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CHITOSAN NANOPARTICLES PREPARED FROM *LUCILIA CUPRINA* MAGGOTS AS ANTIBACTERIAL AGENT

By
MOSTAFA I. HASSAN[1*], FATMA A. TAHER[2], ALY F. MOHAMED[3] AND MOHAMMAD R. KAMEL[1]
Department of Zoology and Entomology, Faculty of Science[1] and Department of Chemistry, Faculty of Science (Girls)[2], Al-Azhar University, Nasr City and Applied Researches Sector, Vacsera[3], Giza, Egypt
(*Correspondence: mostafa012@gmail.com)

Abstract

Chitosan were prepared from cuticle of *Lucilia cuprina* maggots with two steps; deproteinization and deacetylation. It was characterized with solubility and Fourier Transform Infrared spectroscopy (FT-IR). Chitosan was ball-milled to obtain the chitosan nanoparticles which characterized with dynamic light scattering (DLS) and transmission electron microscope (TEM). Chitosan nanoparticles with degree of deacetylation (DDA) 80.5% were showed antibacterial activities against *Klebsiella pneumoniae* and *Bacillus subtilis*. The mode of action of chitosan nanoparticles on the tested bacteria was studied by TEM. Leakage of some cell contents, cell deformation and rupture of cell were observed, therefore, the chitosan nanoparticles were observed to be a powerful antibacterial agent.

Keywords: Antibacterial agent, Chitosan nanoparticles, *Lucilia cuprina*, Maggots

Introduction

Chitin is the second most important natural polymer in the world and it not soluble in aqueous acidic media. When the degree of deacetylation of chitin reaches about 50%, it becomes soluble in aqueous acidic media and then is called chitosan (Rinaudo, 2006). Traditionally, the chitosan was prepared chemically from the shell of crustaceans by four steps, while the preparation of chitosan from maggots can be processed by two steps.

The cuticle of the maggots is mainly composed of chitin (Jing *et al*, 2007) that representing the main source of chitosan which is more useful and interesting bioactive polymer (Dutta *et al*, 2004) because it is a high biodegradable, safe & antimicrobial agents. Chitosan nanoparticles have been developed as a natural antimicrobial agent, and *in vitro* results provide promising use for disease treatment (Ma, 2015).

The present study aimed to prepare the chitosan from *Lucilia cuprina* maggots and investigate the antibacterial activities of chitosan nanoparticles against *Klebsiella pneumoniae* and *Bacillus subtilis* and their mode of action using transmission electron microscope.

Materials and Methods

Collection and Rearing of flies: *Lucilia cuprina* (Diptera: Calliphoridae) was collected and reared after Hassan, *et al.* (2013).

Preparation of chitosan: Chitosan powder prepared according to the method of Oduor-Odote *et al.* (2005) with some modifications as the following: Second and third larval instars of *Lucilia cuprina* were collected and stored in freezer at -20 °C until homogenization in blender, then dried in oven at 50°C. The homogenized maggots (Chitin) were sieved and weighted. Deproteinization of chitin: 200g from chitin were stirred in 4 liters of sodium hydroxide 2.5 % (w/v) by magnetic stirrer for 2h at 50°C, then filtrated and washed with distilled water until became neutral, and finally washed with absolute ethanol. Step of deprotenization was repeated twice then dried in oven at 50°C for 12h. Deacetylation of chitin: 100g deproteinized chitin were left in 2 liters of sodium hydroxide 50% (w/v) for overnight, and then stirred by magnetic stirrer for a total of 7h at 110°C, then filtrated, washed with distilled water and absolute ethanol. After that, the

chitosan left for draying in oven at 50°C for 12h and then weighted.

