA	Mannitol Salt Agar
Min	Minutes
MW	Molecular weight
N.A	Nutrient agar
N.B	Nutrient broth
NC	Nanochitosan
RD	Rhabdomyosarcoma
S	Streptomycin
SEM	Scanning Electron Microscope
TPP	Tri-polyphosphate
XRD	X- Ray Diffraction

Chapter One

Introduction And Literature Review

1. Introduction and Literature review

1.1- Introduction

Nanobiotechnology is now regards the core of developing all the fields of life sciences and biomedical including the diagnosis, therapy, drugs delivery. In the fields of therapy and drugs delivery, the efficacy of many therapies is almost limited by their potential to reach the target of therapeutic action belong to various problems such as low life time, poor bioavailability, in vivo low stability, solubility, intestinal absorption problems, therapeutic effectiveness, side effects, and exceed the safe dose concentrations. In most cases, only a small quantity of advised dose reaches the target site, while the most of the drug distributes throughout the rest of the human body in accordance with its physicochemical kinetics and biological properties (Ochekpe *et al*., 2009). Streptomycin (S) is a bactericidal antibiotic drug under aminoglycosides group and produced from *Streptomyces griseus* (Singh and Mitchison, 1954). It is known to have toxic effects causing nephrotoxicity and neuroparalysis (Burman *et al*., 2001). Nanobiotechnology applications in drug delivery systems have played new capabilities in direct-targeted effect and slowing the release of drugs. For the continuous release of drug, the one of the important technique is encapsulating the active drug ingredient in polymer components, thus, the dose and frequency of administration would be reduced (Das *et al*., 2005). Chitosan is derivative of chitin and is a poly aminosaccharide with many significant biological like biodegradable, biocompatible, bioactive and chemical properties. All of these properties make chitosan and its derivatives preferred to use in many biomedical fields (Ding *et al*., 2006). Carboxymethylchitosan (CMC) has received more attention because of its good water solubility, and it is safe to be applied in medicine and pharmaceutics because of their compatibility with human body environment (Zhang *et al*., 2004).

CMC is prepared by means of carboxymethylation, as some of the –OH groups of chitosan were substituted by $–CH_2COOH$ groups. Therefore, the reactive ligands such as (–COOH) and ($–NH_2$) groups are still available to chemical modifications to improve its physiochemical properties (Janvikul and Thavornyutikarn, 2003).

The development of microorganism resistance to antibiotics is a major challenge for microbiologists, antibiotics and doctors because of the need to produce a new generation of antibodies to combat this resistance. Therefore, nanobiotechnology played a large role in the allocation of antibiotics in the areas of infection exclusively and accurately the causing the infection without spreading to other areas in the human body (Singh *et al*., 2014).

Cancer continues to be a prevalent and lethal disease, despite advances in tumor biology research and chemotherapy development. Nanobiotechnology is widely used to detect and detect early cancer diseases and to induce highly specialized effective treatments to cancer cells itself. Nanobiopolymers are widely used for these purposes (Ediriwickrema and Saltzman, 2015).

Purpose of the study

Preparation and fabrication of nanochitosan derivative to encapsulate streptomycin and test it is susceptibility on pathogenic bacteria and RD cell line.

1.2 Literature Review

1.2.1- *Staphylococcus aureus*

The name *staphylococcus aureus* comes from the Greek words "staphyle" meaning a bunch of grapes, "coccus" which mean round-shape and "aureus" for golden because most colonies have a characteristic orange-yellow coloring on the traditionally used agar plates (Samanthamoorthy, 2007). *Staphylococcus aureus* belongs to the Micrococcaceae family and is part of the genus *staphylococcus*, which contains more than 30 species such as *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*. Among the staphylococcal species, *S.aureua* is by far the most virulent and pathogenic for humans. *S.aureus* is a 1µm in diameter, gram-positive cell that in the laboratory may be observed as single cells, in pairs or as grape-like irregular cluster, it is characterized as coagulase and catalase positive, non-motile, non- spore forming and as facultative, anaerobic it grows in yellow colonies on nutrient rich media and is referred to as the yellow staphylococci (winnwashington, 2006).

S. aureus causes a wide range of infections from a variety of skin, wound and deep tissue infections to more life threating conditions such as pneumonia, endocarditis, septic arthritis and septicemia. In addition, *S. aureus* may also cause food poisoning, scalded skin syndrome and toxic shock syndrome, through the production of different toxins (winnwashington, 2006). The pathogenicity of *Staphylococcus aureus* is due to the toxins and antibiotic resistance. *S. aureus* is a major cause of nosocomial and community-acquired infections (Bhatia and Zahoor, 2007). *S.aureus* expresses many potential virulence factors such as surface proteins that promote colonization of host tissues invasions that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production), immunological disguises (Protein A, coagulase), Membrane-damaging toxins that

lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin), exotoxins that damage host tissues or otherwise provoke symptoms of disease and inherent and acquired resistance to antimicrobial agents (Sibbald *et al*., 2006; Podbielska *et al*., 2011).

1.2.2- *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of genus pseudomonas, which belong to the Psedomonadaceae family. Its gram-negative, aerobic rod, glucose none fermenting (Kiss *et al*., 1997). *P. aeruginosa* is 0.5-1.5µm in diameter and 1.5-3.0µm in length, not spore forming, some of these require minimal nutrient as carbon source and others require for growth (Vissillier *et al*., 2001). All strains are motile by means of a single polar flagellum, commonly found in soil and water. *P. aeruginosa* resistant to high concentrations of salts and dyes, weak antiseptic and many commonly used antibiotics (Todars, 2004). This type of bacteria produce a number of pigments that inhibit growth of other bacteria, the most important pigment pyocin (blue soluble in water) and pyoverdin (green yellowish also called pseudobactin) that are toxic to the host cell, also produce other pigments such as pyorubin (red) and pyomelanin (brown) (Ravel and Cornelis, 2003). *P. aeruginosa* is an opportunistic pathogen and causes urinary tract, respiratory tract and skin infections (Stover *et al*., 2000). In addition, soft skin infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly with a patient who are immunosuppressed.

P. aeruginosa is responsible for the nosocomial infections worldwide (Blance *et al*., 1998). Often these infections are hard to treat due to the natural resistance of the species, as well as to it is the remarkable ability to acquire further mechanisms of resistance to multiple groups of antimicrobial agents.

P. aeruginosa represents a phenomenon of antibiotic resistance and demonstrates practically all known enzymatic and mutational mechanisms of bacterial resistance (Pechere and Kohler, 1999).Virulence determinants of pathogenic *Pseudomonas aeruginosa* include the adhesions fimbria, polysaccharide capsule (glycocalyx) and alginate slime (biofilm); Invasions elastase, alkaline protease, hemolysins (phospholipase and lecithinase), cytotoxins (leukocidin), siderophores and siderophore uptake systems, and pyocyanin diffusible pigment; Motility/chemotaxis, flagella; toxins, exoenzyme S, exotoxins A and lipopolysaccharide (Todars, 2004).

1.2.3- Streptomycin

Streptomycin is a water-soluble aminoglycoside derived from *Streptomyces griseus*. It is marketed as the sulfate salt of streptomycin. Streptomycin was first isolated in 1943 by Albert Schatz. Aminoglycosides bind to the 30S ribosome and block protein synthesis. The aminoglycosides also slow down protein synthesis that has already initiated and induce misreading of the mRNA. Aminoglycosides are useful primarily in infections involving aerobic, gram- negative bacteria, and some gram- positive bacteria. Streptomycin Sulfate Injection This product is used in the treatment of acute infections caused by various kinds of sensitive bacteria strains, like respiratory tract infections (pneumonia, laryngopharyngitis and bronchitis), urinary tract infections (Amano *et al*., 2008).

The efficiency of many drugs is often limited by their potential to reach the site of therapeutic action due to various problems such as poor bioavailability, *in vivo* stability, solubility, intestinal absorption, sustained and targeted delivery to site of action, therapeutic effectiveness, side effects, and inconstancy of drug concentration in plasma which either fall below the minimum effective concentrations or surpass the safe therapeutic concentrations. In most cases, only a

small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biological properties (Ochekpe *et al*., 2009).

1.2.4- RD cell line

Rhabdomyosarcoma (RD) is a malignancy that arises from skeletal muscle precursors (Saab *et al*, 2011). It is the most common type of soft tissue sarcoma in children and adolescents less than 20 years old, with an incidence of 4.5 cases per million children/adolescents per year around the world (Ognjanovic *et al*, 2009).

There are two major types of RD, embryonal and alveolar, which differ markedly in their outcomes. Embryonal RD usually presents in children less than 10 years old and has a 5-year survival of close to 75%. On the other hand, Alveolar RD occurs at about the same incidence throughout childhood and adolescence and is associated with a poorer prognosis with a 5-year survival rate of less than 50%. Patients with relapsed RMS have an even worse prognosis, with survival ranging from 10 to 30% (Pappo *et al*., 1999).

1.2.5- Chitosan

Chitosan is a polymer of N-acetyl glucosamine that can be obtained through deacetylation of chitin (Sevda and McClureb, 2004). The amine and hydroxyl(OH) groups give chitosan many special properties making it applicable in many areas and easily available for chemical reactions chitosan is safe, nontoxic and can interact with polyanions to form complexes and gels (Sunil *et al*., 2004; Se and Niranjan, 2005). As shown in figure (1-1)

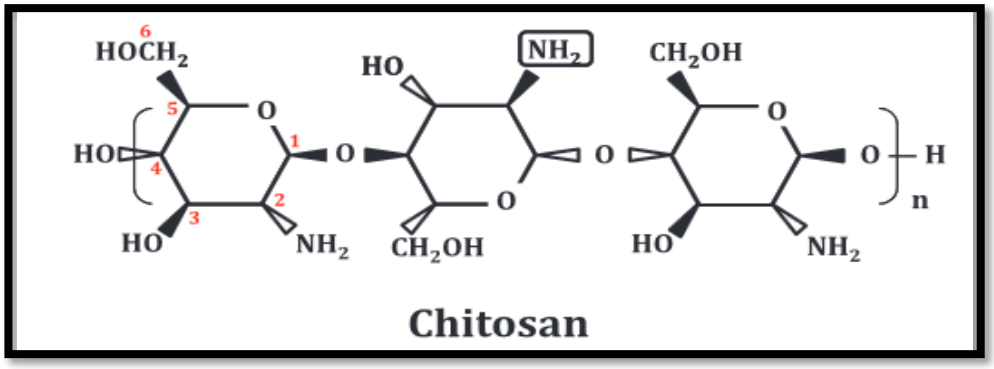


Figure (1-1): Chemical structure of chitosan (Pradip *et al*., 2004)

It possesses many significant biological (biocompatible, biodegradable, bioactive) and chemical properties (polycationic, hydrogel, reactive group such as OH and NH_2) all of these properties make chitosan and its derivatives widely used in many biomedical fields (Ding *et al*., 2006).

