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The Influence of Titanium Dioxide Nanoparticles on the Ultrastructure of Bacterial and Fungal Cells, And Evaluation of Their Cytotoxicity

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ABSTRACT

The nanoparticles of titanium dioxide (Tio_2NPs) are among the most promising nanomaterials which widely used at the commercial level, especially in cosmetics, medicine and food industry. In our study, the antimicrobial effect of titanium dioxide nanoparticles on pathogenic micro-organisms was explained by transmission electron microscopy, which was used to investigate the mode of action of TiO_2NPs on bacterial and fungal cells. *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. and *Penecillium* spp. were greatly affected by TiO_2NPs. The cytotoxicity assay of Oral Epithelial cell (OEC) lines was studied using water soluble tetrazolium salt (WST-1 assay) for TiO_2 nanoparticles at different concentrations 0.01 mg/ml, 0.1 mg/ml, 0.3 mg/ml, 1 mg/ml and 10 mg/ml. The cell viability of the oral epithelial cell lines were 96.26 %, 83.16 %, 81.82 %, 76.94 % and 55.8 % respectively, and IC_{50} of TiO_2NPs was greater than 10 mg/ml concentration. The obtained results recommended the non-toxic nature of titanium dioxide nanoparticles and their antimicrobial activity against different food borne pathogens that would has a potentiality in the context of food preservation.

Keywords: Antimicrobial activity, Transmission electron microscopy, Cytotoxicity assay, WST-1 assay, IC_{50}.

1. Introduction

TiO_2 nanoparticles occupy a special position among various nanomaterials as they are distinguished by their high availability, high photocatalytic properties, non-toxic and favorable price which make them widely used in food, paints, printing inks, papers, plastics, biomedical and cosmetic products (Rashid *et al*., 2021). Biologically synthesized TiO_2NPs can efficiently inhibit both gram-negative and gram- positive bacteria (Marimuthu *et al*., 2013).

Transmission electron microscopy (TEM) allows the examination of microbial cells by providing a high resolution image of their internal macromolecular structures. For example, TEM can provide images of the bacterial cell envelopes, ribosomes, and nucleic acids, and this can help to characterize the damage appeared in the internal structures of the organisms after exposure to nanoparticles (Feliciano *et al*., 2012).

Cytotoxicity test is a rapid, standardized test that is very used to determine whether the nanoparticles produced are harmful or benign on cellular components. Different cell cytotoxicity assays were used among them WST-1 assay. The toxicity of nanoparticles in cell lines was quantified by determining IC_{50} values in WST-1 assay, which means the inhibitory concentrations that effected 50 % growth inhibition (Tu *et al.*, 2009; Rosli *et al*., 2015).

Investigation of the physicochemical properties of nanoparticles, and their performance, is necessary to assess their potential hazardous and toxic effects on biological systems and the human body. The toxicity of nanoparticles and their effect on the human body and surrounding biosystems depend on crystalline form (anatase and rutile), particle size, agglomeration of the nanoparticles, cells

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involved, cytotoxicity assay and concentration of the nanoparticles (Jin *et al*., 2008; Nasrollahzadeh and Sajadi, 2019; Hashem and Al-Karagoly, 2021).

The World Health Organization (WHO) stated that TiO_2NPs were poorly absorbed from the gastrointestinal tract which is the main route of exposure for the general population and they posed low hazard effect in mammals or aquatic species (*Oncorhynchus mykiss* and *Daphnia magna*). So it is accepted that TiO_2NPs were health friendly and nontoxic to biological environment (Warheit *et al*., 2007).

The objective of this study was to evaluate the effect of Tio_2NPs on pathogenic micro-organisms (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. and *Penecillium* spp.) with the help of TEM as an important tool for a better understanding. Also this study evaluated the cytotoxicity assay of Tio_2NPs.

