

Research Article

Antimicrobial effect of Chitosan and Nano-Chitosan against some Pathogens and Spoilage Microorganisms

Warda M. Abdeltwab¹, Yasser F. Abdelaliem², Wedad A. Metry¹ and Mahmoud Eldeghedy¹

¹Dairy Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.

²Agricultural Microbiology Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.

Abstract: An experiment was conducted to investigate the antimicrobial effect of chitosan and nano-chitosan. Two Gram-negative, three Gram-positive bacteria and three fungal strains were used as test microorganisms. The obtained results indicated that 88% of nano-chitosan particle size was in the range of 93.76nm and 12% in 405nm. Nano-chitosan showed maximum antibacterial activity against *S. aureus* and *L. monocytogenes* with inhibition zone of 30mm (23µg/ml concentration) and the lowest 23mm with *E. coli* at the same concentration. Other tested bacteria were affected in different degrees. The MIC and MLC ranged between 64 to 256 and 128 to 512µg/ml, respectively. The highest effect was against *S. aureus* at 23.04µg/ml. Chitosan solution was found to have less antifungal activity against *C. albicans* when compared to nano-chitosan. MIC and MLC for chitosan and nano-chitosan were recorded at 64 and 128µg/ml with chitosan and 23.04 and 46.08µg/ml with nano-chitosan. The highest nano-chitosan activity was recorded against *S. cerevisiae*, 7 and 16µg/ml for MIC and MLC, respectively. Nano-chitosan at concentrations 3.0 and 4.5µg/ml were the most effective to retard fungal activity.

Keywords: Chitosan, Nano-chitosan, Antimicrobial activity, Pathogenic and Spoilage Microorganisms.

1. Introduction

Chitosan is a non-toxic, biodegradable polymer of high molecular weight and is very much similar to cellulose, a plant fiber. The only difference between chitosan and cellulose is the amine (-NH₂) group in the position C-2 of chitosan instead of the hydroxyl (-OH) group found in cellulose. However, unlike plant fiber, chitosan possesses positive ionic charges, which give it the ability to chemically binding with negatively charged fats, lipids, cholesterol, metal ions, proteins and macromolecules [1]. Chitosan is a substance derived from chitin, which is found in shellfish shells such as crab, lobster and shrimp. It is also found in common foods we eat such as grain, yeast, bananas, and mushrooms. The chitin is deproteinized, demineralized and deacetylated. It is a dietary fiber, meaning that it cannot be digested by the digestive enzymes of a person [2]. In this respect, [3] reported that chitin and chitosan have attained increasing commercial interest as suitable resource materials due to their excellent properties including biocompatibility, biodegradability, adsorption, ability to form films and to chelate metal ions.

Nano-chitosan is a natural material with excellent physicochemical properties. It is environmentally friendly and bioactive. Nano-chitosan have been prepared by several approaches, including physical crosslinking by ionic gelation between chitosan and specific negatively charged macromolecules such as pentasodium tripolyphosphate [4]. Moreover, chitosan and chitosan nanoparticle films and coatings can be used as a vehicle for incorporating natural or chemical antimicrobial agents, antioxidants, enzymes or functional substances such as plant extracts, probiotics, minerals or vitamins [5]. However, chitosan nanoparticles exhibited higher antimicrobial activity than chitosan during the storage period [6]. Chitosan coating containing chitosan nanoparticles can be increased cheese shelf life [7].

Competition in food industry is increasing day by day to satisfy the needs of consumers. Require a search for new processes to prolong shelf life and guarantee safety and quality of food. Nowadays, nanotechnology could be the solution in both food and dairy industry. The antibacterial action modes of chitosan and its derivatives have been highlighted by many researchers regarding this point.

One of the mechanisms is that chitosan works as a chelating agent [8]. The second is that chitosan of molecular weight can enter the nuclease of the cell [9]. Then interacts with DNA and interferes with mRNA synthesis of protein, thus inhibits the action of various enzymes. The third noticed by [10]. Indicates that chitosan would result in greater changes in the cell wall structure and in the permeability of the cell membrane as it works on the negatively charged cell surfaces. Consequently, it will have a great effect on gram-negative bacteria.