When chitosan has a high degree of protonation of the amine functions, it displays the capacity to form hydrogels in presence of specific polyanions. This process derives from inter- and intramolecular cross-linkages mediated by the anionic molecules (Janes *et al*. 2001, Terbojevich and Muzzarelli 2009) and has been used to produce chitosan-based nanoparticles by ionic gelation or polyelectrolyte complexation. It should be noted that *ionic gelation* is the preferred term when chitosan gelation is induced by small anionic molecules, such as phosphate, citrate, sulfate, while a polyelectrolyte complexation is considered to occur when anionic macromolecules are used instead of small molecules (Bhattarai *et al*. 2010).

1.2.5.1- Chitosan derivatives

1.2.5.1.1- Carboxymethylchitosan

Carboxymethyl chitosan is water soluble chitosan derivative is prepared by means of carboxymethylation, as some of the OH groups of chitosan were substituted by carboxymethyl (CH2COOH) groups (Chen *et al*, 2005). Since the chitosan is insoluble in neutral to alkaline pH and the solubility observed only in the acidic aqueous solution below pH 6.5, the solubility of chitosan can be improved by depolymerization and chemical modifications (Cravotto *et al*., 2005). Figure (1-2) Shows the chemical structure of CMC by Reem and Riham **(**2013). Many of water-soluble derivatives have been prepared but compared with other water-soluble chitosan derivatives; carboxymethyl chitosan has been widely studied because of its ease of synthesis and possibilities of broad of applications (Mourya *et al*., 2009). The properties and application of carboxymethyl chitosan are dependent on its structural characteristics mainly the average degree of substitution and the locus amine or hydroxyl groups of the carboxymethylation (Fernanda *et al*., 2005).

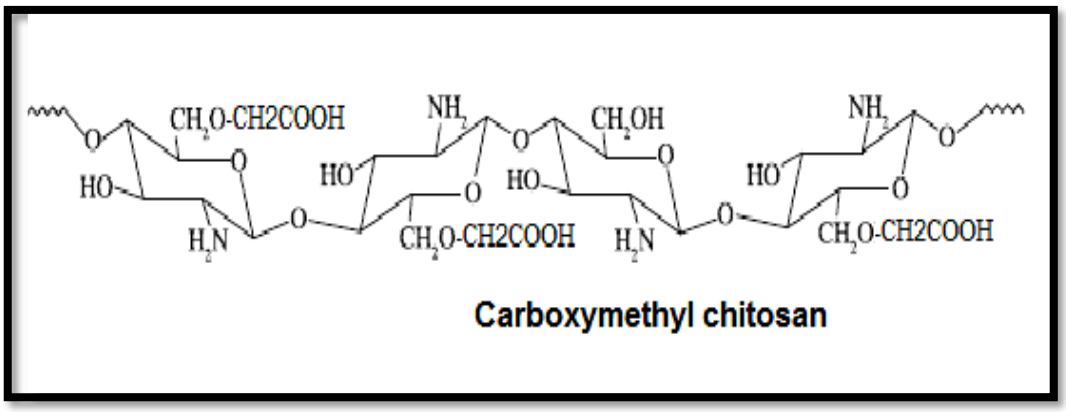


Figure (1-2): Chemical structure of carboxymethyl chitosan (Reem and Riham, 2013).

Carboxymethylated chitosan has received more attention because of its good water solubility and it is more convenient to be applied in medicine because it fits

the neutral environment of the human body (Zhang *et al*., 2004; Chen and Park, 2003). Chitosan and derivatives are used in various fields: pharmaceutical (Ng and Swami, 2005) and biomedicine (Berger *et al*., 2004) as carrier of controlled drug delivery, cosmetics (Rinaudo, 2006), agriculture (Chung *et al*., 2003) and food industry (Beysseriat *et al*., 2006).

1.2.6- Nanotechnology

Nanotechnology is the design, characterization, production and application of materials, devices and systems by controlling shape and size in the nanoscale (Abad *et al*., 2005). Nanotechnology is derived from the Latin word "nano" which means dwarf (Gulsun, 2009). Nanotechnology is a multidisciplinary field, as it combines the knowledge from different disciplines: chemistry, physics and biology amongst others (Schmid, 2006; Schmid, 2010). The rough estimate for defining nanotechnology is the measurement of a material or devices at one or more dimensions in the range of 1-100 nm. To put this into perspective, "a human hair is approximately 80,000 nm wide and a red blood cell approximately 7000 nm wide (Shand Hope and Kathy Jo Wetter, 2006).

Nanoparticles are less than a few 100 nm this reduction in size brings about significant change in their physical properties with respect to those observed in bulk materials. They can be metallic, polymer-based or a combination of materials (Ran and Kalaichelvan, 2013). The increase in the surface area to volume ratio is also a consequence of the reduction in size. It leads to the appearance of surface effects related to the high number of surface atoms as well as to a high specific area, which are important from the partial point of view (Salata, 2004).

The absence of generally accepted, strictly established definition of the term nanotechnology is explained by a wide spectrum of various technologies that nanotechnology covers, which are based on various types of physical, chemical and

biological processes realized on the nano level. The absence of generally accepted, established time span for the beginning of nanotechnology development is explained by the fact that nanotechnology has its background in the distant past when people use it without knowledge of it (Tolochko, 2009).

The first time of purposely created and applied technological processes and means. Which were subsequently termed nanotechnology is usually, connected with the well-known lecture of Mr. Richard Feynman a professor of California institute of technology delivered in 1959 at the session of the American physical society. In this lecture called, "there is a lot of space down there" for the first time the possibility to create nano-sized products with the use of atoms as building particles were considered. Nowadays this lecture is referred to as the origin of the nanotechnological paradigm. Norio Taniguchi introduced the word "nanotechnology" for the first time into a scientific world in 1974. The idea of nanotechnological strategy. Which were put forward by Feynman, were developed by Eric Drexler in his book "vehicles of creation: the arrival of the nanotechnology era" published in 1986 (Tolochko, 2009).

Nanotechnology is being developed at several levels: materials, devices and systems. The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications (Salata, 2004). Although much of nanotechnology is still in the research and development phase, nanomaterials are expected, to be used in a wide variety of applications ranging from biomedical drug delivery to electronics, pollution remediation and less toxic mode of manufacturing (Braid and Vogt, 2004).

1.2.7- Synthesis of nanomaterials

The methods used in the fabrication of nanomaterials meaning materials with at least one dimension at the nanoscale level 1-100nm. These include nanostructured surfaces, nanoparticles, etc. methods for fabricating nanomaterials can be generally divided into two groups:

A- Top-down approach:

The top-down approach involves the breaking down of bulk material into nano-sized structure and particle. These approaches are inherently an extension of those that have been used for producing micron-sized products. They involve the slicing or successive cutting off a bulk material to get nano-sized particles or to produce their structure with appropriate properties. This technique is useful to produce nano-sized particles on a large scale by using mechanical force. The problems associated with this are their imperfection of surface structure, surface dislocation, the formation of aggregates and clustering together with difficulty in the controlling morphology of the particles (Kumar, 2014).

B- Bottom-up approach

While nanomaterials have been generated by top-down or in the other word physical methods the bottom-up or in the other word, the chemical approach is found to be more useful. It involves a more effective build up of material from the bottom atom by atom, molecule by molecule or cluster by cluster (Kumar, 2014). Bottom-up methods work in the opposite direction the nanomaterial, such as a nano-coating is obtained starting from the atomic or molecular precursors and gradually assembling it untile the desired structure is formed (Filipponi and Sutherland, 2013). The major advantages of the bottom-up approach lie in the production of nanostructures with fewer defects, more homogeneous chemical composition better control of size and shape (Kumar, 2014).

1.2.8- Nanobiotechnology

The nanotechnology revolution continues today and provides many beneficial tools, which allow us to generate individual molecules at the nanoscale for the application. In nanbiotechnology this combination of nanotechnology and biology refers to scientific applications by involving nano-sized devices, new biological applications and new methods in medical treatments (Garalleh, 2013). Nanobiotechnology is a multidisciplinary field that covers a vast and diverse array of technologies. It is the combination of fields that has led to the birth of a new generation of materials and methods of making them (Shoseyov and Levy, 2008). Therefore, the association of these two technologies "nanobiotechnology" can play a vital role in developing and implementing many useful tools in the study of life (Fakruddin*et al*., 2012). Nanobiotechnology deals with the properties and characteristic of nanoscale individual molecules and their applications involving the interaction of biological nanostructures and their interfacing between biological, chemical and physical systems (Garalleh, 2013).

1.2.8.1- Advantages of nanobiotechnology

Advantages of nanobiotechnology include the effectively of nanoparticles that can be used to deliver and transport drugs (Andrieux *et al*., 2005); various nano products can be accumulated at higher concentrate than normal drugs (Vasir *et al*., 2005). Nanosystems have a capacity of selective localization in inflamed tissues (Allen and Cullis, 2004). Increased vascular permeability coupled with an impaired lymphatic drainage in tumors improve the effect of the nanosystems in the tumors or inflamed tissues through better transmission and retention (Maeda *et al*., 2000). Moreover, drug loading onto nanoparticles modifies cell and tissue distribution, leads to more selective delivery of biologically active compounds to enhance drug efficacy and reduces drug toxicity (Feng *et al*., 2004; Villarroya *et al*., 2004).

Application of nanotechnology in drug delivery system has opened up new possibilities in the sustained and targeted release of drugs (Dinauer *et al*., 2005). Specifically designed nanoparticles can reach less accessible sites in the body by escaping phagocytosis and entering tiny capillaries. Controlled release of the drug from the nano formulations could maintain more constant levels of the drug in the blood stream for longer durations. Sustained release of drug can be achieved by encapsulating the active ingredient in a polymer matrix such that drug find its way through the restrictive cavities in the matrix Thus, the dose and frequency of administration would be reduced (Das *et al*., 2005; Patil *et al*., 2011).

1.2.9- Types of nanobiomaterials

Nanomaterials have defined as materials having sizes smaller than 100 nanometers ($1nm=10^{-9}$) along at least one dimension (length, width or height). (korotcenkov, 2010). Nanomaterials are a new step in the evolution of understanding and utilization of materials. They are investigated as promising tools for the advancement of the diagnostic biosensor, drug delivery and biomedical imaging for their unique physicochemical and biological properties (Dong *et al*., 2012). Nanomaterials have a relatively larger surface area when compared to the same volume or mass of the material produced in a larger form. When the given volume is divided into smaller pieces the surface area increases. So the particle size decreases a greater proportion of atoms are found at the surface compared to those inside. Hence Nanoparticles have a much greater surface area per given volume compared with larger particles. It makes materials more chemically reactive (Houdy *et al*., 2006). Many properties of nanomaterials such as size, shape, chemical composition, surface structure, aggregation and solubility can greatly influence their interaction with biomolecules and cells. Suitable control of these properties and responses of nanostructures can lead to new devices and technologies (Dong *et al*., 2012).

