The Republic of Iraq

Ministry of Higher Education

and Scientific Research

University of Kufa

Faculty of Sciences

Department of Biology

Synergistic Effect

of Biogenic Chitosan Nanoparticles and Methotrexate drug Against Breast Cancer and *Staphylococcus aureus*

A Research Submitted to

The Council of the Department of Biology, Faculty of Science University of Kufa, as Part of the Requirements for Obtaining The Bachelor's Degree in Biology Science

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2022 A.D **1443A.H.**

﴿ وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا ﴾

صدق الله الْعَلِيُّ الْعَظِيم

سورة الإسراء الآية رقم ٨٥

Dedication

To you, my master, O the remnant of God, who is the guardian of our unseen matter (may God hasten his reappearance), I raise this little effort, hoping for acceptance, recital the Almighty's saying:

﴿ يَا أَيُّهَا الْعَزِيزُ مَسَّنَا وَأَهْلَنَا الضُّرُّ وَجِئْنَا بِبِضَاعَةٍ مُزْجَاةٍ فَأَوْفِ لَنَا الْكَيْلَ وَتَصَدَّقْ عَلَيْنَا ۖ إِنَّ اللَّهَ يَجْزِي الْمُتَصَدِّقِينَ ﴾

To the dearest people to us, the biggest supporters to us, to those who gave us love and affection, and the most encouragement to us, my father and mother, who have endured all the difficulties to facilitate it for us, especially the cost of my study.

For those who supported me with encouragement (my brothers, friends).

Jaafar & Mortada

Acknowledgment

First and foremost I would like to thank Allah to help me overcome difficulties and facilitate the research task. when I complete writing dissertation, I realized how great this gift is for me. You gave me the power to believe in my abilities and pursue my dreams. I could never have done this without the faith I have in You, the Almighty.

I would like to express my gratitude to my project supervisor Prof. Dr. Nawfal Hussein Aldujaili through providing his valuable advice, guidance, support and encouragement throughout my undergraduate. He had a very distinguished effort and the role in instructing and scientifically supervising me throughout research.

I'd also like to pay my thankfulness to Head of Biology Department and the Biology Department teachers and to everyone in the faculty of science for my assist to complete part of practical research.

My sincere thanks are to my uncle Dr. Ali Abdul Hakim Albakaa for his encouragement and support to complete this Search.

Jaafar & Mortada

Summary

TO study clarify the effect of combination (Chitosan nanoparticles + Methotrexate) on breast cancer cell line (MCF-7 Cell) and Inhibiting the growth of antimicrobial resistance (AMR) bacteria (*S.aureus*).

Three strains of bacillus (*B.clausii*, *B.coagulans*, *B.Subtilis*) were tested as chitosan nanoparticles (NPs) produce , Was selected *B.coagulans* was selected For biosynthesis of Chitosan nanoparticles on based color change, biological activity and UV-visible Spectroscopy. Chitosan nanoparticles consists of naturally occurring amino polysaccharide, derived as a deacetylated form of chitin. the biological, physical and chemical properties have led to great interest in its use in biomedicine and other fields.

The study included the manufacture and characterization of Chitosan nanoparticles by UV-Visible spectrophotometry was used to detect the synthesis of chitosan nanoparticles with an absorption peak at 280nm, Scanning Electron Microscope (SEM) analysis showed the spherical, homogenous with average sizes from 30.94 to 95.51 nm, The elemental analyses of biogenic chitosan nanoparticles with Energy Dispersive Spectroscopy (EDS) showed 53.14% carbon, 30.76% Oxygen, 1.17% Sulfur, 0.63% chloride, 0.24% Calcium , phosphorus 0.49% , 13.34% nitrogen and 0.23% sodium, X-ray Diffraction (XRD), Atomic force microscope appeared at 375^{o}, Fourrier Tranceform Infrared Spectroscopy (FTIR) showed the presence of bands similar from proteins, fatty acid and other chemical compounds, Atomic Force Microscope (AFM) showed the lateral dimensions and The average diameter was of the chitosan NPs (71.414 nm).

Where the effect of each of the chitosan nanoparticles , Methotrexate drug(MTX) and combination (Chitosan NPs + Methotrexate) against cancer cells was evaluated using the ELISA assay at different concentrations (512 - 256 - 128 - 64 - 32 - 16 - 8) μg/ml.

Summary

As well as studying the effect against Antimicrobial resistance (AMR), *Staphylococcus aureus* which poses a future threat to the lives of patients. The study aims to use Chitosan nanoparticles as an alternative or adjunct to the work of antibiotics. infection specimens were collected from (Euphrates cancer Hospital) in AL-Najaf Province. Isolation and identification of *Staphylococcus aureus* using morphological and biochemical tests in VITEK2 compact system with a 91 % probability.

Where the effect of each of the chitosan nanoparticles, Methotrexate drug and combination (Chitosan NPs + Methotrexate) against (*Staphylococcus aureus*) using agar well diffusion methods at different concentrations (200 , 100 , 50 μg/ml).

The results of this study. Among the three compounds, the combination (Chitosan NPs + Methotrexate) gave the best results Where the highest growth inhibition area against MCF-7 cell cultures. was at absorbance (OD = 0.162) with a concentration (512 μg/ml) and the lowest growth inhibition area at absorbance (OD = 0.143) with concentration (8 μg/ml).

The combination (Chitosan NPs + Methotrexate) had a high synergistic effect with different concentrations (200 , 100 , 50 μg/ml) showed high inhibition activities against tested bacteria. The highest inhibition zone of combination (Chitosan NPs + Methotrexate) observed in Gram positive bacteria was (26mm) in *S.aureus* with concentration (200 μg/ml).

The results of this study are suggested. was an activity of combination (Chitosan NPs + Methotrexate) on MCF-7 cell cultures . It showed acceptable results for less cytotoxicity and its effect to inhibit high growth at low concentrations and the nontoxic to cells even at high concentration.

Combination (Chitosan NPs + Methotrexate) is a good option for the most common causes leading to Methotrexate intoxication for breast cancer patients are inappropriate dosage, and concomitant use of drugs, such as antibiotics. For this reason, the Methotrexate drug was combined with chitosan nanoparticles to reduce the toxicity of the Methotrexate drug and worked it on inhibition on microbes.

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Chapter One

Introduction

1. Introduction

Cancer remains one of the leading causes of death in the world. According to the statistics of the World Health Organization (WHO), 10 million deaths from cancer were reported in 2020 breast (685,000 deaths) and more than 13 million cancer deaths may occur in 2030 (Zhang *et al*.,2017). in 2020, Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%)(Yuan *et al*.,2022). According to the Iraqi Ministry of Health, the annual rate of cancer in Iraq is 2500 cases, including 20% incidence of breast cancer. Despite the advances in our understanding of molecular and cancer biology, discovery of cancer biomarkers and conventional surgical procedures, radiotherapy and chemotherapy such as Methotrexate drug , the overall survival rate from cancer has not significantly improved in the past two decades (Jemal *et al*.,2008). Most of these treatments of cancer cause side effects, therefore, it showed the necessity for effective new therapies against cancer particularly, those who are ready to evade drug resistance and different important side effects (Vultur *et al*., 2014).

Gram-positive organisms are the major cause of invasive bacterial illness in cancer patients. A wide variety of gram-positive bacteria produce significant opportunistic infections from bacteremia in cancer patients, with *Staphylococcus aureus* bearing the worst burden of disease. was shown to account for between 1.3% and 12% of all instances of bacteremia in cancer patients. the mortality rate for both pneumonia and bacteremia exceeded 50%. vancomycin is still the basis of MRSA treatment; although, substantial vancomycin failure rates have been documented in patients with cancer and MRSA bloodstream infection. Because of the evolution of cancer therapy and the shifting epidemiology of important gram-positive pathogens, continual efforts are required to understand and limit the impact of these bacteria in cancer patients through The creation of novel antibacterial, optimization of treatment techniques (Holland *et al*., 2014).

Nanobiotechnology is in part, the science of synthesizing molecular sized material that can range between 1-100nm. It is concerned with the advancement of pioneer materials in health, medicine, the environment,

economics, research and technology, and other fields, resulting in a significant change in the production of technologies. Which led to the development of molecular-Nano biotechnology, which is concerned with the study of cellular pathways involved in Nano biosynthesis at the molecular level and the construction of complex molecular machines capable of performing specific tasks. There are several number of physical, chemical, and biological methods available for synthesizing different types of nanoparticles, physical and chemical methods which are more expensive, energy consuming and potentially toxic to the environment (Palmqvist, 2017).

Chitosan NPs is a biopolymer that has been extensively researched for its potential applications in a variety of fields due to its biocompatibility, biodegradability, nontoxicity, mucoadhesion, and in addition to its natural origin and low cost. Chitosan NPS in this area can be a very interesting material because it has got low toxicity on the normal cells, while it has a toxic effect on tumor cells, the local action, it also causes low ability to move and it can induce apoptosis And its role in inhibiting Antimicrobial resistance (AMR) (Tang *et al*., 2016).

1.2. Aim of the study

The aim of the study was to clarify the effect of combination chitosan nanoparticles with Methotrexate drug on :

i. breast cancer cell line (MCF-7 Cell)
ii. Inhibiting the growth of antimicrobial resistance (AMR) bacteria (*S.aureus*). through the following **objectives**:

1. Synthesis of chitosan nanoparticles through different bacteria
2. characterization of chitosan nanoparticles with different techniques such as UV visible, SEM, XRD, AFM, EDS and FTIR.
3. Investigation the effects each of chitosan NPs, , Methotrexate drug (MTX) and MTX- Chitosan NPs is combination (chitosan NPs + MTX) on breast cancer cell MCF-7 by evaluating effects by ELIZA assay.
4. Investigation the effects each of chitosan NPs, Methotrexate drug (MTX) and combination (Chitosan NPs + Methotrexate) to Inhibiting the growth of antimicrobial resistance (AMR) bacteria (*S.aureus*).

Chapter Two

Literature review

2.1. Breast cancer incidence and mortality

Breast cancer is one of the most common cancers in women worldwide, and it is the leading cause of cancer-related deaths in women and is considered the first most frequently diagnosed form of the disease which considered as the leading cause of cancer death in women (Yuan *et al*.,2022).

And Breast cancer is the most common cancer among women in Arab countries. Breast cancer accounts for approximately 1/3 of the registered female cancers. In a study Alwan reported a trend for breast carcinoma to affect younger age group. The incidence of breast cancer in Iraqi women increased in the last two decades and forms one of the major threats to female health (Alwan and Nada,2016). Iraqi National Programs for early detection of breast cancer were developed in order to decrease morbidity and mortality of breast cancer (World Health Organization, 2002). Age at which breast cancer diagnosed is with important implications since cancer in younger age group is more aggressive. in 2020, Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%). There is a huge difference in breast cancer survival rates worldwide, with an estimated 5 - year survival of 80% in developed countries to below 40% for developing countries (Yuan *et al*.,2022).

2.1.1. Risk Factors for the Development of Breast Cancer

Risk factors for breast cancer include : Female sex, older age, genetics, lack of childbearing or nursing, greater levels of estrogens, specific dietary habits, radiation exposure, positive family history of breast cancer, and obesity are all risk factors for breast cancer. Tobacco use appears to raise the risk of developing breast cancer. Long-term smokers face a 35% to 50% increase in risk. Oral contraception may be a risk factor for the development of premenopausal breast cancer (Biswas *et al*.,2015).

2.1.2. Treatment of breast cancer

The treatment of breast cancer is determined by a variety of factors, including the cancer's stage and the patient's age. Breast cancer is often treated with surgery, followed by chemotherapy, radiation therapy, or both. Hormone receptor-positive tumors are frequently treated for years with hormone-blocking treatment. Monoclonal antibodies or other immunomodulators may be given in advanced stages with distant metastasis (Masood ,2016).

