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# The Effect of Chitosan as an Antimicrobial Agent

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**Abstract:**
This study investigates the antimicrobial properties of chitosan nanoparticles against *Proteus mirabilis*, which is the primary cause of urinary tract infection. Chitosan nanoparticles were synthesised, which are 10–65 nm in size, exhibiting a spherical morphology. Characterisation techniques, including Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray Diffraction (XRD), confirmed their properties. One hundred urine samples were collected from patients at Al-Hindiya Teaching Hospital, Karbala, Iraq, between February and April 2024. Out of these, 63 were positive for *Proteus mirabilis*, which were confirmed through PCR detection of the ureR gene. Antimicrobial activity of chitosan nanoparticles was evaluated at concentrations of 0.5, 1.0, 1.5, and 2 mg/ml, with inhibition zones ranging from 10 mm to 18 mm. These results implied that chitosan nanoparticles may be effective alternatives to traditional antibiotics.

## Introduction

Chitosan is a cationic biopolymer produced through chitin's complete or partial deacetylation. This polysaccharide is linear composed of “1-4 linked 2-amino-2-deoxy-β-D-glucopyranose” obtained by deacetylating “N-acetyl-D-glucosamine” [1, 2]. The term "chitosan" does not specifically refer to a singular substance; instead, it denotes a series of co-polymers, characterised by their degree of deacetylation, molecular mass, polymerisation, acid dissociation constant, and viscosity [3]. Chitosan made from microbial sources is seen as a good option because it allows for better control over the process, leading to a clean and uniform product with specific beneficial features. This biopolymer is highly versatile and demonstrates a diverse array of uses in medicine, agriculture, the food sector, cosmetics, and wastewater treatment [4].

The biopolymer chitosan is derived from chitin, a substance found in crab shells that has been known for a long time to kill microbes. For a long time, Chitosan has served as the optimal material for the fabrication of nanoparticles for many applications. Natural chitosan-based nanoparticles (CNPs) are

better than nanoparticles from other sources because they can replenish, are biocompatible, biodegradable, nontoxic, have high permeability towards biological membranes, and are safe for the environment. This is why they are preferred for many uses, such as antimicrobial activities [5]. Chitosan nanoparticles stick better to the membranes of germs and can easily get inside the cell of a pathogenic bacteria [6].

As an opportunistic bacteria in the Enterobacteriaceae family, *Proteus mirabilis* causes many health problems in both humans and animals, especially infections of the reproductive system and the digestive system [7]. It is the third most important bacteria in medicine, after *Escherichia coli* and *Klebsiella pneumoniae*, because it causes health problems and pathological infections of many body systems, especially the reproductive system and the digestive system [8]. The bacterium *Proteus mirabilis* is rod-shaped, Gram-negative, and can live without oxygen. It moves in swarms and has urease activity. 90% of all Proteus illnesses in people are caused by *Proteus mirabilis*. It can be found in a lot of places on Earth and water [9]. Swarming constitutes a form of collaborative group locomotion that Proteus mirabilis can utilise to traverse the outer layer of solid media or apparatus. Most of the time, *Proteus mirabilis* is linked to urinary tract infections, especially those that are difficult or caused by a catheter [10].

The study's goal is to look into how chitosan nanoparticles can be used to kill microbes in textiles. Chitosan nanoparticles are very active because They have an exceptionally high area of coverage and volume ratio, and they should have good bioactivity even at very low amounts. At first, the physical, chemical, and thermal features of synthesised nanoparticles compared to bulk chitosan were studied in more depth. A comparison study was also done to see how the bioactivity of nano-chitosan-treated polyester was better than bulk chitosan-treated polyester at the same concentration.

## 2. Materials and Methods

### 2.1. Equipment and Instruments

- X-ray diffraction (XRD) (Bruker) - Germany
- Autoclave (Labtech) - Korea
- Balance (Electrical Denver) - Canada
- Centrifuge (Hitachi) - Japan
- Digital Camera (Sony) - Japan
- Distillator (GFL) - Germany
- Fourier Transform Infrared (FT-IR) Spectroscopy (Perkin-Elmer 1725x) - Japan
- Hood (Biological Laboratory) - Korea
- Incubator, Oven (Memmert) - Germany
- Light Microscope (Olympus) - Japan
- Micro and Cooling Centrifuge (Hermle Labortechnik) - Germany
- ELISA system (Beekman) - Austria
- Micropipette (Eppendorf) - Germany
- pH meter (Orient) - United States
- Refrigerator (Beko) - Korea
- Field Emission Scanning Electron Microscope (FEI) - Netherlands
- Shaking Incubator (Gallenkamp) - England
- Thermocycler (Bio-Rad) - USA
- Vortex Mixer (Thermolyne) - United States
- Digital Magnetic Stirrer - United States
- Ultrasonicator UP400St - Germany.