1.2.9.1- Nanoparticles

Nanoparticles are defined as solid colloidal particles that include both nanospheres and nanocapsules. They can prepare by both polymerization methods and synthesis with performed polymers (Fatal and Vauthie, 2002; Vauthier and Bouchemal, 2008). In addition, displaying a sub-micrometer size preferably smaller than 500 nm (Couvreur, 2013). Nanoparticulate systems show promise as active vectors due to their capacity to release drugs (Cruz *et al*., 2006; Amaral *et al*., 2007). Their subcellular size allows relatively higher intracellular uptake than other particulate systems (Furtado *et al*., 2001). They can improve the stability of active substance (Ourique *et al*., 2008). Moreover, can be biocompatible with tissues and cells when synthesized from materials that are either biocompatible or biodegradable (Guinebretiere *et al*., 2002).

1.2.9.2-Nanocapsules

Nanocapsules are defined as nano-vesicular systems that exhibit a typical core-shell structure in which the drug is confined to a reservoir or within a cavity surrounded by a polymer membrane or coating (Letchford and Burt, 2007; Anton *et al*., 2008). The cavity can contain the active substance in liquid or solid form or as a molecular dispersion (Radtchenko *et al*., 2002). Likewise, this reservoir can be lipophilic or hydrophobic according to the preparation method and raw materials used.

Drug-loaded polymeric nanocapsules have exhibited potential applications in the field of drug delivery systems in recent years (Rong *et al*., 2011). Polymeric nanocapsules, which are a specific type of polymeric nanoparticles used for the improvement of biological effects (Frank *et al*., 2015). Advantages of nanocapsule systems as active substance carriers include high drug encapsulation efficiency due to optimized drug solubility in the core, low polymer content compared to other nanoparticulate systems such as nanosphere (Pinto *et al*., 2006; Anton *et al*., 2008).

Different advantages to the use of nanocapsules like, increasing of drug efficiency and reducing of toxicity and tissue irritation (Fessi *et al*., 2010).

1.2.9.3- Nanomicelle

Nanomicelles are self-assembling nanosized (usually with a particle size within a range of 10-100 nm) colloidal particles with a hydrophobic core and hydrophilic shell (Trivedi and Kompella, 2010). Nanomicelles solubilize hydrophobic drugs by entrapping the drugs within a mixed micellar hydrophobic core with a corona composed of hydrophilic chains extending outwards (Velagaleti *et al*., 2010). Nanomicelle are currently successfully used as a pharmaceutical carrier for water insoluble drugs and demonstrate a series of attractive properties. They have demonstrated a variety of favorable properties including biocompatibility, longevity, high stability in vitro and in vivo, capacity to effectively solubilize a variety of poorly soluble drugs, changing the release profile of the incorporated pharmaceutical agents and the ability to accumulate in the target zone based on the enhanced permeability and retention effect (Movassaghian *et al*., 2015). These polymeric micellar nanocarriers have applications in drug delivery primarily such as anticancer therapy, to the brain to treat neurodegenerative diseases, antifungal agents, ocular drug delivery (Kshirsagar *et al*., 2011).

1.2.10-Nanotherapeutics

Nanotherapeutics is important discipline in the field of medicine; especially nanoparticle-based drug delivery for cancer therapy is spreading rapidly which can overcome the limitations of conventional drug delivery systems. Nanometric drug carriers of optimum size and surface characteristics are highly stable and possess high carrying capacity. Moreover, the feasibility of incorporation of both hydrophilic and hydrophobic substances and feasibility of variable routes of administration allow controlled drug release from the matrix and improved drug bioavailability (Gelperina *et al.*, 2005).

1.2.11-Characterization of Nanomaterials

There are various techniques for detecting, measuring and characterizing nanomaterials:

A- Imaging characteristics

1- Scanning electron microscope (SEM)

The electron microscopy-based technique determines the size, shape and surface morphology with direct visualization of the nanoparticles. Therefore, scanning electron microscopy offers several advantages in morphological and sizing analysis. However, they provide limited information about the size distribution and true population average. During the process of SEM characterization, a solution of nanoparticles should be initially converted into a dry powder. This dry powder is then further mounted on a sample holder Whole sample is then analyzed by scanning with a focused fine beam of electrons. Secondary electrons emitted from the sample surface determine the surface characteristics of the sample (Jores *et al*., 2004).

2- Transmission electron microscope (TEM)

Transmission electron microscopy techniques can provide imaging, diffraction and spectroscopic information, either simultaneously or in a serial manner, of the specimen with an atomic or a sub-nanometer spatial resolution. TEM operates on a different principle than SEM, yet it often brings the same type of data. The sample preparation for TEM is complex and time-consuming because of its requirement to be ultra-thin for the electron transmittance. During the TEM characterization nanoparticles dispersion is deposited onto support grids or film then, a beam of electrons is transmitted through an ultra-thin sample it interacts with the sample as it passes through the surface characteristics of the sample is obtained (Molpeceres *et al*., 2000).

3- Atomic force microscopy (AFM)

This technique is also known as scanning force microscopy (a technique that forms images of surfaces using a probe that scans the specimen), AFM is ideal for quantitatively measuring the nanometer scale surface roughness and for visualizing the surface nanotexture on many types of material surfaces including polymer nanocomposite (Joshy *et al*., 2003). The topographical map is generated by tapping the probe onto the surface across the sample. Advantages of AFM are derived from the fact that the AFM is nondestructive technique and this allows the imaging of delicate biological and polymeric nano and microstructures Moreover, the AFM has a very high three-dimensional spatial resolution (Shi *et al*., 2003).

B- Analytical characteristics

1- Scanning UV- visible microscopy.

Among the techniques of nanoparticles characterization, the most commonly used are UV-VIS spectroscopy (Zimbone *et al*., 2011). The theory and mathematical basics UV-Vis techniques are already well known (Evanoff and Chumanov, 2005). In the case of UV-Vis spectroscopy, the intensity of light that is passing through the sample is measured. Nanoparticles have optical properties that are very sensitive to size, shape, agglomeration, and concentration changes. UV-Vis spectroscopy is fast and easy to operate techniques for particles characterization, especially for colloidal suspensions (Leung *et al*., 2006; Huang *et al*., 2007). There are several advantages of UV-Vis techniques: simplicity, sensitivity and selectivity to NPs, short time of measurement, Therefore, these techniques are increasingly used for NPs characterization in many fields of science and industry (Sato-Ber´ru *et al*., 2009; Brar and Verma, 2011).

2- Fourier transform-infrared (FTIR) spectroscopy

FTIR is one of the most widely used tools for the detection of functional groups in pure compounds and mixtures and for compound comparison. The infrared study is related to the vibrational motion of atoms or molecules. FTIR spectroscopy is widely used to study the nature of surface adsorbents in nanoparticles since the nanoparticles possess large surface area, the modification of the surface by a suitable adsorbate can generate different properties. The FTIR spectra of the nanoparticles, which contain some adsorbates, possess additional peaks in comparison with the FTIR pattern of a bare nanoparticle. So the property change with different adsorbates can easily be detected with FTIR spectroscopy. The compounds absorb electromagnetic energy in the infrared region of the spectrum. The position of a particular absorption band is specified by a particular wave number (Tourintio et al., 1998).

3- X-Ray Diffraction (XRD)

X-Ray Diffraction is a primary characterization tool for obtaining critical features such as crystal structure and size. X-rays are electromagnetic radiation similar to light but with a much shorter wavelength (Joshi *et al*., 2008). A beam of x-rays is sent into the sample and the way the beam is scattered by the atoms in the path of the x-ray is studied. It has been widely applied in the characterization of nanoparticles including those synthesized using biological agents (Giannini *et al*., 2016).

4- Particles size distribution

Current research demands the fastest and most popular method of determining particle size. The fastest and most popular techniques like dynamic light scattering (DLS), widely used to determine the size of nanoparticles in colloidal suspensions in the nano and submicron ranges. In this technique, monochromatic light exposure hits the moving particle, which results in changing the wavelength of the incoming

light. The extent of this change in wavelength determines the size of the particle. Dynamic light scattering (DLS) offer the most frequently used technique for estimation of the particle size and size distribution (Mosqueira *et al*., 2008).

5- Zeta potential

Surface charge and intensity determines the interaction of nanoparticles with the biological environment as well as their electrostatic interaction with bioactive compounds. Stability of colloidal material is usually analyzed through zeta potential of nanoparticles. Zeta potential is an indirect measure of the surface charge. Zeta potential values (either positive or negative) are achieved in order to ensure stability and avoid aggregation of the particles. Zeta potential values can be utilized in evaluating surface hydrophobicity and the nature of material encapsulated within the nanocapsules or coated onto the surface (Pangi *et al*., 2003).

Chapter Two

Materials And Methods

2- Materials and Methods

2.1- Materials

2.1.1- Apparatus and equipment

The following apparatus and equipment were used in this study:

Apparatus and equipment	Company /origin
AFM microscope	Angstrom Advanced Inc. /USA
Analytical balance	Sartorius /Germany
Autoclave	Express /Germany
FTIR	Shimadzu /Japan
Heat magnetic stirrer	SCO tech /India
High-speed cooling centrifuge	Eppendorf /Germany
Hood	ESCO /USA
Incubator	Sanyo /USA
Lyophilizer	Christ /UK
Microscope	Olympus/ Japan
Particle size analyzer	Brookhaven /USA
pH meter	Hanna /Italy
Refrigerator	CONCORD /Lebanon
SEM microscope	INSPECT S50 /UK
UV-Visible Spectrophotometer	Spectroscan 80 DV /UK

Vortex	Bohemia /USA
Water distillater	GFL /Germany

2.1.2- Chemicals and reagents

The following chemicals were used in this study:

Chemicals	**Company/Origin**
Chitosan (high MW)	Xi'an Lyphar Biotech Co., Ltd/China
Chitosan (low MW)	Himedia /India
Ethanol (Absolute)	Avantor /Netherlands
Glacial acetic acid	Analar /England
Isopropanol	Solvochem /UK
Methanol(Absolute)	LOBA /India
Monochloroacetic acid	BDH/England
Sodium hydroxide (NaOH)	Merck /Germany
Tri-polyphosphate (TPP)	BDH /England
Gram stain kit	Local company

2.1.3 Culture media

The following media were used in this study:

Item	Company/Origin
Mannitol salt agar (MSA)	Himedia/ India
Nutrient agar (NA)	
Nutrient broth (NB)	
Muller Hinton agar (MHA)	Salucea/ Netherlands

The above media were prepared according to the instructions by the manufacturing companies and sterilized by autoclaving at 121ºC for 15min.

2.1.4 Bacterial isolates

Bacterial isolates used in this study are illustrated bellow:

Bacterial isolate	Infection type	Source
S. aureus	Skin infection	Department of Biotechnology/ AL- Nahrain university
P. aeruginosa	Burn infection	

2.1.5 Antibiotic

Discs (10μg) and powder vials of streptomycin were supplied from Hi-media Company (India) and local pharmacies, respectively.

2.1.6 Cell line

The following cell line was used in this study**:**

Cell line	Source
RD cell line	Biotechnology Research Center/AL- Nahrain university

2.2-Methods

2.2.1- Bacterial isolates activation

The bacterial isolates included pathogenic G+ve *S. aureus* and G-ve *P. aureginosa* were activated by inoculating the loopfull of colonies from slants in NB medium prepared in the screw-cupped tube and incubated at 37 ° C for 24h. The culture of *S. aureus* was streaking on MSA and *P. aureginosa* on NA incubated at the same conditions. They were spreading and purified by sub culturing on nutrient agar at the same conditions. Pure colonies were selected, staining with Gram stain, and examined under a microscope. Selected colonies were sub cultured in plates or slants of NA and incubated at the same conditions. The cultures were stored in the refrigerator.