2.1.3. Chemotherapy

Chemotherapy can be given before surgery, after surgery, or instead of surgery for inoperable cases. The process of killing cancer cells by using certain medicines is termed as chemotherapy (Masood ,2016).Table (2-1) describes example of the more commonly used chemotherapeutic agents and their mechanisms of action.

Table (2-1): Example of chemotherapeutic agents and their mechanisms of action.

Chemotherapeutic	Mechanism of Action
Methotrexate	inhibits enzymes responsible for nucleotide synthesis DNA & RNA

However it has various side effects. Metastatic or secondary breast can is difficult to treat but it can be controlled and sometime for various years (Müller *et al*.,2001). Chemotherapy can be prescribed to manage metastatic breast cancer to minimize or sluggish its development.

2.1.3.1. Methotrexate

Methotrexate (MTX), is used in the treatment of breast, head and nose, lungs and Non-Hodgkin Lymphoma cancers, and considered one of the most studied folate antagonist .MTX inhibits dihydrofolate reductase (DHFR), which leads to accumulation of polyglutamated folates, causing further inhibition of thymidylate synthase and glycinamide ribonucleotide formyltransferase. Subsequently, the lack of reduced folate substrates

impairs synthesis of purine nucleotides, thereby inhibiting DNA & RNA synthesis which leads to decreased proliferation growth of cancer cells (Gharebaghi *et al*,2017).

2.1.3.2. Side effects of Methotrexate

Methotrexate is a well-tolerated immunosuppressive medication, However, its application is limited due to low solubility, high toxicity ,rapid metabolism and side effects. In addition, the variability and unpredictability of the function of drug causes the treatment failure. Since a large amount of MTX metabolizes quickly in liver and being excreted through the kidneys, its half-life in plasma is short and the concentration in target tissues decreases (Gharebaghi *et al*,2017). The most common causes leading to MTX intoxication are inappropriate dosage, and concomitant use of drugs, such as NSARs, antibiotics or salicylates, new initiation of MTX or re-instatement or increase of dosage (Knoll *et al*.,2016).

2.2. Invasive *Staphylococcus aureus* bacteria in breast cancer

At least half of all microbiologically verified opportunistic infections in cancer patients are caused by Gram-positive bacteria. Immunosuppression caused by the underlying cancer or its attendant therapy, such as neutropenia, and mucosal barrier breakdown, such as that seen after long-term vascular catheter placement or during graft against host disease, combine to make cancer patients especially vulnerable to gram-positive infections. Due to healthcare-associated exposure and selection from antibiotic prophylaxis, such infections are frequently caused by resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA).

S.aureus has a significant clinical influence on cancer patients. *S. aureus* was shown to account for between 1.3% and 12% of all instances of bacteremia in cancer patients in a systematic review of bacteremia studies published since 2008. However, focusing just on bloodstream infections may result in a major underestimating of the impact of *S. aureus*. Skin and soft tissue infection (26.7%) and pneumonia (25.4%) were the most common forms of infections caused by *S. aureus* among nonneutropenic cancer

patients in 9 Asian nations, while bacteremia accounted for only 14.0%. Having pneumonia was an independent risk factor for mortality, and the observed mortality rate for both pneumonia and bacteremia exceeded 50%, which is much greater than the rate reported in non-cancer patients. The prevalence of methicillin-resistant *S. aureus* isolates among cancer patients varies geographically but appears to be increasing overall.

Invasive methicillin-Resistant *S. aureus* (MRSA) infections should be treated with a beta-lactam anti-staphylococcal such as cefazolin or nafcillin. Treatment of MRSA bacteremia with vancomycin, rather than a beta-lactam, was related with increased mortality in a matched case control trial in which about 40% of patients had cancer. Vancomycin is still the basis of MRSA treatment; nevertheless, substantial vancomycin failure rates have been documented in patients with cancer and MRSA bloodstream infection. So far, studies of modern anti-staphylococcal antibiotics (e.g., daptomycin, ceftaroline) in cancer patients have been few, but usually positive (Holland *et al*., 2014).

2.3. Nanobiotechnology

Nanotechnology is a multidisciplinary science that deals with the synthesis, design, characterization, and use of materials, as well as various elements of research and technology at the nanoscale. One nanometer equals one thousandth of a micrometer = 10^9 meters). Nanotechnology is not a single growing scientific discipline in and of itself, but rather , a meeting of different traditional sciences, such as, chemistry, physics, materials science and biology, to bring together the required collective expertise needed to develop these novel technologies. Nanobiotechnology is a new advanced field of nanotechnology that combines biological principles with chemical and physical procedures to manufacture nano-sized particles with specific functionalities and represents an economical alternative to chemical and physical methods of nanopaticle formation. The applications of nanotechnology include fluorescent biological labels, drugs and gene delivery, bio-detection of pathogens, detection of protein, probing of DNA structure, tissue engineering, tumor detection, separation and purification of

biological molecules and cells, MRI contrast enhancement and phagokinetic studies (Natarajan *et al*., 2010).

2.3.1. Chitosan Nanoparticles

Chitosan the second most abundant next to cellulose, naturally occurring amino polysaccharide, derived as a deacetylated form of chitin. Its nontoxic, biocompatible, antibacterial and biodegradable properties have led to significant research towards biomedical and pharmaceutical applications, such as drug delivery, tissue engineering, wound-healing dressing and anticarcinogenic etc. Chitosan is readily modified by a variety of methods due to the presence of hydroxyl and amino groups on its backbone. Chitosan has been modified to improve its physicochemical properties in order to broaden its application possibilities. Numerous alteration techniques using chemical, physical, and enzymatic processes have been extensively reported. For example, chitosan is often de-polymerized to oligosaccharides and/or monomers, which may restrict its applications under certain biological conditions. De-polymerization is usually accomplished by enzymatic modification with a chitinase enzyme. Many methods for modifying chitosan have been reported, including quaternization, N-alkylation, hydroxyl-alkylation, carboxy-alkylation, thiolation, and glycation. Physical modifications include the use of electric radiation and sonication. Modified chitosan with improved properties, such as excellent solubility in aqueous solutions at various pH, modulated surface charges, better absorption performance, and the inclusion of new cross-linking sites, have found novel applications until adapted to a specific use (Ali and Ahmed,2018).

2.3.2. Nanoparticle Synthesis Methods

There are two important techniques for preparing nanomaterials: top-down (size reduction from bulk material) and bottom-up (synthesis of materials from atomic level) Figure(2-5) (Bhagyaraj *et al*.,2018).

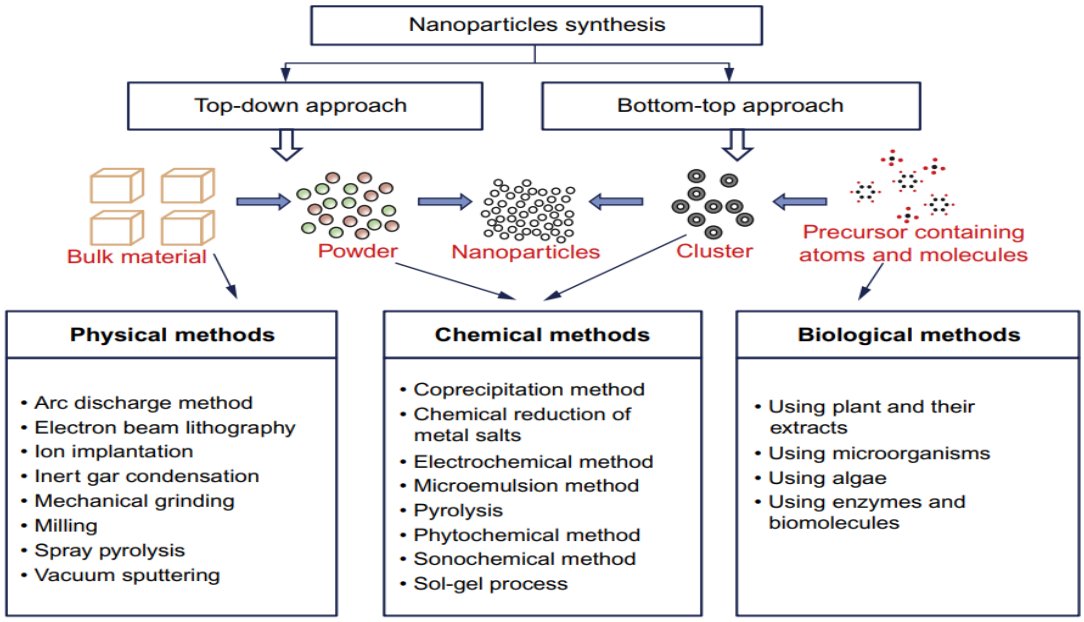


Figure (2-5) ,Top-down and bottom-up approach for synthesis of nanoparticles.

2.3.2.1. Synthesis of Nanoparticle by bacteria

One approach to achieving this goal is to synthesis NPs utilizing natural processes such as carbohydrates, microbial enzymes, vitamins, polysaccharides, biodegradable polymers, microorganisms, and biological systems. One promising technique is based on bacterial NP production. bacteria have an outstanding potential for heavy metal ion reduction, making them a good candidate for nanoparticle manufacturing. Some bacterial organisms, for example, have acquired the ability to use specialized defensive mechanisms in response to environmental pressures such as heavy metal ion or metal toxicity. It was observed that some of them can live and grow in situations with extraordinarily high metal ion concentrations (Dhanker *et al*., 2021).

2.3.3. Biomedical Applications of Chitosan Nanoparticles

Chitosan NPs has been commonly applied in drug delivery systems as a carrier substance. Dependent on their main amino groups, the cationic character of the chitosan provides the polymer with its distinct biophysical

and biochemical properties above all others. Chitosan NPs have their specific advantages compared to other forms of delivery systems, including gradual and regulated drug release, increasing drug uptake and enhanced drug bioavailability, reduced drug side effects, etc. Protein-based medications are quickly hydrolyzed in the gastrointestinal tract by enzymes. These drugs are less vulnerable to oxidation by gastric enzymes when encased in chitosan nanoparticles. Additionally, chitosan NPs can significantly increase the safety of drugs. Chitosan NPs modulate drug release, enhance protein biodegradation, and facilitate hydrophilic compound assimilation through the epithelial layer. They are being investigated for their potential role in the delivery of drugs that act on the stomach (Zhao *et al*.,2018).

2.3.3.1. Chitosan NPS Applications in the Treatment of Breast Cancer

To overcome the existing challenges connected with cancer treatment safety and efficacy, numerous scientists have worked tirelessly to develop smart tailored therapy using breakthrough nanobiotechnology to combat the terrible killer cancer. When compared to unmodified drugs, nanobiotechnology-based treatments have shown substantial advantages such as chitosan NPS, including enhanced half-lives, retention, targeting efficiency, and fewer patient adverse effects (Deshpande,2016).Nanosystems have four unique properties that distinguish them from other cancer therapeutics:

(i) the nanosystems can themselves have therapeutic or diagnostic properties and can be designed to carry a large therapeutic (payload);

(ii) nanosystems can be attached to multivalent targeting ligands, which yield high affinity and specificity for target cells;

(iii) nanosystems can be made to accommodate multiple drug molecules that simultaneously enable combinatorial cancer therapy and

(iv) nanosystems can bypass traditional drug resistance mechanisms. The nanocarriers can obtain greater intracellular concentrations of medicines in

cancer cells while limiting toxicity in normal cells, improving anticancer effects and reducing systemic toxicity.

