Current Science International
Volume: 13 | Issue: 03| July – Sept.| 2024

EISSN:2706-7920 ISSN: 2077-4435
DOI: 10.36632/csi/2024.13.3.33
Journal homepage: www.curresweb.com
Pages: 382-398

Competitive Evaluation on Antioxidant and Antimicrobial Properties for Three Different Molecular Weights of Commercial Chitosan Biopolymer

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Received: 20 July 2024 **Accepted:** 25 August 2024 **Published:** 15 Sept. 2024

ABSTRACT

Chitosan is a nontoxic copolymer that is a plentiful natural biopolymer derived from the exoskeletons of crustaceans and arthropods. Three distinct macromolecular weights of commercial chitosan i.e high, medium, and low were screened for their significant properties as antioxidant and antimicrobial. Because chitosan can protect important cell macromolecules, reduce free radical generation, and prevent oxidative stress, it is likely to have a wide range of medical and physiological uses. The study showed that chitosan can scavenge a wide range of free radicals, including DPPH, $ABTS^{\cdot+}$, hydrogen peroxide, and free radical, Fe^{3+} reduction. When compared to the other molecular weights of chitosan, *in-vitro* antioxidant tests revealed that low molecular weight chitosan was the best antioxidant molecular weight The following was the order of the comparative antioxidant properties (IC_{50}) of various molecular weights of chitosan: ABST< H_2O_2 <FRPA < DPPH. Chitosan (LMW, MMW, and HMW) was tested in vitro against a panel of G+ve and G-ve bacterial pathogens and fungi. The results showed that chitosan exhibited broad and prolonged antibacterial action against G+ve and G-ve bacteria, fungi, and yeast, based on the concentration (400 µg/ml). Additionally, MIC tests revealed moderate antimicrobial activity against *Salmonella typhi* and *Escherichia coli* and strong antimicrobial activity against Gram positive bacteria (*Staph aureus* and *Bacillus subtilis*); MIC values against *Fusarium solani, F. oxysporium,* and *A. niger* ranged from 100 to 150 µg/ml.

Keywords: Commercial chitosan- antioxidant- antimicrobial- Free radicals- IC_{50} (half-maximal inhibitory concentration)- bacteria- fungi- MIC (minimum inhibitory concentration).

1. Introduction

Chitosan is a naturally occurring polymer that is biodegradable, non-antigenic, non-toxic, and biocompatible. It is generated from chitin and has various health benefits, including strong antioxidant and antibacterial properties (Feng *et al*., 2008 and Muxika *et al*., 2017 and Sarfraz *et al*., 2024).

Commercial chitosan is made by partially deacetylating marine chitin that comes from shrimp, lobster, and crab shells (Fig. 1). Due to its high acetyl concentration and lack of free amine groups, chitin has a low water solubility and a low inclination to react. Because of these drawbacks with chitin, chitosan is a better polymer because of its hydrophilic nature and free amine groups, which provide it greater solubility and reactivity and its potential incorporation into gels or nanoparticles (Negm *et al.,* 2020). Because chitosan donates a lot of OH and NH_2 groups as H atoms, it has garnered a lot of interest in its development as a natural antioxidant and antimicrobial (Guarnieri *et al*., 2022).

While chitin and chitosan are examples of extremely basic polysaccharides, the majority of naturally occurring polysaccharides, such as cellulose, dextran, pectin, alginic agar, agarose, and carragenans, are neutral or acidic in nature. Chitin and chitosan can form films, chelate metal ions,

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and have optical structural characteristics because of their special qualities (Hirano,1999 and Kulawik, 2023). In addition to being used in agriculture, water treatment, tissue engineering, and the pharmaceutical, medical, cosmetic, and food sectors, chitosan and its derivatives' physical and chemical characteristics also support their usage in these other fields (Sahariah, 2017).

Chitosan's molecular weight and degree of N-deacetylation define its physicochemical properties. The N-deacetylation of chitosan can be defined in two ways. either as the degree of deacetylation (DDA) or acetylation (DA). The more widely used term, the DA, represents the ratio of N-acetyl glucosamine monomers (Fig.1) to the total number of units of the polymer. It is an essential element since chitosan's superior properties over chitin are a result of N-deacetylation (Negm *et al.,* 2020), also due to primary amine protonation makes chitosan soluble in aqueous acidic media, whereas chitin's abundance of acetylated residues keeps the polymer from dissolving in such conditions (Kim, 2018).

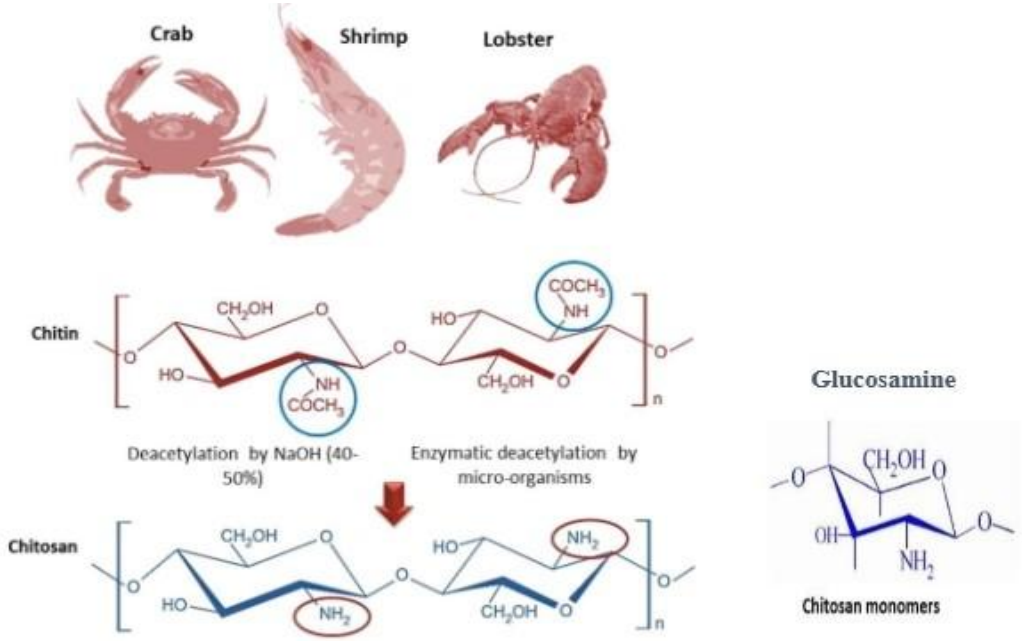

Fig. 1: Commercial sources and chemical structure of chitin, chitosan and chitosan monomer (glucosamine).

There are up to 17,000 citations on this topic in the Scopus database. The significant number of citations indicates a specific concern regarding the chemistry and usage of chitosan (Friedman and Juneja 2010). One type of fine biomaterial is chitosan. Numerous studies have examined the antimicrobial qualities of chitosan and its derivatives (Shahidi *et al.,* 1999 and Rabea *et al*., 2003). Studies have been done on the antioxidant qualities of chitosan (Xing *et al.*, 2005).

Thus, the goal of this investigation was to assess the antioxidant and antimicrobial (bacteria, yeast and fungi) activities of three distinct molecular weights of commercial chitosan LMW, MMW, and HMW.