### 2.2. Chemicals and Biological Materials

- MacConkey agar
- Brain Heart Infusion (BHIB) (Himedia, India)
- Clean plastic containers (50 ml)
- 70% ethanol alcohol
- Muller Oxoid Hinton agar (UK)
- Brain Heart Infusion agar (BHIA) (Himedia, India)
- Fe3O4
- NH3
- Deionised water
- Chitosan (possessing a medium weight in molecules and 85% deacetylation. Sigma Chemical "St. Louis, United States")
- Petri dish (Al Hanoof, Jordan)
- Loop (China/Al-rawan)
- Agarose (Concord, USA)
- Crystal violet powder (BDH, England)
- Ethidium Bromide Solution (Canada, Bio Basic)
- DNA Ladder 100 bp (United States, Promega)
- Ethanol (96%) (UK, BDH)
- DNA loading dye (United States, Promega)
- Glycerol (Sigma, USA)
- Gram stain (Himedia, India)
- Methanol (BDH, England)
- Phosphate buffer saline (PBS) (Bioworld, USA)
- Tris-EDTA buffer (Bio Basic, Canada)

### 2.3. Sample Collection

Approximately 100 urine specimens were collected in the period between 1 February 2024 and 30 April 2024, at the Al-Hindiya Teaching Hospital in Karbala Governorate, Iraq. Samples were taken from people of all ages and genders who had been diagnosed by experts with urinary tract infections. Clean plastic containers that hold 50 ml were used for this purpose, and the bacteria were cultured by the streaking method on MacConkey agar in Petri dishes for testing [11].

### 2.4. Urine Culture

MacConkey differential agar was used to grow the bacteria, and the plates remained at 37 degrees Celsius for twenty-four hours. The result was 63 samples positive and 37 samples negative. The colonies were identified by their shape and colour; they were pale because they weren't lactose-fermenters. The isolates were then moved to new MacConkey agar [12], and samples were named by looking at their shape on MacConkey agar. They were also named by looking at them under a microscope and using a Gram stain [13].


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## 2.5. Genetic Examination

### 2.5.1. Genomic DNA Extraction and PCR Experiments

Using extraction kits from Geneaid and special primers for finding the *ureR* gene, DNA was taken from bacteria that were mixed in Brain Heart Broth solution [14], as shown in **Table 1**:

**Table No. 1: Primer utilised in PCR assay for the identification of Proteus mirabilis**

| Genes | "Primer Sequence (5'-3')" | bp Size |
|---|---|---|
| "*ureR* (Forward)" | "GGTGAGATTTGTATTAATGG" | 225 |
| "*ureR* (Reverse)" | "ATAATCTGGAAGATGACGAG" | |

### 2.5.2. Agarose "Gel Electrophoresis"

PCR on agarose gel was used to find the bacterial DNA after it had been extracted. The gel was made with a concentration of 1% for DNA detection and 2% for PCR [15]. This was accomplished by dissolving 1 or 2 grams of agarose in 100 millilitres of the TBE buffer solution, which had already been made. The gel was melted by heating the fluid to its boiling point, and it was then left to cool at 50 °C. Ultimately, two microliters of ethidium bromide at an ultimate amount of 0.5 micrograms per millilitre were included. The acarose gel was carefully poured into the tin so that bubbles wouldn't form. It was allowed to solidify for thirty minutes. Finally, the TBE buffer solution was added so that it covered the gel's surface [15].

**Table No. 2: Interaction components of ureR *Proteus mirabilis* gene detection.**

| Components | Size (µl) |
|---|---|
| Primer (forward) | 0.75 |
| Primer (reverse) | 0.75 |
| DNA | 1.5" |
| Deionising water | 17 |
| Total size | 20 |

**Table no.3: Optimal conditions of Polymerase Chain Reaction (PCR).**

| Steps | "Temperature°C | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 94 | 4 second | 1 |
| denaturation | 94 | 40 minutes | 40 |
| Annealing | 58 | 1 second | |
| Extension | 72 | 20 minutes | |
| Final extension | 72 | 10 second | 1" |

## 2.6. Chitosan Nanoparticle Synthesis

The synthesis and optimisation of chitosan nanoparticles were performed in two steps (16), as follows:

### 2.6.1. Preparation of magnetic core nanoparticles

- Fe3o4 was produced and developed as "magnetic core nanoparticles".
- Aqueous solutions of "Fe (II) and Fe (III) chloride, at a molar ratio of 1:2", were produced and introduced into a free of oxygen while nitrogen was purged through them.
- After agitating the mixture for thirty seconds, we quickly added twenty ml of NH3, calibrating its ph value to 9.