Antibacterial activity of chitosan nanoparticles and copper-loaded nanoparticles were investigated against *E. coli*, *S. choleraesuis*, *S. typhimurium*, and *S. aureus* using the calculation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Results showed that chitosan nanoparticles and copper-loaded nanoparticles could inhibit the growth of various bacteria tested. Their MIC values were less than 0.25 µg/ml, and the MBC values of nanoparticles reached 1 µg/ml. Atomic force microscopy (AFM) revealed that the exposure of *S. choleraesuis* to chitosan nanoparticles led to the disruption of cell membranes and the leakage of cytoplasm [11].

Chitosan prepared from fresh shrimp head was found to be of good quality and comparable to that of commercially available chitosan. Procedures employed for the preparation of chitosan influenced its molecular weight, as well as its degree of deacetylation (DD). Low-molecular-weight (LMW) chitosan affected growth, development and morphology of *Aspergillus parasiticus in vitro*. It increased the production of total aflatoxin B1 in corn with a reduction in the *A. flavus* production within 8 days. Chitosan gave a good level of radial growth inhibition against *A. parasiticus* [12].

Chitosan nanoparticles were prepared from low-molecular-weight (LMW), high-molecular-weight (HMW) chitosan and its derivative, trimethyl chitosan (TMC). The zeta potential of the particles (from 1 to 3mg) ranged between +22 to +55mV. *C. albicans* and *F. solani* showed to be sensitive to LMW and HMW. At the time *A. niger* resisted the inhibitory effect of these particles except those from HMW. It could be included that the parent compound could be formulated and used as a natural antifungal agent into nanoparticles [13].

The antibacterial activity of chitosan nanoparticle derivatives prepared on Vancomycin-resistant *Enterococcus*, *S. aureus* and *E. coli* was examined by well diffusion and microtiter method according to [14]. The highest inhibition zone was recorded against *S. aureus*, lower diameter of inhibition zone was shown against *E. faecalis* and *E. coli*, respectively. MIC and MBC results showed the highest inhibitory effect of chitosan nanoparticles against *S. aureus*. This nanoparticle can be used as an antibacterial agent [15].

On the basis of the above-mentioned information and in the light of the previous studies, the present study has been designed for the preparation of nano-chitosan from chitosan and determination of the antimicrobial activity of nano-chitosan and chitosan against some foodborne pathogens and spoilage microorganisms.

2. Material and Methods

2.1. Materials

Chitosan (CS) powder (low-molecular-weight, 75–85% deacetylated), and chemicals were obtained from El-Nasr, Merck, Sigma and Loba Chemie companies. All chemicals used for this study were analytical grade.

2.2. Microorganisms

For the purpose of *in vitro* testing of antimicrobial activity of chitosan and nano-chitosan particles with thyme, the following standardized bacterial and fungi cultures were used two gram-negative bacteria *E. coli* ATCC 25922 and *S. enteritidis* ATCC 13076 and three gram-positive bacteria *L. monocytogenes* ATCC 15313, *B. cereus* ATCC 10876 and *S. aureus* ATCC 8095, *Candida albicans* and *Penicillium roqueforti* were obtained from the culture collection of Agricultural Microbiology Department, Faculty of Agriculture, Fayoum University and *Saccharomyces cerevisiae* was obtained from Dairy Microbiology Laboratory, National Research Center (NRC), Dokki, Giza, Egypt.

2.3. Methods

2.3.1 Activation of microorganisms

Luria-Bertani agar medium (LB) is a semisynthetic medium used for the general cultivation for all indicator bacteria, potato dextrose agar medium and Czapek's agar is semisynthetic media used for the general cultivation of yeasts and fungi (Difco, USA).

2.3.2 Experimental design

2.3.2.1 Preparation of nano-chitosan: Chitosan was dissolved in 0.5% (w/v) in acetic acid 1% (v/v), the pH was raised to 4.6–4.8 with 10N NaOH. Nano-chitosan formed spontaneously upon addition of 1ml of an aqueous tripolyphosphate solution (0.25%, w/v) to 3ml of chitosan solution under magnetic stirring. Nanoparticles were purified by centrifugation at 9000g for 30 min. supernatants were discarded and the nano-chitosan were extensively rinsed with distilled water to remove any sodium hydroxide and then freeze-dried before further use or analysis [11].