Methotrexate (MTX) acts as an antagonist of folic acid, which is necessary for DNA synthesis, and has a therapeutic effect on many types of cancer cells that overexpress folate receptors on their surfaces However, its clinical efficacy is often compromised by the acquisition of resistance in cancer cells, due to cellular efflux of the molecule. In this context, the encapsulation of antitumor drugs such (MTX) in nanoparticulated systems like polymeric NPs, which retain a higher drug concentration within the cell, might overcome the shortcomings associated with conventional drug delivery strategies (Nogueira *et al*.,2013).

2.3.3.2. Nanobiotechnology applications as a Antimicrobial resistance (AMR)

Nanobiotics as antibacterial complementary to antibiotics are highly promising and are gaining large interest as they might fill the gaps where antibiotics frequently fail. nanoparticles are now considered a viable alternative to antibiotics and seem to have a high potential to solve the problem of the emergence of bacterial multidrug resistance.

The particle's matrix may have antibiotic properties or carry an antibiotic medication within or on its surface that can act on a bacterial target. The antibacterial process of chitosan NPs can be divided into two distinct mechanisms. The first is that chitosan NPs forms a coating of the polymer on the cell surface by adsorption. The membrane prevents nutrients from entering the cells and functions as a bacteriostatic and decontamination barrier; The second mechanism is that chitosan NPs permeates cells through osmosis, adsorbs anionic cytoplasm within the cells, and induces floccus formation The antibacterial function of chitosan differs depending on its the bacteria that operate on it, growth stage, pH, and concentration. is particularly effective against *S.aureus*, exhibiting similar properties to antibiotics (Atay, 2019).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Material

3.1.1. Chemicals and Biological materials

Table (3-1): Chemical and biological materials used in present study.

No.	Items	Company	Country
1.	Chitosan	Reagent world	USA
2.	Dimethyl sulfoxide (DMSO)	Santacruz Biotechnology	
3.	Methotrexate (MTX),Tables	Ebewe	Austria
4.	Acetic acid	Chem- lab	Belgium
5.	Trypsin/EDTA	Capricorn	Germany
6.	Deionized water	Sigma	Sigma

3.1.2. Instruments

Table (3-2) list; the instruments used in this study

No.	Instruments	Company	Country
1.	Cooling centrifuge	Hettich	Germany
2.	Incubator	Memmert	
3.	fluorescence microscope	Leica	
4.	Roswell Park Memorial Institute (RPMI 1640)	Capricorn	
5.	Biosafety cabinet	Genex	

6.	Shaking incubater	Genex	
7.	Cell culture plates	Santa Cruz Biotechnology	USA
8.	Phosphate buffer saline (PBS)	Bioworld	
9.	Methyl-Thiazolyl Tetrazolium (MTT stain)	Bio-World	
12.	CO2 incubator	Cypress Diagnostics	Belgium
13.	Centrifuge	CYAN	
14.	Deep Freeze	LG	
15.	Microwave oven	LG	Korea
16.	Falcon Culture Flask	Guangzhou Jet Bio-Filtration	China
17.	Vitek-2	Biomerieux	France

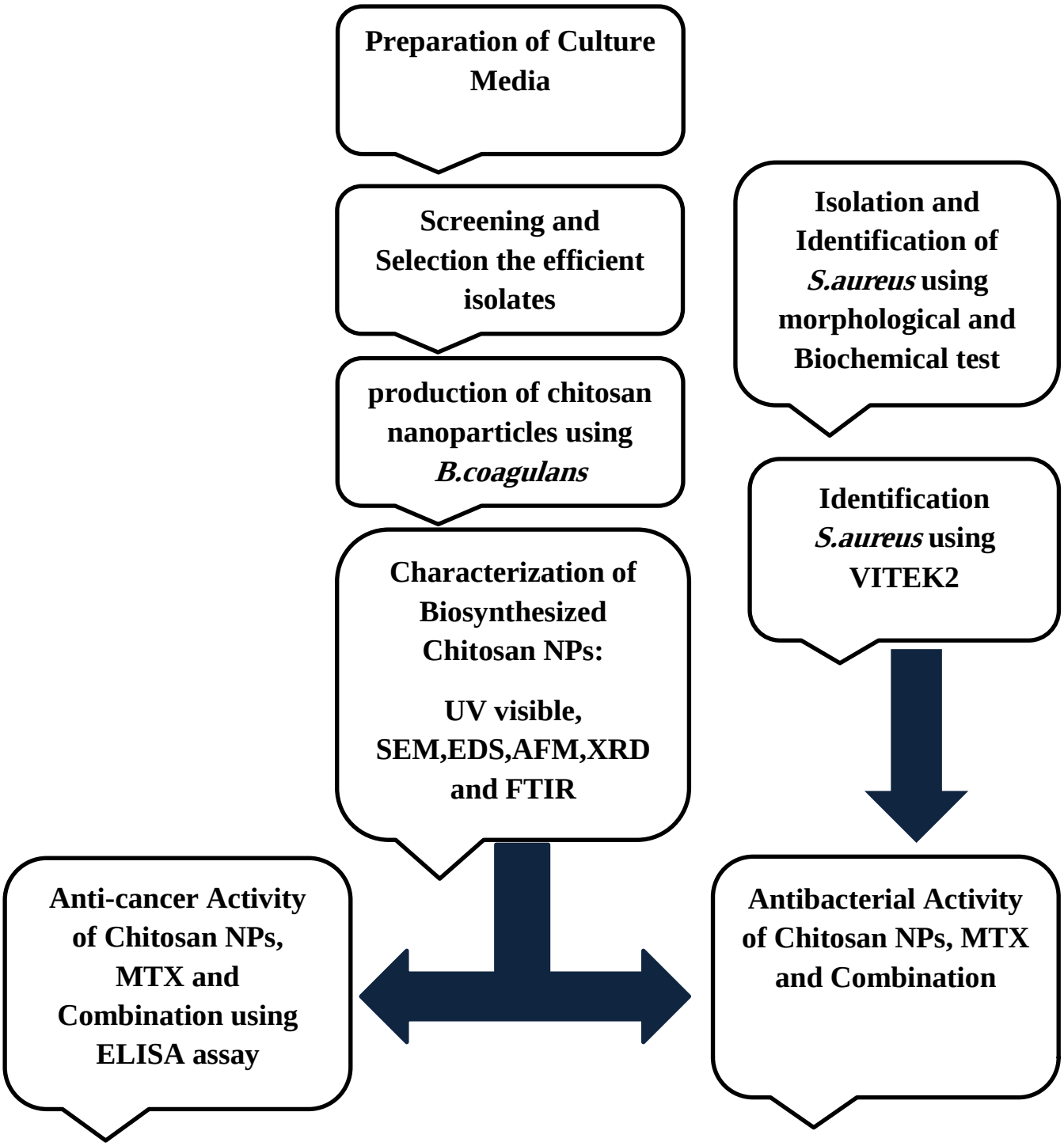
3.1.3. Culture Media

Table (3-3): Culture media used in the study

No.	Culture Media	Manufacture Company	Country
1.	Brain heart infusion broth	Himedia	India
2.	Macconky agar		
3.	Mannitol salt agar		
4.	Muller Hinton agar		
5.	Blood agar		
6.	Nutrient agar		

3.2. Methods

3.2.1. The study design



3.2.2. Preparation of Culture Media

reparation of Culture Media brain heart broth 3.7 mg . have been prepared according to the manufacturer's instructions. The constituents are dissolved in pure water 100 ml and completely dissolved in a microwave oven. The media is sterilized by autoclaving at 121°C for 20 minutes and then dumped into sterilized screw tubes for broth media inside a laminar hood for 24 hours at 37°C to preserve sterility (MacFaddin, 2000.)

3.2.3. Isolation and identification of *Staphylococcus aureus* using morphological and biochemical tests

staphylococcus aureus infection specimens were collected from (Euphrates cancer Hospital) in AL-Najaf Province. Swabs from patients were obtained and transported to the laboratory, where they were streaked on blood agar, Macconkey agar and Mannitol salt agar

3.2.4. Screening and Selection of Isolates

The specimens were inoculated on Nutrient agar, blood agar, Macconkey agar, and Mannitol salt agar and incubated at 37°C for 24 hours. Purification and subculture were then performed on the isolated colonies. The pure colonies of the isolates were selected using nutrient agar and sheep blood agar (Hemraj *et al*., 2013). According to Bergey's Manual of Systematic Bacteriology and identification was performed with the VITEK2 compact system.

3.2.5. Identification *Staphylococcus aureus* using VITEK2

Compact System suspension of bacteria was made according to the manufacturer's instructions. Transferring an overnight pure culture and suspending it in 3.0 ml of sterile saline in a 12 x 75 mm clear (polystyrene) test tube yielded an acceptable number of colonies. The turbidity was reduced to 0.5 McFarland. Making use of a Densi-Chek turbidity. The identical suspension was utilized in Gram positive-ID with the VITEK-2 compact system. Finally the Gram positive -ID cassettes were put into the vitek-2 chamber with the specimen suspension tubes (karagoz *et al*., 2015).

The VITEK2 compact system was used to achieve antibiotic sensitivity for the tested isolate.

3.2.6. Selection the efficient isolate that producing Chitosan NPs

First, carry out the process of cultivating the types of bacteria (*B.clausii*, *B.coagulans* , *B.Subtilis*) in the brain heart infusion broth, taking into account the methods of sterilization during the process of planting the medium with bacteria, then put the tubes in the vibrating incubator for 24 hours at a temperature of 37 °C.

After incubation, the colloidal suspension was centrifuged at 4500 rpm for 10 minutes, In The raw material for the samples to find out which samples were activated better. take 1mg/1ml of chitosan, where the brain heart infusion broth medium is 5ml in a test tube.

weigh the chitosan by 0.005mg of it. The chitosan is dissolved by acetic acid, where it is diluted by 5%, where the solution is equal to the amount of the broth medium in a test tube. put the solution consisting of chitosan and acetic acid on the mixing device to dissolve the chitosan well in the acetic acid. add the solution to the culture medium, mix it well, then leave it in the shaking incubator for 24 hours at a temperature of 37 °C. After 24 hours of incubation, determine which type of bacteria was activated, and depend on that on the activity of the bacteria where Based on colour change, UV visible spectrum, and antibacterial activity against pathogenic bacteria (*s.aureus*) as an indicator strain, was chosen as an efficient isolate.

3.2.7. Production of chitosan nanoparticles using *B.coagulans*

B.coagulans has the ability to biosynthesize nanoparticles extracellular (Rudek *et al*.,1976).

After identifying the activated sample, which is *B.coagulans*, transplant the bacteria into the brain heart broth in a larger amount by 500 ml and leave it in the incubator for 24 hours at a temperature of 37 degrees Celsius.

Then make a solution of chitosan with acetic acid and add it to the culture medium containing the bacteria in an equal amount, then leave it in the incubator for 24 hours at a temperature of 37 C.

After incubation, perform the centrifugal process 3000 rpm for 20 min For the culture medium that contains bacterial cells, divide the total amount into small packages, which are unrein cup of 50ml Repeat the process until all the quantity is used up.

After the centrifugation of the samples is completed, get rid of the supernatant and take the precipitate containing the nanomaterial.

Collect the sedimentary samples in a unrein cup and doing the process of washing the samples using distilled water where fill the bottle with water and put it on the mixing device and then put it in the centrifuge for 30 minutes to 3000 revolutions per minute where doing the washing process twice.

After completing the second washing process, get rid of the water and take the sediment only, then dry it by placing it in the incubator until it dries at a temperature of 40 °C.