- Within 45 minutes, the synthesised nanoparticles adhered to the magnet, then readily separated, rinsed with deionized water at a pH of 7, and subsequently dried at 37 degrees Celsius.
- After dissolving 0.02 g of "magnet nanoparticles" in fifty millilitres of deionised water and mixing it with 50 ml of tri-sodium citrate solution, the mixture was sonicated for 30 minutes.

#### 2.6.2. procedure

- Approximately 500 milligrammes of chitosan were solubilised in 50 millilitres of a solution containing 1 per cent acetic acid and agitated at 1000 revolutions per minute for 25 minutes at the surrounding temperature till the solution is clarified.
- The resultant solutions were then sonicated, subsequently titrated with Naoh or HCl solution set to ph 5 and filtered through a 0.2 µm mesh.
- In this coating process, five millilitres of nano-magnetic solution were incorporated into 75 millilitres of deionised water and subjected to sonication for 10 minutes.
- A solution of chitosan was introduced and subjected to sonication for 5 minutes. The resultant solution was transparent.

## 3. Characterisation of Chitosan Nanoparticles

### 3.1. Scanning Electron Microscope (SEM)

The shape of the "chitosan nanoparticles" was looked at using "field emission scanning electron microscopy (FE-SEM) (AMRAY1910)," which had a special detector for backscattered electrons working at 15-30 kv. The specimens were sputter-coated with roughly 15 nm of gold using a Polaron coater device for scanning electron microscopy pictures [17].

### 3.2. Fourier Transform Infrared Spectra

On a "Perkin-Elmer FTIR spectrometer (spectrum 1000)" with KBr pellets at a precision of 4 $cm^{-1}$ to check how chitosan and TPP are linked. The "chitosan nanoparticles were combined with KBr" in a 1:150 ratio, and they were then pounded by hand in a mixer. Four times the weight of the powder was used to press it into pellets. The "IR absorbance" scan was looked at from 400 to 4000 $cm^{-1}$ to see how strong the sample peaks were [18].

### 3.3. Diffraction of X-rays

We employed a "Rigaku D/max-2500 X-ray" diffractometer utilising the radiation from Cu K to examine the phase and crystallinity throughout a 2-hour range from 10° to 80° [19].

### 3.4. Application of chitosan nanoparticles against Proteus mirabilis

#### 3.4.1. Method of Disc Diffusion

The altered disc diffusion technique was employed to assess the antibacterial efficacy of nanoparticles of chitosan over the isolates of bacteria. This technique was executed using Mueller-Hinton agar media [20]. Six millimetres sterile paper discs infused with chitosan nanoparticles at concentrations of 0.5, 1, 1.5, and 2 mg/ml, individually. The proliferation of Proteus mirabilis on the agar used for brain heart infusion (BHIA) facilitated the activation and sustenance of the bacteria. Subsequently, chitosan nanoparticle samples were applied to an infected Muller-Hinton agar plate,

which was incubated for 24 hours at 37ºC. The inhibition zone was seen post-incubation, and the diameter of the bacterial development inhibition halo surrounding the samples was quantified in millimeters.

## 4. Results

The study demonstrated that chitosan nanoparticles exhibit significant antimicrobial activity against *Proteus mirabilis*, with inhibition zones ranging from 10 mm to 18 mm at concentrations of 0.5 to 2 mg/ml, respectively. Chitosan nanoparticles were synthesized with sizes between 10–65 nm and characterized using SEM, FTIR, and XRD, which confirmed their spherical morphology, functional group presence, and crystalline structure. Out of 100 urine samples collected from patients with urinary tract infections, 63 were positive for *Proteus mirabilis*, confirmed by PCR detection of the ureR gene. The results highlight the dose-dependent effectiveness of chitosan nanoparticles as an antimicrobial agent, suggesting their potential as a cost-effective alternative to traditional antibiotics, especially given the rising concerns of antimicrobial resistance.