2.3.3 Methods of Analysis

2.3.3.1 Determination of chitosan molecular weight (M_v): One percent (w/v) of the chitosan was prepared

by dissolving 0.5g of purified chitosan in 50mL of 1% acetic acid with stirring using magnetic stirrer for about one hour. The viscosity of the solution was determined using Brookfield viscometer with RV number 6 spindle. The average molecular weight was calculated using "Equation 1".

$$[\eta] = K_m M_v^a \quad (1)$$

The equation shows the relationship of viscosity and average molecular weight, where $[\eta]$ is the intrinsic viscosity, $K_m = 1.81 \times 10^{-3}$ (ml/g) and $a = 0.93$ are the empirical Mark-Houwink viscometric constants that are specific for a given polymer [16].

2.3.3.2 Some characteristics of nano-chitosan

2.3.3.2.1 Electrical charge and size of nano-chitosan:

Particle size, electrical distribution, uniformity and the zeta potential of nano-chitosan were determined using Dynamic light scattering (DLS) or Zetasizer instrument (Malvern). Zeta potential, that is, surface charge, can greatly influence particle stability [15].

2.3.3.3 Transmission Electron Microscopy (TEM):

Transmission electron microscopy (TEM) was used to observe the morphology of the nano-chitosan. Samples were placed on copper grill, covered with nitrocellulose. They were dried at room temperature and examined using TEM without being negatively stained [17].

2.3.3.4 Microbiological examinations

2.3.3.4.1 Determination of the antibacterial activity of chitosan and nano-chitosan:

Agar well diffusion method (qualitative test) was used as described by [14]. Chitosan or Nano-chitosan powder was accurately quantified and added to 0.25% acetic acid according to [11]. The plates were left at 4-5°C for 2h to allow diffusion of the substances and then incubated aerobically for 24h at temperature optimum for each indicator organism. Absence or presence of inhibition zones, as well as their diameters, were recorded.

2.3.3.4.2 Determination of MIC and MLC of chitosan and nano-chitosan on some pathogenic bacteria:

MIC and MLC values of chitosan and nano-chitosan on some pathogenic bacteria were determined by broth dilution method [18], using *E. coli*, *S. enteritidis*, *L. monocytogenes*, *B. cereus* and *S. aureus* as indicator bacteria.

2.3.3.4.3 Bacterial broth dilution method for determination of MIC and MLC for chitosan and nano-chitosan

- a. Different concentrations of chitosan and nano-chitosan were used for this method, which

previously prepared in serial two-fold concentrations, were placed in tubes of LB broth medium. Chitosan and nano-chitosan were prepared in concentrated solutions and then diluted to the appropriate concentrations in broth.

- b. One ml inoculum of the pathogenic organism ($\sim 2 \times 10^5$ CFU/ml) was added individually to tubes containing growth media with chitosan in serial two-fold dilution (4, 8, 16, 32, 64, 128, 256, 512 and 1024 $\mu\text{g/ml}$ broth medium) and nano-chitosan in serial two-fold dilution (1.44, 2.88, 5.76, 11.52, 23.04, 46.08, 92.16, 184.32 and 368.64 $\mu\text{g/ml}$ broth medium). Control tube was free from chitosan and nano-chitosan.
- c. Tubes were examined for turbidity as growth indicator after 24 hours. The lowest concentration of chitosan and nano-chitosan that inhibits growth of the organism, as detected by lack of visual turbidity (comparing with control), is considered the minimum inhibitory concentration (MIC).
- d. The minimum lethal concentration (MLC), the lowest concentration of chitosan or nanoparticles that kills 99.9% of bacteria, was determined by assaying the live organisms in those tubes from the MIC test that showed no growth. A loop full of each of those tubes was inoculated on LB agar and examined for signs of growth. Growth of bacteria demonstrates the presence of these bacteria in the original tube. On the contrary, if no growth was observed, the original tube contained no living bacteria, and the chitosan and nanoparticles were considered as being bactericidal at that concentration.

