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Evaluation of Chitosan Activity Produced Via Biotransformation of Chitin Against Some Pathogens Associated with Burn Infection *in Vitro* and *in Vivo*

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Abstract

Burn infections are a serious concern for patients with burn injuries, as they can lead to significant morbidity and mortality. The present study was designed to evaluate the chitosan activity against five pathogens collected from burn infections (*Escherichia coli ZE15, Acinetobacter bummani ZA2, Klebsiella pneumonia ZK3* and *Pseudomonas aeruginosa ZP17*, *Staphylococcus aureus ZS2*). Chitosan was produced in solid-state fermentation using corn meal supplemented with 2% of shrimp shell chitin. The degree of deacetylation of chitosan was 79.5% and the FT-IR spectrum (4000 to 400) cm-1 gave IR spectra similar to that of the commercial chitosan. The produced chitosan showed a significant inhibitory ability from the concentration of 10 mg/ml to 2 mg/ml. The M.I.C. and S.I.C were varied between the isolates; for *P. aeruginosa* ZP17 and *Staph. aureus* ZS2, the M.I.C and S.I.C were 1.6 mg/ml and ~~of~~ 1.4 mg/ml respectively, while for *E. coli* ZE15, *Kl. pneumonia* ZK3 and *A. bumannii* ZA2 were 1.4 mg/ml and 1.2 mg/ml. Furthermore, this study showed ~~an~~ the important role of the produced chitosan as an antibiofilm agent to reduce the biofilm formation for the tested isolates. However, the strongest antibiofilm activity was against *E. coli* Z.E15 with 55.2%. Moreover, four potential formulas containing chitosan with concentration of 1.6 mg/ml were tested. A formula containing polyvinyl alcohol, vaseline and chitosan to create a cream showed the best results in comparison with the four-formula used in this study. This formula was added to pads of thick cellulosic filter paper to treat a mouse model with full thickness cutaneous burn to assess the benefits of treating with a chitosan dressing. The healing process was examined and results showed a signs of remodeling stage by day four and ~~a~~ scar tissue formation by day nine.

Keywords: chitosan; Burn infections; antimicrobial activity; formula

تقييم فعاليه الكيتوسان الناتج من التحول الاحيائي للكايتين ضد بعض مسببات الامراض المرتبطه باصابات الحروق داخل وخارج الجسم

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تعد الالتهابات مصدر قلق كبير للمرضى الذين يعانون من إصابات الحروق ، حيث يمكن أن تؤدي إلى مضاعفات خطيرة تؤدي الى الوفاة. صممت الدراسة الحالية لتقييم نشاط الشيتوزان ضد خمسة من مسببات الالتهابات التي تم جمعها من عدوى الحروق (*E. coli ZE15*، *Acinetobacter bummani ZA2*، *Klebsiella pneumonia ZK3* و *Pseudomonas aeruginosa ZP17*، *Staphylococcus aureus ZS2*). تم إنتاج الشيتوزان باستخدام تخمير الحالة الصلبة باستخدام مستخلص الذرة و 2٪ من الكايتين المحضر من قشور الروبيان. بلغت درجة نزع الأسيتيل من الشيتوزان 79.5٪ ، وقد أعطى طيف FT-IR من 4000إلى 400) سم -1 أطياف الأشعة تحت الحمراء مماثلة لتلك الموجودة في الشيتوزان التجاري. وجد ان للشيتوزان المنتج القدرة على التثبيط من تركيز 10 مجم / مل إلى 2 مجم / مل. وجد ان نسب MIC و SIC للشيتوزان المنتج للعزلات *P. aeruginosa ZP17* و *Staph. aureus ZS2* هي 1.6 مجم / مل و 1.4 مجم / مل على التوالي ، بينما كانت نسب MIC و SIC للعزلات *Klebsiella pneumonia ZE1. E.coli.* و *ZK3* و *A. bumannii ZA2* هي 1.4 مجم / مل و 1.2 مجم / مل على التوالي. علاوة على ذلك ، أظهرت هذه الدراسة دورًا مهمًا للشيتوزان المنتج كعامل مضاد حيوي لتقليل تكوين الأغشية الحيوية (biofilm) للعزلات المختبرة. ومع ذلك ، كان أقوى نشاط مضاد حيوي ضد *E.coli Z.E15* بنسبة 55.2٪. تم اختبار أربع تراكيب صيدلانية محتملة تحتوي على الشيتوزان بتركيز 1.6 مجم / مل. أظهرت التركيبة المحتوية على كحول بولي فينيل وفازلين وشيتوزان لصنع كريم أفضل النتائج مقارنة بالصيغ الأخرى المستخدمة في هذه الدراسة. تمت إضافة هذه الصيغة إلى قطع من ورق الترشيح السليلوزي السميك لعلاج نموذج فأران مختبريه بحرق جلدي بسمك كامل لتقييم فوائد العلاج بضمادة الشيتوزان. تم فحص عملية الشفاء وأظهرت النتائج علامات على مرحلة إعادة بناء النسيج في اليوم الرابع وتشكيل نسيج ندبي في اليوم التاسع.