**Figure 1: The pale colour of *Proteus mirabilis* colonies.**

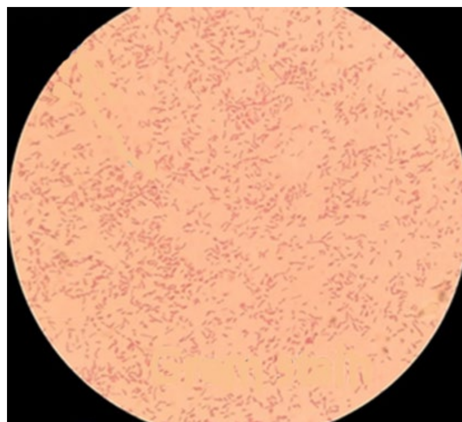

**Figure 2: *Proteus mirabilis* in Gram stain smear.**

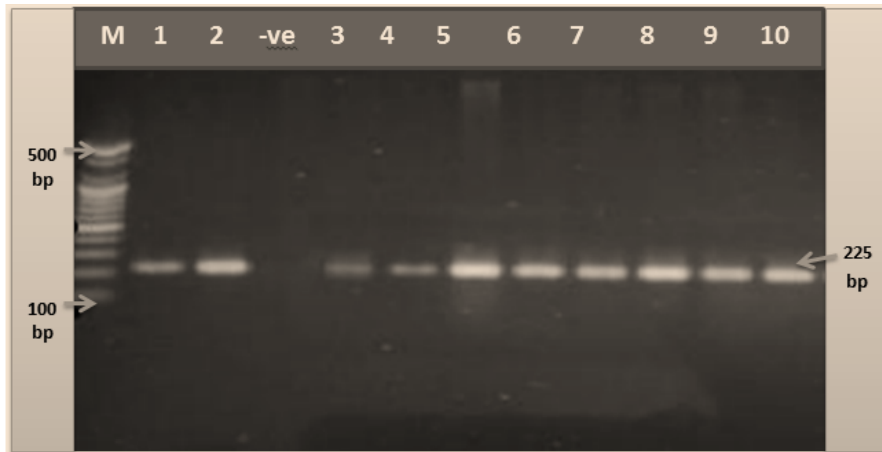


**Figure 3: The PCR result measuring 225 bp was put through "1.5% agarose gel electrophoresis" at 90 V for 1 hour in 1x TBE buffer. The gel was then seen under a transilluminator with UV light after being stained with Red Safe. Lane L: a DNA ladder.**