Characterization of chitosan: The chitosan was characterized with solubility and Fourier transform infrared spectroscopy (FT-IR) analysis. The chitosan sample was tested for solubility in acetic acid 1% (v/v) with a ratio 1g: 100ml. It was analyzed by FT-IR JASCO 4100 in wave range 4000-400cm^{-1} with resolution of 4cm^{-1} at Micro Analytical Center, Faculty of Science, Cairo University. The degree of deacetylation of chitosan was calculated by the equation (1) according to Baxter *et al.* (1992):

$$DDA\ \% = 100 - \left[\frac{A_{1655}}{A_{3450}} \times 115\right] \quad \ldots\ldots. (1)$$

Where:

A_{1655} = Absorption of band at 1655 cm^{-1}

A_{3450} = Absorption of band at 3450 cm^{-1}

A_{Band} = - log (Transmittance)

Preparation of chitosan nanoparticles: Chitosan nanoparticles were prepared by ball-milling for 8h at 3400rpm using Retsch Planetary Ball Mill pm 400 after Zhang *et al.* (2014) at Egyptian Petroleum Research Institute (EPRI).

Characterization of chitosan nanoparticles: The chitosan nanoparticles were characterized with dynamic light scattering (DLS) after Qi *et al.* (2004), and transmission electron microscope (TEM) at Egyptian Petroleum Research Institute (EPRI). The chitosan nanoparticles sample was suspended in distilled water then sonicated for 15 min and measured by Malvern Mastersizer 2000 (Nano-ZS). Morphological characteristics of the chitosan nanoparticles were investigated by transmission electron microscope (Hitachi H-7000, Japan). The chitosan nanoparticles were sonicated in ethanol for 15 min to obtain a dilute suspension. Few drops from a dilute suspension were added on glow discharged carbon-coated microscopy grid and allowed to dry in room temperature then investigated at 200 kV.

Antibacterial activity chitosan nanoparticles: The antibacterial activity of chitosan nanoparticles against *Klebsiella pneumoniae* (RCMB 01002 23-5) as Gram-negative bacteria and *Bacillus subtilis* (RCMB 01001 69-3) as Gram-positive bacteria were evaluated by employing a microdilution method at Unit of Microbiology, The Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Chitosan nanoparticles were dissolved in acetic acid 1% (v/v) and diluted to a concentration of 8 mg/ml, further 1:2 serial dilutions were performed by addition of culture broth to reach concentrations ranging from 8000 to 0.49µg/ml.

A quantity of 5µl of each dilution was distributed in 96 well plates, as well as a sterility control and a growth control (containing culture broth plus acetic acid 1% (v/v), without antimicrobial substance). Each test and growth control well was inoculated with 5µl of microbial suspension (10^5 CFU/ well). All experiments were performed in triplicate and the microdilution trays were incubated at 37°C for 24h after Souza *et al.* (2005). Ten µl of 3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml) were added to each well and the plates were re-incubated for 3h at 37°C.

Fifty µl of Dimethyl sulfoxide (DMSO) solution were added to these wells and microbial growth was detected by optical density (ELISA reader, Tecan, Sunrise Remote/ Touch screen).

Electron microscopy examination: *Klebsiella pneumoniae* and *Bacillus subtilis* were treated with 125µg and prepared for electron microscopy examination (Helander *et al*, 2001).

Results

Solubility: The chitosan sample was soluble immediately without heating or sonication in 1% acetic acid (v/v) with ratio 1g /100ml. This result indicated to the sample was chitosan powder with degree of deacetylation above 50 %.

Fourier transform infrared spectroscopy (FT-IR) analysis: FT-IR spectrum of tested chitosan was shown (Fig. 1) and the main

peaks for chitosan functional groups; stretching of NH & OH Free, asymmetric stretching of CH (-CH2), bending of NH (R-NH2), asymmetric bending of CH (-CH2), symmetric bending of CH (-CH2), stretching of CN or bending of OH, symmetric stretching of (C-O-C), stretching of (C3-O) OH group, stretching of (C6-O) OH group and vibration of pyranose ring skeletal were detected at bands: 3440, 2922, 1596, 1424, 1381, 1253, 1152, 1091, 1035 and 896 cm^{-1}, respectively.

The absence of band absorption at 1540 cm^{-1} indicated successful deproteinization for chitin. The main functional groups which used in differentiation between chitin and chitosan were (C=O → $NHCOCH_3$) amide group at band around 1660 cm^{-1} and (NH_2) amine group at band around 1600 cm^{-1}. The high intensity of band at 1596 cm-1 in FT-IR and absence of absorption band around 1660 cm-1 was indicated to the presence of the NH_2 group and the success of the deacetylation process of chitin. After calculation the degree of deacetylation (DDA) of chitosan by equation (1), the DDA of chitosan sample is 80.5%.