2.3.3.4.4 Determination of antifungal activity of chitosan and nano-chitosan:

P. roqueforti and *A. niger* spore suspension were activated on potato dextrose agar [19]. After incubation of $28 \pm 2^\circ\text{C}$ for 5 days, spores were harvested and collected on tween 80 0.1% (v/v), determined using hemocytometer and adjusted to a final concentration of $1 \times 10^4/\text{ml}$. Different chitosan concentration 6, 8, 10 and 15g/L were prepared by dissolving chitosan and nano-chitosan in 0.04mol/L acetic acid. The pH was adjusted to 5.11 using acetic acid with the same above-mentioned concentration.

Then, added to sterile Czapek agar medium (3:7, v/v), the final pH of the Czapek medium containing chitosan ranged from 5.63 to 5.75. The pH adjustment maintained the same conditions during the experiment without affecting the growth and ensured that all of the

chitosan amino groups were positively charged (pK_a of chitosan is 6.5). The final concentrations of nano-chitosan in Petri dishes were 1.8, 2.4, 3.0 and 4.5g/L [12], the fungistatic index (FI) was calculated according to "Equation 2":

$$FI = (1 - R_i / R_c) \times 100 \quad (2)$$

Where R_c is the mean value of the colony radius in acid control media and R_i is the colony radius in the nano-chitosan amended media.

All the measurements or analyses were carried out in triplicate.

2.3.4 Statistical Analysis

All the experiments were performed in triplicate and the results obtained were analyzed statistically. General Linear Models (GLM) were performed using [20] Windows, version 19 software package according to the following model "Equation 3":

$$Y_{ijk} = \mu + T_i + P_j + TP_{ij} + e_{ijk} \quad (3)$$

Significant differences among treatments, storage period and the interaction mean between them were compared at $P \leq 0.001$ level of significance using Duncan's multiple range test [21].

Relevant details should be given, including experimental design and the technique(s) used and clearly indicate the statistical methods used to summarize information for interpretation.

3. Results and Discussion

3.1 Particle size, zeta potential, and morphology of nano-chitosan

The nano-chitosan was prepared by ionic cross-linking of positively charged chitosan (molecular weight of chitosan is 125.68 kDa) with negatively charged tripolyphosphate (TPP). In this study, ionic gelation method was applied because this method is easy and fast.

The size, dispersion and uniformity of nano-chitosan were determined by Dynamic light scattering (DLS). Table (1) and Fig. (1) showed that, 88% of nano-chitosan particles size was in the range of 93.76nm and the rest 12% in the range of 405nm. It is evident from the same figure that nano-chitosan have narrow size distribution (polydispersity index (PDI) = 0.417) with average particle size of 93.76nm and zeta potential of 14.6mV (Fig. 2). Nano-chitosan was prepared and size of them was verified by DLS and transmission electron microscopy (TEM) Fig. (3), then the results of these methods were confirmed with each other. Also, the results indicated that the diameter of particle was around 100nm. Also, size (including size distribution) and zeta potential are essential characteristic parameters for nanosuspensions [22].

Table 1. The size distribution by number of nano-chitosan by DLS (nm).

Size (d.nm)	Mean Number (%)	Size (d.nm)	Mean Number (%)
0.4000	0.0	68.06	7.6
0.4632	0.0	78.82	23.1
0.5365	0.0	91.28	27.9
0.6213	0.0	105.7	18.6
0.7195	0.0	122.4	8.2
0.8332	0.0	141.8	2.4
0.9649	0.0	164.2	0.4
1.117	0.0	190.1	0.1
1.294	0.0	220.2	0.6
1.499	0.0	255.0	1.2
1.736	0.0	295.3	1.8
2.010	0.0	342.0	2.0
2.328	0.0	396.1	2.0
2.696	0.0	458.7	1.6
3.122	0.0	531.2	1.2
3.615	0.0	615.1	0.8
4.187	0.0	712.4	0.5
4.849	0.0	825.0	0.2
5.615	0.0	955.4	0.1
6.503	0.0	1106	0.0
7.531	0.0	1281	0.0
8.721	0.0	1484	0.0
10.10	0.0	1718	0.0
11.70	0.0	1990	0.0
13.54	0.0	2305	0.0
15.69	0.0	2669	0.0
18.17	0.0	3091	0.0
21.04	0.0	3580	0.0
24.36	0.0	4145	0.0
28.21	0.0	4801	0.0
32.67	0.0	5560	0.0
37.84	0.0	6439	0.0
43.82	0.0	7456	0.0
50.75	0.0	8635	0.0
58.77	0.0	1.000e4	0.0