Introduction

Burn injuries are a significant global health issue that affects millions of people each year [1]. In addition to the initial trauma, burn patients are at high risk for infections, which can result in prolonged hospitalization, extensive scarring, and even death [2]. Burn wound infections are challenging to treat due to the emergence of antibiotic-resistant bacteria, including multi-drug resistant (MDR) bacteria. The emergence of MDR bacteria has led to an urgent need for alternative therapies to treat burn infections [3]. One potential solution is the use of chitosan, a natural polymer derived from the exoskeleton of crustaceans. Chitosan has demonstrated broad-spectrum antimicrobial activity and has been shown to be effective against a range of bacterial and fungal pathogens, including MDR bacteria [4]. Chitosan is biocompatible, biodegradable, and non-toxic, making it an attractive candidate for wound healing applications [5]. Despite promising preclinical results, few clinical studies have evaluated the efficacy of chitosan in treating burn infections, particularly those caused by MDR bacteria. A limited number of studies have reported the effectiveness of chitosan in treating burn infections caused by drug-resistant bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* [6]. These studies suggest that chitosan may be a promising alternative to traditional antibiotics for treating MDR burn wound infections.

Chitosan works by disrupting the bacterial cell membrane, leading to cell death [7]. It also has immune-modulatory properties, which can enhance the host's natural defense mechanisms against infections [8]. Furthermore, chitosan can help promote wound healing by stimulating the production of growth factors, reducing inflammation, and promoting the formation of granulation tissue [9]. Chitosan's unique properties make it a promising candidate for use in the treatment of burn infections, particularly those caused by MDR bacteria. Therefore, several studies ~~were~~ investigated the use of chitosan produced from different sources such as shrimp shells in the treatment of burn infections, including those caused by MDR bacteria [10,11,12].

The primary objective of these studies was to assess the ability of chitosan to prevent and treat bacterial infections in burn wounds. This involved evaluating the antimicrobial activity of chitosan against a range of bacteria commonly associated with burn wound infections, including MDR strains. The studies also evaluated the safety and tolerability of chitosan in burn patients and compare the effectiveness of chitosan to current standard of care treatments [10]. Overall, these researches had the potential to improve the clinical outcomes for burn patients, particularly those with MDR bacterial infections, and provide a new therapeutic option for the treatment of burn infections [13]. This research aims to study the antimicrobial properties of chitosan produced via biotransformation of chitin. The study also investigates the possibility of using it as an effective agent for the treatment of some microbial infections that affect humans, especially burn infections, by finding a suitable drug formula.

Materials and Methods

Collection of pathogens associated with burn infection samples

From 1st September 2021 to 20th November 2021,178 burn samples (swaps) were collected from individuals in burn center of medical city hospitals (Baghdad) in order to isolate pathogenic bacteria. Then, the swaps were inoculated into brain-heart infusion (BHI) and incubated for 16-18 hrs. at 37C [14]. Bacterial growth Samples were transformed into blood and MacConkey agar media and then, Colonies representing different morphologies were isolated from the plates and sub-cultured into nutrient agar slants for further identification process [15]. Then applied the samples for biochemical tests and gram stain for identification.

Antibiotic Susceptibility Test

Kirby-Bauer method [16] was used to examine the antibiotic susceptibility of isolates. The antibiotic disks (Amikacin, Azetreonam, Cefepime, Gentamycin, Tobramycin, Tetracycline, Collistin, Levofloxacin, penicillin, Imipenem, ampicillin, chloramphenicol, vancomycin, Nirofurantoin and Tetracycline.)were chosen according to CLSI M100 2020.

Biofilm formation assay (quantitative)

The Biofilm formation among pathogenic isolates was achieved by using the microtiter plate assay based on the method described by [17], [18]

Preparation of chitosan

An isolate of *Aspergillus niger* was used in this study for the Preparation of chitosan. This isolate was already obtained from the College of Science, Department of Biotechnology/University of Baghdad. Production of chitosan was achieved based on the conditions and strategy developed by [19]. as follows:

In Erlenmeyer flasks, an isolate of *A. niger* was cultivated on 10g of corn supplemented with 2% commercial shrimp shell chitin (100 ml). A 1:1 ratio of mineral salt solution was used to moist the flasks (w: v). 0.05M sodium phosphate buffer was used to get the pH to 7. The flask was inoculated with 2 ml of spore suspension containing 1×10^6 spores/ml after sterilising at 121Co for 20 min. The flasks were then incubated at 30 °C for 6 days. Chitosan was extracted as follows: the whole solid-state biomass was first homogenized with 1M NaOH (1:30 w/v) in an autoclave at 121°C, 15 psi for 15 min. After that, alkali insoluble materials (AIMs) were gathered using cotton gauze and rinsed with distilled water until the pH reached a neutral state. The cleaned AIMs were put in glass Petri dishes, weighed, and dried in an oven at 60°C overnight. Chitosan was extracted from AIM using 1% acetic acid (1:40 w/v), followed by centrifugation at 10,000 rpm for 15 minutes and an autoclave for 15 minutes. AIMs were discarded, and 4M NaOH was used to adjust pH to 10. Repeating the centrifuge after an

overnight period allowed the precipitate to be collected, cleaned with distilled water, and weighed. It was cleaned once more using acetone (1:20) (w/v) and 95% ethanol (1:20) (w/v). Chitosan precipitate was weighed (mg chitosan/g chitin) after being dried at 60 ˚C.