3.2.8. Characterization of Biosynthesized Chitosan NPs

The physical characteristics of biosynthesis nanoparticles were characterized by UV-Visible Spectroscopy, Scanning Electron Microscope, Energy Dispersive X-Ray Spectroscopy, X-ray Diffraction, Atomic force microscope, and Fourrier Tranceform Infrared Spectroscopy.

3.2.8.1. UV visible Spectroscopy of Chitosan NPs

The Chitosan NPs formation and their size estimation within the composite suspension were confirmed by UV-Visible spectroscopy (shimadzu UV-visible 1800) in Alameen center for Advanced Biotechnology and research.

3.2.8.2. SEM Analysis of Chitosan NPs

SEM was used to determine the surface morphology and scale of nanoparticles in composite films. Chitosan NPs were examined at the University of Tehran. The specimen was prepared by grinding chitosan nanoparticles, preparing a suspension of the nanoparticles, and attaching a droplet of the suspension to the fixing matrix. Prior to SEM characterization, the samples were further air dried and stored in a desiccator. The imaging was carried out at an accelerating voltage of 15 KV (Biao *et al*., 2017).

3.2.8.3. EDS Analysis of chitosan NPs

Energy dispersive x-ray spectroscopy (EDS) was used to perform an elemental analysis of the chitosan NPs. At an accelerating voltage of 10- 12 KV, a low vacuum mode, a spot size of 5mm, and working distances of 5-10mm, point compositional analysis were investigated (Biao *et al*., 2017), At the University of Tehran.

3.2.8.4. AFM of Chitosan NPs

AFM was used to investigate the dispersion and aggregation of nanomaterials, as well as their shape and size. A sufficient amount of biogenic chitosan NPs was sent to the University of Tehran for atomic force microscopy.

3.2.8.5. XRD Analysis of Chitosan NPs

Biogenic chitosan NPs were sent to Tehran for X-ray Diffraction (XRD) Analysis, XRD is a common analytical technique for analysing molecular and crystal structures, qualitative detection of different molecules, quantitative resolution of chemical species, calculating crystallinity, isomorphous substitutions, and particle sizes (Biao *et al*., 2017), At the University of Tehran.

3.2.8.6. FTIR of Chitosan NPs

FTIR The transmittance of the prepared formulations was accomplished by FT-IR spectrophotometer, Biogenic chitosan NPs was sent for FTIR at the University of Tehran.

3.2.9. Anti-cancer Activity of Chitosan NPs , MTX and Combination (Chitosan NPs + Methotrexate) using ELISA assay.

Maintenance of cell cultures MCF-7 cells have the following characteristics **(** Primary tumor (invasive breast ductal carcinoma), non-mutant P53 ,estrogen receptor present, proliferative response to estrogens , Presence of progesterone receptors**)** (Lacroix and Leclercq, 2004). MCF-7 Cell line was provided by Iraqi Center for Cancer and Medical genetic research\ University of AL-Mustansiriyah, and was maintained in RPMI-1640 supplemented with 10% fetal bovine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C (Sulaiman *et al*.,2018).Transplant the breast cancer sample, and after it is completed and reactivated, take it out from the incubator, and change the culture medium by withdrawing it using the pipette and adding PBS Phosphate buffer saline at a concentration of 5 ml .

And add trypsin at a concentration of 1 ml to the falcon containing breast cancer cells to remove the living cells stuck on the wall of the falcon and put it in the incubator for 3 minutes, then take it out and add PRMI 4ml to stop the trypsin action and prevent it from decomposing the cells .

Mix the solution consisting of RPMI, trypsin and PBS Phosphate buffer saline together, then withdraw it by means of the pipette and put it in the centrifuge tube in order to obtain a precipitate for 5 minutes at 3000rpm .

After the centrifugation process, get rid of the supernatant and take the precipitate and add RPMI to it at a concentration of 1 ml. prepare a slide for counting white blood cells and add 10 microliters of 1 ml of the mixture to it. After counting under the fluorescence microscope of breast cancer cells,

adding them, dividing them by 4, and then multiplying them by 10,000, it appears that there are 190,000 cells per 1 milliliter .

After counting and not getting the required number of cells per 1 milliliter, add RPMI to increase the dilution and reach the required number, which is 6000 cells, according to the following equation ;

Cell concentration = 190000 cells

Numbers of cell = 6000 cells in every well

Numbers of well = 30

total cell count = 30 × 6000 = 180000

total cell count/Concentration of cells in the well=180000/190000=0.947ml

of cells and complete the volume to 6 milliliters, assuming that put 200 microliters in each well.

200 × 30 = 6000 ul ÷1000 ul = 6 ml

Then put in the plate of Cell culture test the solution containing the live cells after completing the formation of 6000 cells in each well with an amount of 200 µl where 21 wells were consumed. Then put the plate of Cell culture in the incubator for 24 hours at a temperature of 37 °C.

Start with the dilution of the materials to be tested for their effect on breast cancer cells, which are chitosan and MTX, and the combination of the two materials together (MTX + Chitosan).

Use the dilution method in tubes, where make 7 dilutions for each material starting from (512 - 256 - 128 - 64 - 32 - 16 - 8) µg/ml.

And take 7 Eppendorf tubes for each subject so that the total is 21 and write on each group the name of the sample.

Put a volume of 1mg/1ml of each material in each of Eppendorf No.(1) and then put a DMSO solution of the materials, which is a material that benefits the process of preserving cells by decomposing them inside and preventing their damage in Deep freeze, where put 10 microliters of it to ensure the

solubility of the material, add RPMI with it at a concentration of 900 microliters to complete the volume to 1 milliliter In Eppendorf No. (1)

And put the RPMI solution with a volume of 500 µl to the rest of Eppendorf from (2-7) and then doing the dilution process.

Start with the dilution process, where the concentration of the solution in Eppendorf (1) is 1000 µl, where 500 µl is withdrawn from the first Eppendorf that contains the concentrated solution and add it to 500 µl to it in a tube (2) to complete the concentration to 1000 µl and so on until reach the last Eppendorf No. (7).

After completing the dilution process, bring the plate that slide containing the samples after they have been incubated for 24 hours and empty 21 wells from the medium after making sure that the cells are activated and their growth inside the wells by examining them with an fluorescence microscope where the samples are in the form of a sticky precipitate down the well .After emptying the pits, add the dilute solutions that were previously prepared in Eppendorf from the materials chitosan and MTX and the combination material between them, where add 200 µl of solutions to each well with horizontal lines for each material, where each material has 7 wells, then put the plate in the incubator for a period 24 hours at 37 °C.

After incubation, MTT is added to the solution in the plate with a volume of 20 µl per well. The importance of it correlates with the living cells and differentiates them from the dead cells affected by the drugs. Then the incubation is done for 4 hours in a CO2 incubator. After incubation, MTT solution is removed and then add DMSO in a volume of 100 µl to each well where it binds with the MTT and gives a violet color to separate the unaffected cells from the cells affected by the materials.

3.2.10. Antibacterial Activity of Chitosan NPs , MTX and Combination

The antibacterial activity of Chitosan NPs , MTX and combination (Chitosan NPs + Methotrexate) was carried using agar well diffusion methods against strain of resistance bacteria (*S.aureus*).

Preparation of three dilutions, wherein Eppendorf No.(1) contains concentrated material in a volume of 200 μg/ml and the preparation of Eppendorf (2) and (3) with half of the previous dilution (100,50 μg/ml) respectively. Applying the previous steps of each dilution of three materials , Chitosan, MTX and combination, we get nine dilutions.

Preparation of three agricultural Petri-dishes containing the culture medium Müller agar, where the first three Petri-dishes are diffusion methods by *S.aureus*. Making three pores for each Petri-dishes using a sterile cork borer ,Pores (7 mm diameter) in the agricultural medium.

Fill with three pores in the first dish containing *S.aureus* Chitosan NPs from Eppendorf (1, 2, 3) and Repeat the last step on each from the material MTX and combination (Chitosan NPs + Methotrexate) then The Petri-dishes were then incubated at 37°C for 24 hours. All previous steps were performed under high sterilization conditions.

Chapter Four
Results and Discussion

4. Results and Discussion

4.1. Identification of *Staphylococcus aureus* isolate Using VITEK2

VITEK 2 automated compact system with Gram positive-ID cards and 43 biochemical assays was used to identify isolates. After 6 hours, the isolates were identified as *S.aureus* with a 91 % probability (Table 4-1).

Table (4-1) Biochemical identification of *S.aureus* isolated from breast cancer infection's using VITEK2 compact system.

Biochemical Details			*Staphylococcus aureus*													
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	(-)
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	lLATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	+	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

4.2.1. The biosynthesis of chitosan NPs by *B.coagulans*

B.coagulans, which was used in the biosynthesis of chitosan NPs, displayed extracellular biosynthesis ability by using cell free supernatant as a substrate after the addition of chitosan NPs under previously established conditions. The colour changes of the reaction mixture from light to dark, as shown in (Figure 4-1), after 24 hours of shaking at 3000 rpm, as well as the colour change and antibacterial behavior against bacteria (*S.aureus*), served as markers for the biosynthesis of chitosan NPs by *B.coagulans*, as shown in (Figure 4-2).

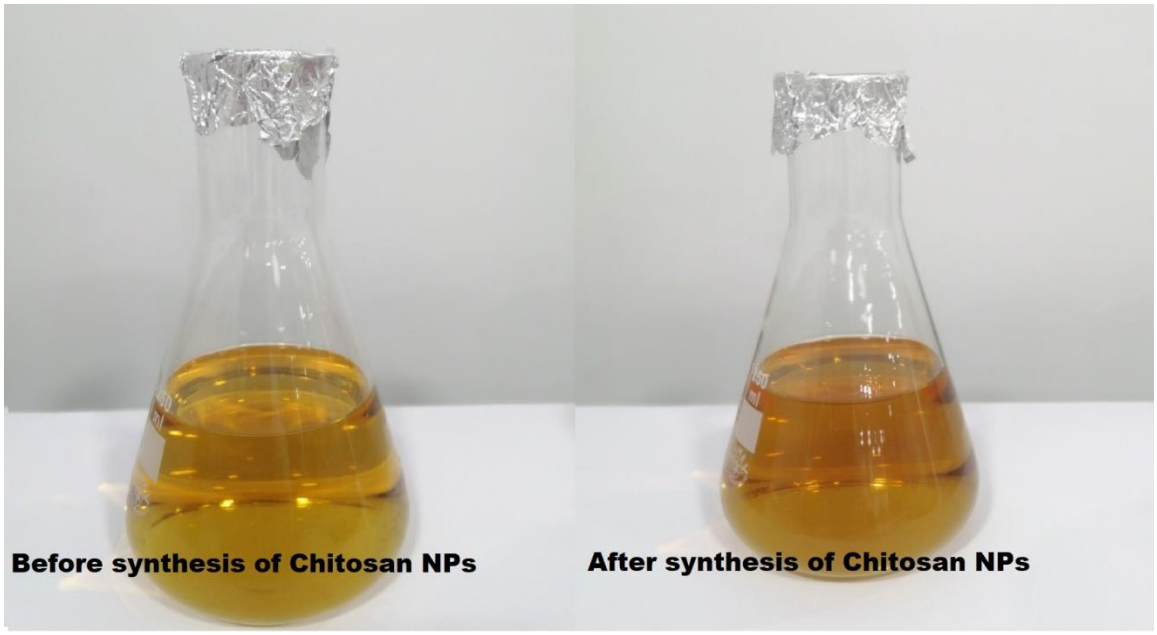


Figure (4-1) Biosynthesis of Chitosan NPs in BHIB using *B.coagulans* at optimum conditions. Colour change from light to dark after synthesis

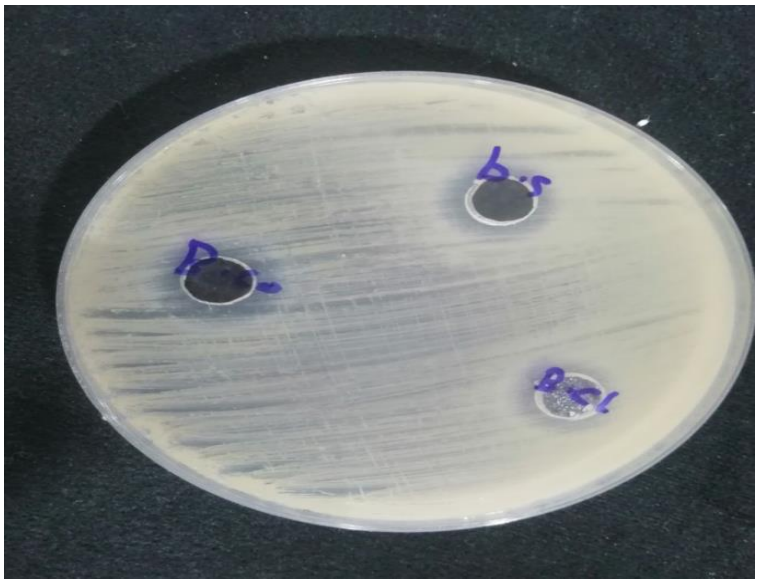


Figure (4-2) Anti-bacterial activity of Three strains of bacillus (*B.clausii* 12mm, *B.coagulans* 14mm, *B.Subtilis* 14mm) against bacteria (*S.aureus*).