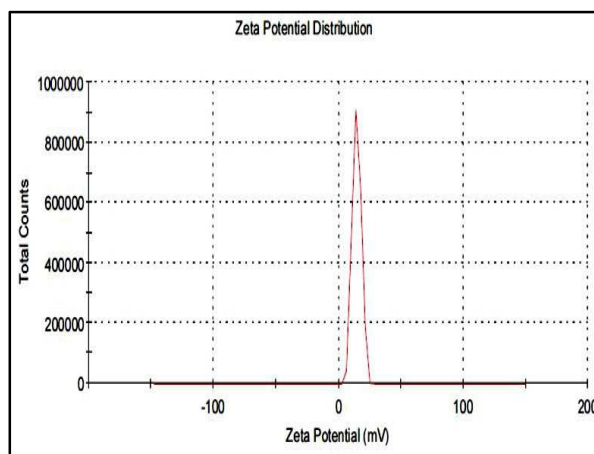


Fig. 1. The size of nano-chitosan by dynamic light scattering DLS (nm).

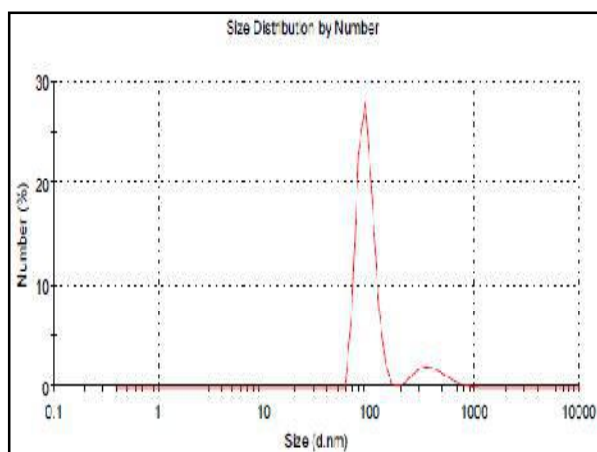


Fig. 2. Electrical charge of nano-chitosan by Zetasizer (mV).

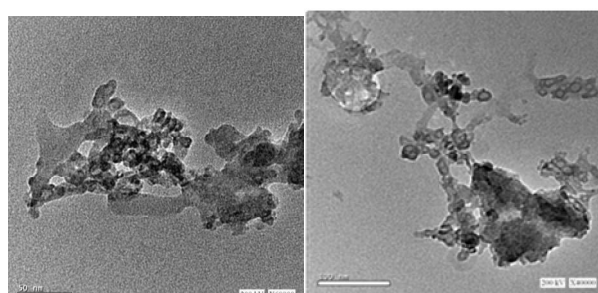


Fig. 3. Transmission electron microscopy images of nano-chitosan.

3.2. Antimicrobial activity of chitosan and nano-chitosan

3.2.1 Antibacterial activity

3.2.1.1 Agar well diffusion: The results in Table (2) and Figs. (4, 5 and 6) revealed that all the chitosan concentrations were potent antimicrobials against all tested microorganisms. Nano-chitosan showed higher degree of inhibition than that done by chitosan. Nano-

chitosan showed maximum antibacterial activity against *S. aureus* and *L. monocytogenes*, maximum inhibition zone diameter recorded 30mm at concentration 23µg/ml and the lowest with *E. coli* 23mm at the same concentration. Similarly, chitosan showed maximum inhibition zone with *L. monocytogenes* diameter of 25mm at concentration 64µg/ml, but *S. aureus* recorded the lowest inhibition zone (21mm) at the same concentration. More specifically, nano-chitosan represented highest susceptibility to all tested bacterial strains.