Dynamic light scattering (DLS) analysis: The number-weighted distributions of particle size and particle size distributions of chitosan nanoparticles batches were measured by dynamic light scattering, and it showed one major peak at 53.59 nm that represented 98.2 % from the sample (Fig. 2).

Transmission Electron Microscope (TEM) analysis: The investigation of chitosan nanoparticles by TEM showed the homogenous cubic shape particles with width ranged between 16.33 nm to 30.73 nm (Fig. 3).

Chitosan nanoparticles showed antibacterial activities against *Klebsiella pneumoniae* and *Bacillus subtilis*. The effect of chitosan nanoparticles on *Klebsiella pneumoniae* was began at concentration 1.95µg/ml and showed 16.75 % inhibition. The completely inhibition of *K. pneumoniae* was recorded at concentrations 250, 500, 1000, 2000, 4000 and 8000 µg/ml, while, the effect of chitosan nanoparticles on *Bacillus subtilis* was recorded at concentration 31.25µg/ml and increased with the increasing of concentration until showed 100 % inhibition at concentrations 4000 and 8000 µg/ml (Tab. 1; Fig. 4).

Table 1: Antibacterial activities of chitosan nanoparticles against *Klebsiella pneumoniae* and *Bacillus subtilis*

Concentration (µg/ml)	Inhibition (%)	
	Klebsiella pneumoniae	*Bacillus subtilis*
8000	100	100
4000	100	100
2000	100	90.68
1000	100	87.66
500	100	70.17
250	100	56.75
125	93.13	45.02
62.5	83.68	16.36
31.25	68.37	7.65
15.63	48.77	-
7.81	37.86	-
3.9	27.81	-
1.95	16.75	-
0.98	-	-
0.49	-	-

Electron microscopy examination: Morphological changes were observed in treated bacteria; *Klebsiella pneumoniae* (RCMB 01002 23-5] and *Bacillus subtilis* (RCMB 01001 69-3) with chitosan nanoparticles (Fig. 5 B & D), respectively. Chitosan nanoparticles increased the permeability of the bacteria and led to leakage of cell contents,

cell deformation and rupture of cell. Both longitudinal section (LS) and transverse section (TS) of untreated (Control) *Klebsiella pneumoniae* and *Bacillus subtilis* were shown in figure (5 A and C), respectively. Intact and apparent bacterial cell walls (CW) and cell membrane (CM) were observed. Also, concentrated bacterial contents (BC) were clearly observed.

Discussion

Solubility: Generally, solubility in organic acid such as acetic acid is primary analysis for differentiation between chitin and chitosan, where, the chitosan has the ability to soluble in organic acid while the chitin has not this ability.

According to the experimental procedures, both chitosan and chitosan nanoparticles samples were dissolved in 1 % acetic acid. Hence; in agreement with Peter, (1995), the solubility of chitosan in acetic acid 1 % was indicated the chitosan has degree of deacetylation above 50 %.

Fourier Transform Infrared (FT-IR) spectroscopy analysis, agreed with Gylienë *et al.* (2003); Zvezdova (2010); Zhang *et al.* (2011); Liu *et al.* (2013); Song *et al.* (2013); Hafsa *et al.* (2014); Wanule *et al.* (2014) and Kaya *et al.* (2015) the broad peak around 3440 cm^{-1} was represented the stretching vibration of NH and OH groups in chitosan.

The asymmetric stretching vibration of CH ($-CH_2$) was observed at peak around 2922 cm^{-1}, this result agreed with Dong *et al.* (2001); Gylienë *et al.* (2003); Zvezdova (2010); Liu *et al.* (2013); Song *et al.* (2013); Wanule *et al.* (2014) and Kaya *et al.* (2015).

The detection of the bending vibration for NH ($R-NH_2$) at beak around peak 1596 cm^{-1} was matched with Jia and Xu (2001); Gylienë *et al.* (2003); Liu *et al.* (2013); Song *et al.* (2013); Subhapradha *et al.* (2013) and Kaya *et al.* (2015).