4.2.2. Characterization of biosynthesized Chitosan NPs

4.2.2.1. UV-visible Spectroscopy

UV-Visible spectrophotometric analysis is a tested method for detecting nanoparticles. After 24 hours of incubation, the reaction mixture's colour changed, indicating the synthesis of nanoparticles in the reaction mixture. Visual observation and measurement of the absorbance band using UV-visible spectroscopy can validate the biogenesis of nanoparticles. The absorption spectra of the reaction mixture's nanoparticles displays a peak at 280nm (Figure 4- 3).

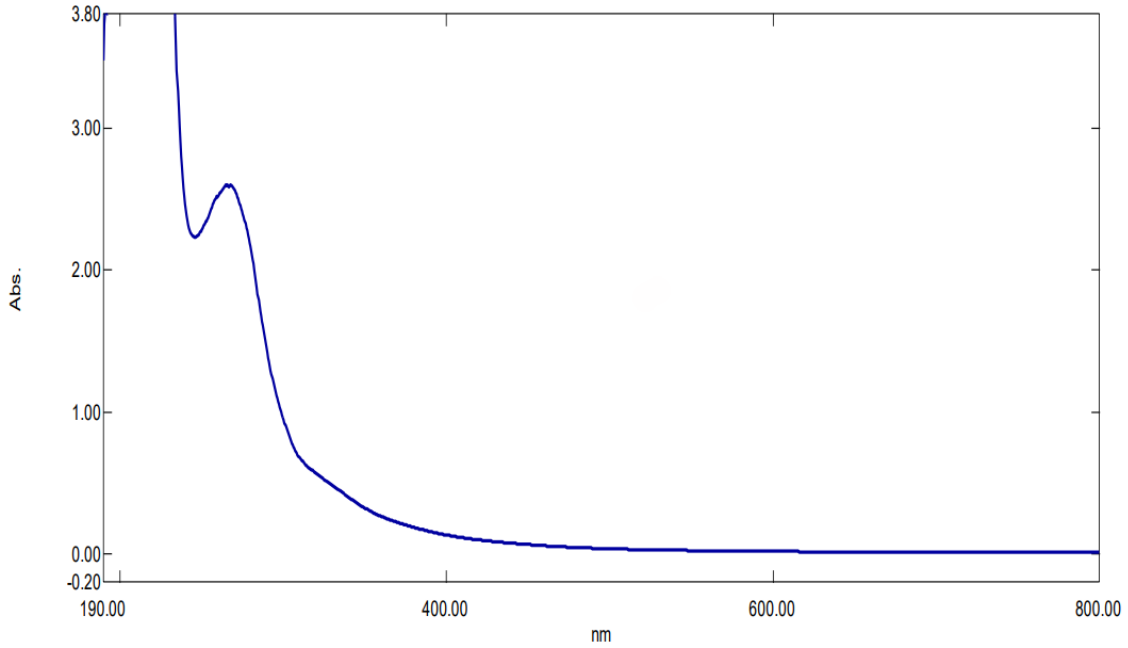


Figure (4-3) UV-visible spectroscopy analysis of Chitosan NPs synthesis by *B.coagulans*

A UV-Visible spectrophotometer was used to evaluate the synthesis of nanoparticles, which revealed a single peak at 280 nm, indicating the presence of chitosan NPs in the reaction solution. The creation of unique yellowish-brown colour was indicative of the production of chitosan nanoparticles within the chitosan matrix.

4.2.2.2. Scanning Electron Microscopy Analysis (SEM)

SEM was used to validate the morphology and size of the biogenic chitosan NPs and well dispersed and spherically shaped chitosan nanoparticle generated by *B.coagulans* with a size ranging from 30.94 to 95.51 nm)(Figure 4-4).

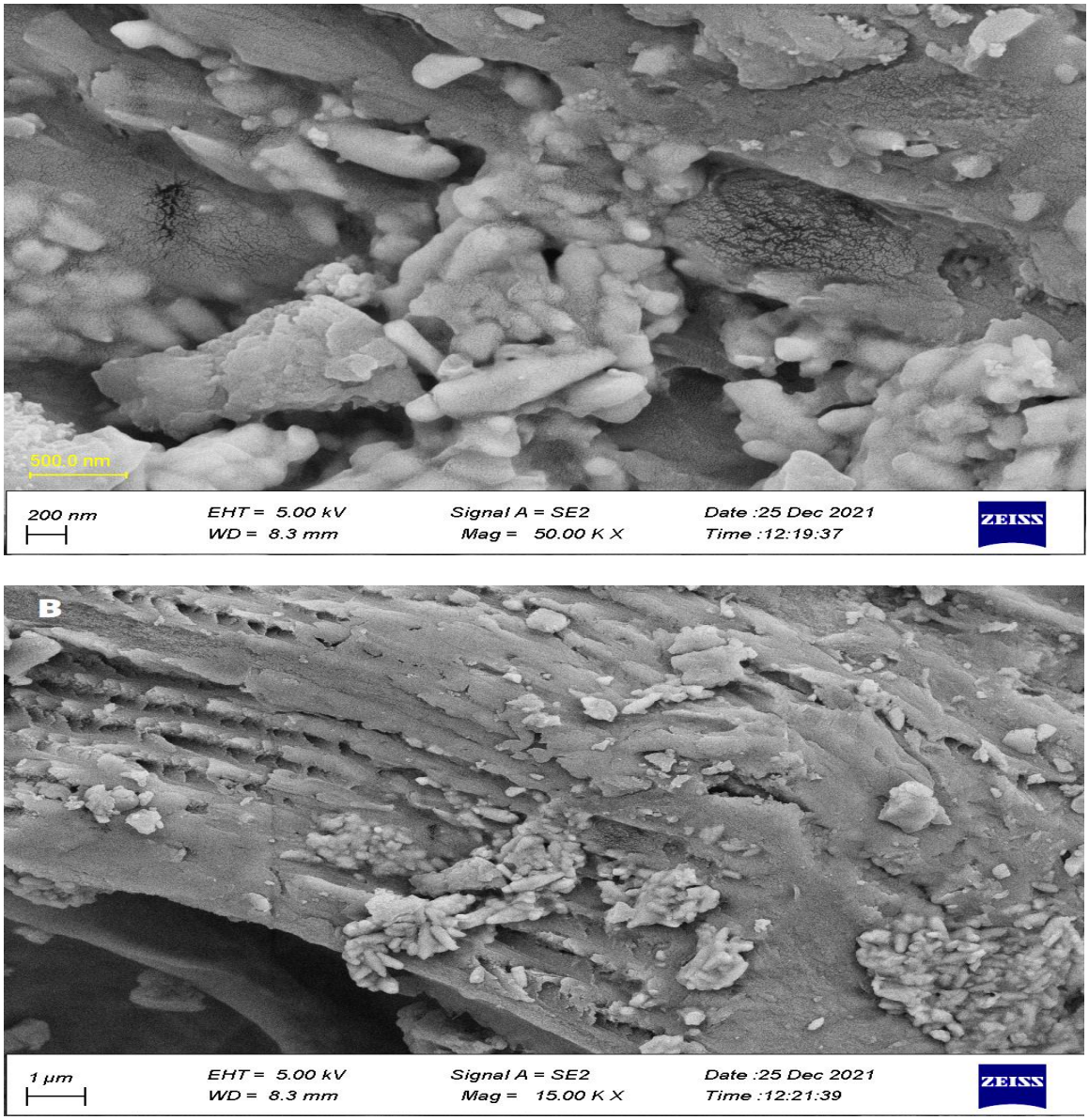


Figure (4-4) SEM micrograph of biogenic Chitosan NPs synthesis by *B.coagulans* showed Chitosan NPs with spherical well dispersed with size (30.94 – 95.51 nm) at A: 200 nm, and B: 1 nm

SEM micrographs of the Chitosan NPs revealed that they were about uniform spheres. The unmodified Chitosan NPs were made up of clusters of nanoparticles.

4.2.2.3. EDS analysis of biogenic Chitosan NPs

The optical absorption peaks of elements were observed using Energy Dispersive Spectroscopy (point and mapping analysis) to quantify the presence of chitosan NPs. Elemental analysis show that the rate of components constituting chitosan NPs production by *B.coagulans* was as follows (Figure 4-5) and (Table 4-2). The energy dispersive spectroscopy analysis identified components, enabling for quantitative and qualitative study of the elements constituents in the manufactured formulations.

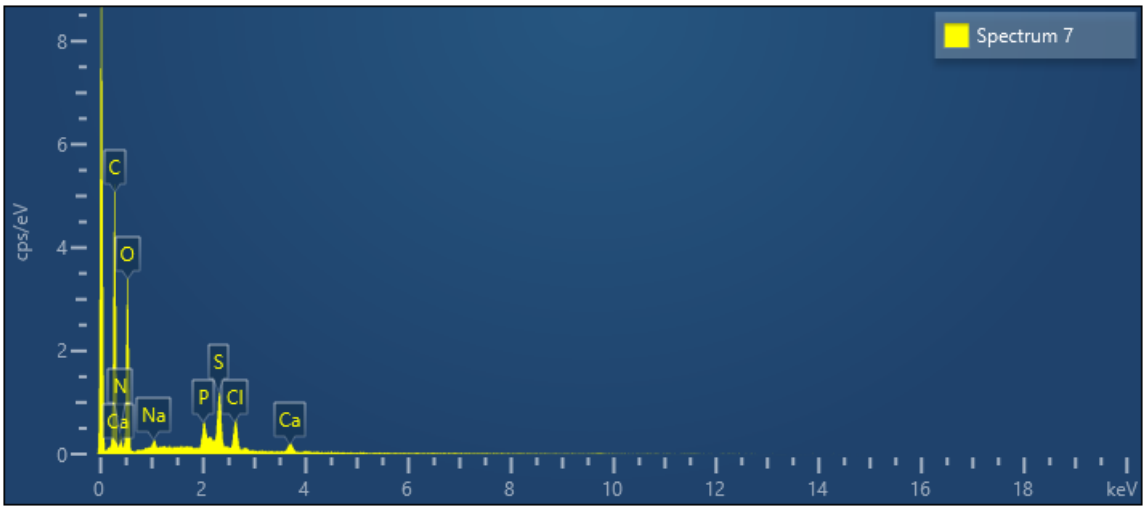


Figure (4-5) EDS analysis of Chitosan NPs synthesis by *B.coagulans*

Table (4-2) Elemental analysis of Chitosan NPs synthesis by *B.coagulans*

Spectrum 7				
Element	**Line Type**	**Weight %**	**Weight % Sigma**	**Atomic %**
C	K series	45.35	0.77	53.14
O	K series	34.97	0.65	30.76

S	K series	2.67	0.08	1.17
Cl	K series	1.59	0.06	0.63
Ca	K series	0.68	0.05	0.24
P	K series	1.08	0.06	0.49
N	K series	13.28	1.20	13.34
Na	K series	0.37	0.04	0.23
Total		100.00		100.00

4.2.2.4. Atomic Force Microscope (AFM)

B.coagulans synthesises chitosan nanoparticles using atomic force microscopy (AFM). Although the form of the probe influences the lateral dimensions, the morphology of chitosan NPs has been described. The height measurements can provide a high degree of quality and accuracy in the elevation of nanoparticles as shown in (Figure 4-6), which included three-dimensional pictures and granularity accumulation distribution charts of Chitosan NPs and The average diameter was of the chitosan NPs (71.414 nm) (Figure 4-7).