2. Materials and Methods

2.1. Materials.

2.1.1. Chitosan

Three different molecular weights of chitosan (HMW, MMW and LMW) average of 600, 300 and 120 kDa respectively, and with a degree of deacetylation in the range 70–95%, were obtained from Sigma-Aldrich company.

2.1.2. Tested microorganisms

Five bacterial strains, three fungal strains, and two yeast strains were among the harmful microorganisms that were evaluated. They came from the American Type Culture Collection (ATCC, Rockville, USA) and were reference strain.

Two G-ve bacterial strains, *E. coli* ATCC25922, *Salmonella typhi* ATCC27853, and three Gram +ve bacterial strains, *Bacillus subtillus* ATCC6633, *Staphylococcus aureus* ATCC25922, and *lactobacillus* ATCC4356. Three pathogenic fungi, *Aspergillus niger* ATCC16888, *F. oxysporium* ATCC62705, and *Fusarium solani* ATCC3631, are present together with two yeast strains, *Candida albicans* ATCC9002 and *Candida tropicalis* ATCC750. Fungal isolates were collected from the culture collection of the Department of Chemistry of Natural and Microbial Products, National Research Center, Cairo, Egypt, while strains of bacteria and yeast were obtained from the American type culture collection. In the National Research Center Laboratory, where the antimicrobial tests were conducted, they were kept at 4°C on agar slants. The strains were cultivated for 24 hrs on new, suitable agar plates before being subjected to any antibacterial tests.

2.2. Methods

2.2.1. Preparation of soluble chitosan.

2.5% (w/v) chitosans were dissolved in 1.0% (v/v) acetic acid solution to prepare chitosan solutions. Using 10M NaOH, the pH was brought down to 5.8, which is the most suitable pH for chitosan solubilization without having any antimicrobial effects. The solutions were autoclaved for 15 mins after being stirred all night (Ying and Zhu 2003).

2.3. Evaluation of antioxidant scavenging radicle capacity.

2.3.1. ABTS radical cation decolourization assay

The ABTS procedure was used to determine the antioxidant activity (Chen *et al*., 2018). The working solution for the ABTS assay was prepared by mixing an equal amount of 2.45 mM potassium persulfate and 7.4 mM ABTS. This solution was then incubated for 12 to 16 hrs in the dark to form an active ABTS radical, which was then reacted with the different Mws of chitosan to measure the antioxidant activity. After mixing 50µl of the sample with 1.9 milliliters of ABTS solution, it was left to dark-incubate for six mins. At 734 nm, absorbance was measured following incubation. The efficiency of capturing free radicals was estimated as follow.

$$ABTS^{\cdot+} \text{ scavenging activity (\%)} = \frac{A0 - A1}{A0} \times 100$$

where A0 is the absorbance of control, A1 is the absorbance of sample.

2.3.2. DPPH scavenging method

The usual technique was used to assess the DPPH scavenging activity of chitosans (Nakkala *et al*., 2016). The ethanolic 0.1 mM DPPH solution was prepared. One milliliter of soluble chitosan at various molecular weight and an equal volume of ethanol were added to three milliliters of DPPH stock solution. After 30 mins of incubation, absorbance at 517 nm was measured. The following equation was used to represent the DPPH scavenging capacity.

$$\%\ inhibition = \frac{blank - sample}{blank} \times 100$$

2.3.3 H_2O_2 scavenging method

Using H_2O_2, the antioxidant activity of chitosans was assessed. 0.4 ml of soluble chitosan, 0.6 ml of 40 mM H_2O_2, and 3.4 ml of 0.1 M phosphate buffer were added. This combination was allowed to sit at room temperature for ten mins. Following incubation, absorbance at λ_{max} 230 nm was recorded in comparison to a blank solution. The standard was ascorbic acid (Mohan and Kakkar, 2020). Using the equation, the percentage of H_2O_2 scavenging was determined.

% Scavenged [H_2O_2] = [(AC – AS)/AC] x 100 Where AC is the absorbance of the control and AS is the absorbance in the presence of chitosan.

2.3.4. Assay for reducing power (FTPR)

Chitosan has the power of reducing. The ability of chitosan to convert FeC_{l3} to $FeCl_2$ was tested using the procedure described by Debnath *et al*., 2021. One milliliter of each concentration of chitosan (ranging from 1 to 5 mg/ml) was added, along with 2.5 milliliters of 0.2 M phosphate buffer and 2.5 milliliters of 1% w/v potassium ferricyanide. These were prepared by serial dilution. These mixtures were incubated at 50ºC for 20 mins. After cooling, 2.5 ml of 10% trichloroacetic acid (TCA) was added to each previous mixture, and the mixtures were centrifuged. The top layer was then removed, yielding 2.5 ml, which was then mixed with 0.5 ml of 0.1% ferric chloride and 2.5 ml of distilled water. The absorbance at 700 nm was measured after a 30-minute reaction at room temperature. Higher absorption suggested stronger reduction power. Distilled water served as the experiment's positive control. Each determination was performed three times.

2.4. Evaluation natural commercial chitosan biopolymer (LMW, MMW and HMW) efficiency by calculation of their IC_{50}.

A linear regression of the concentration–response curve of the percentage of inhibition versus the antioxidant concentrations was used to get the IC_{50} value for each molecular weight (Martinez *et al*., 2020). The mean and standard deviation of the data are displayed. With Excel software, the linear regression analyses were performed. Consequently, the goal of determining an IC_{50} value that is unaffected by radical concentrations is to accurately assess natural chitosan's antioxidant capacity.

2.5. Antimicrobial susceptibility testing

2.5.1. Determination of antimicrobial activity by agar well diffusion method

Using the agar well diffusion method, the antimicrobial activity of each strain of bacteria, fungus, and yeast was evaluated (Ramasamy *et al*., 2011; No *et al*., 2002 and Annaian *et al*., 2016). For the antibacterial test, nutrient agar medium was prepared and autoclaved for 15 mins. In the sterile media, individual species of bacteria, fungus, and yeast were injected, and they were then incubated for 24 hrs at 37°C. Wells with a diameter of 5 mm were created in the infected plates. The varied concentrations of 3 distinct molecular weights chitosan (Stock—400µg/ml and 200µg/ml) from this stock solution 50µl were placed in the appropriate wells. The plates were incubated for 24 hrs at 37 °C while upright, and the inhibition zones were noted.

2.5.2. Determination of the minimum inhibitory concentration (MIC)

The lowest chitosan concentrations at which micro-organisms are incapable of growing are known as MICs. The conventional approach (Ramasamy *et al*., 2013 and Ruparelia *et al*., 2008) was used to calculate the minimum inhibitory concentration (MIC) of chitosan with high, medium, and low molecular weights. A stock solution of 10 mg/ml was made for this procedure. In order to get different concentrations ranging from 400 to 100 µg/ml, this was serially diluted. 50µl of every dilution with varying amounts was added to the wells. Following that, the petri dish and control were all incubated for 48 hrs at 37°C. The zone of inhibition was measured and the experiment was run in duplicate.