3.2.1.2 Minimum inhibitory concentrate and minimum lethal concentration (MIC and MLC):

Table (3) summarizes the MIC and MLC of chitosan and nano-chitosan against tested microorganisms. The MIC and MLC of chitosan were ranged between 64 to 256 and 128 to 512µg/ml, respectively, with all tested microbes while, the MIC and MLC of nano-chitosan ranged between 8 to 184.32 and 16 to 368µg/ml, respectively. Also, the results indicated that nano-chitosan was more effective against tested microorganisms compared with chitosan. Nano-chitosan MIC and MLC results showed the highest inhibitory against *S. aureus*. Different concentrations of MIC were undertaken on different strains. The highest inhibitory effect was obtained with *S. aureus* at concentration 23.04µg/ml with MIC and MLC. In *S. aureus*, perhaps the chitosan nanoparticle with positive charge can interact with the cell surface or essential nutrients so, as to inhibit the growth of bacteria or can interfere with anionic channels [15]. The lowest inhibitory effect was obtained against *S. enteritidis* and *E. coli* at 184.32µg/ml with MIC and 368.64µg/ml with MLC. The more sensitive of chitosan was recorded of MIC 128µg/ml and MLC at concentration 256µg/ml with *S. enteritidis* and *L. monocytogenes*.

Table 2. Antimicrobial activity of chitosan and nano-chitosan using agar well diffusion assay.

Treatments	Conc. (µg/ml)	Inhibition zone diameter (mm) against indicator microorganisms						Concentration Effect
		<i>E. coli</i>	<i>S. enteritidis</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>C. albicans</i>	
Chitosan								
Control	0	8.0 ^l	12.0 ^g	12.0 ^g	10.0 ^h	10.0 ^h	12.0 ^g	10.7 ^D
1	64.0	22.0 ^c	23.0 ^b	25.0 ^a	21.0 ^d	22.0 ^c	22.0 ^c	22.5 ^A
2	32.0	20.0 ^{de}	21.0 ^d	22.0 ^c	18.0 ^f	21.0 ^d	22.0 ^{de}	20.3 ^B
3	16.0	20.0 ^{de}	18.0 ^f	20.0 ^{de}	18.0 ^f	20.0 ^{de}	22.0 ^{de}	19.3 ^C
4	8.0	19.0 ^e	18.0 ^f	21.0 ^d	19.0 ^e	20.0 ^{de}	18.0 ^f	19.2 ^C
Microorganisms effect		17.8 ^C	18.4 ^B	20.0 ^A	17.2 ^D	18.5 ^B	18.4 ^B	
Nano-chitosan								
Control	0	8.0 ^p	11.0 ⁿ	12.0 ^m	10.0 ^o	10.0 ^o	12.0 ^m	10.5 ^E
1	23.2	23.0 ^g	24.0 ^f	30.0 ^b	30.0 ^b	27.0 ^d	33.0 ^a	27.8 ^A
2	11.6	22.0 ^h	23.0 ^g	25.0 ^e	25.0 ^e	20.30 ^g	30.0 ^b	24.7 ^B
3	5.8	21.0 ⁱ	20.0 ^j	24.0 ^f	20.0 ⁱ	23.0 ^g	28.0 ^c	22.7 ^C
4	2.9	22.0 ^h	19.0 ^k	21.0 ^j	18.0 ⁱ	22.0 ^h	22.0 ^h	20.7 ^D
Microorganisms effect		19.2 ^E	19.4 ^E	22.4 ^B	20.6 ^D	21.0 ^C	25.0 ^A	

A, B, ... and E: means within the treatments and storage period effect having different capital superscripts are significantly different ($P \leq 0.001$)
a, b, ... and p: means within the interaction having different small superscripts are significantly different ($P \leq 0.001$).

