Antimicrobial, antibiofilm, and antioxidant activity of chitosan nanoparticles synthesized by *E. coli*

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Abstract:

The aim of this study was to study the antimicrobial, antibiofilm, and antioxidant activity of Chitosan nanoparticles synthesized by *E. coli*. Twenty-five samples and investigations were achieved during the period from November 2018 to April 2019. Wound infection samples were collected from two different hospitals in Najaf province (Al-Sadder medical city). The A5 (*E. coli*) efficient isolate was diagnosed by conventional and molecular methods. Molecular study showed the *E. coli* isolate gave positive results (1270bp) for 16S rRNA assay. Data of *E. coli* strain at the locus (MN197856), accession (MN197856), version (MN197856.1), defined as (*Escherichia coli* strain Kafeel-1 16S ribosomal RNA gene, partial sequence. The results showed the gram positive bacteria highly resistant to novobiocin and nitrofurantoin with the diameter of inhibition zone 27 and 20mm respectively, and low resistant to amoxicillin/calvulanic acid with the diameter of inhibition zone (0mm). Where gram negative bacteria showed highly resistant to imipenem with the diameter of inhibition zone 22mm and low resistant to antibiotic includes ciprofloxacin and amoxicillin/calvulanic acid with the diameter of inhibition zone 5 and 0mm respectively. Biogenic chitosan expressed high significant antibiofilm activity with increasing concentration of chitosan NPs. DPPH reducing activity of nanoparticles which increased with increase concentration of biogenic chitosan NPs.

Keywords: Antimicrobial, antibiofilm, antioxidant Chitosan, and *E. coli*
1 Introduction

Many types of bacteria synthesize nanoparticles differ in their characteristics. Most such syntheses are extracellular binding of metal nanoparticles to bacterial biomass. Strategies for NPs characterization can be assorted into biological, and physiochemical, biological characterization include antimicrobial activity, genotoxicity, and antibiofilm activity. In turn, physicochemical methods represented by spectrophotometry, electron microscopy, and X-ray analysis.

Chitosan is obtained through partial deacetylation of chitin, creating a polysaccharide composed of glucosamine (2-amino-2-deoxy-D-glucose) and N-acetyl glucosamine (2-acetamido-2-deoxy-D-glucose) units linked by β(1→4) bonds. The presence of amino groups in its structure confers it a cationic nature which, in low pH settings, grants chitosan biological activity, namely through interaction with negatively charged compounds present in bacterial cells, such as proteins, polysaccharides, and phospholipids. Chitosan nanoparticles have been shown to interact with bacterial membrane proteins, intracellular proteins, phosphate residues in DNA, and to interfere with cell division, leading to bacterial cell death. The antibacterial study of chitosan was carried out on human pathogenic Escherichia coli by various researchers.

The antibiofilm effects of chitosan on Bacillus cereus, Staphylococcus aureus, Salmonella enterica, and Cryptococcus neoformans have been investigated. The studies of chitosan combined with other substances were carried out for the use of prevention and treatment of biofilm, for example chitosan was combined with antibiotics.

Antioxidants act as defense mechanisms that antagonize the effects of excessive oxidations, and the need to measure antioxidant activity is well documented. In humans, oxidative stress resulted from an imbalanced antioxidant situation (reactive oxygen species versus defense repair mechanisms).

Chitosan is suitable to deliver drugs through different routes, such as oral, ocular, nasal, vaginal, buccal, parenteral, and intravesical, etc.

2 Materials

2.1. Patient and sample collection

Twenty-five sample and investigations were achieved during the period from November 2018 to April 2019. Wound infection samples were collected from two different hospitals in Najaf province (AL-Sadder medical city). The experiment to detect the microorganism resistant to chitosan nanoparticle was conducted by agar well diffusion method on the brain heart agar culture media to six isolates of microorganisms.
2-2 Bacterial strain

Depending on the color change and antibiotic activity recorded the result, finally the one (A5) efficient isolate was selected.

Bacterial isolate (A5) was identified depending on morphological and microscopic examination, automated biochemical identification (VITEK and 16srRNA gene sequencing 12,13.