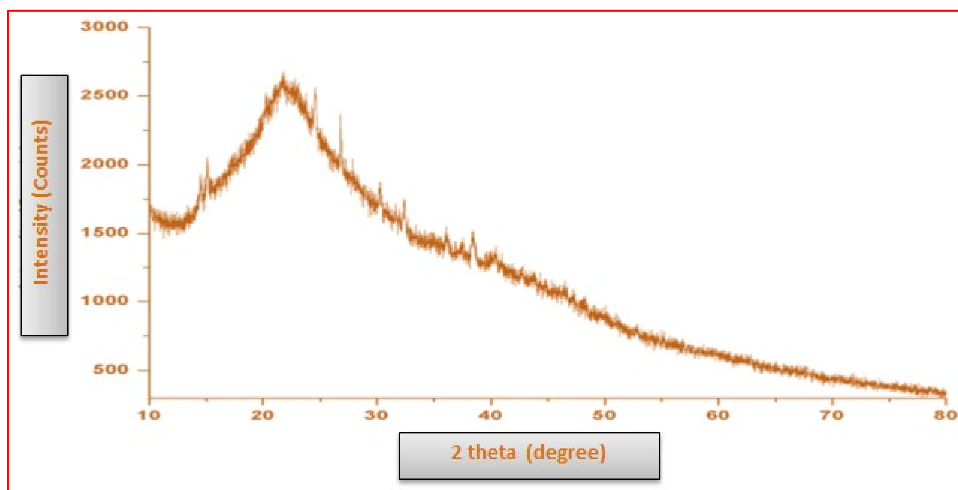


**Figure 4: XRD pattern of "chitosan nanoparticles"**

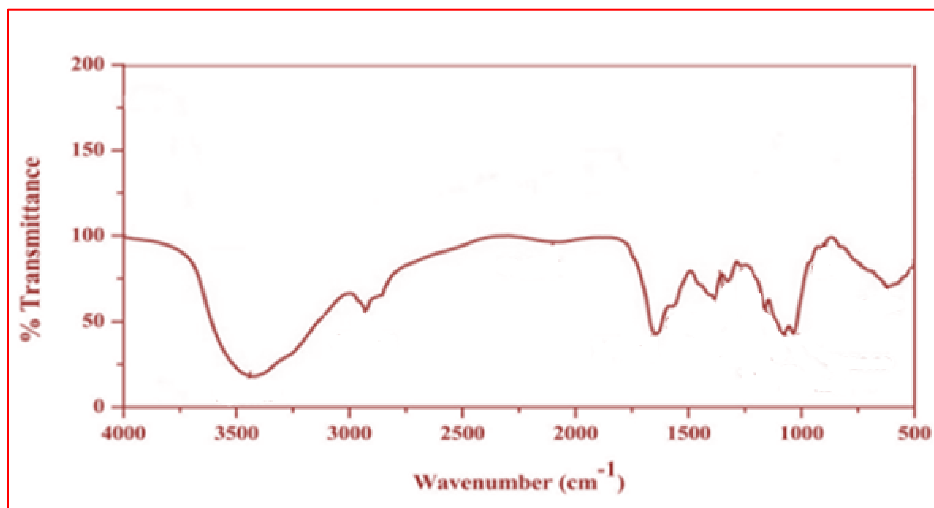


**Figure 5: FTIR of "chitosan nanoparticles"**

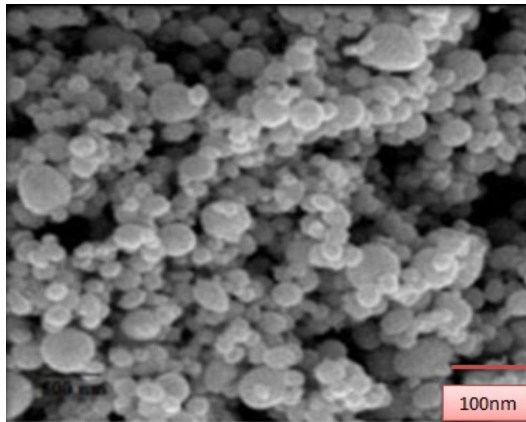


**Figure 6: SEM of "chitosan nanoparticles"**

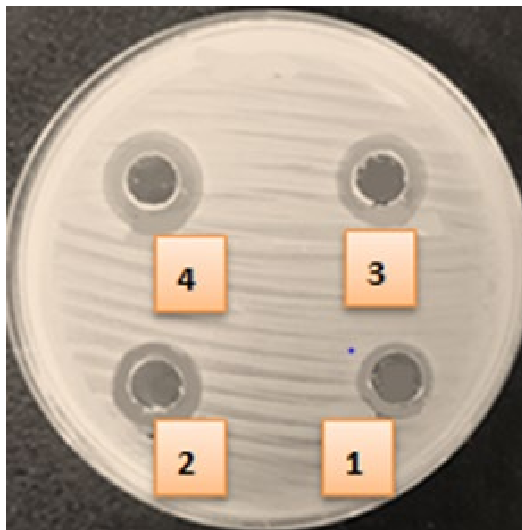


**Figure 7: Inhibition zone of nanoparticles of chitosan against Proteus mirabilis**

**Table No. 4: Inhibition zones (mm) of nanoparticles of chitosan against "Proteus mirabilis"**

| Number | Chitosan Nanoparticles Concentration (mg /ml) | Zone of Inhibition (mm) |
|---|---|---|
| 1 | 0.5 | 10 |
| 2 | 1.0 | 15 |
| 3 | 1.5 | 15 |
| 4 | 2 | 18 |

## 4. Discussion

Proteus mirabilis, a bacillus within the Enterobacteriaceae family, is a gram-negative facultative anaerobe that can ferment maltose but cannot ferment lactose. The bacterium Proteus mirabilis has swarming behaviour and can self-elongate and synthesise a type of polysaccharide during interaction with solid surfaces, thereby enhancing adherence and effective motility across surfaces (e.g., medical devices). The flagella of Proteus mirabilis enhances its motility, assists in colonisation, contributes to biofilm formation, and improve resistance against host defence and likely pharmaceuticals [21]. The positive *Proteus mirabilis* isolates that appeared as shown in Figure 1 were pale in colour and were inspected using a light microscope after performing the Gram stain test shown in Figure 2. The *ureR* gene is deemed significant for the identification of *Proteus mirabilis*, as illustrated in Figure 3,

as it is responsible for the production of the urease enzyme in samples obtained from urinary tract infection cases across various age groups.

