Antibacterial and antiviral activities of chitosan nanoparticles from the American cockroach, *Periplaneta americana*

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**ABSTRACT**

Globally, bacteria, parasites, and viruses are the most common causes of infectious diseases that endanger human health. The present work aimed to evaluate the antibacterial, antiviral, and virucidal activities of chitosan nanoparticles (CNPs) that were prepared from the American cockroach body, *Periplaneta americana*. The chitosan was prepared chemically with a 74.51% degree of deacetylation and then characterized by Fourier transform infrared spectroscopy. CNPs were obtained by ball-milling of chitosan and then characterized by dynamic light scattering and transmission electron microscope (TEM). CNPs inhibited the growth of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Bacillus subtilis*. By TEM investigation, CNPs caused the deformation and rupture of the selected bacteria. Also, CNPs protected the Vero cells and significantly decreased the infectivity titers of human adenovirus 40 and coxsackie B4 virus. In conclusion, this study indicated that applying the CNPs from the American cockroach is significant in decreasing the risk of infectious microorganisms.

**INTRODUCTION**

Researchers are studying more renewable materials to satisfy the needs of the global population and the international community in nutrition, medicine, and industry. In medicine, infectious disease prevention and treatment have got a lot of attention (Meng et al., 2021). Bacteria, fungi, parasites, and viruses are the most common causes of infectious diseases (Lashley, 2004). Antimicrobials discovered in constitutive or immune-stimulated insects have paved new paths in the antimicrobial study (Mosaheb et al., 2018).

Generally, insects like cockroaches have recently drawn the attention of researchers due to their enormous biomass, antimicrobial peptide, and potential as protein, lipid, and chitin sources (Kamal et al., 2020; Lee et al., 2019; Mohan et al., 2020). The American cockroach (*Periplaneta americana*) is a globally widespread species that can be found in tropics, subtropics, and temperate climates. Furthermore, since certain inland regions lack fishery services, *P. americana* would act as a complement to the chitin raw material (Chen et al., 2021).

Chitin is the second natural polymer after cellulose over the world, and chitosan is chitin that has been deacetylated. Biocompatibility, nontoxicity, and biodegradability are just a few of the benefits of chitosan (Kim et al., 2008; Leeta et al., 2013). Chitosan has antibacterial, antifungal, and antiviral properties (Hassan et al., 2016a). These properties vary depending on the origin source of chitosan (Marei et al., 2016), as well as the degree of deacetylation (DDA%) and molecular weight (Chatlet et al., 2001; Vinsova and Vavrikova, 2011). Chitosan nanoparticles (CNPs) are attracting a lot of attention for developing new pharmacological and therapeutic drugs with better biodistribution, increased specificity and sensitivity, and lower pharmacological toxicity (Peniche and Peniche, 2011).

The literature on the antiviral activity of chitosan against human viruses is still limited, and most pieces of literature focus on the effect of chitosan on plant viruses. Therefore, the present work aimed to prepare CNPs from the American cockroach, *P. americana*, and investigate the antiviral and virucidal activities of it against human adenovirus 40 (adeno-40) and coxsackie B4 virus, as well as the antibacterial activity.

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As for the antiviral activity, Vero cells (precultured at 37°C, 5% CO2, 100% humidity, 12 h/12 h light/dark cycle) were incubated (17.5°C) for 24 hours at 96-well plate. Another set was handled with culture media (as the control group), while another set was handled with a safe concentration of CNPs (as the treated group), while the handled cells were incubated for 24 hours at 37°C, washed by 2% (w/v) sodium dodecyl sulfate (SDS) × 115.