After isolation of single colony of A5 on brain heart infusion agar, it was observed for their shape, size, texture, and arrangements of colonies. Then, single pure colonies were transferred to a microscopic slide, fixed well and stained with gram stain in order to identify their response to this stain microscopically under oil immersion with magnification power 100x in order for microscopic identification 14.

2-3 Molecular identification

Sequencing of 16sRNA gene has been used in the present study for identification and differentiation of the isolates and detecting the phylogenetic relationship among the isolated bacteria 15.

Favor Prep™ Genomic DNA Mini Kit was used to extract genomic DNA from bacterial isolates following the manufacturer's protocol (DNA Extraction Mini Kit Favorgen / Korea).

PCR assay was performed to molecular identification using universal primers (Table 2-1). these primers synthesized by (Macrogen/Korea). Primers were dissolved using sterile dd water.

Table (2-1): 16S rRNA primer pair

| Primer name | Sequence 5’ -3’ | Reference |
|-------------|-----------------|-----------|
| 27F         | AGAGTTTGATCCTGGCTCA | Loy et al., 2002 |
| 1492R       | GGTTACCTTGGTACGACTT |           |

2-4 Antimicrobial activity of chitosan nanoparticles

The antibacterial activities of chitosan was tested against multidrug resistance bacteria, Gram-negative (P. mirabilis) and Gram-positive (S. aureus) isolated
from wound infection, bacteria using the agar diffusion method. The agar plates were inoculated with 100 μl of bacterial suspensions. Then, bores (5 mm diameter) were made using a sterile borer and were loaded with 60 μl of each sample (10 mg/ml). After that, the Petri-dishes were kept firstly for 2 h at 4 °C, and then incubated for 24 h at 37 °C. Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones in millimetres and the values were presented as the means of triplicate analysis.

**2-5 Antibiofilm activity of chitosan NPs**

Microtiter plate method was used for *in vitro* antibiofilm activity. Three concentrations 1, 2 and 4 mg/ml of each biogenic chitosan NPs and commercial chitosan NPs was prepared. 0.1ml of cell suspension having 0.5 O.D at 600nm have been inoculated in 1.9 ml BHIB broth medium, 150ul of the cultured BHIB broth then transferred into each well of 96- well microtiter plate in use. An amount of 50ul of each 4X concentration was added to the corresponding wells to obtain the final concentrations. An amount of 50ul of BHIB broth was added one well corresponding to each test bacteria used as control to confirm production of biofilm by bacteria and inhibition of Biofilm formation by chitosan NPs.

An amount of 200μl of autoclaved distilled water was added in peripheral wells (to reduce the water loss). Microtiter plate was incubated for 16 h at 37°C. Planktonic cells then aspirated, and fixed with 99% methanol. Plates then Washed twice with phosphate buffer saline or sterile saline water and air-dried. About 200 μl of crystal violet solution (0.2%) then added to all wells. After 5 min, excess crystal violet was removed and washed twice. After that the plate was air dried and the cell bound crystal violet was dissolved in 33% acetic acid. Biofilm growth was read at 570 nm using micro plate reader. Modified from, 16.

**2-6 Antioxidant activity of chitosan nanoparticles**

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was used to evaluate the ability of the chitosan. The method described by (Harborne and Baxter,1995) with some modification was used. The chitosan was added into DPPH (0.1mM) at concentration ( 1mg/ml, 2mg/ml and 4 mg/ml), as well as 0.5ml of both supernatant and bacterial growth of bacteria was added to 2ml of metabolic solution of DPPH (0.1mM).

The reaction mixture was incubated for 30 min in dark room at 37°C and the absorbance (A) was read at 517 nm. The experiment was repeated for three times
DPPH solution was used as a control (without sample) and ethanol 99.8% as blank. The inhibition of the DPPH radical by chitosan was calculated according to the following formula:

\[
\% \text{ of Inhibition} = \frac{A_0 - A_1}{A_0} 
\]

3 Results & Discussion:

3-1 Screening clinical samples (wound infections) and bacterial isolation

*Escherichia coli* (A5)

The morphological examination of *E. coli* on MacConkey agar and nutrient agar after 18 hours of incubation at 37°C produces large, circular, low convex, grayish white, moist, smooth, opaque or partially translucent colonies (smooth or S forms). The microscopically examination under 100x oil immersion showed Gram negative rod bacteria, *E. coli* produces bright pink flat colonies due to lactose fermentation. The biochemical identification was done depending on the result of each test, either change the color such as urease test or producing bubbles such as catalase test. The results were explained in Table (1-1).