2.2.2-Maintenance of bacterial isolate

Bacterial isolates were maintained according to Han (1995) as follows:

A- Short- Term Storage:

Bacterial isolates were maintained for few weeks when cultured on nutrient agar. The plates were wrapped with Parafilm and stored at 4°C.

B-Medium- Term Storage:

Bacterial isolates maintained for few months by stabbing nutrient agar slants in screw-capped tubes containing 5 mL of nutrient agar medium and stored at 4 °C.

2.2.3-Preparation of nanochitosan (NC)

Soluble and insoluble chitosan (Chitosanamine hydrochloride chitosan) were used in preparation nanochitosan (NC) using the method proposed by Tang *et al*., (2007). Based on the information on the label, low MW:180KDa soluble chitosan and deacetylation degree 90% and High MW insoluble chitosan has an MW 220KDa and deacetylation degree 75%. Steps of the preparation are as follow:

a) Dissolve 0.2 mg of chitosan in 100 ml of 2% glacial acetic acid and mix for 30 min at 400 rpm using heat magnetic stirrer.
b) TPP (17.8 mg) was added in the ratio 5:2 (Chitosan: TPP) and mix were continuing for 2 hrs.
c) After mixing, the reactants was centrifuged at 12000 rpm for 15 min in a high-speed cooling centrifuge. Supernatant discarded and the deposit was washed twice with deionized water.
d) The precipitate was lyophilized in a lyophilizer.

This procedure was conducted also in preparation NC from 2 and 20 mg chitosan.

2.2.4- Preparation of Carboxymethylnanochitosan (CMNC)

Carboxymethylnanochitosan was prepared from nanochitosan by acid–base treatment method proposed by Zennat *et al*., (2013) and modified in some steps and as follow:

-Three grams of NC powder prepared in paragraph 2.2.3 was transferred into the 500 mL conical flask.

- A liquate of 65 mL of isopropanol was added and mixed well for 20 min until uniform suspension was obtained.

- 20.4g of 40% sodium Hydroxide solution added to the conical flask at room temperature.

- 14.4g of monochloroacetic acid/ isopropanol (1:1) solution added to the reactants above in conical flask and mixed for 24 hrs at room temperature to complete the reaction.

- After that, the suspension was produced. Instead of filtration, the suspension was centrifuged at 14000 rpm for 15 min in a high-speed cooling centrifuge.

- The supernatant was discarded and the deposit suspended in absolute methanol and neutralized by glacial acetic acid.

- Centrifugation was repeated and the supernatant was discarded.

- Finally, the deposit washed with 80% ethanol and dried at room temperature and stored in clean screw-capped tube at room temperature.

2.2.5- Preparation of Carboxymethylnanochitosan with streptomycin (CMNC-S)

During the preparation the CMNC and after adding 20.4g of aqueous NaOH (40%) and 14.4g of monochloroacetic acid/isopropanol solution to the nanochitosan then the streptomycin (S) was added to the solution in ratio 1:5 (CMNC: S) and mixed well for 24 hours at room temperature. The reactant was centrifuged at 14000 rpm for 10 min in a high-speed cooling centrifuge. The supernatant was discarded and the deposit was suspended with methanol and neutralized with 10% glacial acetic acid. The deposit was washed with 80% ethanol and dried at room temperature.

2.2.6- Determination of loading efficiency of carboxymethylnanochitosan with streptomycin (CMNC-S)

2.2.6.1- Lambda maximum (λ_{max}) measurement (David Harvey, 2000)

For determination the λ_{max} for S, CMNC and CMNC-S, 1000 µg/ml of each compound was used by dissolving in deionized water and made scanning from 100 nm – 850 nm wavelength in UV-visible spectrophotometer (Spectroscan 80 DV /UK). The values of absorption (Y- axis) are plotted against the values of wavelength (X-axis).

2.2.6.2- Standard curve

The standard curves for S, CMNC and CMNC-S were conducted as follow:

Preparation the serial concentrations 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000µg/mL for each compound in deionized water. The absorption for these concentrations was determined spectrophotometrically using the λ_{max} values, which determined in paragraph 2.2.6.1. The absorption values were plotted against the concentration values. Reliability values were determined by excel liner equations. The absorbency (α) for each compound was appointed by calculating the (tanθ) from standard curve using the equation (David Harvey, 2000).

Absorbance (α) = Δ Abs $_{nm}$ / Δ conc. (µg/mL)

2.2.6.3- Loading efficiency (Xu *et al*., 2015)

It was calculated according to the following formula:

$$\text{Loading efficiency \%} = \frac{\text{Total amount of S added (mg)} - \text{Unbound S (mg)}}{\text{Total amount of streptomycin (mg)}} \times 100$$

The amount of streptomycin encapsulated within CMNC was determined by centrifuging the solution of CMNC-S and the supernatant was analyzed for streptomycin content by measuring the absorbance at 275 nm using UV Spectrophotometer (Spectroscan 80 DV /UK). The loaded S into CMNC was a measurement at 270 nm**.**

2.2.7- Characterization

Characterization of synthesis nano-compounds NC, CMNC and CMNC-S was conducted in Chemistry and Physics Departments of College of Sciences/University of Al- Nahrian and Nanotechnology center/ the University of Technology. The characteristics include images and analytical methods and as follow:

2.2.7.1- Fourier transform-infrared (FTIR) spectroscopy (Vellingiri *et al*., 2013)

The suspension of CMNC and CMNC-S was centrifuged at15000rpm for10min and the supernatant discarded. The deposit was dried at room temperature and serial concentrations was prepared in deionizes water. 10mg from each CMNC and CMNC-S was used for analysis. FTIR spectra were recorded in KBr disc in instrument supplied from Shimadzu company/Japan and under conditions of dry air at room temperature within the wave number range of 500–4,000cm^{-1}.

2.2.7.2- Scanning Electron Microscope (SEM) (Bhavin *et al*., 2013)

It is imaging method accomplished using SEM (INSPECT S50 device, UK). A drop of aqueous solution with (10µg/mL of CMNC and CMNC-S was putted on a holder until dried at room temperature and analyzed by SEM.

2.2.7.3- Atomic force microscope (AFM) (Borm *et al*., 2006)

This instrument was used for topographic imagination and particle size analysis of prepared nanochitosan and their derivative (CMNCS and CNMC-S).

One drop from each prepared CMNC and CMNC-S, with concentration 10µg/mL, and nanochitosan (as mentioned in 2.2.3) prepared from different concentrations of soluble and insoluble chitosan (0.2, 2 and 20mg/100mL) was dried at room temperature and analyzed. The surface morphology of samples was observed by using atomic force microscopy (Angstrom Advanced Inc, USA).

2.2.7.4-Particle size distribution (Bhavin*et al*., 2013)

Particle size and size distribution of the prepared CMNC, CMNC-S (10µg/ml) were analyzed using laser scattering technique in device Brookhaven Instruments, USA. Light scattering was measured at 25 ◦C and with the angle of 90∘.

2.2.8- Sensitivity tests

2.2.8.1- Diffusion test (Atlas, 1995)

Disc diffusion method was used to test the sensitivity of bacterial isolates against streptomycin (S). Active cells suspension was prepared for each isolate from active colonies selected from NA cultures after 18h of incubation. 0.1mL of the suspension (10^6 colony forming unit /ml) was spreading by L- shape on the surface of Muller-Hinton agar (MHA). The plates were left for 30min and then, the disc of S with diameter 6mm distributed over the surface of culturing plates and incubated at 37 ◦C.

2.2.8.2- Dilution test

It was made as follow:

- The concentrations of chitosan (0.002, 0.02 and 0.2 µg/ml) were prepared form the soluble and insoluble chitosan.

- Serial concentrations (10, 50,100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000µg/ml) of S, CMNC and CMNC-S were prepared in sterile deionized water.

- MHA medium was prepared and 20mL of media was poured in sterile Petri-dishes.

- Suspensions of active cells of *S. aureus* and *P. aeruginosa* were prepared.

- A liquate of 0.1mL of active cells (10^6 colony forming unit /ml) from each isolate was inoculated on the MHA plates.

- Wells were made in MHA cultures plates by sterile cork borer.

- A liquate of50µl of each concentration was added into the wells.

- The plates were incubated at 37°C for 24h.

- The inhibition zone was measured.

2.2.9-*In Vitro* cell line viability (Freshney, 2012).

This test was conducted in Biotechnology research center/ University of Al-Nahrian. The anticancer efficacy of S, CMNC and CMNC-S against RD (Rhabdomyosarcoma) cell line was evaluated. The colorimetric cell viability MTT assay was used. Steps of the test were conducted as follow:

-A liquate of 100 µl/well of RD cells (10^6 cell/ mL) was cultured in 96-well tissue culture plate and incubated with 5% CO_2 at 37 °C for 24 hrs.

-Different concentrations of S, CMNC, CMNC-S (50 and 100µg/ml) were prepared by dissolving in sterile Distilled Water (DW).

-A liquate of (100) µL of various concentrations was added to each well and incubated at 37°C for 24 hrs.

- After the incubation, 10µL of MTT solution (5 mg/ mL) was added to each well and incubated at 37°C for 4 hrs.

-Finally, 50 µL of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min.

-RD cells were cultured in complete medium without S, CMNC and CMNC-S as a positive control, and in a complete medium without cells and test solutions as a blank.

-The absorbance was measured for each well at 620 nm using an ELISA reader.

The inhibition ratio was calculated according to the formula:

$$\text{Inhibition}\,(\%) = \left(\text{AC} - \textit{A}\text{S} \;/\; \text{AC}\right) \times 100$$

Where AC and AS are the optical density for positive control and test samples,

Chapter Three

Results And Discussion

3. Results and Discussion

3.1- Synthesis of chitosan derivatives

3.1.1-Nanochitosan

Nanochitosanwas synthesized by ionic gelation method proposed by (Tang et al., 2007) using two types of chitosan, the soluble chitosan (low molecular weight) and insoluble chitosan (high molecular weight as well as the deacylation percent). Gelation method is the suitable technique to synthesis chitosan nanoparticles and this process derives from inter- and intramolecular cross-linkages mediated by the anionic molecules (TPP) (Terbojevich and Muzzarelli, 2009).

Nanochitosan was obtained, and because the quantities produced from nanochitosan using 0.2 mg of chitosan were few and did not meet subsequent testing requirements, therefore, 2mg and 20 mg of chitosan were used, and the same percentage of TPP and acetic acid (2%) remained. Nanochitosan were obtained using 2 mg which was the best concentration for nanochitosan preparation as well as the results of the characterization that confirmed this.