2. Materials and Methods

2.1. The investigated microbes

The tested pathogenic bacteria isolated from food (*Staphylococcus aureus, Bacillus cereus* and *Salmonella*) were isolated from mozzarella cheese, shipsy and tuna respectively. The tested bacteria with 10^6cfu/ml were inoculated into flasks of nutrient broth media (100 µl into 100 ml) then 100 µl of TiO_2NPs with different concentrations (500 µg/ml, 1 mg/ml and 2 mg/ml) were added to each flask. The flasks were incubated in shaker incubator 150 rpm at 37 °C for 24 hrs. One flask with bacteria only used as a control (untreated with Tio_2NPs). All flasks were sent to electron microscope unit of Faculty of Science of Alexandria University to be examined and photographed by Transmission electron microscope. The tested pathogenic fungus *Penecillium* spp. was isolated from orange juice. The plates with the largest inhibition zone (which tested by well agar diffusion assay) and the control plate were sent directly to be examined and photographed by Transmission electron microscope.

2.2. Titanium dioxide nanoparticles

Titanium dioxide nanoparticles were biosynthesized by *Lactobacillus bulgaricus* which was kindly provided by Bacteriology Unit, Botany Department, Faculty of Science, Tanta University.

2.3. Preparation of samples for TEM analysis

- Microbial cells were collected after 24 hours of incubation and washed three times with saline solution, the samples were centrifuged at 10000 rpm for 10 min and the supernatant decanted.
- Cells were fixed in 2.5% buffered glutaraldehyde which prepared in 0.1 M phosphate buffer solution (PBS) at pH 7.4 for 2 hours at 4°C and centrifuged the at 10000 rpm for 5 min. The supernatants were decanted.
- The pellets rinsed three times in PBS for 10 min each time and centrifuged for 15 min at 3000 rpm.
- Cells were post fixed in 1% Osmic acid for 30 min.
- Cells were washed three times with PBS (10 min. each), then centrifuged for 5 min at 2000 rpm and resuspended.
- The fixed cells were cut into 1 mm cubed blocks, then stored in the buffer overnight at 4 °C, then the cells were rinsed two times in distilled water and put into 1% uranyl acetate for 90 min. They were rinsed two times in distilled water before dehydration
- With ascending series of ethyl alcohol (30, 50, 70, 90% and absolute alcohol), these cells were dehydrated with each concentration for 30 min and then Infiltrated with acetone for 1 hour at 37 °C.
- In TEM, after dehydration samples were embedded in Araldite 502 resin.

The plastic molds were cut in the LEICA Ultracut UCT ultra-microtome, stained with 1% toleudine blue. After examination of semi-then sections ultra-thin sections were cut, stained with 2% aqueous uranyl acetate for 20 min, washed with distilled water, then counter stained with lead citrate for 15 min and washed again with distilled water. After air drying, TEM images of the cells were examined and photographed using JEOL-JEM-100 SX electron microscope, Japan (Reynolds, 1963; Feliciano *et al*., 2012).

2.4. Cytotoxicity assay of TiO_2 nanoparticles

Cell culture

Human Oral Epithelial Cell (OEC) line was obtained from Nawah Scientific Inc., (Mokatam; Cairo, Egypt). Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin, 10% of heat-inactivated fetal bovine serumin humidified and 5% (v/v) CO_2 atmosphere at 37 °C (Huth *et al*., 2006).

2.4.1. Cytotoxicity assay

Cell viability was assessed by water soluble tetrazolium salt (WST-1) assay using Abcam® kit (ab155902 WST-1 Cell Proliferation Reagent). Aliquots of 50 µL cell suspension ($3x10^3$cells) were seeded in 96-well plates and incubated in complete media for 24 hrs. Cells were treated with another aliquot of 50 µL media containing drugs (Tio_2NPs) at serial concentrations (0.01, 0.1, 0.3, 1, 10 mg/ml). After 48 hours of drug (Tio_2NPs) exposure, cells were treated with 10 µL WST-1 reagent and the absorbance was measured after one hour at 450 nm using a BMG LABTECH® - FLUOstar Omega microplate reader (Allmendgrün, Ortenberg) (Huth *et al*., 2006; Tu *et al*., 2009).