Corresponding with Dong *et al.* (2001); Gylienë *et al.* (2003); Zvezdova (2010); Liu *et al.* (2013) and Kaya *et al.* (2015), the bending vibration of CH ($-CH_2$) was represented at bands around 1424 cm^{-1} and 1381cm^{-1}.

Stretching of the C–O–C was appeared around band 1152 cm^{-1} which agreed with Dong *et al.* (2001); Zvezdova (2010); Liu *et al.* (2013); Subhapradha *et al.* (2013); Hafsa *et al.* (2014) and Kaya *et al* (2015).

According to the results of Dong *et al.* (2001); Zvezdova (2010); Zhang *et al.* (2011); Liu *et al.* (2013) and Kaya *et al.* (2015), the stretching of hydroxyl groups of C-OH was observed at bands around 1091cm^{-1} and 1035 cm^{-1}.

The stretching of pyranose skeletal ring was appeared at band 896 cm^{-1}, this result was corresponded with Dong *et al.* (2001); Zvezdova (2010); Zhang *et al.* (2011); Kaya *et al.* (2015). According to the previous discussed data for the well-defined chitosan, the Fourier transform infrared (FT-IR) confirmed the presence of main functional groups of chitosan in tested sample.

According to dynamic light scattering (DLS) analysis, the mean diameter of chitosan nanoparticles is 53.59 nm which indicated the successful preparation of chitosan nanoparticles with a Polydispersity Index (Pdi) value of 0.465. It was also confirm a very good monodispersity and quality of the chitosan nanoparticulate suspensions (Melo *et al*, 2000; Chattopadhyay *et al*, 2007). The number-size distribution of chitosan nanoparticles was in a narrow range of 37.84 to 91.28 nm.

The number-size distribution of chitosan nanoparticles consisted of one major peak of 53.59 nm for 98.2 % of sample which indicated with transmission electron microscope (TEM) results and a very small secondary peak represented 1.8 % of the sample which was a result of the bimodal droplet size distribution of starting emulsions. The chitosan nanoparticles size determined by dynamic light scattering (DLS) is slightly larger than that by TEM since DLS measures a hydrodynamic diameter.

Similarly with the results of Liu *et al.* (2001) and Younes *et al.* (2014), the *Klebsiella pneumoniae* was inhibited with chitosan. The positive result of antibacterial

activity of chitosan nanoparticles against *Bacillus subtilis* in this study was in accordance with the results which have been reported against *Bacillus subtilis* (Jing *et al*, 2007; Tayel *et al*, 2010; Benhabiles *et al*, 2012) and *Bacillus cereus* (Liu *et al*, 2001; Tao *et al*, 2011; Benhabiles *et al*, 2012; Younes *et al*, 2014).

The morphological changes which observed in treated bacteria; *Klebsiella pneumoniae* and *Bacillus subtilis* with chitosan nanoparticles involved leakage of cell contents, cell deformation (irregularly shaped) and rupture of cell. The results were corresponded with those of Liu *et al.* (2004) on *Escherichia coli* and *Staphylococcus aureus*, Didenko *et al.* (2005) on *K. pneumoniae* and *Staphylococcus aureus*, Li *et al.* (2010) on *Escherichia coli* and Wang *et al.* (2012) on *Xanthomonas axonopodis* pv. *poinsettiicola* strain R22579. In agreement with the data of Tsai and Su (1999); Zheng and Zhu (2003); Chung *et al.* (2004); Liu *et al.* (2004); Li *et al.* (2010) and Wang *et al.* (2012), the electrostatic interaction between poly cations of chitosan (Amino groups - NH_3^+ in acetic medium) and anions on bacteria cell wall led to increase the cell permeability and leakage of intracellular substances and finally cell death.

Besides, difference between the diameter of bacteria (~ 500nm) and size of chitosan nanoparticles (~ 50nm) played a co-effect in the mode of action of chitosan nanoparticles. That means the chitosan nanoparticles are ten times smaller than the bacteria which facilitates the penetration of antibacterial agent (chitosan nanoparticles) through the bacteria cell wall and cell membrane.