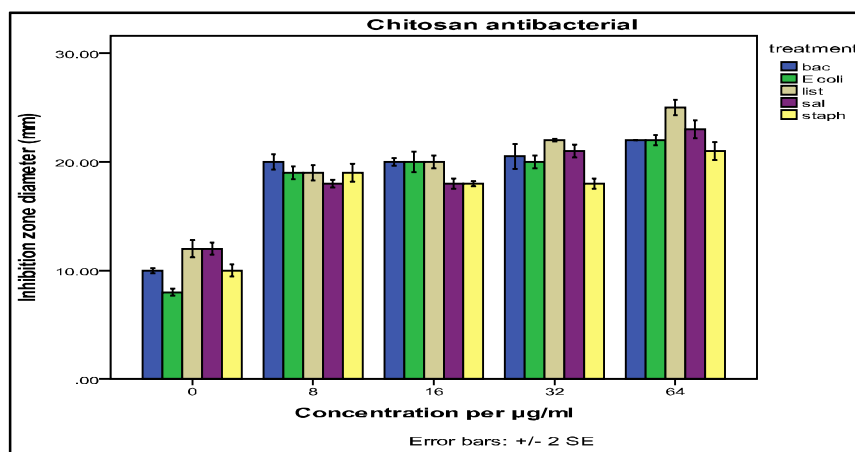


Fig. 4. Antibacterial effect of different concentrations of chitosan against some pathogenic bacteria.

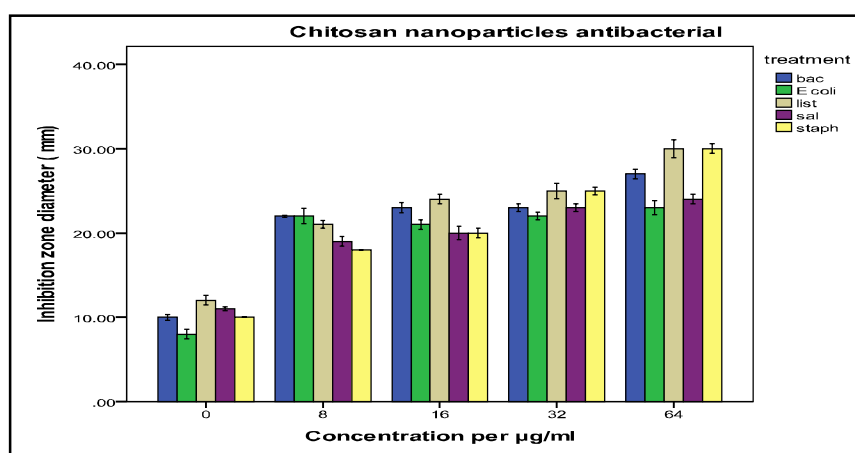


Fig. 5. Antibacterial effect of different concentrations of nano-chitosan against some pathogenic bacteria.

According to previous studies [23,24,25], the antibacterial activity of chitosan under acidic environment may result from its polycationic structure due to the protonation of $-NH_2$ on the C-2 position of the D-glucosamine repeat unit. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells.

3.2.2 Antifungal activity

The results obtained in Table (3) and Fig. (6) Showed that, chitosan solution was found to have less antifungal activity against *C. albicans* when compared with nano-chitosan. The MIC and MLC for chitosan and nano-chitosan were recorded as 64 and 128µg/ml with chitosan and 23.04 and 46.08µg/ml with nano-chitosan, respectively. The highest inhibition activity of nano-chitosan was recorded against *S. cerevisiae* compared with the other microorganisms where MIC and MLC were 8 and 16µg/ml, respectively.

Table 3. MIC and MLC (µg/ml) of chitosan and nano-chitosan against some microorganisms.

Strains	Chitosan (µg/ml)		Nano-chitosan (µg/ml)	
	MIC	MLC	MIC	MLC
Gram-negative bacteria				
<i>E. coli</i>	256	512	184.32	368.64
<i>S. enteritidis</i>	128	256	184.32	368.64
Gram-positive bacteria				
<i>L. monocytogenes</i>	128	256	46.08	46.08
<i>S. aureus</i>	256	512	23.04	23.04
<i>B. cereus</i>	256	512	92.16	184.32
Yeasts				
<i>C. albicans</i>	64	128	23.04	46.08
<i>S. cerevisiae</i>	128	256	8	16

As shown in Table (4) and Fig. (7), it could be noticed that nano-chitosan at concentrations 3.0 and 4.5g/L were effective against *P. roqueforti* where the concentration showed the growth of the fungus to great extent. Fungistatic values recorded were 90 and 100%, respectively, while chitosan was less effective. This finding is an agreement with those obtained by [11] who demonstrated that nano-chitosan exhibited higher affinity to bind to fungal cells.

