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Evaluation of Antibacterial Activity of Chitosan against Different Bacterial Strains

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THE PRESENT study is related to new green and low cost technical method for wastewater treatment using chitosan as natural material. Chitosan was prepared from chitin of the seafood's shell (*Procambrus clarkia*). Preparation of chitosan was carried out by a chemical process involving demineralization, deproteinization and deacetylation. Chitosan is largely known for its activity against a wide range of microorganisms. Antibacterial effect of different doses of chitosan (0.09, 0.2, 0.4, 0.6 and 0.8 g) on *Escherichia coli* (*E. coli*) bacteria in the secondary treated wastewater samples was studied. The secondary treated wastewater was collected from Zenein wastewater treatment plant. Also the effectiveness of chitosan on *Enterococcus faecalis, Bacillus subtillus, Staphylococcu saureus* (gram +ve bacteria), *Escherichia coli*, *Enterobacter aerogenes* (gram –ve) was evaluated by the reduction in total bacterial count of each strain in synthetic media. The study was extended to measure the antibacterial activity (inhibition zone) of chitosan on *E. coli* by the poured plate method. The results indicated that the optimum conditions at which 99.98% removal for all types of bacteria were 0.6 g chitosan, contact time 30 min and shaking 250 rpm. According to the obtained results, the most acceptable antimicrobial mechanism is found to include the presence of charged groups in the polymer backbone and their ionic interactions with bacterial wall constituents. It could be concluded that chitosan could be used for disinfection of the secondary treated wastewater.

Keywords: Chitosan, *Escherichia coli*, Antimicrobial activity, Bioremediation, Biodegradability

Introduction

Many human pathogens can be transmitted by water contamination with wastewater effluents, which should be properly treated to prevent the spread of pathogenic microorganisms **[1]**. Wastewater disinfection is applied to provide protection to humans against exposure to waterborne pathogenic microorganisms **[2]**. The reuse of treated wastewater is one of the main options being considered as a new source of water in regions where water is scarce. Chlorine is a conventional disinfectant that commonly used in wastewater treatment plants. However, effluent of chlorination produces a carcinogenic-mutagenic disinfection byproducts (DBPs) as a result of the reaction between the chlorine and organic compounds in wastewater. Some of these substances have proven to be carcinogenic for humans and animals **[3]**. Numerous adsorbents have been applied for the removal of pathogens, such as chitin, chitosan and cellulose, which are

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not only ecofriendly and cost effective but are also effective in the remediation of common effluents present in wastewater **[4, 5]**.

Some water purification plants worldwide are using chitosan for removal of oils, grease, heavy metals and the fine particulate matter that cause turbidity in water streams **[6].** Chitosan is the second most abundant polysaccharide in nature and is considered as a partially N-deacetylated of a fiber-like substance derived from chitin a homopolymer of ß-(1-4)-linked N-acetyl-D-glucosamine. Each glucosamine unit contains a free amino group, and these groups can take on a positive charge which gives amazing properties of chitosan **[7, 8, 9]**. Chitosan and chitin are a similar in their chemical structure. Chitin consists of a linear chain of acetylglucosamine groups while chitosan is prepared by removing some acetyl groups (CH_3-CO) from chitin. The molecule and the resultant product is found to be soluble in most diluted acids. The acetyl contents of both polymers are different. Chitosan, having a free amino group, is the most useful derivative of chitin **[10]**.

Chitosan is acclaimed for its nontoxic nature, biodegradability, polycationic, antitumor activity, antioxidative activity, anticholesterolemic, hemostatic and analgesic effect. Chitosan possesses a wide range of antimicrobial activity against bacteria, filamentous fungi, yeast and even virus. Studies have reported antibacterial activity of chitosan nanoparticles against *Escherichia coli*, *Staphylococcus aureus, Streptococcus mutans, Salmonella Typhimurium, Salmonella choleraesuis,* and *Pseudomonas aeruginosa* **[11, 12, 13].** The aim of this study is to evaluate the use chitosan (natural polycationic material) as disinficatant to inhibit the growth of some pathogenic bacteria during wastewater treatment.