Conclusion

The chitosan nanoparticles were prepared and showed antibacterial activity against the tested bacteria; *Klebsiella pneumoniae* and *Bacillus subtilis*. The chitosan nanoparticles were increased the permeability of the bacteria and led to leakage of cell contents, cell deformation and rupture of cell, therefore, the chitosan nanoparticles were observed to be a powerful antibacterial agent.

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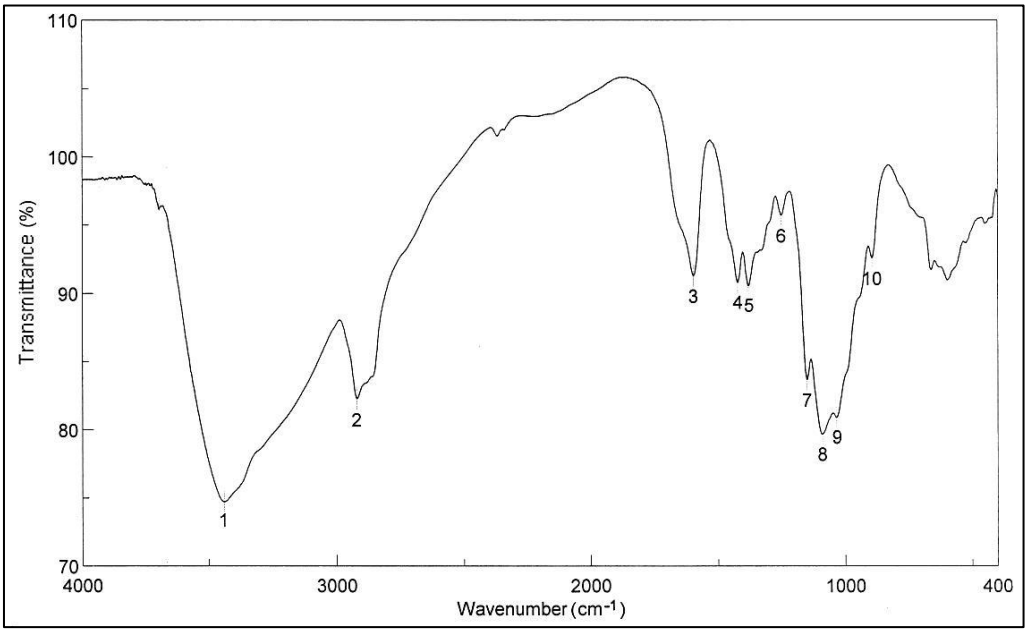

Fig. 1: FT-IR spectrum of chitosan sample prepared from *Lucilia cuprina* maggots.

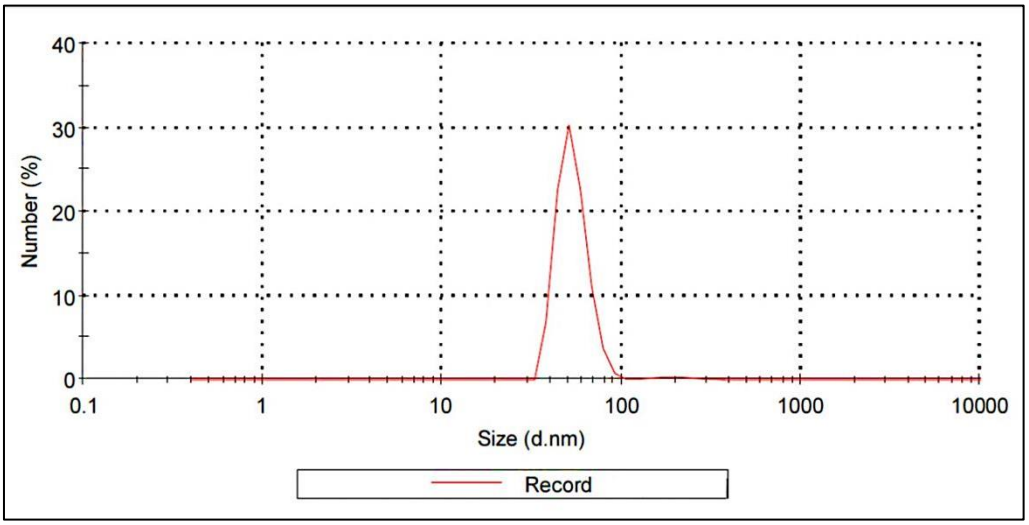

Fig. 2: Sample-size distribution of chitosan nanoparticles batches by dynamic light scattering.