3.1.2-Caroxymehtylnanochitosan (CMNC)

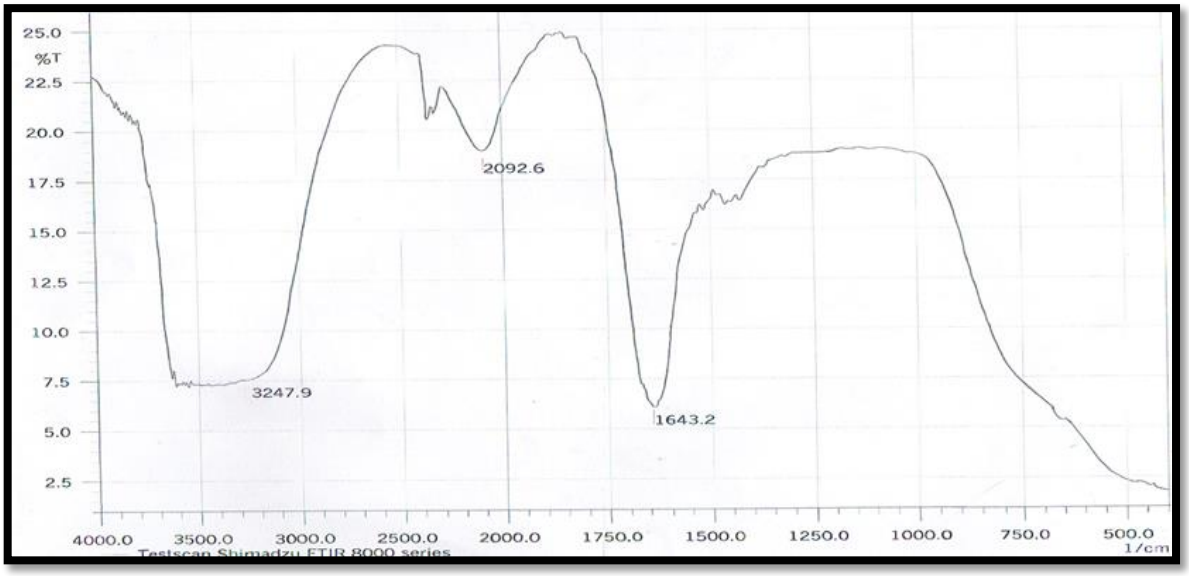
CMNC was synthesized from nanochitosan by the steps of the method proposed by Zennat *et al*., (2013) to synthesize carboxymethyl chitosan by acid-base reactions, as mentioned in 2.2.4. The main reason was the poor solubility of chitosan and nanochitosan when pH is more than 6.5 and this represent a serious barrier in many of its possible applications and the CMNC is soluble in a wide range of pH mentioned by Fernanda and Campana-Filho (2005). The yield was 420 mg dry weight of CMNC from 3g of NC.

3.2- Characteristics of nanochitosan derivatives

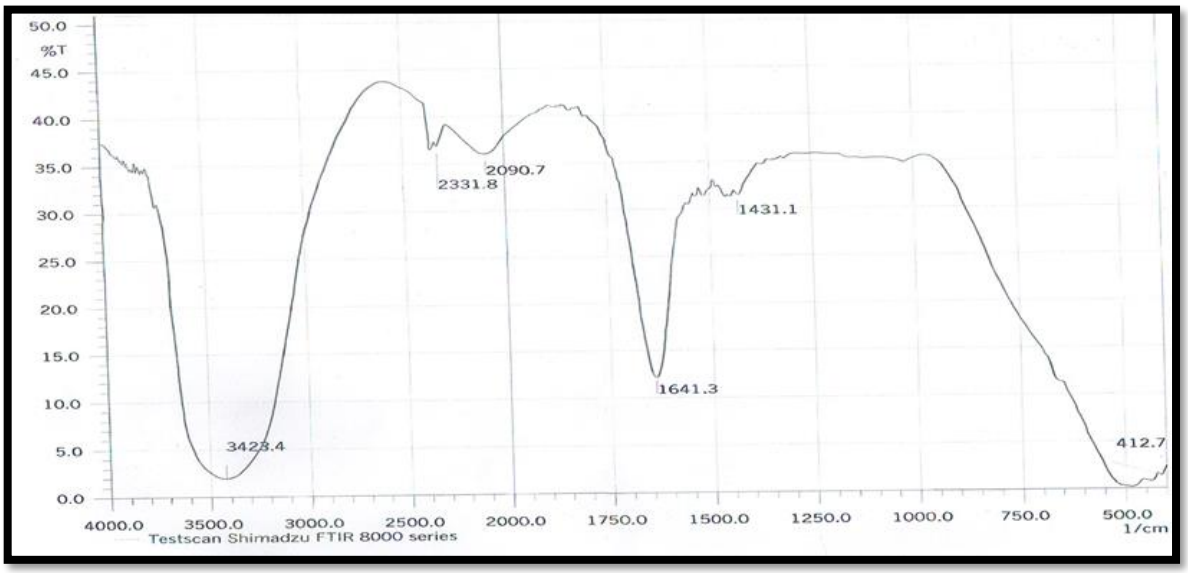
3.2.1- FTIR

Figure (3-1) shows FTIR spectroscopy of CMNC and CMNC-S. It seems that there are no noticeable differences between the results of analysis of CMNC and CMNC-S. Stationed absorption peaks at the same vibration 3000 - 3800 cm^{-1} with the change in stretching of the absorption percent increased sharply in figure (3-1). FTIR spectra of CMNC show a weak peak at 1431.1 cm^{-1}which might be to the symmetrical COO^- group stretching vibration. The not symmetrical stretching vibration of the COO^- group near 1550 cm^{-1} is interfered with the deforming NH2vibration at 1600 cm^{-1} exhibiting a very strong peak. The broad peak in CMNC at 3200–3700 cm^{-1}may due to both (O–H) and (N–H) stretching vibrations. FTIR spectral scanning of the CMNC-S a broad strong absorption peak at 3000–3800 cm^{-1} and which related with the O–H stretching from the inter and intramolecular hydrogen bonds as approved by (Gilbert, 1999). These results indicate the encapsulation of CMNC to the antibiotic as well as the occurrence of aggregates for both compounds within the same frequencies.

The pure chitosan spectrum has several defined peaks at 3360, 2919, 2874, 1640, 1592, 1375, 1153, 1061 and 893 cm^{-1} as mentioned by (Kumar *et al*., 2012). The characteristic peaks of the chitosan derivative at 1640 cm^{-1} is due to (C=N) group formed by a cross-linking reaction between the amino group and the aldehydic group of S. The intensity O-H group of CMNC-S became high in comparison with CNMNC this result of analysis agreed to somewhat to Kumar and Koh (2012).



A- CMNC



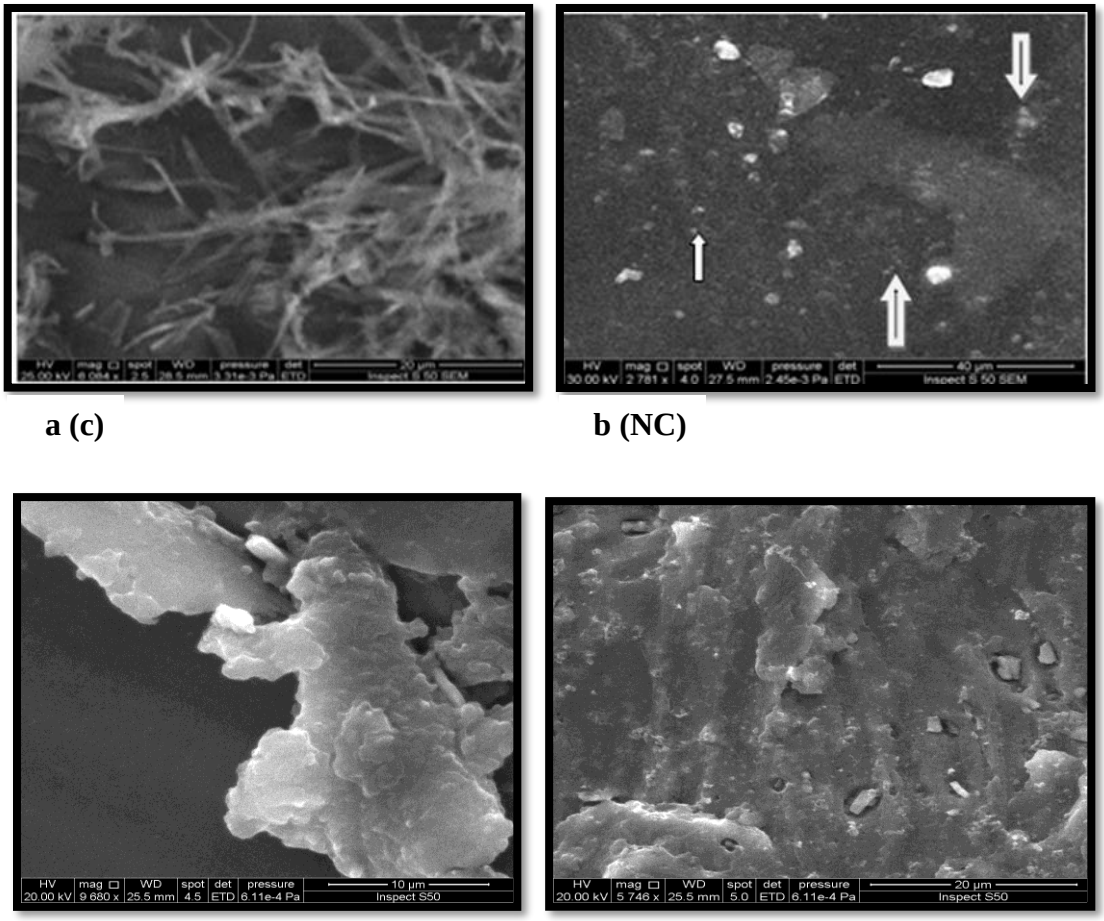
B- CMNC-S

Figure (3-1): Fourier transform infrared (FTIR) spectra of A) CMNC and B) CMNC-S.

SEM

Figure (3-2) shows Scanning Electron Microscope (SEM) three-dimensional morphological structures of chitosan(C), NC, CMNC and CMNC-S, while Figure (3-2) (a) shows the fibril structure of chitosan and (b) the nano-spherical structure of nanochitosan (Ali *et al*., 2016).

Figure(3-2 c) displays an aggregation of CMNC before crushing.The low magnification of SEM instrument was the barrier for deepening in explanation the results. And (d) illustrates encapsulate of streptomycin (S)in the CMNC by encapsulation and attachment. The efficiency of the encapsulation and attachment will be illustrated determined in loading efficiency. There are no consistent results for the morphology in the references for many reasons related to the environmental conditions and the modification in the methods of preparation.



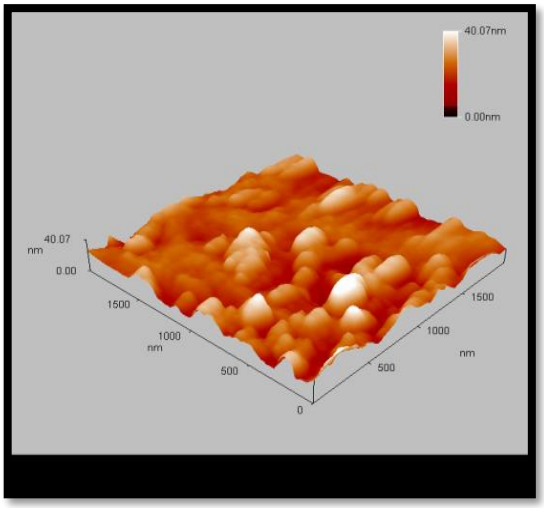
a (c) **b (NC)**

c (CMNC) **d (CMNC-S)**

Figure (3-2): SEM images a: Chitosan structure with magnification (20µm) using SEM ; b: Nano-chitosan with magnification (40µm) Ali, *et al.*, 2016); c: CMNC (10µm); d: CMNC-S.