2.5.3. Studying the morphology of microorganisms by using light microscope.

Morphological changes in microorganisms treated with chitosan were observed by using modern light microscope. Cell scientists still rely heavily on the light microscope as a fundamental tool because of advancements in technology that make it possible to see ever-more-detailed cell structure. Modern light microscopes can enlarge objects up to around a thousand times. Since the majority of cells have a diameter of 1 to 100 µm, light microscopy can be used to see them as well as some of the bigger subcellular organelles, like mitochondria, chloroplasts, and nuclei (Rost and Oldfield, 2000).

3. Results and Discussion

DD (deacetylation degree) and MW (molecular weight) are two crucial chitosan properties. Pu *et al.* (2019) reported that the modifications of chitosan DD and MW not only decide the amount of -NH_2, -CH_2OH and -CHO groups in chitosan, but also more or less effect the hydrogen bonds, electrostatic interaction and steric interaction, which have been demonstrated to be associated with the reducing, antimicrobial and stabilizing activities of chitosan (Fig. 2).

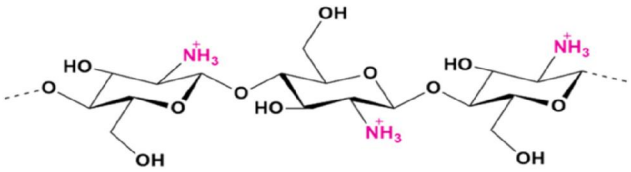

Fig. 2: Structure of chitosan with its active amino groups, after their protonation in acid conditions responsible for the antioxidant, antimicrobial and stabilizing activities.

3.1 Antioxidant activity

Given that free radicals have a harmful function in both biological systems and food, it is well known that scavenging free radicals is an extremely important activity (Bursal and Köksal, 2011).

The antioxidant activity of the three distinct molecular weights was assessed using the scavenging activity of ABTS, DPPH, H_2O_2, and FRAP.

As seen in Fig. 3, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging capacity assays are based on electron and hydrogen atom transfer, while FRAP (ferric antioxidant power) and hydrogen peroxide scavenging activity assays are based on an electron transfer reaction.

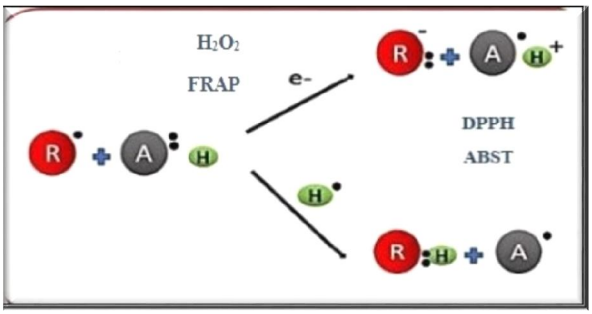

Fig. 3: Different techniques for antioxidant scavenging activity, where R represent free radicle and A represent chitosan.

3.1.1. ABST (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolourization assay

The two primary types of free radicals found in the body are physiological and non-physiological radicals. As a non-physiological free radical, $ABTS^{\cdot+}$ is created by chemical pollution, tobacco smoke, and pesticide residues. These substances have the ability to quickly start a chain reaction of free radicals and cause significant harm to the body's biological components, including lipids, DNA, and protein. The ABTS test was used to assess total antioxidant capacity. An effective method for assessing the antioxidant activity of chain-breaking and hydrogen-donating antioxidants is to measure the free radical cation ABTS, which is produced when potassium persulfate oxidizes ABTS (Chen, *et al*., 2018).

Three various molecular weight chitosans' $ABTS^{\cdot+}$ scavenging activities are displayed in (Fig. 4), which also demonstrates how chitosans' $ABTS^{\cdot+}$ scavenging activity increased when chitosan concentration rose, independent of chitosan's DD or molecular weight. In contrast to MMC and HMC, the lowest MW demonstrated the strongest $ABST^{\cdot+}$ scavenging activity, reaching 90±2.0% at 5 mg/ml. According to Yen *et al*. (2008), crab chitosan exhibited high antioxidant activities of 79.9-85.2% at 10 mg/ml and moderate to high antioxidant activities of 58.3-70.2% at 1 mg/ml.

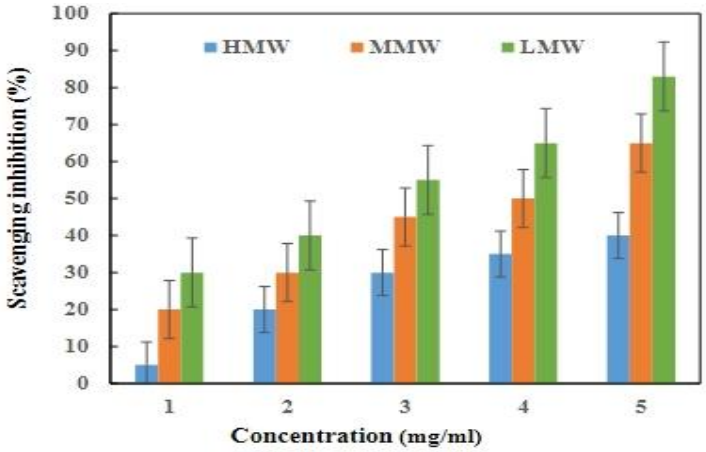

Fig. 4: Estimation of antioxidant potentiality of (LMW, MMW and HMW) chitosan by ABST.+ assay with different concentrations.

3.1.2. Scavenging ability on DPPH radicals (2,2-diphenyl-1-picryl-hydrazyl-hydrate).

Scavenging of hydrogen radicals is one of the key functions of antioxidants. Hydrogen free radicals and a distinctive absorbance at 517 nm are features of DPPH. The study employed DPPH to a scertain the chitosan's capacity for proton scavenging. Fig. 5, displays the DPPH scavenging activity of three distinct chitosan molecular weights with different MWs and concentrations. It is evident that the DPPH scavenging activity rose as chitosan DD and concentration increased and as chitosan MW decreased. This behavior is comparable to the scavenging activity of H_2O_2 and $ABTS^{\cdot+}$ and suggests that the active –OH and $–NH_2$ in the chitosan chains are crucial to the DPPH scavenging process. In solution, chitosan exhibits a compact structure with stronger intra- and intermolecular hydrogen bonding than low molecular weight chitosan. The hydroxyl and amino group activities are weakened by the substantial influence of hydrogen bonding (Li *et al*., 2014). According to Yen *et al*. (2008), at 10 mg/ml, fungal chitosan scavenged DPPH radicals by 28.4-53.5%. It is clear that chitosan derived from crab shells and shiitake stems was ineffective as a DPPH radical scavenger. According to Yen *et al.* (2007) at 10 mg/ml, crab chitosan's DPPH radical-scavenging ability was 28.4%; in contrast, other crab chitosan's scavenging ability ranged from 46.4-52.3%. According to this study, at a concentration of 5 mg/ml, LMW chitosan can exhibit 70±1.5% antioxidant activity.