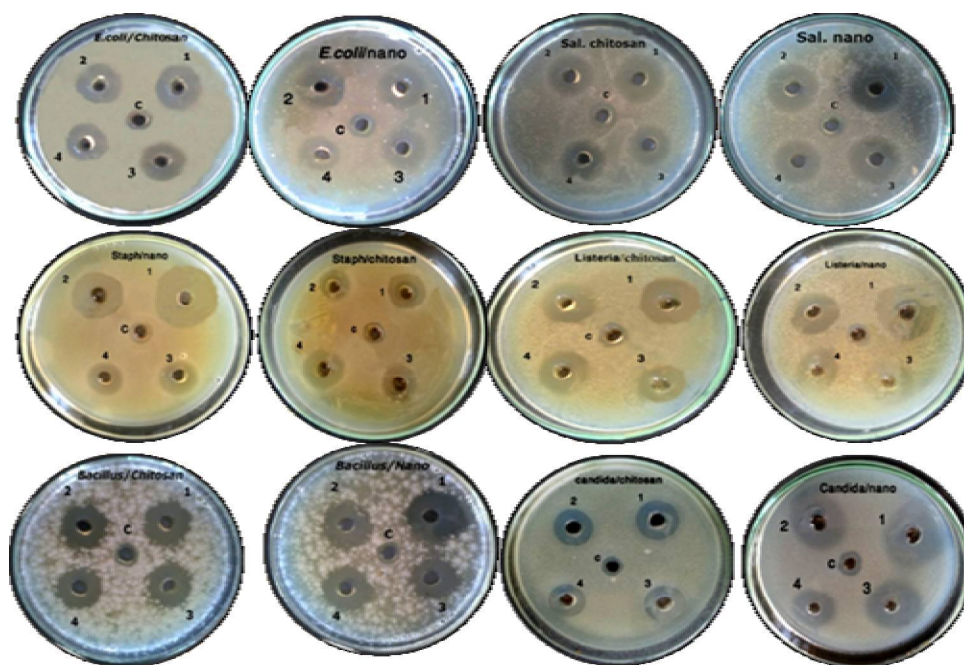


Fig. 6. Inhibition zone produced by chitosan and nano-chitosan by using agar well diffusion.

The fungistatic index of nano-chitosan is shown in (Table 4). Data suggested that nano-chitosan particle might be to diffuse into fungal cells, disrupting the synthesis of DNA as well as RNA, which larger particles of chitosan are located extracellularly. The high resistance ability of *A. niger* could be due to the presence of chitin in its cell wall [26].

Table 4. Fungistatic index of nano-chitosan on *P. roqueforti*.

Treatments	Nano-chitosan	
	Concentration g/L	Fungistatic index (%)
Control	0	0.0
1	1.8	38.9
2	2.4	65.0
3	3.0	91.1
4	4.5	100

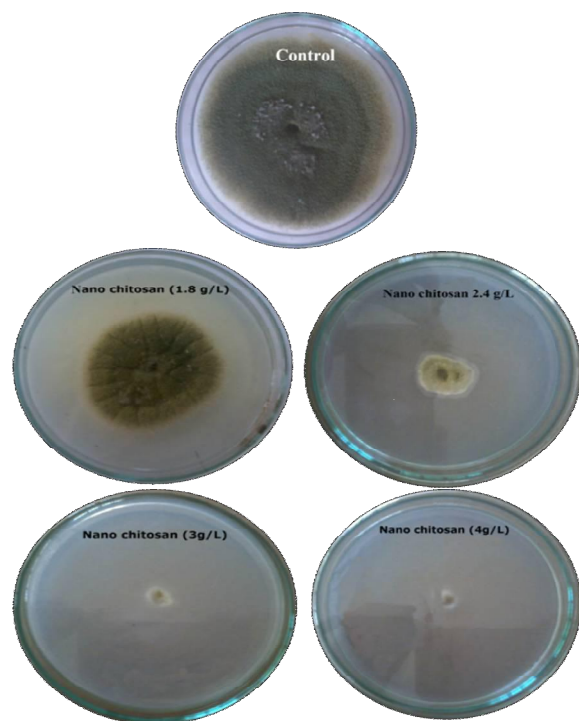


Fig. 7. Antifungal activity of nano-chitosan against *P. roqueforti*.

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