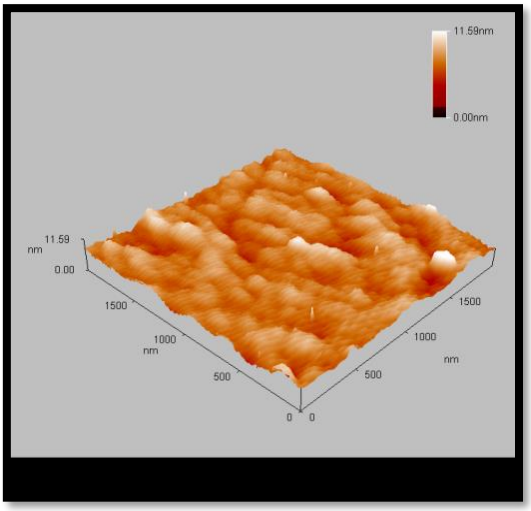
3.2.3- AFM

In the processes of producing nanochitosan particles, different concentrations of chitosan were used versus the constant concentration of TPP for the reason to invest all TPP used for high productivity. The average diameter of nanoparticles and percentage of dominant diameter resulted from AFM image and analysis were chosen for comparison and selection of a suitable concentration of chitosan and the physical state. Figure (3-3 A, B and C) and d show that the concentration 2 mg / 100 ml of 2% glacial acetic acid of soluble chitosan were chosen that gave 54.64 nm average diameter and 90% of nanoparticles with diameter 80 nm. Insoluble chitosan at concentration 2mg/100ml gave an average diameter 105.52 nm and 90% of nanoparticles with a diameter of 170nm. Concentrations of soluble chitosan 20 mg/100 produced nanoparticles have larger diameters but the process was incomplete because of high concentration of chitosan that precipitated at the beginning and remains in suspended state.



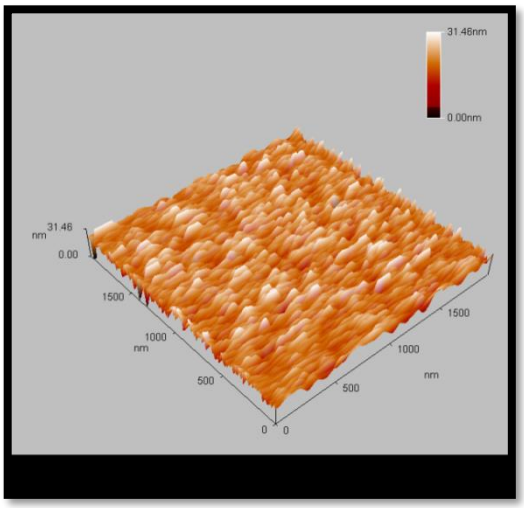
A- (0.2mg soluble nano chitosan)

Avg. Diameter: 86.49 nm
<=10% Diameter: 35.00 nm
<=50% Diameter: 75.00 nm
<=90% Diameter: 140.00 nm



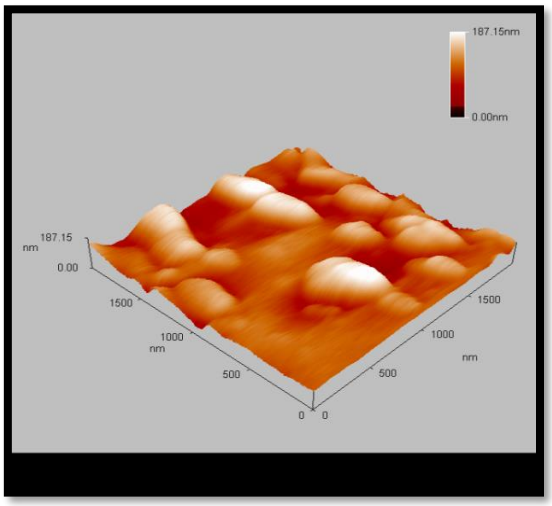
B- (2 mg soluble nano chitosan)

Avg. Diameter: 54.64 nm
<=10% Diameter: 25.00 nm
<=50% Diameter: 50.00 nm
<=90% Diameter: 80.00nm



C- (20mg soluble nano chitosan)

Avg. Diameter: 59.18 nm
<=10% Diameter: 35.00 nm
<=50% Diameter: 55.00nm
<=90% Diameter: 80.00 nm

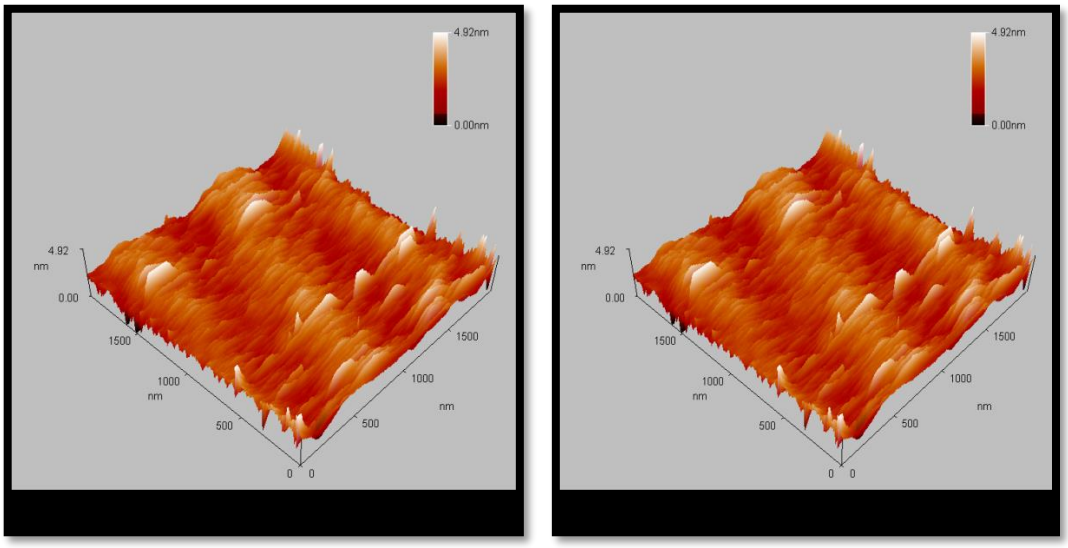


D- (2mg insoluble nano chitosan)

Avg. Diameter: 105.52 nm
<=10% Diameter: 40.00 nm
<=50% Diameter: 90.00 nm
<=90% Diameter: 170.00 nm

Figure (3-3): Atomic Force Microscopic (AFM) 3D images of nanochitosan prepared from chitosan at different concentrations and solubility, a: 0.2mg of soluble chitosan; b: 2 mg soluble chitosan; c: 20 mg soluble d: 2mg insoluble chitosan.

Figure (3-4 A, B) shows the results of 3D AFM images and analysis of CMNC and CMNC-S. According to the process of carboxymethyl nanochitosan preparation, it was obtained nanoparticle size with average diameter 35 nm and 90% of 56 nm diameter. After loading with antibiotic(S), the average of the nanoparticles was increased to reach 37 nm and 90% of particles diameter increased to 62 nm. These results confirmed the loading process of CMNC with S.



A-(CMNC)

Avg. Diameter: 35.76nm
<=10% Diameter: 18.00 nm
<=50% Diameter: 32.00 nm
<=90% Diameter: 56.00 nm

B-(CMNC-S)

Avg. Diameter: 37.39 nm
<=10% Diameter: 18.00 nm
<=50% Diameter: 32.00 nm
<=90% Diameter: 62.00 nm

Figure (3-4): Tapping-mode AFM image and three-dimensional height image of nanoparticles formed by carboxymethylnanochitosan encapsulated streptomycin, A- CMNC; B- CMNC-S.

3.2.4- Particle size distribution

Figure (3-5) shows the particle size analysis of CMNC and CMNC-S. The analysis was made after incomplete crushing of samples because of product texture, so the median particle size of CMNC was 770nm and for CMNC-S 526nm. This result is not compatible with results of AFM analysis, which indicated lower nanoscale diameters and considered more reliable because of the precision 3D scanning of samples.

Approximate sizes were obtained by Chopra *et al*.2012. The particle diameter for the streptomycin carried on chitosan-alginate nanoparticles was 328.4 nm Figure (3-5). It is notable that the diameter of the particles measured by light scattering is higher than the size estimated from microscopy because of the high swelling capacity of chitosan-alginate nanoparticles. Thus, the actual diameter of these particles can be assumed smaller than this.