Material and Methods

Preparation of Procambrus clarkii shells

The Nile *Procambrus clarkii* (Fig.1 A) was used for the experiment for the preparation of chitosan. The Nile *Procambrus clarkii* was brought in fresh condition from local market (Nile, Egypt). The following conditions were chosen as an optimal extractive method **[14, 15]**. The first stage of the extraction process involves a thermomechanical treatments. The shells were separated from cephalothoraxes, scraped free of loose tissue. Then remove the adhesive tissues, and washed individually in lightly saline water, washed thoroughly with distilled water. The washed shells was dried in the sun (25-30°C) for 3 days, and finally dried in an oven at 60 °C for **48 hr** (Fig. 2. B). After that, the dried shells were grinded.

Demineralization

The second stage started with a demineralization process which was carried out using 0.5 M HCl solutions. Typically, 100 g of *Procamrus clarkii* shells powder was immersed in 1000 mL of 0.5 M HCl at ambient temperature

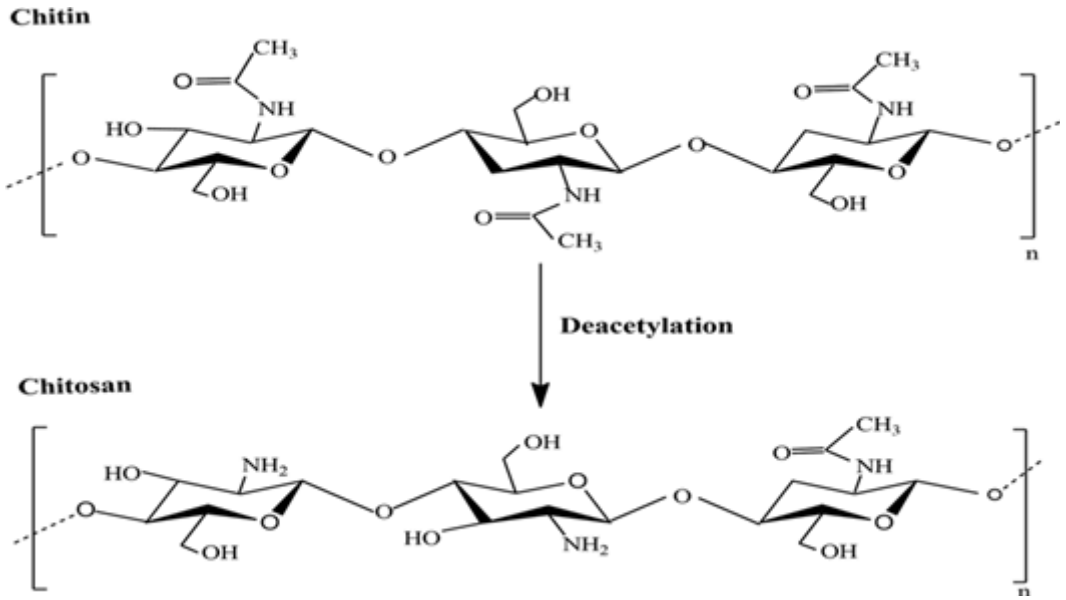

Fig. 1: Transformation of chitin to chitosan by deacetylation process (Nessaa et al., 2010)

(25 ºC) under constant stirring (250 rpm) for 24 h. After filtration, the residue was washed with distilled water until the pH of the rinsed water became neutral, then the residue drid at 60ºC, then weighted **[14, 15]**.

Deproteination

The residue was subjected to deproteination, by immersing in 1000 mL of 1 M NaOH under vigorous stirring at 60 ºC for 24 h. Then the proteins were removed by filtration. Distilled water was used for washing the residue, then dried at 60 ºC and weighted. Finally the extracted chitin was subjected to chitosan preparation **[14, 15]**.