These bacteria were used for nanobiosynthesis of chitosan NPs and identification according to morphological and biochemical characteristics and conventional techniques according to Bergey’s manual of determinative bacteriology.

**Table (3-1): Biochemical Identification of A12**

| Biochemical test                | A5   |
|---------------------------------|------|
| Indol test                      | +    |
| Methyl red test                 | +    |
| Voges–Proskauer Test            | -    |
| Citrate utilization tests       | -    |
| Urease test                     | -    |
| Motility test                   | +    |
| *H*₂*S* production test         | +    |
Lactose fermentation. +

The isolate A12 were identified by VITEK 2 automated compact system with GN/ID card with 64 biochemical tests. The isolates were identified after 6 hours as A12 (*K. pneumonia*) and with probability of 99%. (Table 3-2). These bacteria were used for nanbiosynthesis of chitosan NPs and identification according to morphological and biochemical characteristics and conventional techniques according to Bergey’s manual of determinative bacteriology\(^1\).

**Table (3.2):** The results of biochemical identification of isolate A5 using VITEK2, the results include the probability and confidence estimation of the diagnosis.

Twenty five isolates from wound infections were screened based on the ability of microorganisms for chitosan NPs biosynthesis depended on color change from light to dark (figure 3-1), and biological activities of the chitosan NPs against multidrug resistance bacteria (using *P. mirabilis* as indicator strain), to select the efficient strains . The efficient isolate A12 (figure 3-2).

Chitosan nanoparticles can be prepared using many methods such as ionic gelation, complex coacervation, emulsion cross-linking, spray drying and biological method\(^1\). The use of biological systems for production is an emerging area and the scientists have been viewing with interest microorganisms as probable eco-friendly nano-factories \(^1\).
Figure (3-1): Selection of efficient strain A5 (*E. coli*) Based on color change and antimicrobial activities.

Figure (3-2): Antibacterial activity of biogenic chitosan synthesized by A5 against: A: *P. mirabilis*
3-2 Molecular identification

In addition to biochemical identification, isolate were identified through 16sRNA sequencing by extraction and amplification of this gene. The sample of DNA (clear without fragmentation) was used as templates for PCR during amplification of 16sRNA gene. DNA templates, extracted in were used in the amplification of 16sRNA gene using 16sRNA gene universal primers in (table 2.1). The product then electrophoresed on Agarose gel and documented on gel document. The resulted 16sDNA bands were 1270bp (figure 3.3).

Data of *E. coli* strain made available to ENA in Europe and the DNA Data Bank of Japan at the locus (MN197856), accession (MN197856), version (MN197856.1), defined as (*Escherichia coli* strain Kafeel-1 16S ribosomal RNA gene, partial sequence).

3-3 Antibacterial activity of nanoparticales and antibiotics

The results showed the gram positive bacteria highly resistan
t to antibiotic includes novobiocin and nitrofurantion with the diameter of inhibition zone 27 and 20mm respectively, and low resistant to amoxicillin/calvulanic acid with the diameter of inhibition zone (0 mm). Where gram negative bacteria showed highly resistant to imipenem with the diameter of inhibition zone 22 mm. and low resistant to antibiotic includes ciprofloxacin and amoxicillin/calvulanic acid with the diameter of inhibition zone 5 and 0 mm respectively, as in table (3.2)figure(3.3).
Table (3-2) Antibiotic susceptibility patterns of the Gram positive and negative isolates against Six types of Antibiotic disks.

| Tested bacteria | AMC | IPM | CFM | CPR | F  | NV  |
|-----------------|-----|-----|-----|-----|----|-----|
| S. aureus       | 0   | 25  | 13  | 8   | 20 | 27  |
| P. mirabilis    | 0   | 22  | 5   | 9   | 15 | 11  |

The cell walls of gram-negative bacteria are more complex than those of gram positive bacteria, both structurally and chemically. The structure of gram-negative microorganisms cell contains two layers outside the cytoplasmic membrane, which would represent a greater physical barrier to overcome. This structural complexity would explain the greater inhibitory effect of the derivatives against gram-positive bacteria.²⁰

Figure (3-3): Antibacterial activity of antibiotics against four types of bacteria on the Brain heart agar, 24 hours, n 37°C.