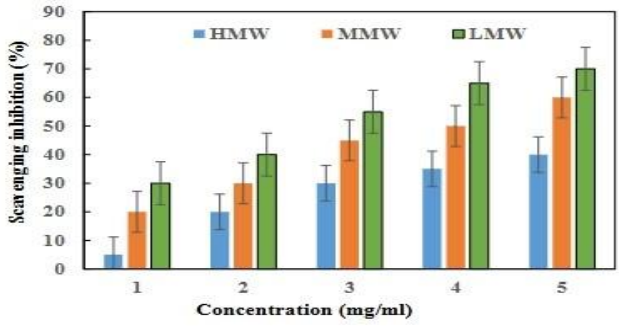

Fig. 5: Estimation of antioxidant potentiality of (LMW, MMW and HMW) chitosan by DPPH assay with different concentrations.

3.1.3. H_2O_2 scavenging method.

Despite being a relatively mild oxidant, hydrogen peroxide breaks down into more potent reactive oxidative species, like hydroxyl and single oxygen radicals, which cause lipids to begin to peroxide (Gupta *et al*., 2022). Within this investigation, chitosan demonstrated a concentration-dependent efficacy in scavenging hydrogen peroxide. The hydrogen peroxide scavenging activity of three

distinct molecular weights chitosans at doses ranging from 1000 to 5000 µg/ml is contrasted in Fig. 6. The activity of chitosans to scavenge hydrogen peroxide increased with increasing concentrations. The maximum hydrogen peroxide scavenging activity was demonstrated by LMW chitosan (80±1.7 % at 5 mg/ml), followed by MMW chitosan (65%) and HMW chitosan, which had inhibited antioxidant activity (50%).

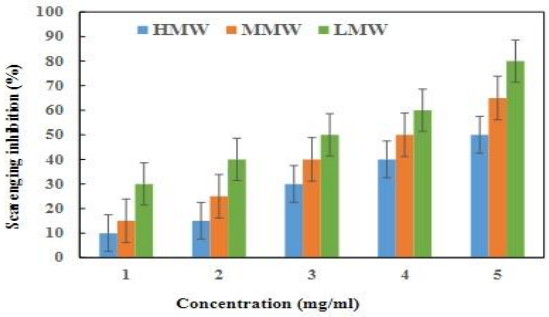

Fig. 6: Estimation of antioxidant potentiality of (LMW, MMW and HMW) chitosan by H_2O_2 assay with different concentrations.

3.1.4. Ferric reducing antioxidant power assay (FRAP)

There has been evidence of a direct relationship between reducing capacity and antioxidant (Hoang *et al*., 2023). The FARP assay was used to evaluate antioxidants' ability to transfer electrons to free radicals by converting ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Pu *et al*., 2019). The FRAP of the three distinct molecular weights of chitosan at various doses is displayed in Fig. 7. As the concentration of chitosan rose, so did the FRAP scavenging activity. In contrast, the chitosan's inhibitory impact ranged from 30±1.6% to 72.8± 2 % at concentrations between 1 and 5 mg/ml for LMW chitosan. When chitosan concentration rose and chitosan MW decreased, FRAP antioxidant capacity of chitosan increased.

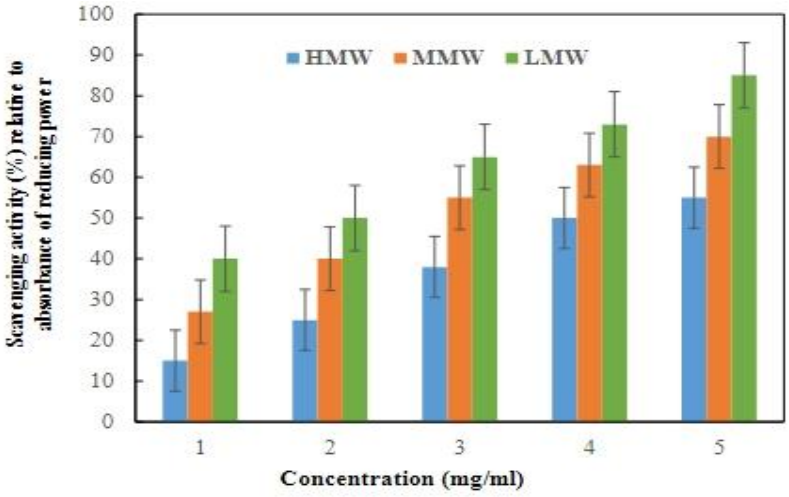

Fig. 6: Estimation of antioxidant potentiality of (LMW, MMW and HMW) chitosan by ferric reducing antioxidant power (FRAP) assay with different concentrations of chitosan.

3.2. IC_{50} of low, moderate and height molecular weights chitosan.

A biomaterial's half-maximal inhibitory concentration (IC50) is the most commonly utilized and useful indicator of its efficacy. It provides a gauge of an antagonist biocompound's potency in biochemical research by showing how much bioactive compound is required to halve a biological process.

The simplest estimate of IC50 is to plot x-y and fit the data with a straight line (linear regression). IC50 value is then estimated using the fitted line, i.e., Y = a * X + b, IC50 = (0.5 - b)/a.
Where, Y: % inhibition; X: Concentration; a: Slope; b: Intercept (the intersection of the lines on the Y axis).

For the four antioxidant scavenging techniques activities, the IC50 of low molecular weight chitosan exhibits the lowest value (Fig. 8); the regression line equations are not displayed. Our findings closely resemble those of Purgiyanit *et al*., 2022. They found that the inhibitory concentrations (IC_{50}) of commercial chitosan on DPPH radicals were 5.2 and 4.25 mg/ml, respectively. Antioxidant activity and IC50 value have an adverse relationship (Prabu and Natarajan 2012).

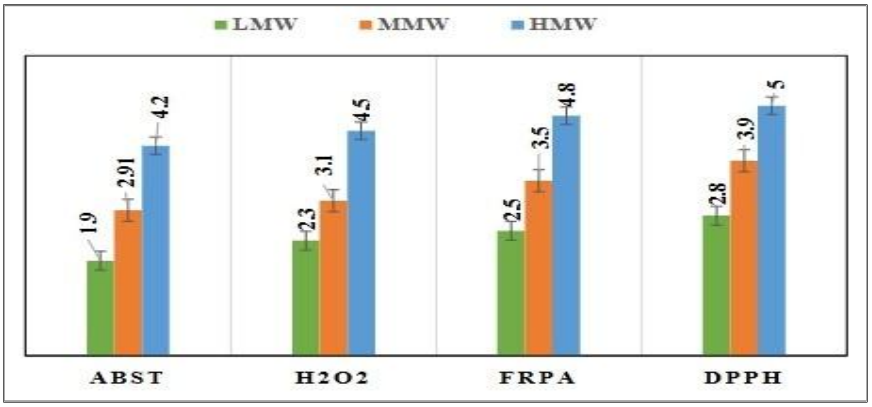

Fig. 8: Comparative antioxidant study of low, moderate and high molecular weights chitosan on the basis of IC_{50} value (mg/ml) by four scavenging assays (ABTS, H_2O_2, FRPA and DPPH).

3.3. Antimicrobial activity

According to research by Rabea *et al*. (2003), chitosan with high, medium, and low molecular weight at concentrations of 400 µg/ml and 200 µg/ml demonstrated good antimicrobial activities against almost all tested pathogenic bacterial, yeast, and fungal strains. Chitosan has a significant antimicrobial influence on a wide range of microorganisms, including bacteria, fungi, and yeast.