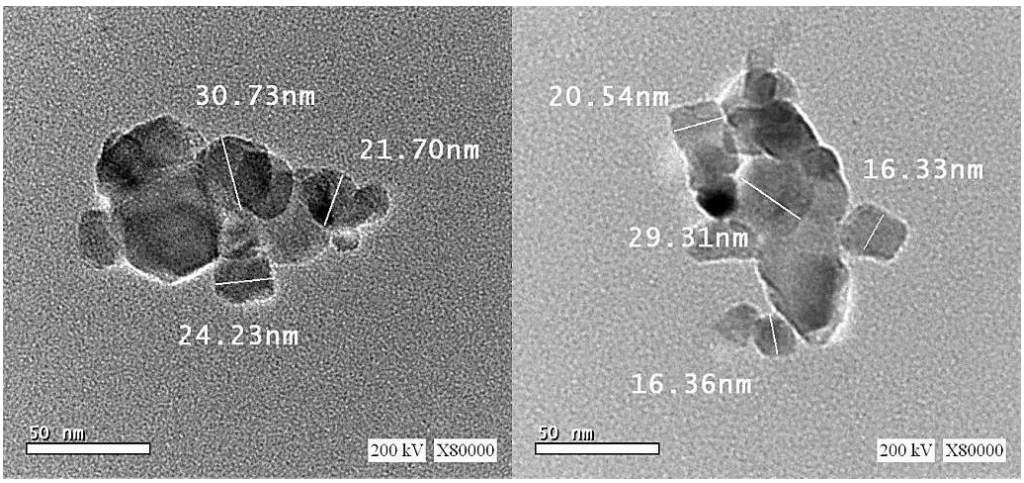

Fig. 3: Transmission electron microscope photomicrograph of chitosan nanoparticles X80000.

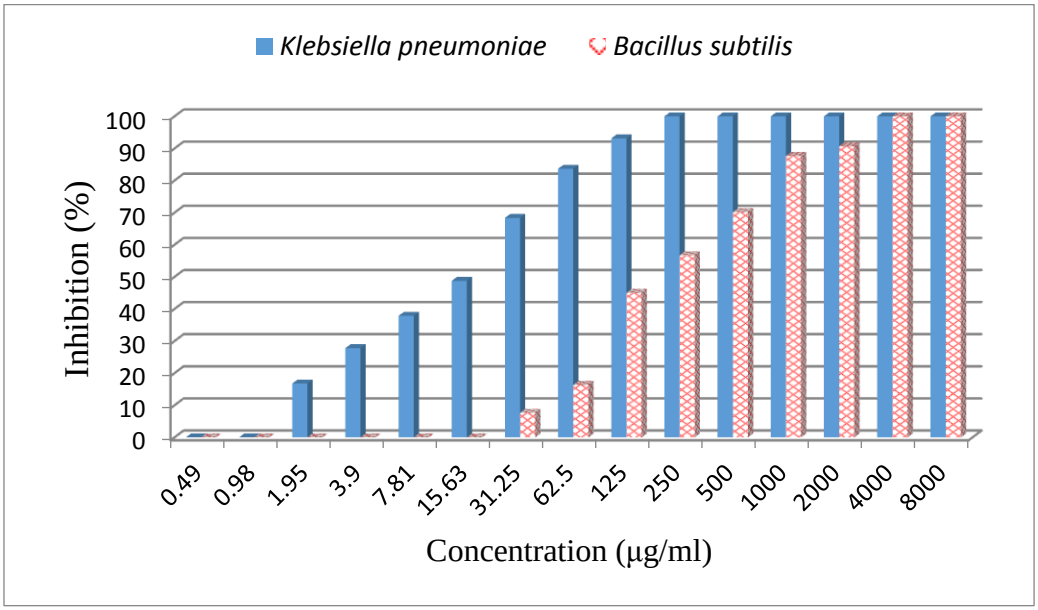

Fig. 4: Antibacterial activities of chitosan nanoparticles against *Klebsiella pneumoniae* & *Bacillus subtilis*