Preparation of chitosan (deacetylation)

The extracted chitin was deacetylated to form chitosan by treating with 50 % NaOH and boiled at 100 ºC with stirring on a hot plate for 2 h, cooled for 30 min at room temperature. After filtration, the residue was washed continuously with the 50% NaOH and filtered. Then the residue was washed three times with distilled water. The crude chitosan was obtained by drying in oven at 60 ºC and weighted (Fig. 3)**[14, 15]**.

Effeciency of chitosan in reduction of E. coli count from wastewater samples

Method

Secondary treated wastewater samples collected from UASB/DHNW combined system that installed in Zenein wastewater treatment plant (ZWWTP) at Giza governorates, Egypt, in clean and sterilized polyproplene bottles [16-17]. The samples collected were tested using MPN technique to evaluate the effectiveness of chitosan on *E. coli* bacteria (before treatment) as control samples, then different concentrations of chitosan (0.09, 0.2, 0.4, 0.6 and 0.8 g) added on 100 ml secondary treated wastewater, then shaked for 30 min at 25ºC at 250 rpm, then filterated. The filterate of each untreated and treated samples with different concentration were examined by MPN

Fig 2: (A) *Procambrus clarkii,* (B) the dried shells of *Procambrus clarkii*

Fig. 3: Chitosan powder after deacetylation of chitin

method using lauryl broth tubes which arranged in three raws, each contain raw of 5 tubes. Serial dilution of each concentration was prepared and used to inoculate the lauryl broth tubes, then incubated at 35 °C for 48 h, positive tubes (gas production-acidic reaction-changing in colour from purple to yellow) used to inoculate tryptone water, then add 0.2-0.3 ml of kovac›s reagent, positive tubes give deep red colour on the upper layer. Calculate MPN from the number of indole positive tubes **[18]**.

The percentage of removal of bacteria is calculated as follows:

% of removal = $[C_o - C_e / C_o * 100]$

Where: C_o is the bacterial count in the untreated sample

C_e is the bacterial count in the treated samples **[19]**.

Effect of chitosan on different bacterial strains

Different concentrations of chitosan powder (0.09, 0.2, 0.4, 0.6 and 0.8) were used to evaluate the bactericidal effect of chitosan on different bacterial strains (*Escherichia coli,* ATCC 25922), *Enterococcus faecalis* ATCC 29212), *Baccillus subtilus* ATCC,6633 *(Staphylococcus aureus*, ATCC 29213), and *Enterobacter aerogenes* ATCC, 13048) at different contact times (10, 20 and 30 min), using nutrient broth agar media for enrichment of each strain, incubate at 35 °C for 24 h.

Pour plate method test

Using plate count agar media and pour plate method to evaluate percentage (%) of bacterial removal

Procedure

10 ml of bacterial suspensions for each enriched strain was separately inoculated in an autoclaved water (1000 ml). Different concentrations of chitosan powder (0.09, 0.2 0.4, 0.6 and 0.8 g) added to 100 ml of diluted bacterial suspension and shaked at 250 rpm for different contact time 10, 20 and 30 min at 25°C, the control wasn't conain chitosan (only diluted bacterial suspension) to calculate the initial concentrations of each bacterial strain. Then add 1 ml of each concentration and the control on steriled petri dish, then pour the cooled agar media and incubate at 35 °C±0.5 for 48 h, the bacterial count was estimated according to APHA, 2017 **[18]**.

The antibacterial effect of chitosan powder on E. coli using amoxilline as positive control

Spread method

Spread 0.1-0.2 ml of bacterial suspension of *E. coli* on the surface of solidify nutrient agar medium **[18]**, the plate was devided into two halves, then by sterilized forceps immerge one disc of amoxilline on one half of the medium and add on the other half concentrations 0.6 and 0.8 g of chitosan directly on the media, then incubated at 35°C for 24 h, after incubation measure the inhibitory zone of chitosan powder comparing with positive control amoxilline **[20]**. All tests were carried out in a laminar flow under aseptic conditions.