Characterizations of chitosan

Determination of degree of deacetylation (DDA)

The degree of deacetylation (DDA) was determined for chitosan based on the method described by Reem *et .al* (2019) [19]

Infrared spectroscopy (FTIR)

The IR spectra of chitosan produced in this study were determined and compared with the IR spectrum of a commercial chitosan. 2 mg sample of chitosan, which already dried overnight at 60 °C under reduced pressure was thoroughly blended with 100 mg of KBr, to produce 0.5 mm thick disks. The disk was then dried for 24 hr. at 110°C under reduced pressure. Infrared spectrometer was recorded with Bruker 66 Spectrometer, using a 100 mg KBr disks as a blank [19].=

Study the in vitro antimicrobial activity of chitosan

Antimicrobial activity assay

The spectrum activity of chitosan was determined against clinical bacterial isolates (*Escherichia coli*, *Acinitobacter bummani, Klebsiella pneumonia* and *Pseudomonas aeruginosa*, *Staphylococcus aureus*). Well, diffusion method was used to evaluate the antimicrobial activity of chitosan [19].

Determination of minimal inhibition concentration of chitosan (M.I.C) and sub inhibition concentration (S.I.C)

This test was performed against the selected pathogenic isolates using microtiter plates. [20]. A 1:100 inoculation of an overnight culture of the clinical isolate, which had about 1×10^8 cells/ml, was placed into each plain tube, which held 20 ml of nutrient broth. For the primary screening dilution, the wells were labeled appropriately, and each well was loaded with 200µl of different chitosan serial dilutions of (10, 8, 6, 4, 2, 1 mg/ml) obtained after dissolving twenty milligrams of chitosan in one milliliter of 1% acetic acid solution as a stock for making the lower strength of chitosan. To acquire the concentrations required for this experiment as a secondary screening dilution, 2g of chitosan was dissolved in 100 ml of 1% acetic acid to create a stock solution at a concentration of 20 mg/ml. Serial dilutions of chitosan were made from stock solution (2, 1.8, 1.6, 1.4, 1.2, 1) mg/ml Then, 100 µl of different chitosan two folds concentrations were added to each well that containing 100 µl of two folds of Muller Hinton broth to get the right concentration in the final volume of 200 µl. After 24h incubation at 37C aerobically, 30 µl of 0.015 % Resazurine dye solution was added to each well and incubated for four hours. For acquiring the M.I.C. and S.I.C., the colour was changed from purple to pink [21] to evaluate the results. To measure the produced chitosan's ability to inhibit the bacterial isolates, With the highest M.I.C. values were identified and prepared for the well diffusion on agar plates. Four wells were made using a 5 mm crock bore, and the first well-received 200 l of chitosan solution was diluted to 1.6 mg/ml. The second well-received 200 l of commercial chitosan (1 mg/ml) for comparison. Moreover, 30 mg/ml of the antibiotic Ceftriaxon was utilised to compare the inhibitory zone and 1% acetic acid solution was employed as a negative control. The plates were incubated at 37Cº for 24hr. Antimicrobial activity was evaluated by measuring the diameter of inhibition zone against the five tested clinical isolates [22].

Antibiofilm activity of chitosan

In order to determine the anti-biofilm activity of chitosan, the strongest biofilm forming isolates of five different species isolates, *Staph. aureus*, *P. aeruginosa*, *E. coli*, *A. baumannii* and *K. pneumoniae*, were selected. First, these isolates were cultured in BHI broth overnight, then each sample was diluted in a 1:100 ratio of TSB media that contain 1.6 mg/ml chitosan. Then, 100 µL were transported into 96 microtiter plates per well with three replicates for each species. The same steps were repeated but with media that does not contain chitosan which is considered a positive control for each bacterium. The direct effect of chitosan on biofilm formation was measured quantitatively by comparing the mean OD for each bacterium with and without the Chitosan. Finally, three wells were filled with 100 µL of media that contain 1.6 mg/mL of chitosan without any bacterial inoculum as a negative control (Blank) [19].

Preparation of pharmaceutical formula including chitosan

Four potential formulas containing chitosan with concentration of 1.6 mg/ml were prepared as follows:

a. ***First formula***

This formula was prepared by mixing 8 ml of chitosan (20 mg/ml) with 92 ml of 1% acetic acid. Then, 5g of Carbopol was added and mixed until its fully homogenized. The mixture was left for 24h to obtain carbopol hydrogel with 1.6 mg/ml chitosan.

b. ***Second formula***

This formula was prepared by adding 8 ml of chitosan (20 mg/ml) to 92 ml of 1% acetic acid. After mixing, 15 g of p*olyvinyl alcohol* (*PVA*) was added and mixed until its fully homogenized. The mixture was then left for 24h to obtain PVA hydrogel with 1.6 mg/ml chitosan.