3.3.1. Antibacterial Activity

With three distinct molecular weights (HMW, MMW, and LMW), chitosan demonstrated a broad spectrum of bioactivity in vitro against Gram-positive tested bacterial isolates S. aureus ATCC25922, *Lactobacillus* ATCC4356, and *Bacillus subtillus* ATCC6633, as well as Gram-negative isolates *E. colli* ATCC25922 and *Salmonella typhi* ATCC27853. Gerasimenko *et al*. (2004) reported comparable findings, showing that chitosans with varying molecular weights prevent the growth of bacteria, both Gram positive and negative. Table (1), Fig. (1) and chart (1) showed that LMW chitosan (400 µg/ml) the strongest antibacterial properties against G+ve bacteria, exhibiting an inhibition zone of 22.6±0.43 mm against *lactobacillus.* These outcomes concurred with those confirmed by (You- Jin *et al*., 2001), who stated that the molecular weight and degree of acetylation of chitosan have been shown to be the primary determinants of both the antibacterial activity and growth inhibitory impact of chitosan. Although LMW Chitosan also produced an inhibition zone of 20.5±0.30 mm for *S. aureus* ATCC25922, these findings were not as good as those of Ahmed *et al*. (2017), who reported an inhibition zone of 33.4 ± 0.53 mm for *S. aureus*.

Table 1: Antimicrobial activities of HMW, MMW and LMW chitosan with concentration 400 µg/ml

	Inhibition zone in (mm)		
Microbial strain	**High molecular weight**	**Medium molecular weight**	**low molecular weight**
1- *Bacillus subtillus* ATCC6633	13.0±0.0430	16.0±0.70	19.0±0.30
2- *S. aureus* ATCC25922	19.0±0.92	17.0±0.20	20.5±0.30
3- *lactobacillus* ATCC4356	19.5±0.82	15.0±0.83	22.6±0.43
4- *E. colli* ATCC25922	22.0±0.50	18.3±0.49	18.5±0.85
5- *Salmonella typhi* ATCC27853	17.5±0.70	16.0±0.20	16.7±0.60
6- *Candida tropicalis* ATCC750	13.8±0.06	15.0±0.50	18.5±0.04
7- *Candida albicans* ATCC9002	15.0±0.60	14.0±0.30	18.3±0. 03
8- *Fusarium solani* ATCC3631	13.5±0.01	17.0±0.40	17.4±0.04
9- *F. oxysporium* ATCC62705	18.3±0.40	19.5±0.30	23.5±0.09
10- *A. niger* ATCC16888	15.5±0.13	16.8±0.02	17.8±0.54

Values are given as mean ± SD of three experiments.

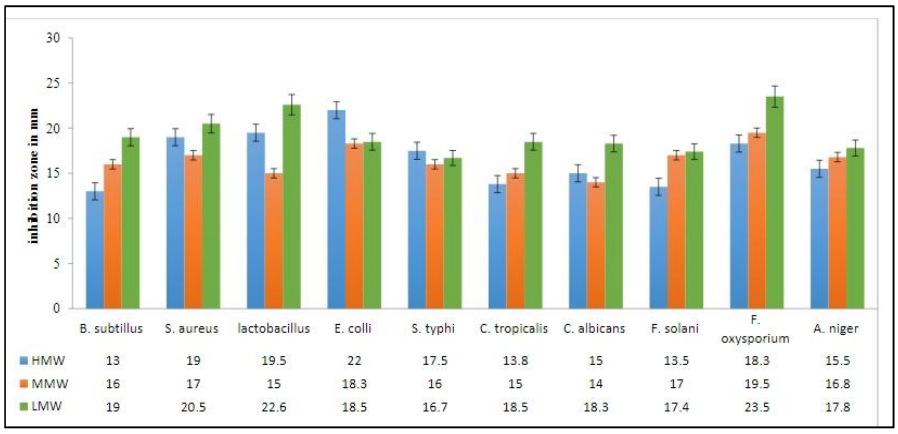

Chart 1: antimicrobial activities of HMW, MMW and LMW chitosan at concentration (400 µg/ml).

Additionally, Salmabi and Seema, 2014 revealed that the ATCC strain of *S. aureus* had the maximum antibacterial activity (30 mm), which was greater than our results. The inhibition zone diameter for *S. aureus* was also reported to be in the range of 14–30 mm. Additionally, the coagulase negative *S. aureus* inhibition zone diameter ranged from 12 to 22 mm, which is smaller than our inhibition zone diameter. These variations in the results show that the antibacterial activities of LMW chitosan depend on strains of the same species in addition to the bacterial strain type and concentration. Conversely, LMW chitosan had reduced antibacterial efficacy against G-ve bacteria, exhibiting inhibition zones of 18.5±0.85 mm and 16.7±0.60 mm against *Salmonella typhi* and *E. coli*, respectively.

With an inhibitory zone of 22.0±0.50 mm against E. colli, HMW chitosan exhibited greater efficacy against G-ve bacteria. Additionally, according to studies (You *et al*. 2001), HMW chitosan exhibited more potent anti-G-ve bacteria activities compared to LMW and MMW chitosan (Darmadji and Izumimot, 1994). The reported growth inhibition values for *lactobacillus*, *S. aureus*, and *Bacillus subtillus* were 19.5±0.82 mm, 19.0±0.92 mm, and 13.0±0.0430 mm, respectively, when compared to G+ve bacteria. Anaian *et al*. (2016) found similar results, noting that the largest inhibitory clear zone against *P. vulgaris* using HMW chitosan was 17 mm, whereas the highest inhibition zone against *S. aureus* was 16 mm. The lowest clean zone, was 13 mm. The effect of HMW against G-ve bacteria showed the lowest activity, with a 12 mm inhibitory zone against *K. pneumonia* and *E. coli* and a 7 mm zone against *Salmonella sp*.

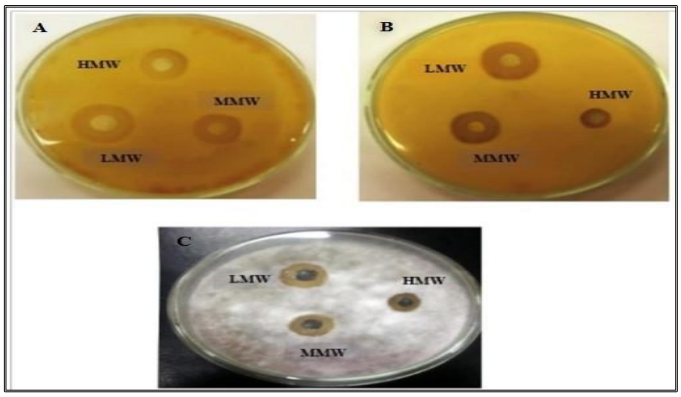

Fig. 9: Antimicrobial activities of HMW, MMW and LMW chitosan with concentration 400 µg/ml against (A) *lactobacillu*s (B) *Candida albicans and* (C) *F. solani.*

The results showed that LMW chitosan had the strongest antibacterial activity against G+ve bacteria lactobacillus, with an inhibitory zone diameter of 12.2±0.03mm. A lower concentration of 200 µ/ml (HMW, MMW, and LMW) chitosan was utilized, and is depicted in Table (2), and Chart (2). MMW chitosan demonstrated the greatest activity against G-ve bacteria, exhibiting an inhibition zone of 9.4±0.13 mm against *Salmonella typhi*. This activity differed somewhat from that of LMW chitosan, which displayed a clear zone of 9.2±0.42 mm. According to several scientific publications, low molecular weight chitosan has a greater antibacterial effect than moderate molecular weight, and for certain microbial species, high molecular weight chitosan has the least inhibition zone; Gerasimenko *et al*. (2004) and Elsherif *et al*. (2024) also noted that LMW's antibacterial activity on *S. aureus* was more successful than HMW in preventing bacterial growth, which is consistent with our findings.