Results

Table 1 shows the relation between the count of *E. coli* before and after treatment secondary treated wastewater using different doses of chitosan. As the dose of chitosan increases the bacterial count of *E. coli* decreases and the removal percentage increases. The doses of 0.09 g/100 and 0.2 g/100 ml of chitosan show 95.3 % and 99.8 %, respectively by MPN method while 0.4, 0.6 and 0.8 g/100 ml show 99.99 % removal.

a) Effect of chitosan on Enterococcus faecalis bacteria

It could be be noted from Table (2) that the removal of *Enterococcus feacalis* was 40.8%, 49.2% and 71.4% by using 0.09 g/100 ml of chitosan at time intervals of 10, 20 and 30 min of shaking, respectively. The removal was increased by increasing the dose of chitosan to 0.2 g/100 ml with corresponding removal rate of 95 %, 97.1 % and 98.5% after 10, 20 and 30 min, consecutively. The removal reached 98.3% at concentration of 0.4 g/100 ml after 10 min of shaking, then became 99.98 % after 20 and 30 min of shaking, respectively. While, the removal of *Enterococcus feacalis* was constant (99.98%) at concentrations of 0.6 and 0.8 g after 10, 20 and 30 min of shaking, respectively. In this study, 0.6 g of chitosan has antibacterial power against *Enterococcus feacalis* as supported by the study of Priscilla et al., 2019 **[21]**, who assessed the antibacterial efficiency using agar diffusion technique using 2% of chitosan.

As the contact time increases, the bacterial strains removal was increased with low concentration. It is believed that the bacteriostatic/ bactericidal activity of chitosan is related to the protonated positive charge number of chitosan and the number of negative charges on the microbial surface **[22,23]**.

b) Effect of chitosan on (E. coli)

In the present study (Table 3), the percentages of removal of *E. coli* was 33.3%, 46.7% and 63.1% at chitosan concentration of 0.09 g/100 ml and contact time of 10, 20 and 30 min consecutively.

TABLE 1: Effect of chitosan on *E. coli* removal from secondary treated wastewater*

Dose of chitosan (g/100ml)	Min	Max	Average	% of removal
Raw wastewater	$2.0x10^5$	$1.70x10^6$	$9.5x10^5$	
0.09	$3.50x10^4$	$5.40x10^4$	$4.45x10^4$	95.3
0.2	$1.0x10^3$	$1.2x10^3$	$1.1x10^3$	99.8
0.4	$3.6x10^2$	$7.8x10^2$	$5.7x10^2$	99.9
0.6	$< 1.8x10^2$	$< 1.8x10^2$	$< 1.8x10^2$	< 99.9
0.8	$< 1.8x10^2$	$< 1.8x10^2$	$< 1.8x10^2$	<99.9

* counts in MPN/100 ml

TABLE 2: Effect of different concentrations of chitosan on *Enterococcus faecalis* by different times (10, 20 and 30 min)*

Dose of chitosan (g/100 ml)	10 min	% Removal	20 min	% Removal	30 min	% Removal
0.09	$1.925x10^3$	40.8%	$1.65x10^3$	49.2%	$1.3x10^3$	71.40%
0.2	$1.6x10^2$	95%	9.5x10	97.10%	5.0x10	98.50%
0.4	5.5x10	98.30%	<1	99.98%	<1	99.98%
0.6	<1	99.98%	<1	99.98%	<1	99.98%
0.8	<1	99.98%	<1	99.98%	<1	99.98%

* Intial count = $3.25x10^3$ cfu/ml

At concentration of 0.2 g and contact time of 10, 20 and 30 min, the percentage of removal of *E. coli* increases to 66.1%, 75.3% and 81.1% consecutively. Then after using concentration of 0.4 g, the percentage of removal reached 94.9% after 10 min and the percentage was 99.98% at both contact time of 20 and 30 min. By increasing the dose to 0.6 and 0.8, the percentage of removal remain constant at 99.98% after contact time of 10, 20 and 30 min. The previous results indicated that, by increasing contact time and the concentration of chitosan the effeciency of bacterial removal increases. Low-molecular weight chitosan enters the cell through cell membrane, because chitosan can absorb the electronegative substances in the cell and flocculate them, it disturbs the physiological activities of the bacteria and consequently kills them **[24, 25]**.