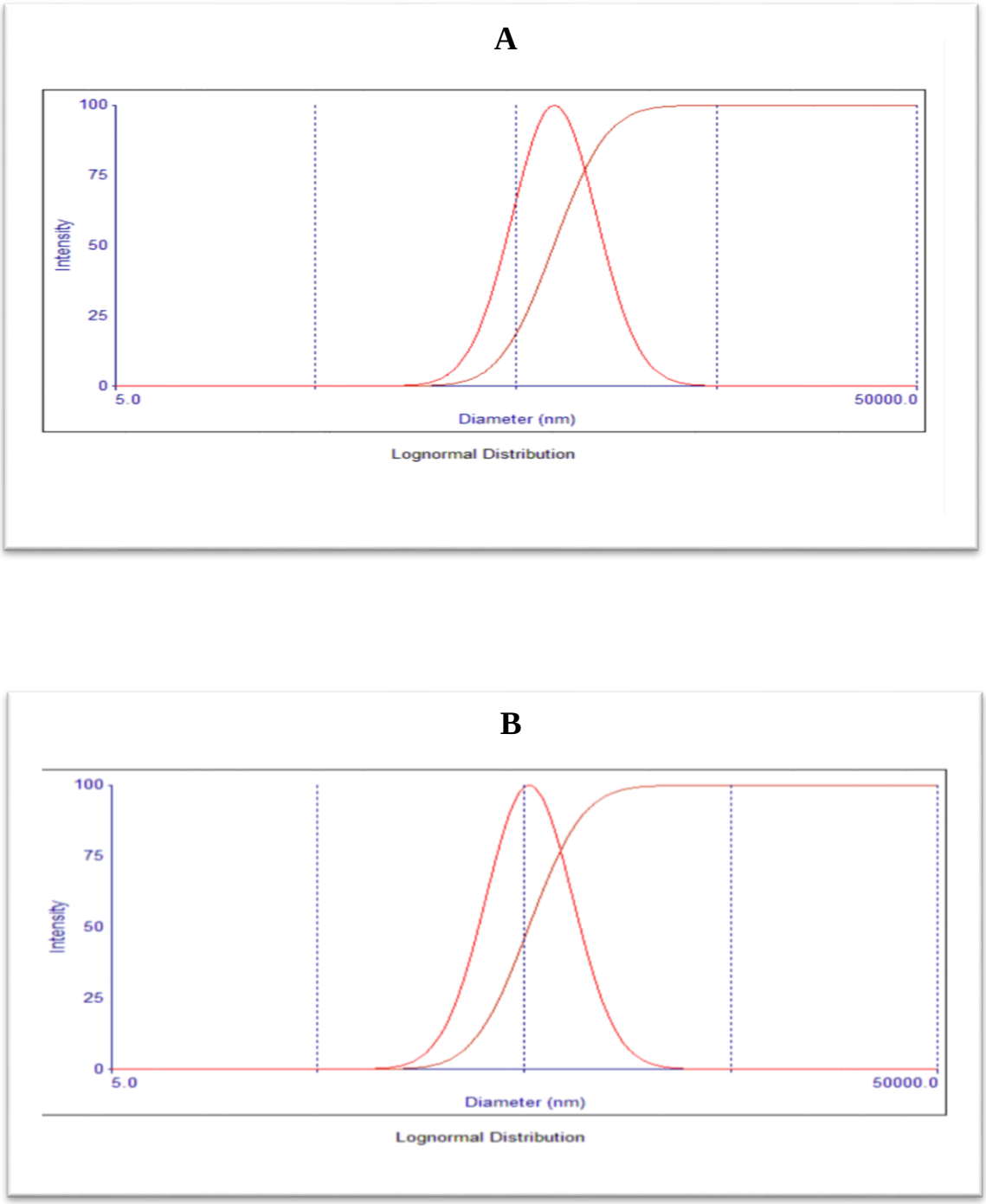


Figure (3-5): Particle size distribution of A: CMNC (Median diameter: 770nm) and B:CMNC-S(Median diameter: 526nm) .

3.3-Determination of lambda maximum ((λ_{max})

Figure (3-6) shows the top peaks of the maximum wavelength absorption of determination the λ_{max} for S, CMNC and CMNC-S. The concentration used for measurement was 1000 μg/mL for each compound in order to obtain suitable and clear curves to avoid any unresponce of them was read the absorbance of them with a spectrophotometer (Spectroscan 80 DV, UK). Figure (3-6) illustrate the λ max for S, CMNC and CMNC-S, which were used to determination the loading efficiency percentage in subsequent steps. Figure (3-6) shows λ max of the S, CMNC and CMNC-S.As shown in the figure that the λ max for streptomycin was 275 nm and 265 nm for CMNC while 270 nm for CMNC-S. These results reflect the loading and attachment processes conducted between CMNC and streptomycin and this return to the reason for that encapsulation S within CMNC as well as the adsorption of the S on the surface of CMNC.

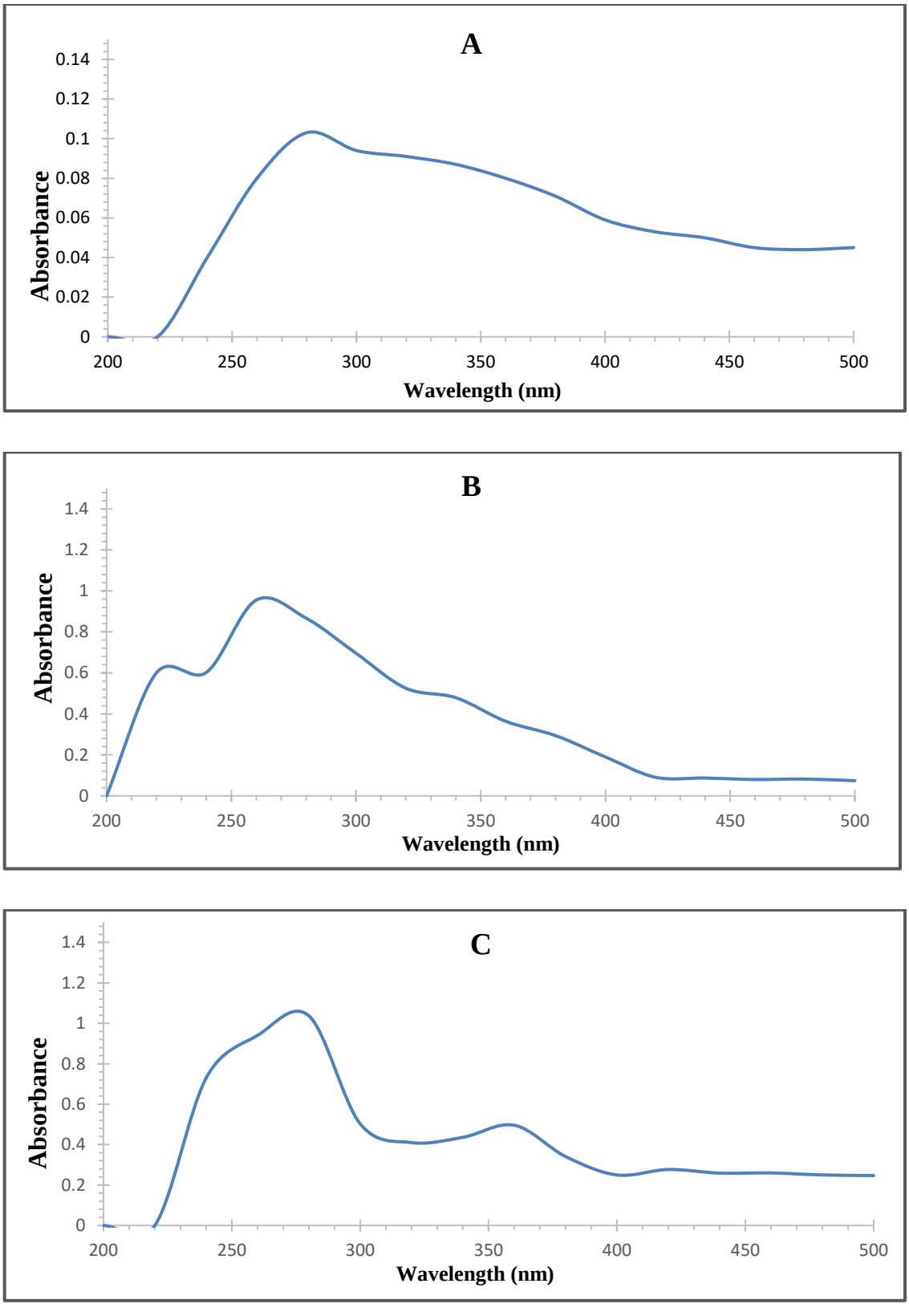


Figure (3-6): Lambda maximum for: A- Streptomycin B- Carboxymethylnanochitosan and C-Carboxymethylnanochitosan with streptomycin.

3.3.1-Standard curve

A standard curve of (S, CMNC and CMNC-S) was plotted by using different concentrations of (S, CMNC and CMNC-S) against the absorbance value.

The absorbency (α) values for S, CMNC and CMNC-S were 0.002, 0.004 and 0.006, respectively were determined from standard curves (figure 3-7). These values were used for determination the concentrations of streptomycin and nanochitosan derivatives for estimation the loading efficiency.

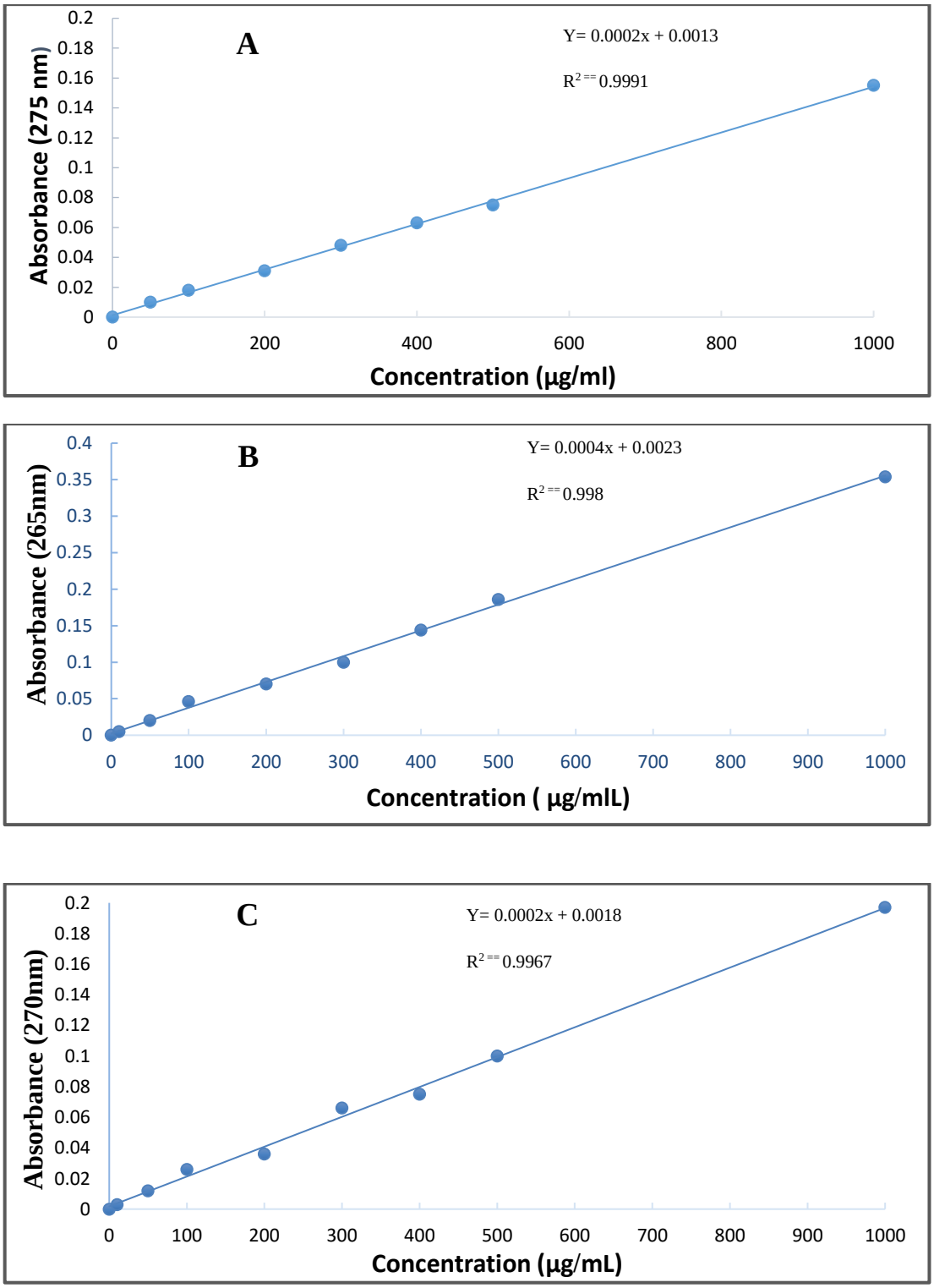


Figure (3-7): Standard curves of A- Streptomycin

B- Carboxymethylna nochitosan and C- Carboxymethyl nanochitosan with streptomycin.