According to Mohamed *et al*. (2006), even at high doses up to 2400 mg mL^{-1}, MW chitosan (MW= 3.60×10^5 Da) demonstrated very little or no antibacterial action on the investigated bacterial strains. This discovery aligns with the findings published by No *et al*. (2002). They found that the antibacterial effects of varying chitosan molecular weight varied depending on the type of bacteria and the gram-negative bacteria (*P. fluorescens*, *Escherichia coli, Salmonella typhimurium*, and *Vibrio parahaemolyticus*) and gram-positive strains (*B. megaterium*, *B. cereus, Lactobacillus plantarum*, and *S. aureus*).

Table 2: Antimicrobial activities of HMW, MMW and LMW chitosan with concentration 200 µg/ml

	Inhibition zone in (mm)		
Microbial strain	**High molecular weight**	**Medium molecular weight**	**Low molecular weight**
1- *Bacillus subtillus* ATCC6633	8.3±0.031	8.9±0.05	10.8±0.04
2- *S. aureus* ATCC25922	9.7±0.45	7.6±0.23	11.5±0.30
3- *lactobacillu*s ATCC4356	8.4±0.032	7.5±0.800	12.2±0.03
4- *E. colli* ATCC25922	7.2±0.23	8.6±0.049	8.5±0.05
5- *Salmonella typhi* ATCC27853	7.5±0.021	9.4±0.13	9.2±0.42
6- *Candida tropicalis* ATCC750	6.8±0.021	7.2±0.032	8.5±0.65
7- *Candida albicans* ATCC9002	6.0±0.26	6.7±0.17	9.3±0.01
8- *Fusarium solani* ATCC3631	6.5±0.076	6.9±0.048	7.4±0.09
9- *F. oxysporium* ATCC62705	6.3±0.040	8.5±0.0530	8.6±0.07
10- *A. niger* ATCC16888	7.5±0.56	8.3±0.084	8.8±0.05

Values are given as mean ± SD of three experiments.

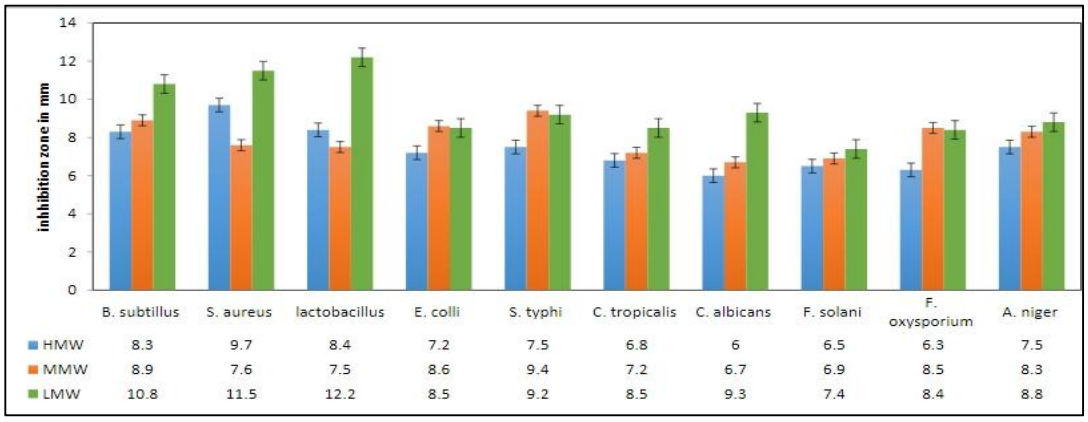

Chart 2: Antimicrobial activities of HMW, MMW and LMW chitosan at concentration of 200 µg/ml.

They also mentioned that chitosan with molecular weight of 7.46×10^5 Da worked better against *E. Coli* and *P. fluorescens* than chitosan with an MMW of 4.70×10^5 Da did against *V. parahaemolyticus* and *S. typhimurium*. Compared to LMW chitosan of MW = 2.8×10^4 Da, which exhibits strong antibacterial activity against *S. typhimurium*, chitosan of HMW = 11.06×10^5 and 2.24×10^5 Da exhibits little to no antibacterial activity. Goy *et al*. (2009) noted that virtually little antibacterial activity was displayed by chitosan with LMW ranging from 10,000 to 100,000 Da.

3.3.2. Antifungal and anticandida activities of chitosan.

Results of an analysis and estimation of the impact of chitosan molecular weight on antifungal activities are summarized in Table 1. Data demonstrated that the studied microorganism and its molecular weight both influence the chitosan's activity. Furthermore, we discovered that the antifungal activity of chitosan varied greatly even within the same species. From the results, our conclusion shown that chitosan's antifungal efficacy with (HMW, MMW, and LMW) at 400 µg/ml, is mostly strain-dependent. As seen in chart (1) and illustrated in table (1), the tested three molecular weights of chitosan exhibited very significant antifungal activity against all of the tested fungal species. The best efficacy was achieved by LMW chitosan, with inhibition zones of 18.5±0.04 mm and 18.3±0.03 mm against *Candida tropicalis* ATCC750 and *Candida albicans* ATCC9002, respectively, when the three different molecular weights of chitosan were tested against yeast. Conversely, HMW chitosan's minimal inhibitory zone against *Candida tropicalis* was 13.8±0.06mm. As opposed to *Candida albicans* ATCC9002, HMW exhibited superior activity, with an inhibitory zone diameter of 15.0±0.60 mm. In contrast, MMW chitosan inhibits the growth of *Candida albicans* ATCC9002 and *Candida tropicalis* ATCC750, measuring 15.0±0.50 mm and 14.0±0.30 mm, respectively.

The maximum inhibition zones measured by LMW chitosan against *F. oxysporium* and *A. niger* were 23.5±0.09 mm and 17.8±0.54 mm, respectively. MMW chitosan had the highest level of efficacy against *Fusarium solani* and *F. oxysporium,* with inhibition zones measuring 19.5±0.30 mm and 17.0±0.40 mm, respectively. While for HMW chitosan the best activity was against *F. oxysporium* then *A. niger* with inhibition zone diameter 18.3±0.40 mm and 15.5±0.13 mm. The minimum antifungal activity was by HMW and MMW chitosan against *Fusarium solani* and *A. niger* with inhibition zone 13.5±0.01 mm and 16.8±0.02 mm respectively.