3. Results and Discussion

3.1. Antimicrobial activity of titanium dioxide nanoparticles

The biosynthesized TiO_2 nanoparticles (rutile, tetragonal with 16.3 nm size) had antibacterial activity against gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and gram negative bacteria (*Salmonella*). Also TiO_2NPs had antifungal activity against pathogenic fungus (*Penecillium* spp.). The antimicrobial activity observed by the formation of inhibition zone with diameter (20 nm, 17 nm, 25 nm and 27 nm respectively). The antimicrobial activity was confirmed by transmission electron microscope (TEM) as presented by Figures (1:9). Electron microscopy was used to investigate the mechanism part of action of TiO_2NPs on microbial cells by determining if there was a differentiation in the cells morphology as a result of TiO_2NPs and compared with untreated cells (control).

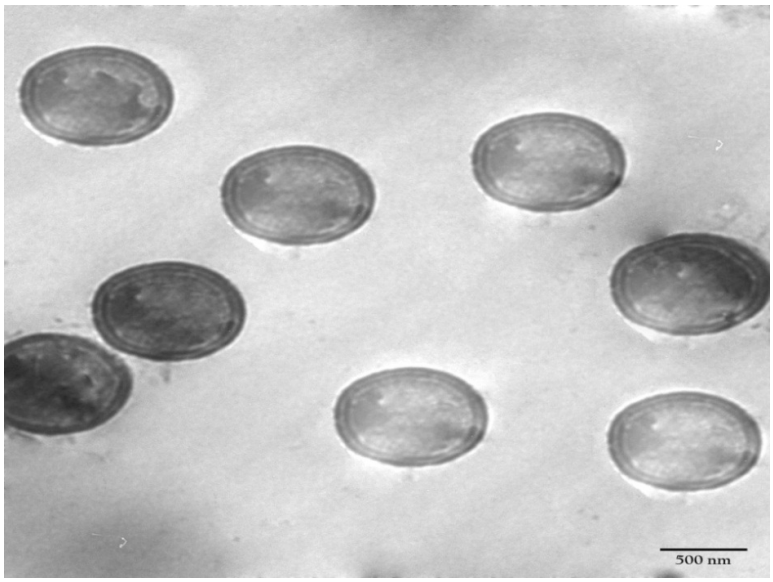

Fig. 1: Photomicrograph of the control (untreated with Tio_2NPs) cells of *Staphylococcus aureus*. It shows that all the cellular constituents appeared normally with intact cell wall.

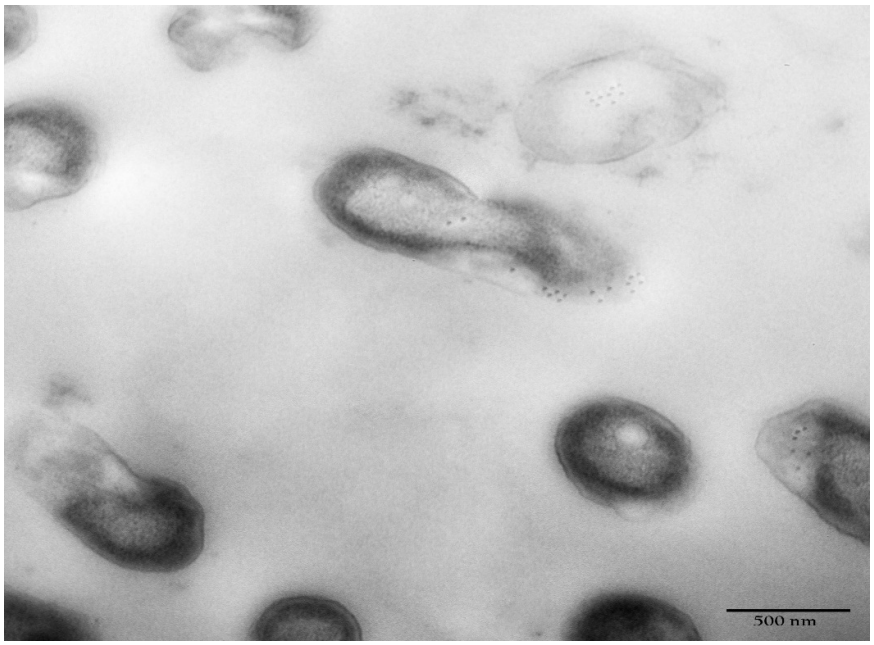

Fig. 2: Photomicrograph of the TiO_2NPs treated cells of *Staphylococcus aureus*. It shows distortion of cocci shape of bacterial cells, cell lysis, releasing of the cell constituents and shrinking of internal components of bacterial cells due to vaculation.