Effect of chitosan on Bacillus subtillus

Table 4 shows the removal perentage of *Bacillus subtillus* bacteria at different dose of chitosan. The removal percentage at dose of 0.09 g/100 ml and different contact time of 10, 20 and 30 min were 52%, 72% and 74%, respectively. Then the removal was increased by dose of 0.2 g/100 ml to be 76%, 78% and 82% at different contact time (10, 20 and 30 min), respectively. When the dose of chitosan increased again to 0.4 g/100 ml, the removal was 87.2 %, 97.6% and 97.6%, at different contact time of 10, 20 and 30min, respectively. While the dose was 0.6, 0.8 g/100 ml of chitosan, the removal percentage was 99.98%. The mechanism of inhibition was proposed where the deduction was made that the chitosan disrupts the outer membrane of the Gram-positive bacteria, leading to the leakage of cellular constituents and cell lysis **[22]**.

Effect of chitosan on Enterobacter aerogenes

It was noted that, when the concentration of chitosan and the time of shaking increased, the removal of bacterial strains increased too (Table 5). The removal percentage of *Enterobacter aerogenes* was 30, 61, 69.4% at dose of 0.09 g/100 ml of chitosan and contact time of 10, 20 and 30 min of shaking (250 rpm), respectively. Then by increasing the dose to 0.2 g/100 ml, the removal was increased gradually to 45, 81.5 and 99.2 % by increasing the time of contact to 10, 20 and 30 min, respectively. At dose of 0.4 g/100 ml of chitosan the removal at contact time of 10, 20 and 30 min and shaking of 250 rpm was 92, 99 and 99.98%, respectively. Then the removal became constant (99.98%) when the dose was increased to 0.6 and 0.8 g/100 ml and at contact

TABLE 3: Effect of different doses of chitosan on *E. coli* removal at different contact time*

Dose of chitosan (g/100 ml)	10 min	% Removal	20 min	% Removal	30 min	% Removal
0.09g	$3.25x10^3$	33.30%	$2.6x10^3$	46.70%	$1.8x10^3$	63.10%
0.2g	$1.65x10^3$	66.10%	$1.2x10^2$	75.30%	$9.2x10^2$	81.10%
0.4g	$2.5x10^2$	94.90%	<1	99.98%	<1	99.98%
0.6g	<1	99.98%	<1	99.98%	<1	99.98%
0.8g	<1	99.98%	<1	99.98%	<1	99.98%

* Intial count = $4.88\ x10^3$ cfu/ml

TABLE 4: Removal efficiency of Bacillus subtillus by chitosan at different contact times

Dose of chitosan (g/100 ml)	10 min	% Removal	20 min	% Removal	30 min	% Removal
0.09	120	52%	70	72%	65	74%
0.2	60	76%	55	78%	45	82%
0.4	32	87.20%	6	97.60%	6	97.60%
0.6	<1	99.98%	<1	99.98%	<1	99.98%
0.8	<1	99.98%	<1	99.98%	<1	99.98%

* Initial count = $2.5x10^2$ cfu/ml

TABLE 5: Effect of different doses of chitosan on Enterobacter aerogenes removal at different contact time*

Dose of chitosan (g/100 ml)	10 min	% Removal	20 min	% Removal	30 min	% Removal
0.09	4550	530%	2535	61%	1986	69.4%
0.2	3575	45%	1200	81.5%	52	99.2%
0.4	520	92%	65	99%	<1	99.98%
0.6	<1	99.98%	<1	99.98%	<1	99.98%
0.8	<1	99.98%	<1	99.98%	<1	99.98%

* Initial count = >6500 cfu/ml

time of 10, 20 and 30 min. It was suggested that chitosan penetrates the cell wall and combine with the DNA, thus inhibiting the synthesis of mRNA and reducing DNA transcription **[26]**. It is also possible that chitosan forms a film around the bacteria, which will inhibit nutrient absorption as described by Zheng and Zhu, 2003 **[25]**.