Our research demonstrated that LMW chitosan demonstrated antifungal activity against *F. oxysporium* with a clear zone diameter of 8.6±0.0530mm, which was superior to that achieved with the effect of MMC chitosan. The best activity was 8.8±0.05 mm against *A. niger,* followed by *F. oxysporium*, and with inhibition zone 8.6±0.07, as shown in Table 2. The lowest activity against the tested fungal strains was observed by HMW chitosan against *F. oxysporium* with inhibition zone diameter 6.3±0.040mm. Additionally, HMW chitosan showed the least action against *Candida albicans* with an inhibition zone diameter of 6.0±0.26 mm, whereas LMW chitosan had the maximum activity against candida strains with a clear zone of 9.3±0.01mm (Fig. 9). MMW chitosan inhibits the growth of *Candida albicans*, with 7.2±0.032 mm inhibition zone.

3.3.3. Chitosan's minimum inhibitory concentrations (MICs) against particular strains of bacteria, yeast, and fungus.

In order to determine the minimum inhibitory concentration (MIC) of chitosan, investigations were conducted and the results are shown in Chart 3. HMW chitosan's minimum inhibitory concentrations (MICs) against *Salmonella typhi,* Lactobacillus, *E. colli*, *Bacillus subtillus*, and *S. aureus* were 120, 120, 120, and 150 µg/ml, respectively. Additionally, the MMW chitosan recorded MICs of 100, 120, 100, <150, and <120 µg/ml. Conversely, the LMW chitosan's MIC values were 100. 100, less than 120. 100, and 120 µg/ml, in that order.

While Liu *et al*. (2006) reported higher MICs of LMW and HMW against *S. aureus* and *E. coli* reaching 5 mg/ml, Kathiresan and Nayak, 2016 observed lower MICs of LMW and HMW against *S. aureus* and E. coli up to 80µg/ml (0.008%). The findings of Annaian *et al*. (2016) were in fairly good agreement with ours; they reported that the minimum inhibitory concentration (MIC) of chitosan against the investigated strains of *Salmonella sp*., *P. aeruginosa, S. aureus*, and *Streptococcus sp*. was 60, 80, 100, and 80 µg/ml, respectively.

HMW chitosan's MIC values against *Candida tropicalis* ATCC750 and *Candida albicans* ATCC9002 yeast were found to be less than 150 and 120 µg/ml, respectively. The MIC values for MMW chitosan were 100 and less than 100 µg/ml, but the MIC values for LMW chitosan were less than 100 and less than 120 µg/ml, (Chart 3).

HMW chitosan was found to have minimum inhibitory concentrations (MICs) of less than 150, 120, and 120 µg/ml against the fungal strains *Fusarium solani* ATCC3631, *F. oxysporium* ATCC62705, and *A. niger* ATCC16888, respectively. Finally, the MIC values of LMW chitosan were reported as 100, 120, and 100 µg/ml, respectively, whereas the MIC values of MMW chitosan were ≥150, 150, and ≥120 µg/ml. Guo *et al*. (2002) found that higher minimum inhibitory concentrations (MIC) for *Fusarium oxysporum* and *Candida albicans*, respectively, at 500 µg/ml and 200 µg/ml.

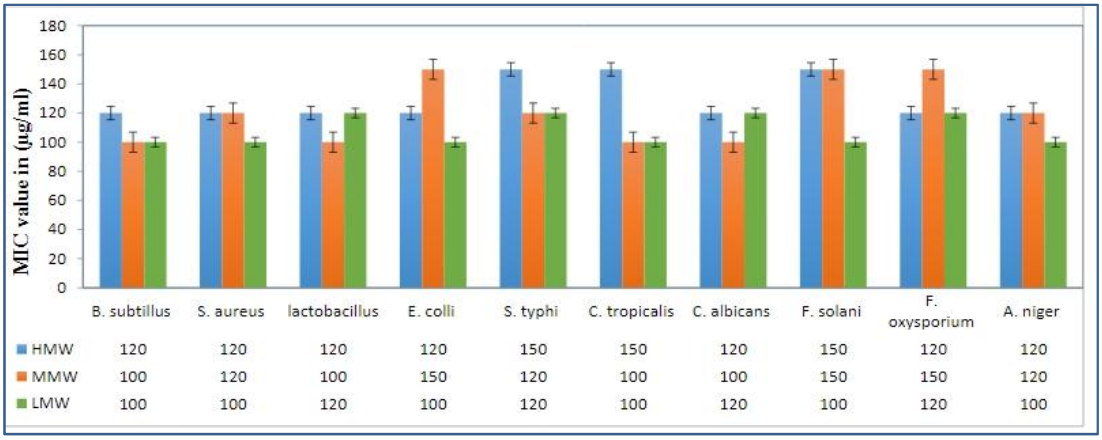

Chart 3: Minimum inhibitory concentration MICs of chitosan against selected bacteria, yeast and fungi strains.

3.4. Microbial cell observation using optical light microscope

As shown in Fig. (10-A), normal *B. subtillus* cells that were prepared for an optical light microscope displayed a smooth, undamaged surface with an ideal rod shape. Furthermore, as illustrated in fig. (10–b), when treated with a minimal concentration of chitosan (MIC), the bacterial cell exhibits a slight shortage and deformation in the form of a rod, along with the formation of endospores to overcome the stress of chitosan. This indicates that the low concentration of chitosan has a bacteriostatic effect on the bacterial cell (Prabu and Natarajan, 2012). Fig. (10-C), showed a shorter, deformed compact cell with thickening in the cell wall and the appearance of dead cells occurred when the chitosan concentration was 200 µg/ml. According to Eaton *et al*. (2001) and Annaian *et al*. (2016), chitosan is a precipitate of water-insoluble macromolecules that form a thick layer around bacterial cells. This layer can block the entry of vital nutrients into the cells, leading to cell death. According to Ming *et al*. (2010), the bacterial cell treated with chitosan exhibited severe

surface deformation, including the production of flagella, fimbriae, and pili protrusion, as shown in our work in Fig. (4-D).

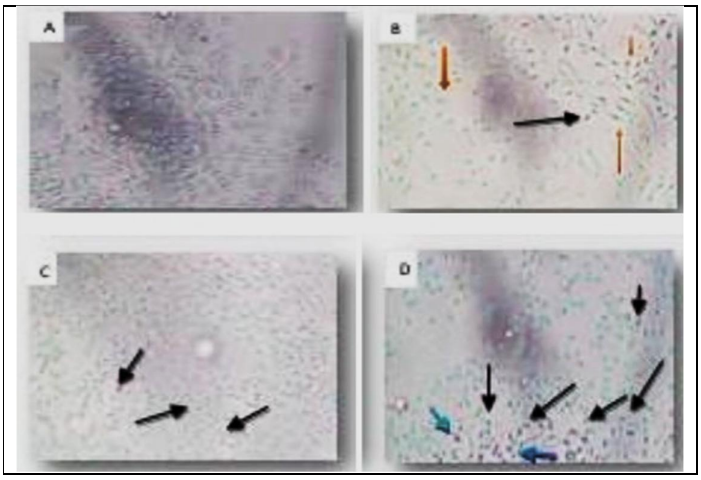

Fig. 10: Micrographs of *Bacillus subtilis*. (A) represent normal cell with uniformly shaped, (B) treated cell with MIC LMW chitosan, as shown by the orange arrow, the bacterial cells remain long, undamaged. At a chitosan concentration of 200 µg/ml, the cells seem shorter and more compact (C). (D) Displays the dead bacterial cells at 400 µg/ml chitosan concentration. The blue arrow indicates a ruptured cell wall and internal organelle leakage, whereas the black arrow indicates dead bacterial cells.