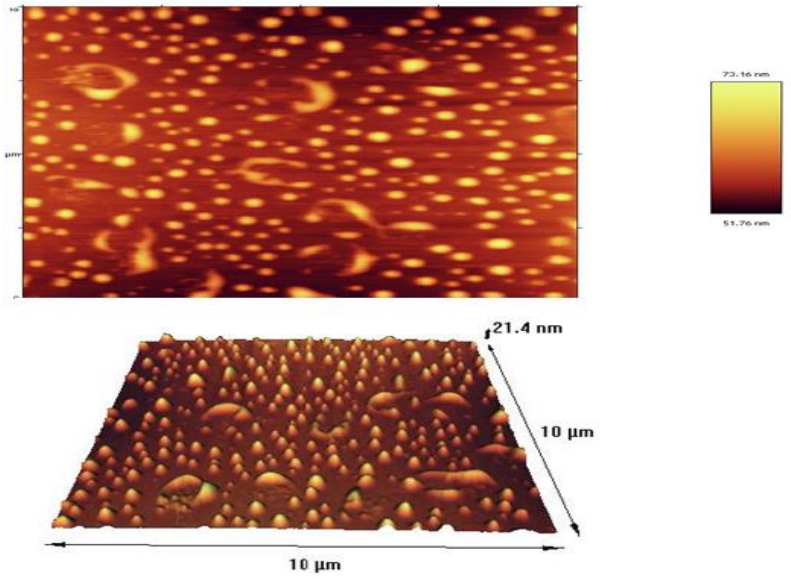


Figure (4-6) AFM analysis of biogenic Chitosan NPs synthesis by *B.coagulans*

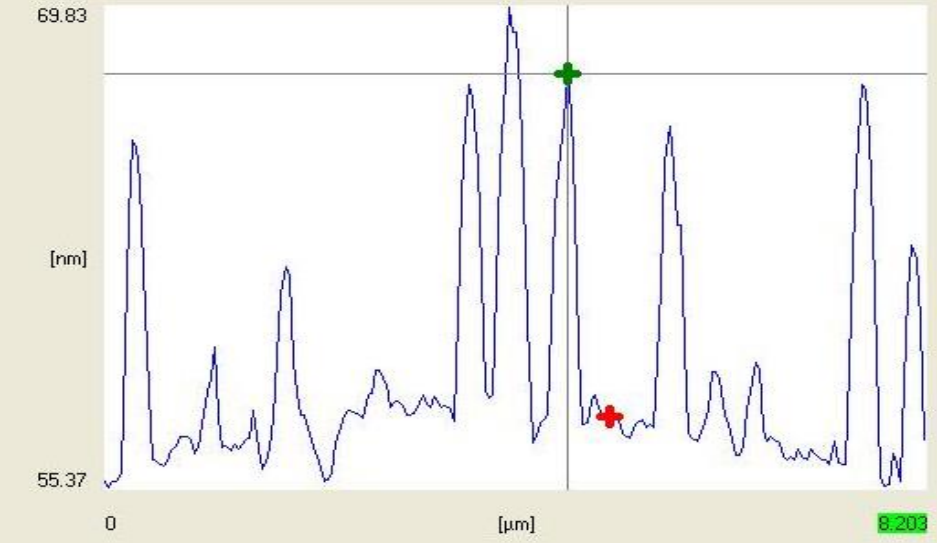


Figure (4-7) Granularity of biogenic Chitosan NPs

4.2.2.5. X-ray Diffraction (XRD) Analysis

The absence of a diffract gram peak in the XRD patterns of biologically produced chitosan NPs suggested an amorphous structure. XRD was used to investigate the crystalline structure of chitosan NPs. The polymorph's diffraction pattern was ascribed to the chitosan polymer's characteristic peaks seen at $2\Theta=375^{\circ}$. At 375^{o}, the XRD spectrum of biogenic chitosan nanoparticles appeared. The presence of bioorganic coupons/proteins in the nanoparticles during the manufacturing process was suggested by the strong peak (Figure 4-8).

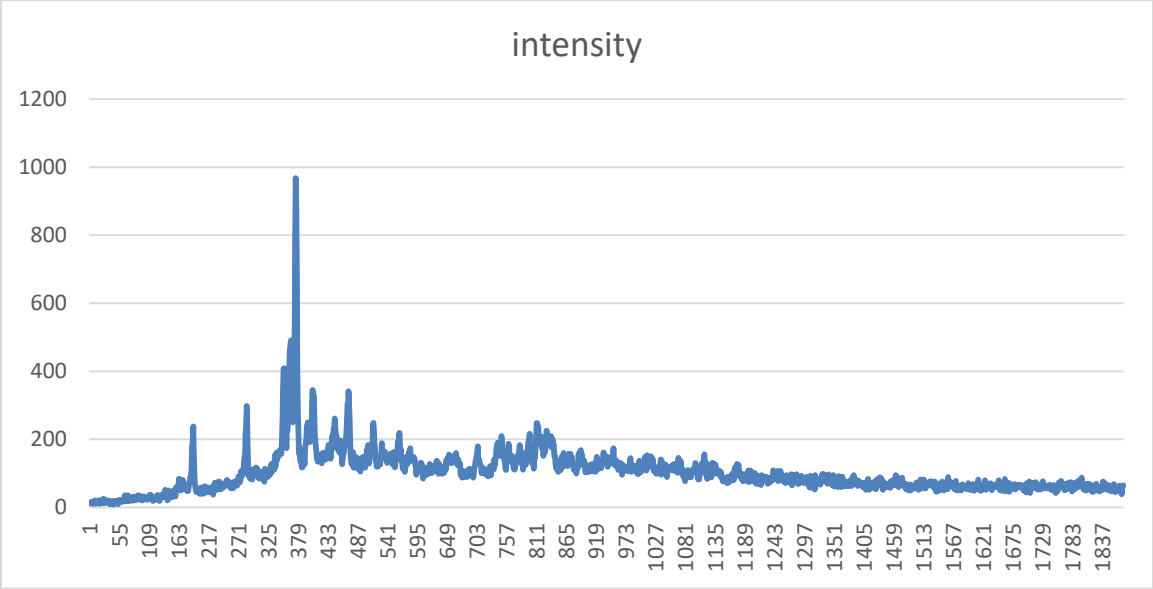


Figure (4-8) XRD analysis of Chitosan NPs synthesis by *B.coagulans* showing the structure and size

Color change, UV-visible spectrophotometer, SEM, and EDS were all utilised to characterise nanoparticles. Nanoparticle elements' form, scale, distribution, and existence were investigated. As a result, *B.coagulans* was chosen as the biosynthesis method for Chitosan NPs.

4.2.2.6. Fourier Transform Infrared Spectroscopy (FTIR)

The fourier transform infrared (FTIR) spectra of chitosan NPs were acquired using an FTIR spectrometer in order to evaluate the complicated surface processes of chitosan NPs. (Figure 4-9) and (Table 4-3) shows the presence of bands similar from proteins, fatty acid and other chemical compounds.

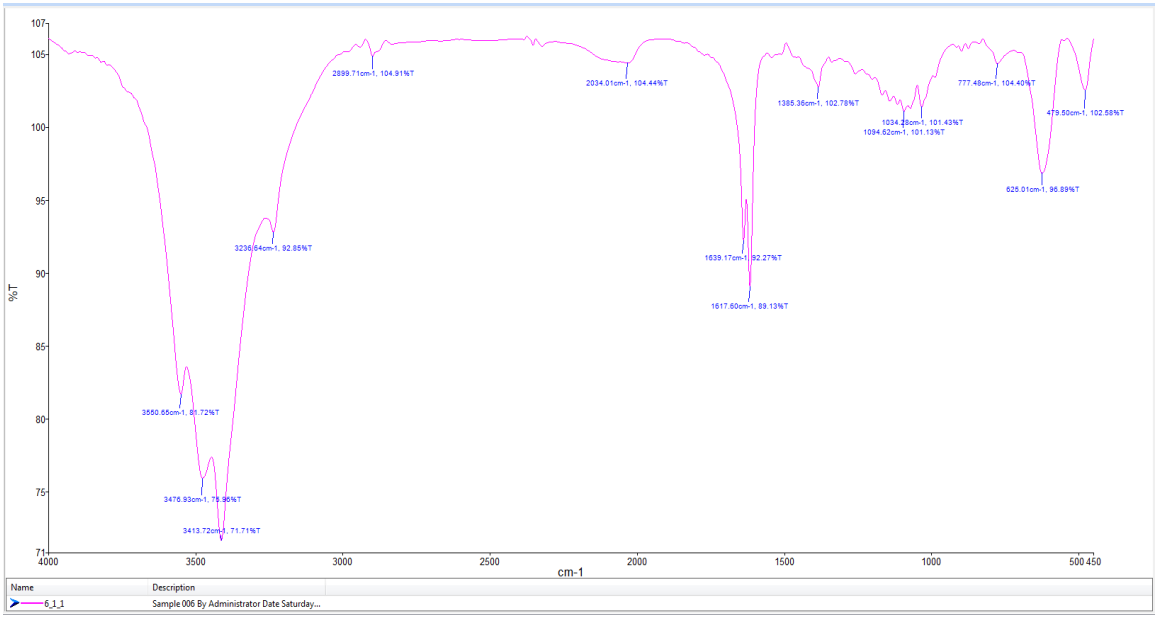


Figure (4-9) Fourier Transform Infrared Spectroscopy of biogenic Chitosan NPs synthesis by *B.coagulans*

Table (4-3) showed the presence of bands of Chitosan NPs synthesis

Peak Number	X (cm-1)	Y (%T)
1	3550.65	81.72

2	3476.93	75.96
3	3413.72	71.71
4	3236.64	92.85
5	2899.71	104.91
6	2034.01	104.44
7	1639.17	92.27
8	1617.60	89.13
9	1385.36	102.78
10	1094.62	101.13
11	1034.28	101.43
12	777.48	104.40
13	625.01	96.89
14	479.50	102.58

4.3.1. Activity of Chitosan NPS on MCF-7 cell cultures

In order to determine the activity of chitosan NPs and their effect on MCF-7 cell cultures, an ELISA assay is performed Figure (4-10).