Third formula

This formula was prepared by adding 8 ml of chitosan (20 mg/ml) to 2 ml of 1% acetic acid. Next, 0.5 ml of tween 80 was added and mixed. Then, 89.5 ml of Vaseline was added to the mixture and mixed well in a water bath until it dissolved completely to obtain Vaseline cream with 1.6 mg/ml chitosan

c. ***Fourth formula***

A cream containing the partially purified chitosan was prepared as follows: in this formula, the aqueous phase was prepared by adding 8 ml of 20 mg/ml of chitosan to 22 ml of 15% of polyvinyl alcohol with stirring and the mixture was left for 24h for full dissolving. Then, the 30ml of the aqueous phase was added to 69.5 ml of melted vaseline and mixed with the addition of 0.5 ml of tween 80 as an emulsifying agent. The mixture was mixed steadily until its fully homogenized.

In vitro evaluation of formula including chitosan

The antibacterial activity of the four-formula containing the prepared chitosan as an active material was investigated by using pads of thick cellulosic filter paper with 1 cm in width and 3cm in length. All pads were placed on Muller Hinton agar which was previously inoculated with 1×10^4 cells/ml of bacterial indicators. Then, plates were incubated for 24 hrs at 37°C. Following the incubation, the diameter of the inhibition zone around each pad indicating to the antibacterial activity**.**

In vivo evaluation of formula including chitosan

Fourteen local white mice were used in the in vivo experiments which were divided into 3 groups as the following:
- A test group that contains 10 mice two mice for each bacterial isolate
- The second group ~~that~~ contains 2 mice as a negative control.
- The third group that contains two mice is considered a Positive control.

After adaptation period of 3 days, mice shoulder and thigh regions were shaved and burned using a soldering iron. Then, each mouse group was infected with one of the bacterial indicators and marked with blue ink. Each group in the experiment was treated as follows: the test group that was infected with bacterial species was treated with the experimenting cream two times daily for a period of 10 days, while the negative group was infected with *Pseudomonas aeruginosa* and left untreated. The last group was the positive group that contain two mice that were infected with *Pseudomonas aeruginosa* and treated with Hamazine 1% cream twice daily for 10 days period. The experiment time ~~was~~ depended on the time of complete healing.

Results and Discussion

This study provides evidence for the prevalence of pathogenic bacteria in burn wounds, the importance of identifying the specific bacteria involved in order to tailor treatment, and the effectiveness of various interventions for preventing and managing burn infections. One hundred seventy-eight isolates of pathogenic bacteria were collected from burn samples from individuals in burn center of medical city hospitals (Baghdad) during the period of September to November 2021. Samples showed 77% bacterial growth while 23% revealed no growth, 16.29% were Gram-positive whereas, 60.67% were Gram-negative. Only five different species of clinical significance were taken into consideration for this study which appears to be 92.7% of the total growth. The various types of bacteria isolated from burn wound culture of total of 178 wound swabs were shown in Table (1) which revealed that the most dominant type is the Gram-negative bacteria starting with *P. aeruginosa* followed by *E. coli*, then come the skin normal habitants *S. aureus* then *Kl. pneumonia* and at least *A. bumannii*. These findings are comparatively close to other local studies [23].

Table 1: The percentage of pathogenic isolates collected from burn patient skin

Bacteria	Frequency	Percentage
Pseudomonas aeruginosa	69	50.3%
E. coli	28	20.4%
Staphylococcus aureus	19	13.8%
Acinetobacter bumannii	3	2.1%
Klebsiella pneumonia	8	5.8%
Others	10	7.3%

As the objective of this research is to evaluate the chitosan antimicrobial properties against some pathogens associated with burn infections. Therefore, a selection was made by subjecting all the collected pathogenic isolates to antibiotic susceptibility test and biofilm formation assay to choose the most common multidrug-resistant isolates that can be used as indicators in the next experiments. According to results, the following isolates were selected to be used as indicators: the first clinical pathogenic isolate chosen was *P. aeruginosa* ZP17, which only showed moderate sensitivity toward colistin according to CLSI 2021 with an inhibition zone of 13 mm, whereas it was resistance to other antibiotics. In addition, this isolate was a strong biofilm producer with OD 2.99. The second isolate was *Staph. aureus* Z.S2 which showed a resistance to most antibiotics examined including Penicillin, Cefepime, Gentamicin, Tobramycin, Amikacin, Levofloxacin, Nitrofurantoin, and Tetracycline. Moreover, this isolate

showed an ability to form strong biofilm, therefore, it was considered as multi-drug resistant and hence it was selected to be used as an indicator in this study. The third clinical pathogenic isolate was *E. coli* ZE15 which showed intermediate sensitivity according to CLSI 2021 toward Levofloxacin, tobramycin and tetracycline, whereas it was resistant to other antibiotics. Furthermore, the isolate *E. coli* Z.E15 showed an ability to form a strong biofilm with OD 2.58. The fourth selected pathogenic bacterium was *K3. pneumonia;* based on results, the most resistant isolate was ZK3 which showed resistance to penicillin, cefibime, Tetracycline, Ampicillin, Colistin sulfate and imipenem. In addition, this isolate showed intermediate sensitivity to levofloxacin, gentamicin and tobramycin according to CLSI 2021. system. Moreover, the isolate *K. pneumonia* ZK3 showed an ability to form moderate biofilm with OD 1.82. The fifth indicator that was selected as multi-drug resistant was *A.* bumanni ZA2, results revealed that ZA2 isolate was sensitive only to Tobramycin, levofloxacin and colistin sulfate. In addition, it showed strong biofilm production with OD 1.92.