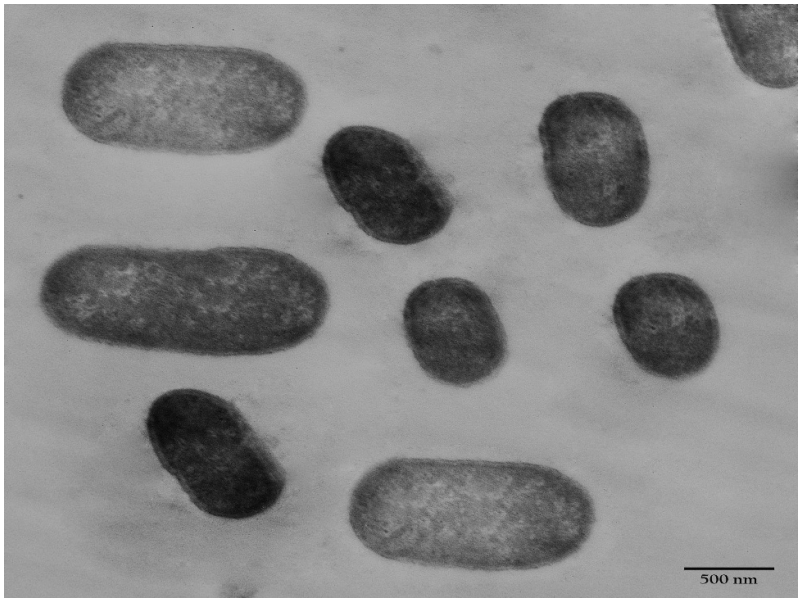

Fig. 3: Photomicrograph of the control (untreated with Tio_2NPs) cells of *Bacillus cereus*. It shows normal bacilli cells, with thick cell wall and homogenous cytoplasm.

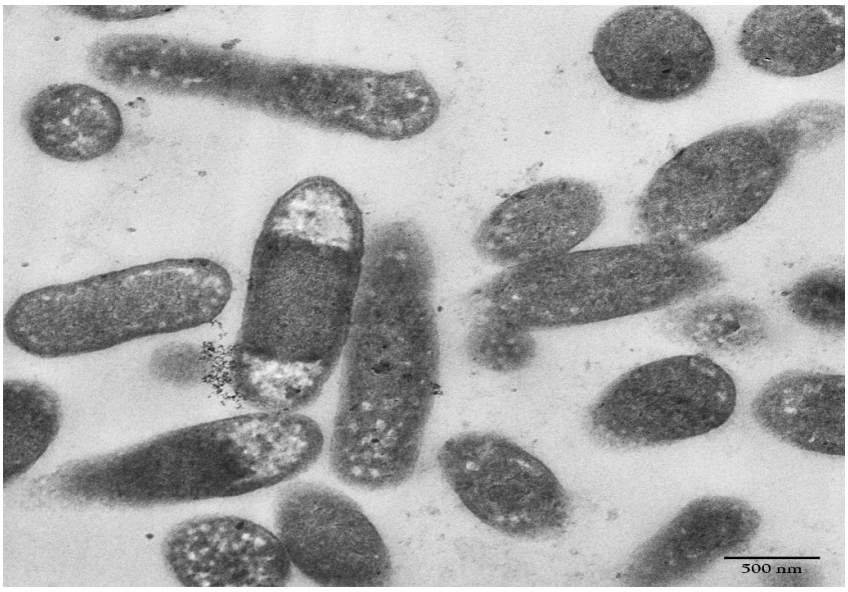

Fig. 4: Photomicrograph of the TiO_2NPs treated cells of *Bacillus cereus*. It shows aggregation of nanoparticles inside and outside the cells which caused distortion, complete lysis of some bacterial cells and partial lysis to the other cells compared with the normal control cells.