The results of chitosan NPs at different concentrations (512 - 256 - 128 - 64 - 32 - 16 - 8) μg/ml. showed its activity against MCF-7 cell cultures. Where the highest growth inhibition area was at absorbance (OD = 0.150) with a concentration (512 μg/ml) and the lowest growth inhibition area at absorbance (OD = 0.102) with concentration (8 μg/ml) as in the table (4-2) and figure (4-11).

A nanoparticles (NPs) are materials with typically overall dimensions less than several hundred nanometers and magnitude smaller than human cells. Because of this unique physical property, NPs demonstrate marvelous interactions with both on surface and inside the cancer cells. Therefore, The surface charge of chitosan was the major factor in the cytotoxic activity due

to the electrostatic ionic interaction between the negatively charged groups of tumor cells and the positively charged amino groups of chitosan (Adhikari,Sharan, and Yadav,2018).

4.3.2. Activity of Methotrexate drug on MCF-7 cell cultures

The results of Methotrexate at different concentrations (512 - 256 - 128 - 64 - 32 - 16 - 8) μg/ml. showed its activity against MCF-7 cell cultures Figure (4-10).Where the highest growth inhibition area was at absorbance (OD = 0.147) with a same concentration (512 μg/ml) and the lowest growth inhibition area at absorbance (OD = 0.117) with concentration (8 μg/ml) as in the table (4-4) and figure (4-11).Recent studies showed that in tumor tissue folate receptors were found higher compared with healthy tissue. The folate re-ceptors are overexpressed on a variety of human tumors, such as breast tumor. MTX, is an analogue of folic acid, which exhibits not only a targeting role as folic acid but also a therapeutic effect to many types of cancer cells that overexpress folate receptors on their surfaces. So it has been utilized for the treatment of several forms of cancers for decades, including leukemias, breast cancer, head and neck cancer, lymphomas and carcinomas (Gharebaghi *et al*.,2017).

4.3.3. Activity of Combination (Chitosan NPs + Methotrexate) on MCF-7 cell cultures

The results of combination (Chitosan NPs + Methotrexate) at different concentrations (512 - 256 - 128 - 64 - 32 - 16 - 8) μg/ml. showed its activity against MCF-7 cell cultures Figure (4-10).

Where the highest growth inhibition area was at absorbance (OD = 0.162) with a concentration (512 μg/ml) and the lowest growth inhibition area at absorbance (OD = 0.143) with concentration (8 μg/ml) as in the table (4-4) and figure (4-11).

Polymeric NPs have shown preferential accumulation at tumor sites, their usage as carriers improves efficacy and reduces side effects. Chitosan NPs has been widely used to prepare nanoparticles drug delivery system since has many good bioproperties and physiochemical characteristics. Chitosan

NPs is a natural polysaccharide which derived from chitin by deacetylation. This cationic polymer is regarded as biocompatible, biodegradable and non-toxic. The cationic properties of Chitosan NPs are particularly valuable for drug delivery systems. For example, ion complexes between Chitosan NPs and anionic drugs i.e. methotrexate (MTX) can be formed to NPs. It showed acceptable results for less cytotoxicity and its effect to inhibit high growth at low concentrations and the nontoxic to cells even at high concentration (Ekinci *et al*.,2015).

Figure (4-10) A: plate of the Cell Culture in the incubator B: plate of the Cell Culture after the incubator for a period 24 hours at 37 °C.

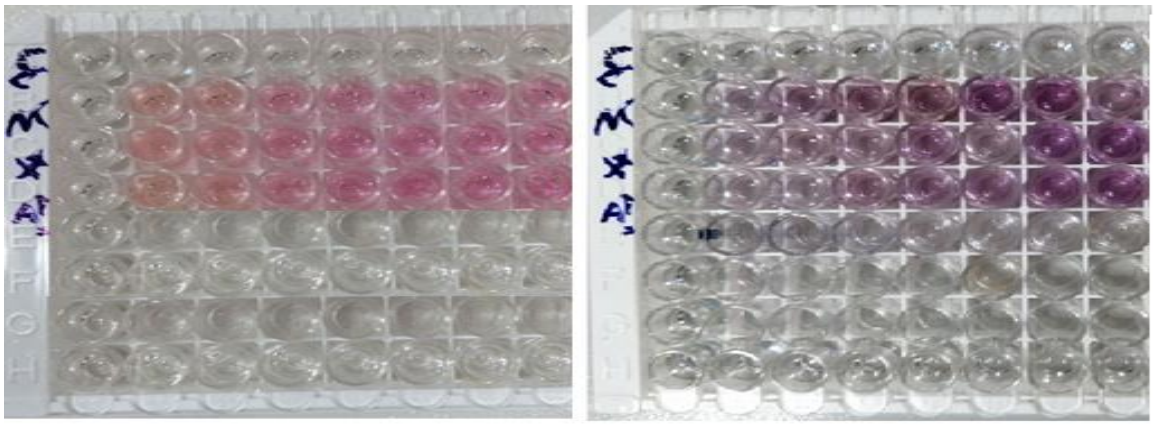


Fig : A **Fig : B**

Table (4-4) The results of the ELISA assay show the effect of the chitosan NPs , MTX and combination (Chitosan NPs + Methotrexate) on MCF-7 cell cultures

samples	1	2	3	4	5	6	7
Con. µg/ml	512	256	128	64	32	16	8
Chitosan	0.150	0.146	0.144	0.142	0.140	0.135	0.102
Methotrexate	0.147	0.146	0.145	0.141	0.133	0.126	0.117
Combination	0.162	0.156	0.153	0.150	0.148	0.145	0.143

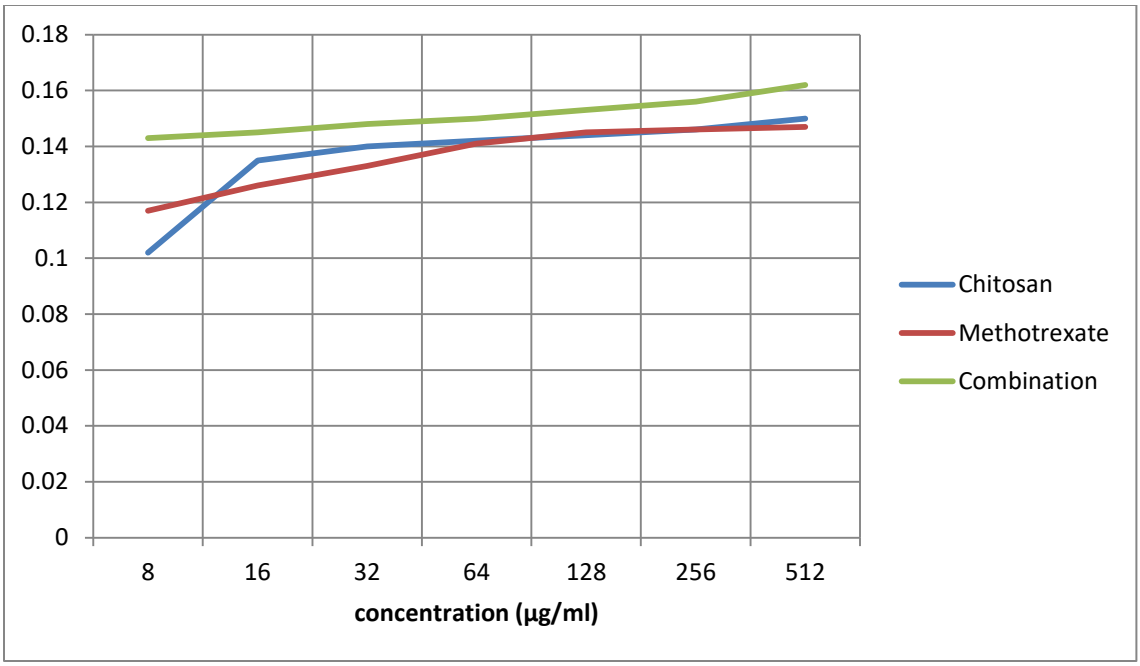


Figure (4-11) Activity of the chitosan NPs , MTX and Combination (Chitosan NPs + Methotrexate) on MCF-7 cell cultures

4.4.1. Antibacterial activity of Chitosan NPS against *Staphylococcus aureus*

Biogenic chitosan NPs synthesized by *B.coagulans* have been evaluated for their antibacterial activity against some resistance bacterial. The agar well diffusion method was used for detecting the antibacterial activity of biogenic chitosan NPs. Chitosan NPS with different concentrations (200 , 100 , 50 µg/ml) showed inhibition activities against all tested bacteria. The highest inhibition zone of Chitosan NPs observed in Gram positive bacteria was (24mm) in *S. aureus* with concentration (200 µg/ml). This inhibitory effect increased with increasing the concentrations of Chitosan NPS, as in (Figure 4-12), and (Table 4-5).The results showed that nanoparticles have the ability to inhibit the bacterial growth of Gram positive (*S.aureus*).

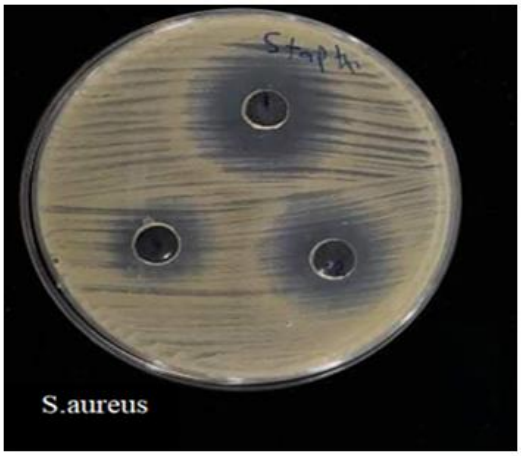


Figure (4-12) Anti-bacterial activity of biogenic Chitosan NPs against bacteria (*S.aureus*). 1(200 µg/ml), 2 (100 µg/ml), 3(50 µg/ml)

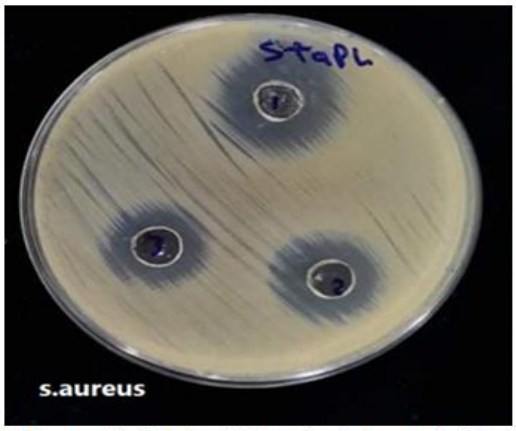
Bacteria	Inhibition zone (mm)		
	(200 µg/ml)	(100 µg/ml)	(50 µg/ml)
S.aureus	24	23	20

Table (4-5) Inhibition zone of Chitosan NPs synthesis by *B. coagulans*

Chitosan NPs are known to have antibacterial properties in Gram positive bacteria such as *S.aureus* . Chitosan NPs were effective bactericidal agents on a broad spectrum of clinically important pathogens. It has been demonstrated that the underlying mechanism of its antibacterial activity is targeting an outer membrane protein, lipopolysaccharide (LPS), causing cell membrane damage that lead to the death of the bacteria at the neutral PH. This effect is greatly enhanced at lower pH suggesting that ionic interaction is critical for antimicrobial activity of chitosan NPs (Jeon *et al*., 2014).

4.4.2.Antibacterial activity of MTX drug against *Staphylococcus aureus*

Methotrexate drug have been evaluated for their antibacterial activity against resistance bacterial. The agar well diffusion method was used for detecting the antibacterial activity of MTX drug. MTX drug with different concentrations (200 , 100 , 50 µg/ml) showed inhibition activities against tested bacteria. The highest inhibition zone of MTX drug observed in Gram positive bacteria was (20mm) in *S.aureus* with concentration (200 µg/ml),. as in (Figure 4-13), and (Table 4-6).The results showed that MTX drug have the ability to inhibit the bacterial growth of Gram positive (*S.aureus*).