On the other hand, chitosan was produced in solid-state fermentation via using corn meal supplemented with 2% of shrimp shell chitin, as a solid substrate [19]. This medium supported maximum production of Chitin deacetylase (CDA) enzyme necessary in the bioconversion process [19]. The isolate *Aspergillus* niger was grown in solid-state fermentation that supports maximum production of CDA [24]. Based on the findings, the bioconversion process produced about 3.8g of chitosan from 8g of shrimp shell chitin (Figure 1), which is approximately near ~~to~~ the value predicted in the experiment carried out by [19]. SSF has been exploited successfully for the cost-effective production of a number of fungal enzymes [25]. A previous study reported that production of commercially potent CDA by SSF ~~is~~ remains unexploited [19]. In this context, fungal chitosan was produced via solid-state fermentation of soybean processing residues using *Mucor rouxii* and the optimum cultivation and extraction conditions were determined for maximum chitosan yield [26].

Figure 1**:** chitosan produced by *Aspergillus* niger in solid-state fermentation (SSF) via bioconversion of chitin

The degree of deacetylation is a major factor since it affects the physicochemical characteristics of chitosan. Chitosan with a high degree of deacetylation has strong positive charges, which makes it more suitable for use in a variety of food and pharmaceutical applications [27]. The amount of deacetylation of the chitosan made in this investigation from commercial shrimp chitin was 79.5%. The highest degree of deacetylation (DDA) was found

in chitosan made from fungal chitin (78.4%), whereas the lowest DDA was found in chitosan made from shrimp shells (74.8%), when compared to chitosan made by [19]. Also, the characteristic functional groups of the generated chitosan were determined and verified using the FT-IR spectra (4000 to 400 cm-1). The isolated fractions showed IR spectra that were comparable to those of commercial chitosan derived from shrimp shells. The FT-IR spectra shown in Figure (2) show a large absorption band that is attributed to O-H stretching vibrations in the 3000–3500 cm-1 region and to the C–O bond at 1400–1650 cm-1 [28]. Moreover, the stretching vibrations of aliphatic C-H, Amide I (-NH deformation of -NHCOCH3), Amide II, Amide III, and C-O-C bonds are assigned to the peaks around 2885, 1650, 1589, 1326, and 1080 cm-1, respectively. The results of IR spectra revealed that the fundamental molecular structure of commercial and produced chitosan was remarkably similar. [29] revealed The FTIR spectrum of the chitosan showed absorption peaks at 3304.74, 2872.84, 1660.75, 1409.97, 1019.88, 852.69 and 713.37 cm−1. The overlapped N-H and O-H stretching vibration peak at 3304.74 cm-1 is attributed to the absorption peak at that position.

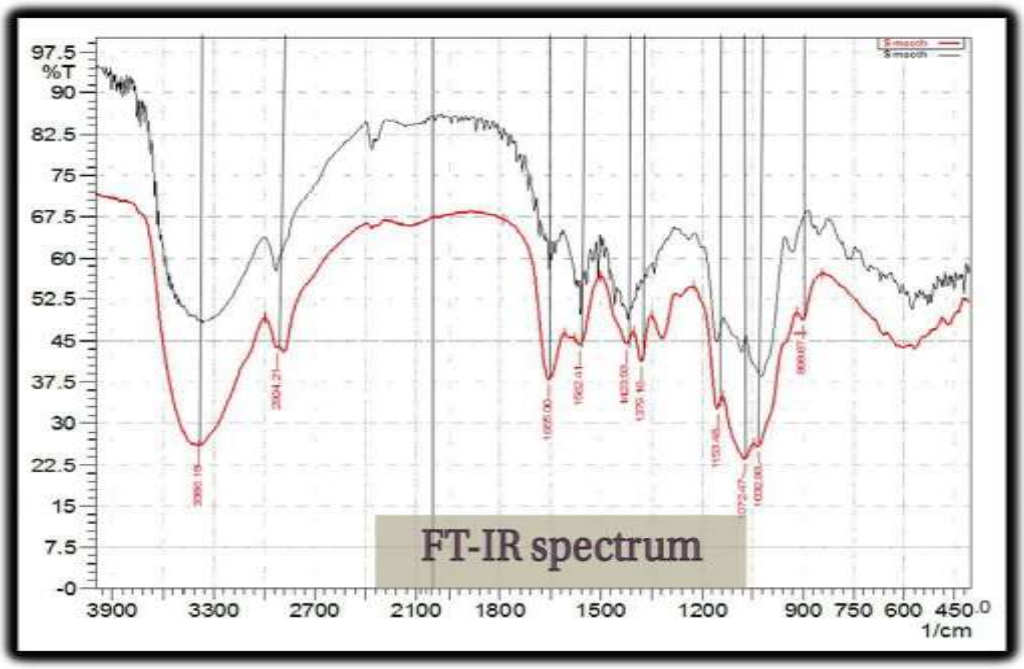


Figure 2: FTIR spectra of commercial chitosan (red color); and chitosan produced by *Aspergillus niger* in SSF (black color).