According to Shakeel *et al.* (2014), a positively charged amino group in chitosan interacts with negatively charged bacterial cell membranes to cause the intracellular organelles of the bacterial cell to leak out, as illustrated in Fig. (4-d), which is one of the antibacterial properties of chitosan that has been reported in numerous studies. When the chitosan concentration reached 400 µg/ml, as shown in Fig. 4-D, the bacterial cells died completely. Furthermore, as seen in Fig. (4-d), chitosan can build up on the surface of the bacterium to create a condensed polymer coating. Chitosan-treated bacteria showed deformed cell membranes this may be due to the formation of chitosan vesicular structures and an additional layer of chitosan, causing the appearance of the thickened cell envelope (Helander *et al*., 2001). Consequently, the thickness of the bacterial cell envelope prevents essential nutrients from entering the cell. Abd El-Hack *et al*. (2020) summarized the mode of antibacterial action by chitosan in Fig. 5. They suggested that the mode of action includes electrostatic interactions between the negatively charged microbial cell membranes and the positively charged NH_3^+ sites of chitosan. Intracellular material is released as a result of the contact changing the microbial cell's permeability.

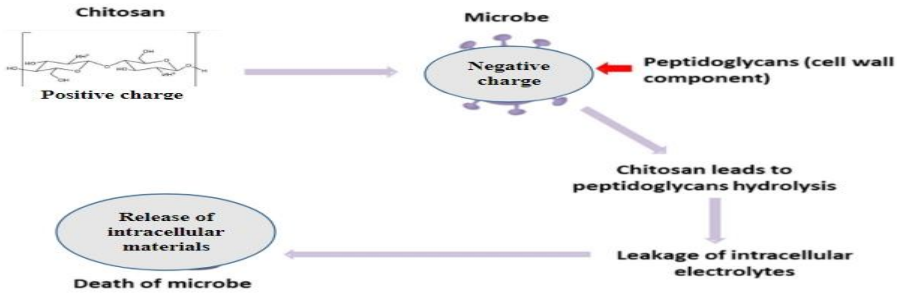

Fig. 11: The proposed antimicrobial mechanism of chitosan

Optical Light and electron microscopic examination was used to determine the morphological differences between normal and chitosan-treated *C. albicans* and C. *tropicalis*. The microscopic examination of normal active cell of candida *albicans* with complete intact uniform mycelia structure as seen in Fig. 6A. On treating with MIC of chitosan the unviable cells appear with thick distorted cell

wall which may be due to the deposition of chitosan molecules on the cell surface leading to the appearance of thick dark cell wall that in turn affect the cell wall permeability as in Fig. 6B leading to the death of the cells (Antonio *et al.,* 2013). On increasing the concentration of chitosan the thickness of the cell wall increase as well as decreasing the no of viable cells, Fig. 6C and 6D, until it reaches to complete cell aggregation in Fig. 6F, (Sarfraz *et al.,* 2024). On the other hand, the mycelia appear deformed with cutting in the cytoplasm as in Fig. 6E.

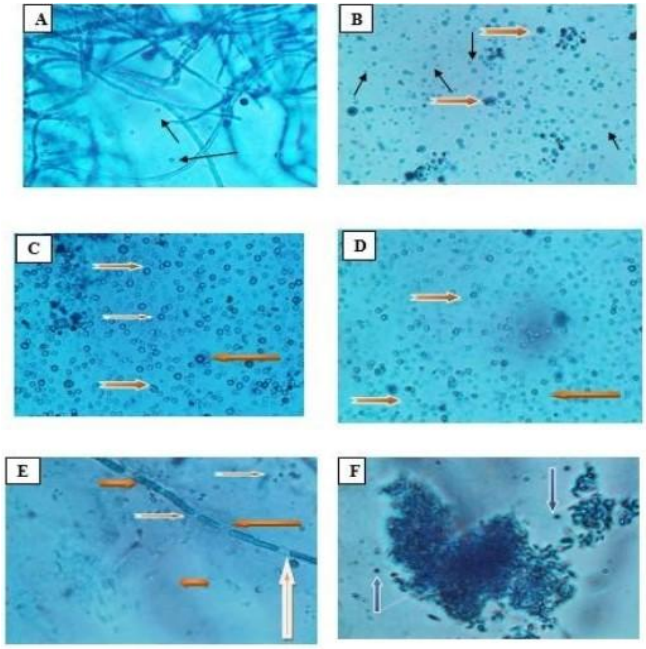

Fig. 12: *Candida albicans* micrographs, brown arrows show the incision in the hyphal cytoplasm and indicate metabolically-inactive or non-viable cells, while black arrows show control mycelia and metabolically-active cells (A). The live cells treated with chitosan exhibit a minor distortion (B). For (C), (D), and (E), 200µg/ml of chitosan was used. (F) non-viable cells treated with 400 µg/ml chitosan.

5. Conclusion

The functions of antioxidants and antimicrobials are crucial to plant and human physiology. Bioactive substances including chitosan can alter the anti-oxidant and antibacterial activity. Distinct levels of antioxidant and antibacterial activity were obtained in this investigation by using three distinct molecular weights with varying degrees of deacetylation. These bioactive substances are essential in preventing the production of free radicals. Using the four radical scavenge capacity methods, antioxidant activity showed that low molecular weight (LMW) chitosan had the most significant antioxidant activity, followed by moderate molecular weight (MMW) and high molecular weight (HMW). The findings showed that the antioxidant activity increased with the chitosan concentration. The degree of acetylation and molecular weight of chitosan were the primary determinants of its antioxidant activity. The IC_{50} value and antioxidant activity have a negative correlation. In essence, the IC_{50} value indicates the amount of antioxidant concentration required to reduce the initial DPPH concentration by 50%. Therefore, higher antioxidant capabilities are reflected by a lower range of IC_{50} values. Using the $ABST^{\cdot+}$ (1.9 mg/ml) and H_2O_2 (2.3 mg/ml) techniques, LMW chitosan displayed the lowest IC_{50} value, suggesting that it may have greater antioxidant properties than MMC and HMW chitosan. Chitosan had clear antibacterial action against both Gram-positive and Gram-negative bacteria, fungus, and yeast, as demonstrated by the successful completion of the agar disc diffusion experiment. Compared to MMW and HMW chitosan, LMW chitosan exhibits a far more promising antibacterial potential because of its solubility in mild acetic acid. Our

findings indicate that the antibacterial properties of chitosan are mostly dependent on the type of microorganisms tested, the degree of deacetylation, and the molecular weight of chitosan.

Acknowledgment

The National Research Center, Dokki, Cairo, Egypt, is gratefully acknowledged by the authors for its financial support of this wok.

Conflict of interest

The publication of this paper does not present any conflicts of interest.

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