3.3.2-Loading efficiency

The loading efficiency was calculated using the equation mentioned in 2.2.6.3. The amount of free drug it was calculated from concentration values obtained from the calibration curves by analysis of the samples using spectrophotometer measured at 275nm (Spectroscan 80 DV, UK). The loading efficiency percentage of S was 90% according to the equation of Xu *et al.,* 2015.Curcumin was entrapped within carboxymehthyl chitosan nanoparticles with an efficiency of 80%.

3.4- Sensitivity test

3.4.1-Sensitivity test of streptomycin discs

Table (3-1) shows the sensitivity test, using disc diffusion technique, of *S. aureus* and *P. aeruginosa* against the streptomycin. *S. aureus* was more sensitive than *P. aeruginosa*. *S. aureus* was regarded sensitive to the streptomycin which gave inhibition zone 15 mm which was higher than standard range (>14mm) according to the limitations administrated by CLSI (2010). *P. aeruginosa* regards resistance to the S <11 mm, (CLSI, 2010).

3.4.2- Sensitivity test of nanochitosan (NC)

Nanochitosan prepared in 2.2.3 was used to evaluate the sensitivity of bacterial isolates. High MW chitosan (insoluble) showed no inhibition zone on bacterial isolates, whereas the low MW chitosan (soluble) show inhibition zone as illustrates in table (3-2). From the table, the *S. aureus* was resist to the nanochitosan at different concentrations, while *P.*

aeruginosa was sensitive to the nanochitosan at concentrations 2 and 20 mg /100mL in comparison with the results of sensitivity against streptomycin as mentioned above (table 3-1).

Table (3-1) Sensitivity of *S. aureus* and *P. aeruginosa* to nanochitosan

Chitosan Concentration (µg/ml)	Inhibition Zones (mm)	
	S. aureus	***P. aeruginosa***
0.002	-----	10
0.02	-----	15
0.2	-----	15

3.4.3- Sensitivity test of (S, CMNC and CMNC-S)

Table (3-2) shows the sensitivity tests of bacterial isolates against different concentrations of nanochitosan derivatives with streptomycin. CMNC was not effective on the response of isolates. *S. aureus* was become sensitive against S starting from concentration 100 µg/mL and more, while *P. aeruginosa* was started their sensitivity at concentration 300 µg/mL which was more resistance than *S. aureus.* The results of sensitivity against CMNC-S were similar to S for each isolate. Therefore, it found that CMNC lost it effects against bacterial isolates in comparison with NC. These results were determined after 24 h of incubation time only. As mentioned in many references the releasing of agents loaded in nanomaterials was happened gradually and takes longer and its effectiveness is cumulative compared to the agents alone, which show their maximum effectiveness at once time (Vellingiri *et al*., 2013).

Table (3-2) sensitivity test of *S. aureus* and *P. aeruginosa* to different concentrations of S, CMNC and CMNC-S

Compound	Concentration (µg/ml)	Inhibition zone(mm)	
		S. aureus	***P. aeruginosa***
S **Streptomycin**	10	15	-------
	50	17	-------
	100	**18**	10
	200	20	13
	300	20	**15**
	400	20	15
	500	21	18
	1000	23	20
CMNC **Carboxymethyl Nanochitosan**	10	-------	-------
	50	-------	-------
	100	-------	-------
	200	-------	-------
	300	-------	-------
	400	-------	-------
	500	-------	-------
	1000	-------	-------
CMNC-S **Carboxymethyl nanochitosan + Streptomycin**	10	10	-------
	50	12	-------
	100	**15**	10
	200	18	13
	300	18	**15**

	400	18	16
	500	18	18
	1000	20	20

3.5- Determination of growth inhibition of RD cell line

It is clear from the results shown in table (3-4), that the best treatment was CMNC-S **(**50 µg/ml) the growth inhibition (GI) was 37%, compared with 23% of the sources S at the same concentration of 50 µg/ml and approaching to the concentration 100 µg/ml of S, note that the loading efficiency is 90% means the real concentration 45 µg/ml. GI% for CMNC-S (100µg/ml) was approximately 50 µg/ml and this may due to the mechanism of the S release which almost the same rate within only 24 hours of culture incubation. Therefore, prolonging the incubation time will increase the percentage of inhibition based on the sentiments of both (Amer *et al*., 2014; Ali *et al*., 2016).

Table (3-3): *In vitro* bioassay of CMNC and CMNC-S against RD cell line

Treatment*	**Mean**	**S.D**	**GI%**
S (50µg/ml)	0.175	0.038	23
S (100µg/ml)	0.140	0.066	38.5
CMNC (50 µg/ml)	0.212	0.045	6.8
CMNC (100 µg/ml)	0.181	0.008	20.6
CMNC-S(50 µg/ml)	0.142	0.012	37.5
CMNC-S(100 µg/ml)	0.147	0.014	35.5
Control	0.228		

*** S: Streptomycin; CMNC: Carboxymethylnanochitosan.**

Conclusions And Recommendations

Conclusions and recommendations:

Conclusions

1. The molecular weight and solubility of chitosan play important role in preparation of nanochitosan.
2. This study showed a high loading of CMNC with streptomycin using encapsulation technique.
3. The CMNC-S was effected in half concentration on *S. aureus and P. aeruginosa* in comparison with streptomycin with full concentration.
4. The CMNC-S, at half concentration, was inhibited the RD cell line at the same percentage of the full concentration of streptomycin.

Recommendations

1. Complete the characterization of polymeric nanoparticles such as zeta potential and X-ray diffraction (XRD).
2. Using other types of antibiotics and studying the antimicrobial activity.
3. Studying the cytotoxicity effects against other types of cancer cell lines.
4. Loading the CMNC with anticancer drug and studying their effects against cell line.

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الخلاصة

تهدف الدراسة الى تحضير الكاربوكسي مثيل كايتوسان النانوي المشتق من الكايتوسان النانوي المحضر من الكايتوسان الذائب ذو وزن جزيئي قليل وتغليف المضاد الحياتي الستربتومايسين ثم توصيفه وفحصه ضد البكتريا المرضية الموجبة والسالبة لصبغة كرام.

تضمنت الدراسة استخدام طريقة الهلام الايونية (Ionic gelation) لتحضير الكايتوسان النانوي من كايتوسان ذائب (وزن جزيئي قليل) وكايتوسان غير قابل للذوبان (وزن جزيئي عالي) و تم استخدام الكايتوسان الذائب لتحضير الكايتوسان النانوي وبتركيز 2% (ملغم/100مل من 2% حامض الخليك) مع نسبة ثابتة من متعدد ثلاثي الفوسفات TPP وذلك لاعطائها افضل ناتج من الكايتوسان النانوي بعدها تم تحضير الكاربوكسي مثيل كايتوسان النانوي من الكايتوسان النانوي باستخدام طريقة حامض- قاعدة (acid-base) وكان المنتج 420 ملغم من الكاربوكسي مثيل كايتوسان النانوي لكل 3غم من الكايتوسان النانوي.

تم تحديد اقصى امتصاصية للمضاد وكانت 275 نانوميترولكاربوكسي مثيل كايتوسان النانوي 265 نانوميتر وللكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد كانت 270 نانوميتر وتم ايضا تحديد المنحنيات القياسية لكل منهم وكانت قيمة الامتصاصية للمضاد 0.002 و0.004 للكاربوكسي مثيل كايتوسان النانوي و0.006 نانوميترللكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد. واظهرت الدراسة ان كفاءة تحميل الكاربوكسي مثيل كايتوسان النانوي للمضاد كانت 90%.

اظهرت نتائج التوصيف باستخدام جهاز FTIR للكاربوكسي مثيل كايتوسان النانويوللكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد ان قمم الامتصاص عند نفس الترددات 3000-3800سم $^{-1}$مع عمق في تمدد نسبة الامتصاص للكاربوكسي مثيل كايتوسان المحمل بالمضاد. اما جهاز SEM فقد اظهرالهيئات المختلفة لتراكيب للمركبات، فقد اظهر تجمع الكاربوكسي مثيل كايتوسان النانوي وتحميله للمضاد. اوضحت نتائج فحص قوة الذرة المجهري(AFM) للتصوير ثلاثي الابعاد، ان تركيز 2ملغم/100مل من الكايتوسان الذائب كان معدل قطره 54.64 نانوميتر وكانت 90% من الدقائق النانوية بقطر 80 نانوميتر اما الكايتوسان الغير ذائب عند نفس التركيز كان معدل قطره 105.52 نانوميتر وكان 90% من الدقائق النانوية بقطر 170 نانوميتر، اعتمادا على طريقة تحضير الكاربوكسي مثيل كايتوسان النانوي، كان

حجم الدقائق النانوية بمعدل قطر 35 نانوميتر وكانت 90% من الدقائق بقطر 56 نانوميتر. اما بعد تحميل الكاربوكسي مثيل كايتوسان النانوي بالمضاد(الستربتومايسين) فقد كان معدل قطر الدقائق النانوية 37 نانوميتر و90%من الدقائق كانت بقطر 62 نانوميتر. بينت نتائج فحص توزيع حجم الجزيئات ان حجم دقائق الكاربوكسي مثيل كايتوسان النانوي كانت 770 نانوميتر وبعد تحميله بالمضاد اصبحت526 نانوميتر.

بينت نتائج الدراسة ان الكايتوسان النانوي المحضر من الكايتوسان غير القابل للذوبان لم تثبيط لنمو البكتريا بينما المحضر من الكايتوسان الذائب فقد اظهر تثبيط لنمو بكتريا *p. aeruginosa* فقط. ومقارنة مع نتائج فحص الحساسية للمضاد، فقد وجد ان الكاربوكسي مثيل كايتوسان النانوي لم يؤثر على حساسية العزلات البكتيرية. اظهرت بكتريا *S. aureus* حساسية للمضاد عند تركيز 100 مايكروغم/مل بينما اصبحت بكتريا *P. aeruginosa* حساسة للمضاد بدأ بالتركيز 300 مايكروغم/مل. اما نتائج الحساسية للكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد فلقد كانت مشابهة لنتائج المضاد لكل العزلات البكتيرية. تم فحص فعالية المضاد (الستربتومايسين) والكاربوكسي مثيل كايتوسان النانوي والكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد مختبريا كمضادة للسرطان باستخدام خط الخلايا (RD) ووجد ان الكاربوكسي مثيل كايتوسان النانوي المحمل بالمضاد وبتركيز 50 مايكروغم/مل كان اكثر فعالية من تركيز100 مايكروغم/مل من المضاد وكان تثبيط النمو للخلايا للكاربوكسي مثيل كايتوسان النانوي37.5% بينما للمضاد كان 38% بعد الحضن لمدة 24 ساعة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تأثيرالكاربوكسي مثيل النانوي المحمل بالستربتومايسين على المكورات العنقودية والزوائف الزنجارية

رسالة

مقدمة إلى مجلس كلية العلوم / جامعة النهرين وهي جزء من متطلبات نيل درجة الماجستير في التقانة الأحيائية

من قبل

منى محمد خيري نجيب

بأشراف

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رجب 1438 هـ

نيسان 2017 م