Effect of chitosan on Staphyllococcus aureus

At concentration of 0.09 g/100 ml of chitosan and different contact time of 10, 20 and 30 min, the percentages of removal of *Staphyllococcus aureus* were 90.2%, 98.3, 99.3%, consecutively. Then by increasing the dose of chitosan to 0.2 g/100 ml the removal increases to 97.4 %, 99.4 % and 99.98 % at contact time of 10, 20 and 30 min, consecutively. Table 6 shows the performance of chitosan for removal of *Staphyllococcus aureus* at different contact time. This results is in agreement

with study of Alaa et al., **[27]** and Lee et al., **[28]** that the chitosan molecule has the ability to interact with bacterial surface and was adsorbed on the surface of the cells and stacks on the microbial cell surface and forming layer around the cell, leading to the block of the channels.

Discussion

Chitosan is the deacetylated form of chitin, an abundant polysaccharide. Its nitrogenous content makes it highly economical material and other physio-chemical properties like biocompatibility, adsorptive ability, etc. Chitosan have been reported to show increased activity due to increased surface area. In the present study, chitosan were synthesized and the antimicrobial activity and it's ability in bacterial count reduction has been detected in detail.

Several models have suggested that the antimicrobial activity of chitosan is attributed to its cationic nature **[29, 30]**. The electrostatic interaction between positively charged R $N(CH_3)^{3+}$ sites and negatively charged microbial cell membranes, is predicted to be responsible for cellular lysis and assumed as the main antimicrobial mechanism **[29, 31]**. Charged chitosan can interact with essential nutrients consequently interfering on microbial growth **[32]**. Consequently, it is expected that polymers with higher charge densities resulted in an improved antimicrobial activity.

Previous studies showed that, the evaluation of chitosan by agar well diffusion method to exhibit the antibacterial activity againt different types of bacterial strains **[33]**. In our study, the used pour plate method was carried out according to APHA, 2017 **[18]**.

In general, the antimicrobial effectiveness of chitosan and its derivative against Gram-positive and Gram-negative bacteria is somewhat controversial. In some published works, the literature represents that unmodified chitosan generally acts stronger on Gram-negative than on Gram-positive strains **[34, 35]**. There are however, contradictory evidences presented by several other authors, for whom greater activities of chitosan and its derivatives over Gram-positive strains were reported as pre-dominant **[36]**. Still many workers demonstrated there were no significant differences observed between the antibacterial activities against the bacterium **[37]**.

Another researches suggested that the chitosan penetrate the cell wall and combine with the DNA, thus inhibiting the synthesis of mRNA and reducing DNA transcription **[26]**. In the case of Gram-negative bacteria it is suggested that the chitosan binds to the outer membrane, affecting the barrier properties. It is also possible that the chitosan forms a film around the bacteria, which will inhibit nutrient absorption as described by **[25]**.

Antibacterial activity of chitosan against E. coli using Amoxilline as positive control

It was detected in this study that by using amoxilline antibiotic (positive control) on nutrient agar media spreaded by *E. coli* bacteria and incubated at 35°C for 24h. the inhibition zone around the disc of amoxilline was 6mm but in case of using chitosan as antimicrobial agent, the inhibition zone of 0.6g of chitosan was 7 mm and for 0.8g the diameter zone was 11 mm (Figs. 4 & 5).

It was observed that no inhibition clear zones were around the membrane because chitosan is unable to diffuse through agar. However, chitosan membranes coupled with honey provided a small

TABLE 6: Effect of different doses of chitosan on *Staphyllococcus aureus* removal at different contact time*

Dose of chitosan (g/100 ml)	10 min	% Removal	20 min	% Removal	30 min	% Removal
0.09	95	90.2%	16	98.3%	6	99.3%
0.2	25	97.4%	5	99.4%	1	99.98%
0.4	<1	99.98%	<1	99.98%	<1	99.98%
0.6	<1	99.98%	<1	99.98%	<1	99.98%
0.8	<1	99.98%	<1	99.98%	<1	99.98%