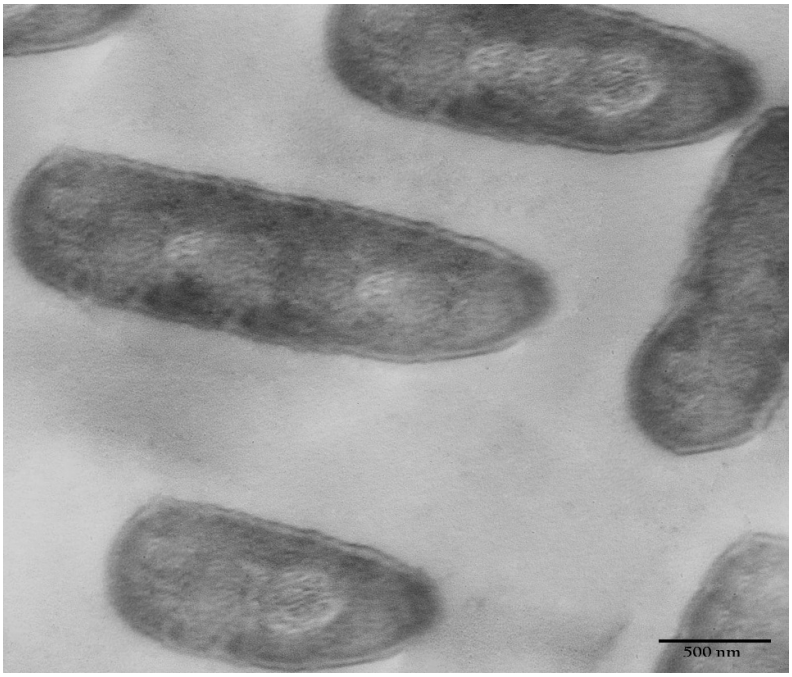

Fig. 5: Photomicrograph of the control (untreated with Tio_2NPs) cells of *Salmonella* spp. It shows normal bacilli cells of *Salmonella* spp. that maintained a uniformly dense internal cytoplasmic cellular component with a continuous smooth cell envelope.

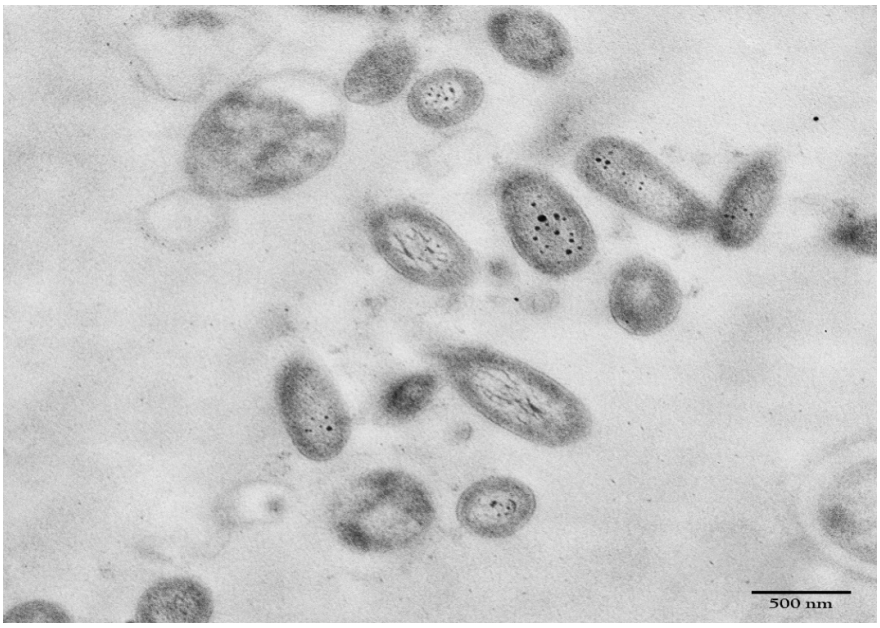

Fig. 6: Photomicrograph of the TiO_2NPs treated cells of *Salmonella* spp. It shows aggregation of nanoparticles inside and outside the cells which caused distortion and complete lysis of the bacterial cells compared with normal control cells. It shows partial lysis of some bacterial cells, rupture of cell wall, irregulation of cell wall of cells and aggregation of nanoparticles inside the cells. The presence of internal components in the surrounding outside the cells is also observed, Swollen of bacterial cell with shrinking in internal components and the other cells appeared completely lysied. Nanoparticles appear in regular arrangement on debris cell wall of bacterial cell.