The antimicrobial activity of the produced chitosan was evaluated against five types of clinical bacterial isolates: four-Gram negative bacteria (*E. coli ZE15, A. bummani ZA2, Kl. pneumonia ZK3* and *P. aeruginosa ZP17*) and one Gram-positive bacteria *Staph. aureus Z.S2*. The antimicrobial activity of the produced chitosan was first examined using well diffusion method. A significant inhibitory ability was observed from the concentration 10 mg/ml to 2 mg/ml, but almost no or less effect with 1 mg/ml with all isolates. It was practically identical for the five isolates to show a similar effect with diameter of sensitivity of about 30 mm with chitosan concentration from 10 mg/ml to 2 mg/ml and growing with concentration of 1 mg/ml (Table 2).

Several investigations have looked into the range of chitosan's action against various types of human pathogenic bacteria in recent years [30]. Chitosan's polycationic nature is thought to interact with anionic groups on the surface of cells to generate an impermeable coating that

prevents the transfer of vital solutes, which is thought to be the major mechanism underlying its antimicrobial activity [31].

Table 2: Primary screening of chitosan activity against pathogenic isolates collected from burn samples

Isolates	Concentrations mg/ml					
	10	8	6	4	2	1
	Diameter in mm					
Escherichia coli ZE15	32	32	31	29	28	8
Pseudomonas aeruginosa ZP17	27	26	23	21	15	5
Klebsiella pneumonia ZK3	27	26	26	25	24	6
Staphylococcus aureus ZS2	34	33	32	29	21	7
Acinetobacter bumannii ZA2	28	28	24	22	18	10

In addition, the spectrum activity of the produced chitosan was further investigated in microtiter plate in order to detect the M.I.C. and S.I.C using the same five types of clinical bacterial isolates. As shown in Table (3), results ~~were~~ varied between the isolates; for *P. aeruginosa* ZP17 and *Staph. aureus* ZS2, the M.I.C of chitosan (1.6 mg/ml) was able to inhibit the growth of these organisms with S.I.C of 1.4 mg/ml, while for *E. coli* ZE15, *Kl. pneumonia* ZK3 and *A. bumannii* ZA2 the M.I.C were 1.4 mg/ml with 1.2 mg/ml of chitosan as S.I.C.

Table 3: Antimicrobial activity of the produced chitosan in comparison with broad-spectrum antibiotic and commercial chitosan

Strain	Diameter of inhibition zone (mm)		
	Commercial Chitosan 3	Produced Chitosan 2	Ceftriaxone disc (30ug) 1
Escherichia coli ZE15	26	25	27
Pseudomonas aeruginosa ZP17	24	21	19
Klebsiella pneumonia ZK3	25	25	26
Staphylococcus aureus ZS2	29	28	23
Acinetobacter bumannii ZA2	22	20	20

For further evaluation, the antimicrobial activity of the produced chitosan was compared with a broad-spectrum antibiotic Ceftriaxone and commercial chitosan. The produced chitosan showed a substantial activity against all tested bacteria at a level approximately comparable to that seen with commercial one, according to the results shown in Figure (3). Intriguingly, the findings also suggested that chitosan's inhibitory activity was somewhat greater than Ceftriaxone disc's antibacterial activity. This finding undoubtedly has significant ramifications for future research. Considering the ways in which harmful bacteria are creating defenses against several classes of antibiotics [32].

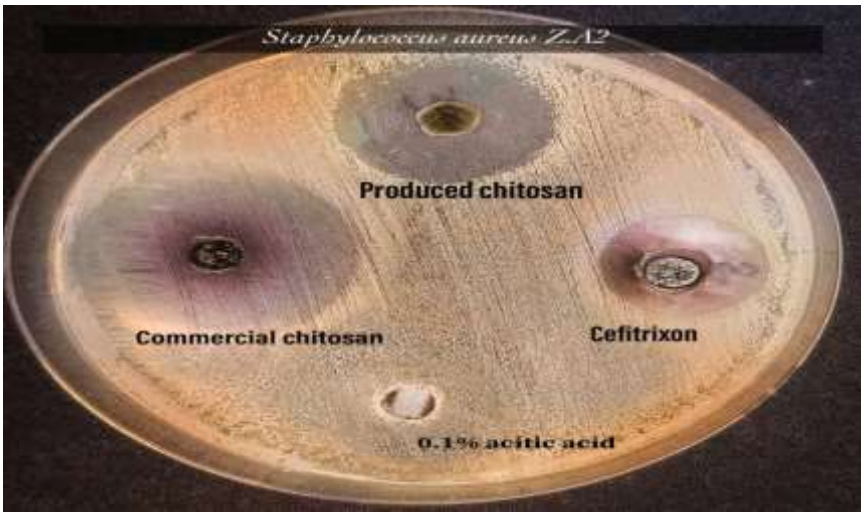


Figure 3: Antimicrobial activity of the produced chitosan in comparison with broad-spectrum antibiotic (cefitrixon 30ug) and commercial chitosan against (*Staphylococcus aureus Z.S2)* isolate