Ninety per cent of Proteus infections are attributed to *Proteus mirabilis* and are categorised as acquired from community illnesses. Although "Proteus species" are not prevalent in nosocomial infections but can induce infections coming from the infected skin and oral tissue of patients as well as healthcare professionals in medical facilities or long-term care settings. People who have had infections from the hospital, ongoing infections, issues with their urinary tract, or previous procedures involving the urethra are more likely to get Proteus infections and other germs like Enterobacter, Klebsiella, Staphylococci, Pseudomonas, and Enterococci. Urinary tract infections (UTIs) happen when bacteria move up the inside surface of the catheter or into the catheter tube from infected urine.

"Chitosan nanoparticles" have been widely employed in biomedical applications. Over the last ten years, it has also been extensively researched because of its capacity to regulate drug release rates, extend therapeutic efficacy, and transport medicines, proteins, DNA, and antigens to specific targeted sites within the body [23]. "Chitosan nanoparticles" exhibit anticancer properties by enhancing the immunological function of the organism. [24, 25]. "Chitosan nanoparticles" conjugated with "metal and metal oxide nanoparticles" exhibit antibacterial properties [26], antifungal [27], anti-biofilm [28], and anticancer [29] properties. Additionally, their applications include pesticides [30], nano-fertilisers [31], eliciting agents [32], and biosensors [33].

In the present study, chitosan nanoparticles were used as an antimicrobial agent against *Proteus mirabilis* in different concentrations, and there were different levels of inhibition zones, as shown in Figure 8, below:

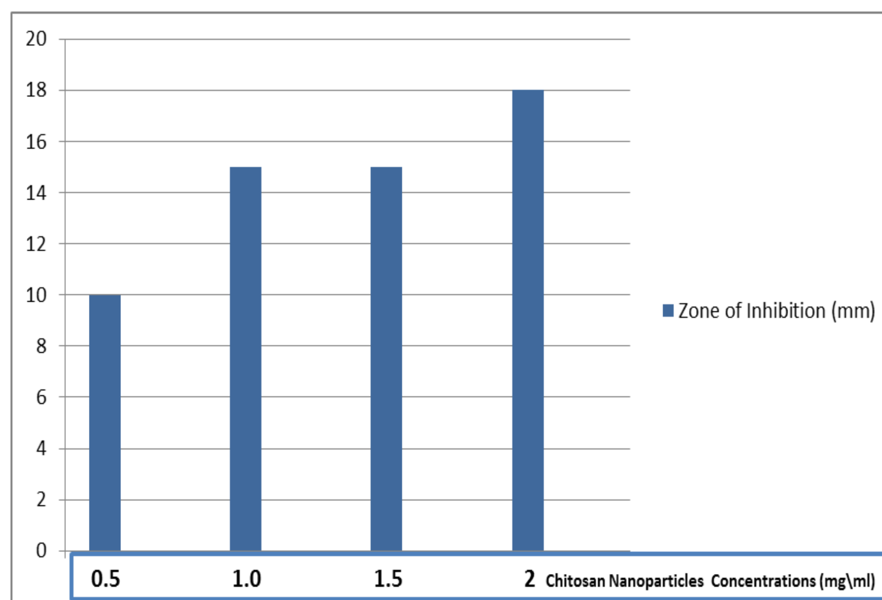


**Figure 8: Chitosan nanoparticles' effect against *Proteus mirabilis* illustrated as a zone of inhibition according to concentrations**

Chitosan nanoparticles can form tiny holes in the membranes of bacteria and fungi, which interfere with the production of proteins and communication inside the cells, possibly leading to cell death [34].

## Conclusion

The current work reveals that chitosan-synthesised nanoparticles may be manufactured economically, have significant antibacterial efficacy, and demonstrate typical features together with great stability when diluted to different concentrations. Chitosan generates nanoparticles with standardised specifications that serve as antibacterial and therapeutic agents, inhibiting the Enterobacteriaceae family of gram-negative, facultative anaerobic bacilli, such as *Proteus mirabilis*. Consequently, employing chitosan nanoparticles as antibacterial agents may serve as an alternative to antibiotics, given that bacteria progressively gain resistance to different types of medications, which frequently result in significant adverse effects globally. Synthetic chitosan nanoparticles serve as preferable and more cost-effective antibacterial agents compared to antibiotics, owing to their straightforward production and the accessibility of requisite raw materials. Our continuous research has demonstrated the efficacy and safety of nanoparticles in medical and biological applications, confirming that they do not adversely impact public health or the human environment.

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