Preparation and characterization of chitosan

The frizzed cockroaches were dried at 50°C and homogenized and sieved to obtain a powder. The powder was deproteinized by stirring with 2.5% (w/v) sodium hydroxide (NaOH) with a ratio of 1 g : 20 ml for 6 hours at 100°C. The stirred powder was filtered, washed, and dried at 50°C for 12 hours. Minerals were removed from the deproteinized powder by stirring with 1 M hydrochloric acid for 2 hours at 75°C. After the washing and drying, the demineralized powder was decolorized with acetone at 50°C for 2 hours and then filtrated, washed, and dried to obtain chitin. Chitosan powder was produced by soaking the chitin in 50% (w/v) NaOH overnight, then stirred for 8 hours at 120°C, filtrated, washed, and dried.

Chitosan powder was analyzed by Fourier transform infrared spectroscopy (FT-IR Jasco 4100) with a frequency range of 4,000–400 cm\(^{-1}\) at res of 4 cm\(^{-1}\). The DDA was calculated from absorption at 3,450 and 1,655 cm\(^{-1}\) by Equation (1) (Baxter et al., 1992):

\[
\text{DDA}\% = 100 - \left[ \frac{A_{3,450}}{A_{1,655}} \times 115 \right]
\]

where \(A_{1,655}\) is the absorption of band at 1,655 cm\(^{-1}\) and \(A_{3,450}\) is the absorption of band at 3,450 cm\(^{-1}\).

Preparation and characterization of CNPs

The chitosan powder was ball-milled (Zhang et al., 2014) for 8 hours at 3,400 rpm to obtain CNPs. This process occurred by Retsch Planetary Ball Mill PM 400. The CNPs were investigated by dynamic light scattering (DLS) (Qi et al., 2004) and transmission electron microscope (TEM). For TEM, a few drops from the suspension were dried on a glow-discharged carbon-coated microscopy grid at 200 kV.

Bacteria, viruses, and cell line

Selected bacterial strains were obtained from Microbiology Unit at the Regional Center for Mycology and Biotechnology (RCMB). Escherichia coli (RCMB 010052) and Klebsiella pneumoniae (RCMB 003-1) as Gram-negative bacteria and Staphylococcus aureus (RCMB 010010) and Bacillus subtilis (RCMB 015-1) as Gram-positive bacteria were used to evaluate the antibacterial activity of CNPs. Vero cells were supplied from the Tissues Culture Unit at VACSERA. Human adeno-40 and coxsackie B4 virus were provided from the Virology Laboratory at VACSERA to investigate the antiviral and virucidal activities of CNPs.

Antibacterial activity of CNPs

Agar diffusion methods were used to evaluate the antibacterial activity of CNPs against the tested bacteria (Valgas et al., 2007). Briefly, the bacterial inoculum (10\(^5\) CFU/ml) was spread on a sterile Petri dish containing Mueller-Hinton agar. 50 µl of 1% CNPs was added to a 6 mm hole in agar. The inhibition zone was measured in mm after incubation at 37°C for 24 hours.

The antibacterial action of CNPs against more sensitive bacteria was investigated using a TEM (JEOL 1010) at the RCMB, Al-Azhar University. The subcultural bacteria were treated with CNPs (500 and 62.5 µg/ml for B. subtilis and K. pneumoniae, resp.) and incubated for 24 hours at 37°C. After the incubation and centrifugation for 15 minutes at 3,000× g, the precipitate was investigated under the electron microscopic examination through fixation, dehydration, embedding, polymerization, sectioning, and staining (Abdel-Razek, 2019).

Cell viability

The CNPs were tested on the viability of Vero cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Bahuguna et al., 2017). Briefly, the Vero cells were incubated in a 96-well plate for 24 hours at 37°C in 5% CO2. By culture media, the CNPs were diluted (2-fold dilution). The incubated cells were treated with CNPs dilution and culture media as treated and control groups, respectively, and then incubated again in the same conditions for the same period. 10 µl MTT solution was added to each well and incubated for 4 hours in the same conditions. Then, 50 µl dimethyl sulfoxide was added to each well and incubated at 37°C for a few minutes, and then by the Enzyme-Linked Immunosorbent Assay reader (BioTek, ELx800), the optical density (OD) was measured at 570 nm. By Equation (2), the cell viability was calculated.

\[
\text{Cell viability}\% = \left( \frac{\text{mean OD of tested cells}}{\text{mean OD of control cells}} \right) \times 100
\]

where OD is the optical density.