Furthermore, this study showed the important role of the produced chitosan as an antibiofilm agent against the clinical pathogenic isolates. The use of microtiter plate allowed to compare the bacterial ability to produce biofilm and hence determine the effect of chitosan as an antibiofilm agent by measuring the OD at 595 nm. The results showed a good ability to reduce the biofilm formation for the tested isolates as seen in the table (4). The strongest antibiofilm activity was against *E. coli* Z.E15 isolate with 55.2% in comparison to the OD of the same isolate without adding the chitosan. Furthermore, *A. bumannii* ZA2 isolate showed a reduction in biofilm formation of about 45.9%, and *Staph. aureus* ZS2 had a 31.5% reduction in activity. While *P. aeruginosa* ZP17 with 29.6% and the least affected bacterial isolate was *Kl. pneumonia* ZK3 with biofilm formation being reduced by approximately 15.8%.

Bacterial biofilms play a key role during infections, which are associated with ~~an~~ increased morbidity and mortality. The classical antibiotic therapy cannot eradicate biofilm-related infections because biofilm bacteria display high drug resistance due to the biofilm matrix [33]. Biofilms in burns are major problems: bacterial communities rapidly develop antibiotic resistance, and 60% of burn mortality is attributed to biofilms. Key pathogens are *P. aeruginosa*, *Staph. aureus*, and multidrug-resistant *A. baumanii* . Chitosan can disrupt the initial stages of biofilm formation by interfering with bacterial adhesion to surfaces. It can also inhibit the formation of mature biofilms by disrupting the communication and coordination of bacterial cells within the biofilm.[34].

Table 4: Antibiofilm activity of chitosan against five pathogenic bacteria collected from burn samples

Isolates	OD without chitosan	OD with chitosan	Reduction percentage %
Escherichia coli ZE15	1.16	0.64	44.8%
Pseudomonas aeruginosa ZP17	2.97	0.88	70.3%
Klebsiella pneumonia ZK3	1.95	0.31	84.1%
Staphylococcus aureus ZS2	2.92	0.92	68.4%
Acinetobacter bumannii ZA2	1.24	0.57	54.1%

Since its discovery approximately 200 years ago, chitosan, as a cationic natural polymer, has been widely used as a topical dressing in wound management owing to its hemostatic,

stimulation of healing, antimicrobial, nontoxic, biocompatible and biodegradable properties. In this work, four potential formulas contain chitosan with concentration of 1.6 mg/ml. All formulas were tested *in vitro* against the pathogenic isolates to evaluate the degree of carrying capacity and the diffusion of chitosan from the formula into the agar plate that contains the pathogenic isolates. For this purpose, all formulas were investigated by adding them to pads of thick cellulosic filter paper with 1 cm in width and 3cm in length which were placed on Muller Hinton ager previously inoculated with bacterial indicators.

As can be seen in Table (5), the fourth formula that contains polyvinyl alcohol, Vaseline and chitosan showed the best results in comparison with the other formula. The combination of PVA and Vaseline has been shown to have several advantages in wound healing. A study by [35] demonstrated that PVA-Vaseline film had excellent mechanical properties and could adhere well to the skin, providing an effective barrier against water and bacteria. The study also found that the PVA-Vaseline film had a significant effect on promoting wound healing, reducing inflammation, and increasing collagen deposition. Another study by [36] showed that the PVA-Vaseline hydrogel could significantly accelerate wound healing in diabetic rats by promoting angiogenesis and re-epithelialization [35].

Chitosan has been studied extensively for its potential use in wound healing. A study by [37] showed that chitosan-based films could significantly accelerate wound healing by enhancing collagen synthesis, increasing angiogenesis, and reducing inflammation. Another study by [38] demonstrated that chitosan-based hydrogels could effectively promote wound healing in diabetic rats by reducing inflammation and promoting angiogenesis [39] Polyvinyl alcohol (PVA), Vaseline, and chitosan are three different materials with distinct properties and applications. However, they can be combined for various purposes, such as in the production of wound dressings or as drug delivery systems. The advantages of combining these materials include enhanced biocompatibility, chitosan is a biocompatible and biodegradable material. When combined with PVA and Vaseline, it can improve the biocompatibility of the resulting composite material, and moisture retention: PVA has excellent water solubility and can absorb large amounts of water, making it useful in the production of hydrogels. When combined with vaseline and chitosan, it can form a dressing that can retain moisture, prevent drying, and promote healing [40], improved mechanical properties: vaseline, a semisolid mixture of hydrocarbons, can improve the mechanical properties of the composite material, making it more flexible and less prone to breakage [41] Overall, the combination of PVA, Vaseline, and chitosan has the potential to produce composite materials with a range of properties that can be useful in various applications, including wound dressings, drug delivery, and tissue engineering [42].