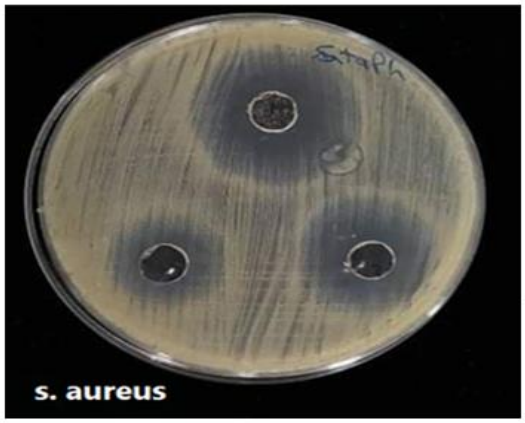
Figure(4-13)Anti-bacterial activity of MTX drug against bacteria (*S.aureus*). 1(200 µg/ml), 2 (100 µg/ml), 3(50 µg/ml)

Bacteria	Inhibition zone (mm)		
	(200 µg/ml)	(100 µg/ml)	(50 µg/ml)
S.aureus	20	18	17

Table (4-6) Inhibition zone of MTX drug

4.4.3. Antibacterial activity of Combination (Chitosan NPs + Methotrexate) against *Staphylococcus aureus*

The synergistic effect of chitosan NPs was examined with MTX drug against the tested bacteria. Combination (Chitosan NPs + Methotrexate) with different concentrations (200 , 100 , 50 µg/ml) showed inhibition activities against tested bacteria. The highest inhibition zone of combination (Chitosan NPs + Methotrexate) observed in Gram positive bacteria was (26mm) in *S.aureus* with concentration (200 µg/ml).The effects were happened by the combination of MTX with chitosan nanoparticles that had antibacterial effect against bacteria. Chitosan NPs increased the activity in combination with MTX where the inhibition activity of the tested bacteria increased, as in (Figure4-14), and (Table 4-7).



Bacteria	Inhibition zone (mm)		
	(200 µg/ml)	(100 µg/ml)	(50 µg/ml)
S.aureus	26	24	21

Figure(4-14)Anti-bacterial activity of combination against bacteria (*S.aureus*). 1(200 µg/ml), 2 (100 µg/ml), 3(50 µg/ml)

Table (4-7) Inhibition zone of combination

It is possible that the mechanism of synergistic antimicrobial activity of MTX and chitosan NPs found in this analysis could be attributable to (i) Chitosan NPs can bind to negatively charged compounds including proteins and anionic polysaccharides. Chitosan NPs has many benefits, including nontoxicity, high biocompatibility, and enhanced drug bio adhesive and payload properties. Because of its high biological ability and biocompatibility, chitosan NPs is a successful choice for a localized anti-bacterial alternative to conventional therapies (Shariatinia ,2019).(ii) but due to the disproportioned amount of MTX, the antidote could not impede toxic effects of the drug. The most common causes leading to MTX intoxication are inappropriate dosage, and concomitant use of drugs, such as NSARs, antibiotics or salicylates, new initiation of MTX or re-instatement or increase of dosage and finally, uncontrolled self-medication. For this reason, the MTX drug was combined with chitosan NPs to reduce the toxicity of the MTX drug and worked it on inhibition on microbes (Knoll *et al*.,2016).

Chapter Five

Conclusions and Recommendations

5. Conclusions and Recommendations

5.1. Conclusions

1- *B.coagulans* revealed ability to synthesis of Chitosan NPs that showed optimal properties UV visible, SEM, AFM, XRD, EDS and FTIR.

2- The anticancer toxic showed effect of the combination (Chitosan NPs + Methotrexate) against breast cancer cell line is dose-dependent.

3- The combination (Chitosan NPs + Methotrexate) showed more cytotoxicity and a highly synergistic effect on breast cancer cells than Methotrexate, and chitosan NPs alone at a lower concentration.

4- The combination (Chitosan NPs + Methotrexate) showed a great inhibition activity against bacteria (*S.aureus*) at concentrations 200 μg/ml.

5.2. Recommendations

1- Testing a larger number of bacteria to find out their ability to produce chitosan NPs.

2- Optimal production condition test to determine perfect conditions such as pH, temperature, etc. for the production of chitosan NPs.

3- Separation and purification chitosan NPS by using advance techniques

4- Toxicity study of Chitosan NPs.

5- Biologically synthesized chitosan NPs could provide more specific cancer treatment instead of chemotherapy to reduce the undesired side effects. These nanoparticles should be further investigated for potential applications as cancer drugs.

6- Studying the therapeutic nanoparticles and their combination (Chitosan NPs + Methotrexate) in vivo in animal tumor models to develop the effective tools for breast cancer therapies.

7- Determining the minimal inhibitory concentration (MIC).

8- Study the antimicrobial activity of chitosan NPs against Antimicrobial resistance (AMR) bacteria exhibiting similar properties to antibiotics.

9- Study the antimicrobial activity of combination (Chitosan NPs + Methotrexate) against Antimicrobial resistance (AMR) bacteria for cancer patients.

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الخلاصة

توضح الدراسة تأثير المزيج من (Chitosan NPs + Methotrexate) على خط خلايا سرطان الثدي (MCF-7 cell) وتثبيط نمو البكتيريا المقاومة للمضادات الميكروبات (AMR) (*Staphylococcus aureus*).

تم اختبار ثلاث سلالات من العصيات (*B.coagulans, B.Subtilis, B.clausii*) كمنتج للجسيمات النانوية من الكيتوسان النانوي (NPS)، تم اختيار (*B.coagulans*) من أجل الحيوي للكيتوسان النانوي بالاعتماد على نشاط مضادات الميكروبات و التغير في اللون والاشعة فوق البنفسجية UV-visible Spectroscopy .

تتكون جزيئات الكيتوسان النانوية من عديد السكاريد الأميني الذي يحدث بشكل طبيعي ، والمشتق على هيئة شكل منزوع الأسيتيل من الكيتين Chitin. أدت الخصائص البيولوجية والفيزيائية والكيميائية إلى اهتمام كبير باستخدامها في الطب الحيوي ومجالات أخرى.

اشتملت الدراسة على التصنيع الحيوي وتوصيف الجسيمات النانوية للكيتوسان بواسطة القياس الطيفي المرئي للأشعة فوق البنفسجية واستخدم للكشف عن تخليق الجسيمات النانوية الكيتوسان مع ذروة امتصاص عند ٢٨٠ نانومتر ، وأظهر تحليل المجهر الإلكتروني الماسح (SEM) كرويًا متجانسًا بمتوسط أحجام من ٣٠.٩٤ إلى ٩٥.٥١ nm، أظهرت التحليلات الأولية للجسيمات النانوية للكيتوسان الحيوي وجود عناصر باستخدام التحليل الطيفي المشتت للطاقة (EDS) (53.14٪ كربون ، ٣٠.٧٦٪ أوكسجين ، ١.١٧٪ كبريت ، ٠.٦٣٪ كلوريد ، ٠.٢٤٪ كالسيوم ، ٠.٤٩٪ فوسفور ، ١٣.٣٤٪ نيتروجين و ٠.٢٣٪ صوديوم. وأظهر حيود الأشعة السينية (XRD)، القوة الذرية عند ٣٧٥ درجة ، والتحليل الطيفي للأشعة تحت الحمراء (FTIR) اظهر وجود نطاقات مماثلة من البروتينات والاحماض الدهنية والمركبات الكيميائية الأخرى، وأظهر مجهر القوة الذرية (AFM) الأبعاد الجانبية وكان متوسط القطر من الكيتوسان النانوي (71.414 nm) .

حيث تم تقييم تأثير كل من جزيئات الكيتوسان النانوية وعقار الميثوتريكسات (MTX) والمزيج بين (Chitosan NPs + Methotrexate) ضد الخلايا السرطانية باستخدام ELISA assay بتركيزات مختلفة (٥١٢ - ٢٥٦ - ١٢٨ - ٦٤ - ٣٢ - ١٦ - ٨) µg/ml.

بالإضافة إلى دراسة التأثير ضد مقاومة مضادات الميكروبات (AMR) ، المكورات العنقودية الذهبية(*Staphylococcus aureus*) التي تشكل تهديدًا مستقبليًا على حياة المرضى. تهدف الدراسة إلى استخدام جزيئات الكيتوسان النانوية كبديل أو مساعد لعمل المضادات الحيوية. جمعت عينات العدوى من (مستشفى الفرات الأوسط للأورام) في محافظة النجف الاشرف. عزل وتحديد *Staphylococcus aureus* باستخدام الاختبارات المورفولوجية والكيميائية الحيوية في نظام VITEK2 المضغوط باحتمال ٩١٪.

الخلاصة

حيث كان تأثير كل من جزيئات الكيتوسان النانوية وعقار الميثوتريكسات والمزيج (Chitosan NPs + Methotrexate) ضد (*Staphylococcus aureus*) باستخدام طرق انتشار الآكار بتركيزات مختلفة (٢٠٠ ، ١٠٠ ، ٥٠ µg/ml).

نتائج هذه الدراسة، من بين المركبات الثلاثة ، أعطى المزيج (Chitosan NPs + Methotrexate) أفضل النتائج حيث كانت أعلى منطقة تثبيط للنمو ضد مزارع خلايا MCF-7 كان عند الامتصاص (OD = 0.162) بتركيز (٥١٢ µg/ml) وأدنى منطقة تثبيط للنمو عند الامتصاص (OD = 0.143) بتركيز (٨ µg/ml).

كان للمزيج (Chitosan NPs + Methotrexate) تأثير تآزري عالي بتركيزات مختلفة (٢٠٠ ، ١٠٠ ، ٥٠ µg/ml) أظهرت نشاطًا عاليًا للتثبيط ضد البكتيريا المختبرة. حيث كانت أعلى منطقة تثبيط للمزيج (Chitosan NPs + Methotrexate) لوحظت في البكتيريا موجبة الجرام كانت (٢٦ mm) في *Staphylococcus aureus* بتركيز (٢٠٠ µg/ml).

تم اقتراح نتائج هذه الدراسة. كان نشاط المزيج بين (Chitosan NPs + Methotrexate) على مزارع خلايا MCF-7 أظهرت نتائج مقبولة لسمية أقل للخلايا وتأثيرها على تثبيط النمو العالي بتركيزات منخفضة وغير سام للخلايا حتى عند التركيز العالي.

يعتبر المزيج (Chitosan NPs + Methotrexate) خيارًا جيدًا للأسباب الأكثر شيوعًا التي تؤدي إلى تسمم الميثوتريكسات لمرضى سرطان الثدي وهي الجرعات غير المناسبة والاستخدام المتزامن للأدوية ، مثل المضادات الحيوية. لهذا السبب ، تم دمج عقار الميثوتريكسات مع جزيئات الكيتوسان النانوية لتقليل سمية عقار الميثوتريكسات وعمله على تثبيط الميكروبات *Staphylococcus aureus*.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة الكوفة

كلية العلوم

قسم علوم الحياة

التأثير التآزري لجسيمات الكيتوسان النانوي الحيوي وعقار الميثوتريكسات ضد سرطان الثدي و *Staphylococcus aureus*

بحث مقدم إلى

قسم علوم الحياة / مجلس كلية العلوم / جامعة الكوفة كجزء من متطلبات الحصول على درجة البكالوريوس في علوم الحياة

من قبل

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