Antiviral and virucidal activities of CNPs

Antiviral and virucidal activities of CNPs were carried out against the tested viruses according to previous work (Abdel-Samad, 2019). As for the antiviral activity, Vero cells (precultured into 96-well plate) were split into two sets. One set was handled with a safe concentration of CNPs (as the treated group), while another set was handled with culture media (as the control group). The handled cells were incubated for 24 hours at 37°C, washed by
Dulbecco's phosphate-buffered saline, and then treated with the virus \([\text{multiplicity of infection (MOI)} = 0.1]\). The titer of virus (in treated and control groups) was determined by the TCID\(_{50}\) standard method \((\text{Reed and Muench, 1938})\) after incubation for 24 hours at 37°C.

For virucidal, equal volumes from the virus (MOI = 0.1) and the safe concentration of CNPs were mixed and incubated for 1, 3, 6, 18, and 24 hours at 37°C. The mixtures were added to Vero cells (previously cultured) and then incubated for 24 hours at 37°C. The virus’s titer was determined by the last-mentioned method.

**Statistical analysis**

The mean and standard deviation (SD) were used to display the data. GraphPad Prism version 8.0.2 was used to investigate the importance of differences between the values of the control and treated groups using a two-tailed independent Student’s \(t\)-test. The threshold for statistical significance was set at \(p < 0.05\).

**RESULTS AND DISCUSSION**

**Characterization of chitosan**

The chemical structure of chitosan was confirmed by the FT-IR spectrum, where the main functional groups were detected.
Successful deproteinization was inferred by absorption absence at 1,540 cm$^{-1}$ (Hassan et al., 2016b). Stretching of NH and OH free was observed at 3,433 cm$^{-1}$ (Kaya et al., 2015), while the bending of NH (NH$_2$) was represented at 1,629 cm$^{-1}$ (Song et al., 2013). The absorption at 1,358 and 1,425 cm$^{-1}$ bands was referred to as the symmetric and asymmetric stretching of CH (CH$_2$) (Gyliene et al., 2003), respectively, whereas the bending of this group was detected at 2,917 cm$^{-1}$ (Liu et al., 2013). The vibration of the pyranose ring skeletal was observed at 899 cm$^{-1}$ (Wanule et al., 2014), and the DDA was 74.51%.

**Characterization of CNPs**

From DLS analysis, the distribution size of CNPs was figured with one main peak representing 99.3% of the whole sample. This peak ranged from 37.84 to 78.82 nm, and the mean diameter was 54.12 nm. There is another tiny peak with a diameter of 272.5 nm (0.7%) (Fig. 2). The polydispersity index of 0.6 indicates the monodispersability of CNPs (Stetefeld et al., 2016).

The TEM image showed the cubic shape of CNPs with a diameter ranging between 22.40 and 36.37 nm (Fig. 3). The slight decrease in the CNPs between DLS analysis and TEM investigation was due to a hydrodynamic diameter (Hassan et al., 2016b).

**Antibacterial activity CNPs**

The CNPs showed antibacterial activity against all tested bacteria, as presented in Table 1. A high inhibition zone was detected with *B. subtilis* and *K. pneumoniae* as Gram-positive and Gram-negative bacteria, respectively. Generally, the
Gram-negative bacteria were more delicate to the CNPs than the Gram-positive ones. Minimum inhibitory concentration values of CNPs were 4,000, 4,000, 1,000, and 500 µg/ml against *S. aureus*, *B. subtilis*, *Escherichia coli*, and *Klebsiella pneumoniae*, respectively.