* Initial count = 975 cfu/ml

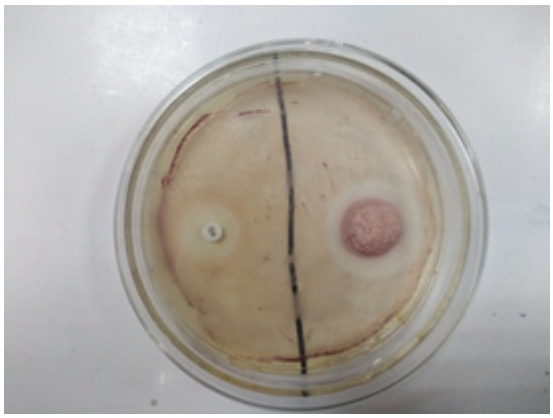

Fig. 4: Inhibition zone of chitosan by a concentration of 0.6g and amoxilline antibiotic disc as (positive control) in culture media inoculated with *E. coli* bacteria after 24h of incubation

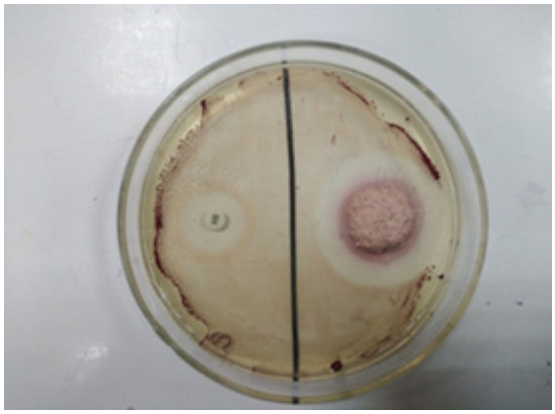

Fig. 5: Inhibition zone of chitosan by a concentration of 0.8g and amoxilline antibiotic disc as (positive control) in culture media inoculated with E. coli bacteria after 24h of incubation

inhibition zone, confirming that chitosan only has antimicrobial effect by direct contact and cannot migrate into the agar.

The antimicrobial properties of chitosan mainly depend on the degree of deacetylation and molecular weight of chitosan **[38, 39]**. El-tahlawy et al., **[40]** and Hosseinnejad and Jafari **[41]** reported that low molecular weight chitosan can penetrate into the cell and inhibits mRNA and protein synthesis. Chitosan oligomers have higher antimicrobial effect due to their shorter chain and free amino groups from D-glucosamine **[42]**.

Similarly, Champer et al., **[43]** reported that the amount of free amino groups affect the antibacterial behavior of chitosan. Likewise, Wang et al., **[44]**, recommeneded that all bacteria have negative charges; consequently, they are easily captured by the protonated amine groups of chitosan and lose their reproductive functions and bioactivity.

The electrostatic interaction results in twofold interference: I) by promoting changes in the properties of membrane wall permeability, thus provoke internal osmotic imbalances and consequently inhibit the growth of microorganisms, II) by the hydrolysis of the peptidoglycans in the walls in the microorganisms, that leads to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase) **[45]**.

Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of cationized amines, the higher will be the

antimicrobial activity **[46]**. This suggests that chitosan has higher activity than that found for chitin and this has been confirmed experimentally **[46]**.

It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation **[22]**.

Conclusion

The results presented inthis study show that chitosan powder has a high antibacterial activity. It was successfully synthesized from *Procambrus clarkii* collected from Nile River, Egypt. According to the results, increasing the concentration of chitosan can affect perfectly on different bacterial strains. It could be postulated that chitosan disrupts the barrier properties of the cell wall structure of Gram-negative and Gram-positive bacteria. This mechanism could be explained by the presence of the free amino groups from the chitosan structure which carry positive charge gives the chitosan cationic properties and so electrostatic attraction between chitosan and the negative cell wall of bacteria occur.Finally, the use of chitosan asan ecofriendly method for treating wastewater can remove pathogenic bacteria for safe use of treated water.

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