Table 5: Evaluation of formula including chitosan against pathogenic bacterial isolates collected from burn samples

Isolates	Diameter of inhibition zone (mm)

	Hydrogel		Cream	
	carbopol + 1.6 mg/ml chitosan	polyvinyl alcohol +1.6 mg/ml chitosan	tween 80, Vaseline + 1.6 mg/ml chitosan	polyvinyl alcohol, Vaseline + 1.6 mg/ml chitosan
Escherichia coli ZE15	11	21	22	24
Pseudomonas aeruginosa ZP17	10	22	22	23
Klebsiella pneumonia ZK3	13	20	21	22
Staphylococcus aureus ZS2	11	22	22	24
Acinetobacter bumannii ZA2	0	20	19	18

Burns are a significant health challenge and healing can result in scar formation. In this study, the produced chitosan was tested for its efficiency to promote wound healing. Based on the results of *in vitro* experiments, formula four which contained the produced chitosan with polyvinyl alcohol and Vaseline to create a cream was used in the in *vivo* evaluation. This formula was added to pads of thick cellulosic filter paper ~~with~~ 1 cm in width and 3cm in length to treat a mouse model with full thickness cutaneous burn to assess the benefits of treating with a chitosan dressing. The experiment involved using fourteen wounded white mice divided into three groups.

Burn injury is a complex process that involves several stages of healing, including the inflammatory phase, proliferative phase, and remodeling phase. Laboratory experiments on mice can help researchers understand the mechanisms of burn injury and develop new treatments for burn victims. Three days after burning and infecting mice with the pathogenic isolates, the first stage was attained for the three groups of tested animals with a positive control group and a negative control group that was infected with *P.aeruginosa Z.p17* which is considered the highly MDR bacteria and strongest biofilm producer based on previous experiments. The healing process was examined day by day for all groups. Results showed that chitosan treated group that applied twice daily as well as 1% Hamazine-treated positive group that also applied twice daily showed ~~a~~ signs of remodeling stage by day 4. It was found that the inflammation signs were reduced as the swelling and redness of skin is getting better. Whereas, in the untreated negative group, these signs still existed with pus formation as the burn ulceration process continued with swelling and redness. By day 9, the chitosan and 1% Hamazine groups started to show ~~a~~ scar tissue formation as they entered the remodeling stage in comparison with the negative group of untreated animals that showed slow healing and were still in the process of proliferative stage (figure 4). Wounds are classified as acute and chronic as a result of a thermal, physical, or chemical injury. Wound healing takes place in five stages: hemostasis, inflammation, migration, proliferation, remodeling, and maturation. The healing periods for acute and chronic wounds are 8–12 days, respectively (Alavi *et al.*, 2019). On the other hand, burn wound represents a susceptible site for opportunistic colonization by organisms of endogenous and exogenous origin. Patient factors such as age, extent of injury, and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, and motility determine the likelihood of invasive burn wound infection [43] Pathogens of specific concern in the burn population include MDR strains of *P. aeruginosa*, *Acinetobacter baumannii* and *S. aureus* [44]. Also study [45] reported that chitosan accelerates cutaneous wound healing and full-thickness skin wounds were created on the backs of rats. The researchers found that chitosan treatment accelerated wound closure and improved

the quality of the healed tissue. These studies suggested that chitosan may have potential as a therapeutic agent for promoting burn infection healing. However, further research is needed to fully understand the mechanism of action and to evaluate its safety and efficacy in humans.

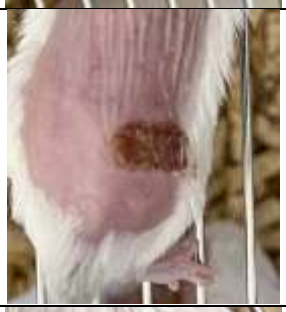
Animal status	**Day 1**	**Day 4**	**Day 9**
E. coli **treated with chitosan**			
S. aureus **treated with chitosan**			
A. bumannii **treated with chitosan**			
K. pneumonia **Treated with chitosan**			
Animal status	**Day 1**	**Day 4**	**Day 9**

Figure 4: *In vivo* experiment for applying a formula including chitosan to treat a group of mice infected with pathogenic bacteria.

Conclusion

The results achieved in the current study demonstrated that chitosan produced via the transformation of shrimp shell chitin has broad-spectrum antimicrobial activity, including multi drugs resistant bacteria commonly implicated in burn infections. This study also showed the important role of the produced chitosan as an antibiofilm agent against the clinical pathogenic isolates as well as the anti-inflammatory and wound-healing properties, which may help to promote the healing of burn wounds. Moreover, the combination of PVA and Vaseline in chitosan formula has several advantages in accelerating wound healing in burn wounds by promoting angiogenesis and re-epithelialization. Finally, the present study supports the idea of using chitosan as a dermal cream or ointment for the treatment of skin infections and as an alternative method to kill the widespread ~~of~~ multi-drug resistance.

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