As shown in Figure 4, the CNPs caused morphological changes on treated *B. subtilis* (Hassan et al., 2016b) and *K. pneumoniae* (Didenko et al., 2005; Hassan et al., 2016b). The bacterial contents were leaked due to increasing the cell permeability that also led to bacterial deformation and rupture of bacteria (Abdel-Razek, 2019; Hassan et al., 2016b; Liu et al., 2004). The cell permeability was increased as a result of the electrostatic interaction between the positive charge of CNPs and the negative charge of the cell (Li et al., 2010; Wang et al., 2012). Moreover, the contrast between the size of CNPs and the bacterial size may have an impact on the antibacterial activity of CNPs (Hassan et al., 2016b).

**Cell viability**

Concerning cytotoxicity assessment of the CNPs, the Vero cells viability is contrarily corresponding with CNP concentration.
Antiviral and virucidal activities CNPs

Infectivity titers of adeno-40 and coxsackie B4 viruses as control were 4.6 and 5.4 log (10)/0.1 ml, respectively.

For antiviral activity, the infectivity titers of adeno-40 and coxsackie B4 viruses were reduced by a safe concentration of CNPs (80 µg/ml). As shown in Figure 6, this concentration substantially reduced adenovirus infectivity titer to 4.2 log (10)/0.1 ml (p < 0.05) and coxsackie B4 virus infectivity titer to 4.4 log (10)/0.1 ml (p < 0.01). The reduction percentages were about 5 and 26 for adeno-40 and coxsackie B4 viruses, respectively.

In the virucidal activity, the CNPs (80 µg/ml) demonstrated a significant reduction in adeno-40 and coxsackie B4 virus’s infectivity titers after incubation for 6 and 24 hours. Typically, after 1, 3, 6, and 24 hours of incubation with this concentration, the adeno-40 virus titer decreased to 4.6, 4.5, 3.6, and 3.3 log (10)/0.1 ml, respectively, while the titer of coxsackie B4 virus was 5.2, 4.7, 3.9, and 3.5 log (10)/0.1 ml, respectively (Fig. 7).

In the present study, the CNPs from American cockroaches protected the Vero cells from the cytopathic effect of adeno-40 and coxsackie B4 viruses, and it showed antiviral activity and suppressed the viral infection (Ai et al., 2012; Chirkov, 2002; Hassan et al., 2016). Also, the chitosan can protect from 3 influenza strains: H7N9, H1N1, and H9N2 (Zheng et al., 2016), and it has been proposed as a potential universal antiviral agent. Moreover, the chitosan derivatives may be used as new HIV drug candidates (Artan et al., 2008). Davydova et al. (2011) noted that the antiviral activity of chitosan is influenced by DA. Chitosan was discovered to significantly decrease the infectivity of the feline calicivirus FCV-F9, as well as the bacteriophages MS2 and phiX174 (Davis et al., 2012). Beta-chitosan has a promising development in veterinary medicines to treat Newcastle disease (He et al., 2016).

CONCLUSION

This study shows the effect of CNPs prepared from American cockroaches on E. coli, K. pneumoniae, S. aureus, and B. subtilis, as well as adeno-40 coxsackie B-4 viruses. CNPs were prepared by ball-milling from 74.51% DDA chitosan. The chitosan showed antibacterial activity and caused the deformation and rupture of bacteria. Furthermore, the CNPs protected the Vero cells and decreased the infectivity titer of adeno-40 and coxsackie B-4 virus. We recommend applying CNPs from American cockroaches as an antimicrobial agent in the management of infectious diseases.

AUTHOR CONTRIBUTIONS

Mahmoud T Mahboub: data acquisition; drafting manuscript; admin, technical, or material support; final approval. Mostafa I. Hassan: concept and design; critical revision of the manuscript; supervision; final approval. Ahmed S. Bream: supervision; final approval. Aly F. Mohamed: data acquisition; data analysis/interpretation; statistical analysis; admin, technical, or material support; supervision; final approval. Mohammad R. K. Abdel-Samad: concept and design; data acquisition; data analysis/interpretation; critical revision of the manuscript; statistical analysis; admin, technical, or material support; supervision; final approval.

CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

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ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

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