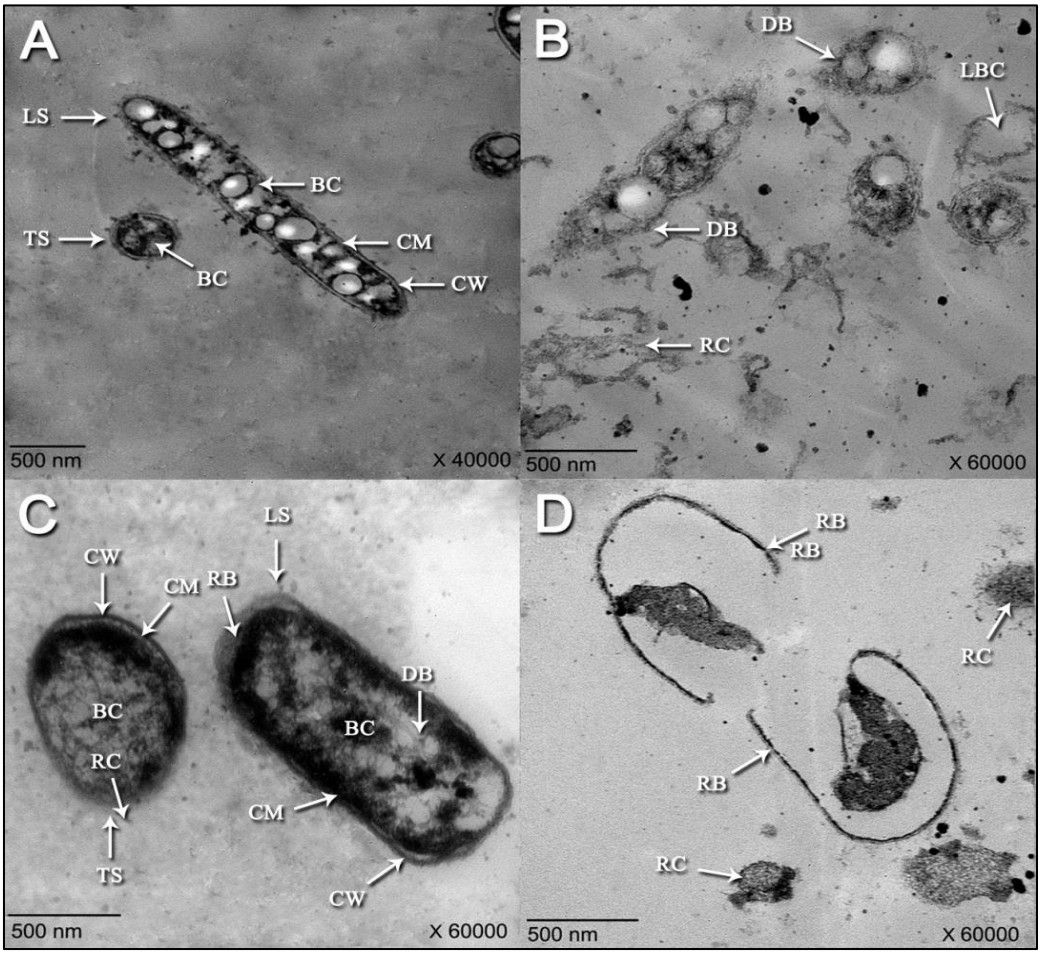

Fig. 5: Transmission electron photomicrographs of treated and untreated *Klebsiella pneumoniae* and *Bacillus subtilis* bacteria with chitosan nanoparticles: A- untreated *K. pneumoniae* (Control) X40000, B- treated *K. pneumoniae* X60000, C- untreated *B. subtilis* (Control) X60000 and D- treated *B. subtilis* X60000. LS = longitudinal section, TS = transverse section, CW = cell wall, CM = cell membrane, BC = bacterial contents, RB = ruptured bacteria, DB = deformed bacteria, LBC = leakage of bacterial contents and RC = released contents.