At the concentration of 80μg/ml, biogenic chitosan nanoparticle showed the largest inhibition zone against S. aureus (35mm) and lower inhibition zone at the
concentration of 10μg/ml, where in gram negative bacteria, P. mirabilis have been recorded to have the largest inhibition zone (17mm) and lower inhibition zone at the concentration 10, 20 μg/ml. Results showed that nanoparticles have the ability to inhibit the bacterial growth of both gram positive and gram negative and the gram positive bacteria were higher sensitivity than gram negative bacteria to biogenic chitosan NPs as shown in table (3.3) and figure (3.4).

Table (3-3): Inhibition zone of the effect the CHNPs against pathogenic bacteria.

| Tested bacteria          | Inhibition zone (mm) produced by chitosan NPs |
|--------------------------|-----------------------------------------------|
|                          | 10μg/ml | 20μg/ml | 40μg/ml | 80μg/ml |
| S. aureus                | 22      | 25      | 27      | 35      |
| P. mirabilis             | 14      | 14      | 15      | 17      |

Figure (3-4): antibacterial activity of biogenic chitosan NPs against pathogenic bacteria (multi drug resistance) media is brain heart agar 24h and 37°C.
3-4 Antibiofilm Activity of biogenic nanoparticles

Chitosan NPs produced by *K. pneumonia* were examined for antibiofilm activity using different concentrations 1mg/ml, 2 mg/ml, and 4 mg/ml of CHNPs via plate method against six strains of bacteria including G +ive and G –ive. Results of antibiofilm activity varies according to the pathogenic bacteria.

Biogenic chitosan expressed high significant antibiofilm activity with increasing concentration of chitosan NPs, at concentration of (1, 2, and 4mg/ml) the absorbance will increase from (0.354 to 0.045), (0.245 to 0.124), (0.255 to 0.175), and (0.175 to 0.061) respectively for the tested bacteria as table 3.4.

Table (3-4): Antibiofilm activity of chitosan NPs at Four concentrations by absorbance at 630 nm against tested pathogenic bacteria.

| Tested Bacteria | Absorbance of chitosan NPs (mg/ml) |
|-----------------|-----------------------------------|
|                 | 1           | 2              | 4               |
| *S. aureus*     | 0.354       | 0.146          | 0.045           |
| *S. pneumonia*  | 0.245       | 0.203          | 0.124           |
| *Proteus mirabilis* | 0.255   | 0.180          | 0.175           |
| *P. aeruginosa* | 0.175       | 0.092          | 0.061           |

Biofilms are complex bacterial populations that resist the action of antibiotics and the human immune system. Due to the lack of effective antibiofilm antibiotics. Nanoparticles were used to resolve this problem. One potentially important candidate treatment uses NPs to show anti-biofilm activity21. However, in most cases antibiotics fail to eradicate these cells, as the concentration of antibiotics required to eradicate bacteria in biofilms is much higher than MIC of planktonic cells 22.
3-5 Antioxeant Activity of Biogenic chitosan Nanoparticles

The inhibition proportion of DPPH scavenging free radicals by bacterial growth was (87%). DPPH reducing activity of nanoparticles which increased with increase concentration of biogenic chitosan NPs. It was 87.9% in 0.75mg/ml, 88.60% in 1mg/ml, 93.41% in 2mg/ml, 97.96% in 4mg/ml, 98.12% in 6mg/ml, and 99.1% in 8 mg/ml, for chitosan NPs (figures 3.5 and Figure 3.6).

![DPPH assay](image)

Figure (3-5): antioxidant activity of chitosan nanoparticles using DPPH assay.
Figure (3-6): Antioxidant activity of chitosan NPs detected by change of color after 30 minutes.

The different mechanisms involved in the radical-antioxidant reactions may explain the different in scavenging potentials of the compounds. The mechanisms of antioxidants are not only by scavenging free radicals, but also by inhibiting production of free radicals.

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