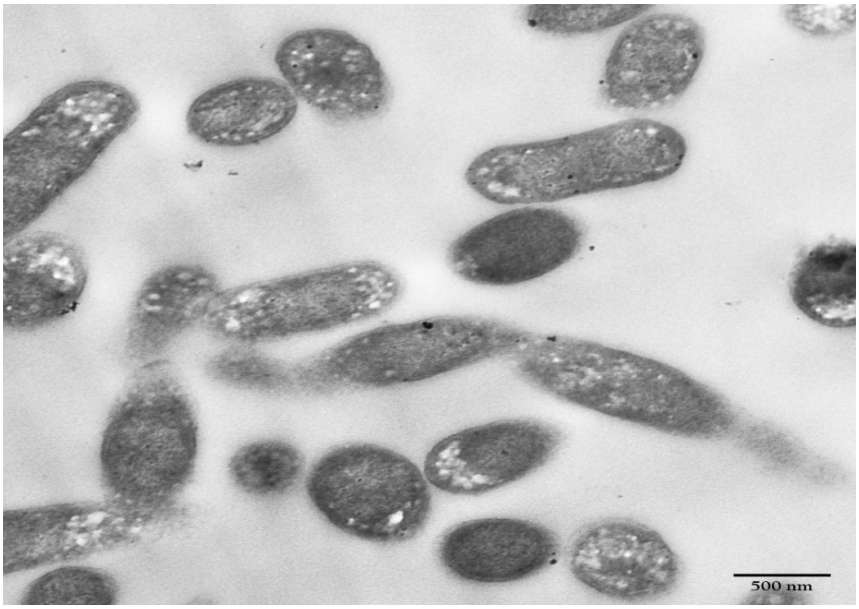

Fig. 7: Photomicrograph of TiO_2NPs treated cells of *Salmonella* spp. It shows small inner vacuoles, disappearance of septa between some cells, rupture of cell wall of most of cells and releasing of internal components outside the bacterial cells. All cells in the figure appeared abnormal compared to control cells.

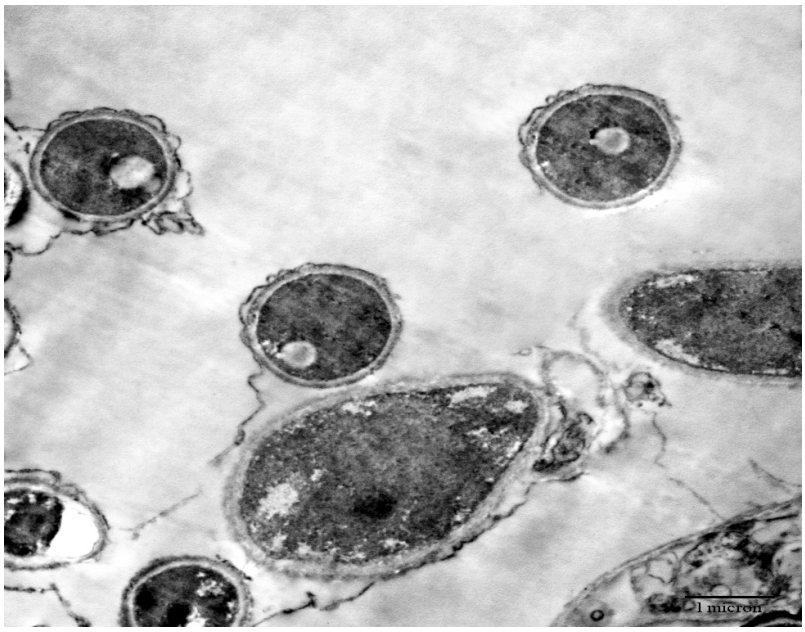

Fig. 8: Photomicrograph of the control (untreated with Tio_2NPs) spores of *Penecillium* spp. It shows normal internal components (air vacuole in all spores, mitochondria, lamellar body and homogenous cytoplasm). All spores with intact, regular and thick cell wall.

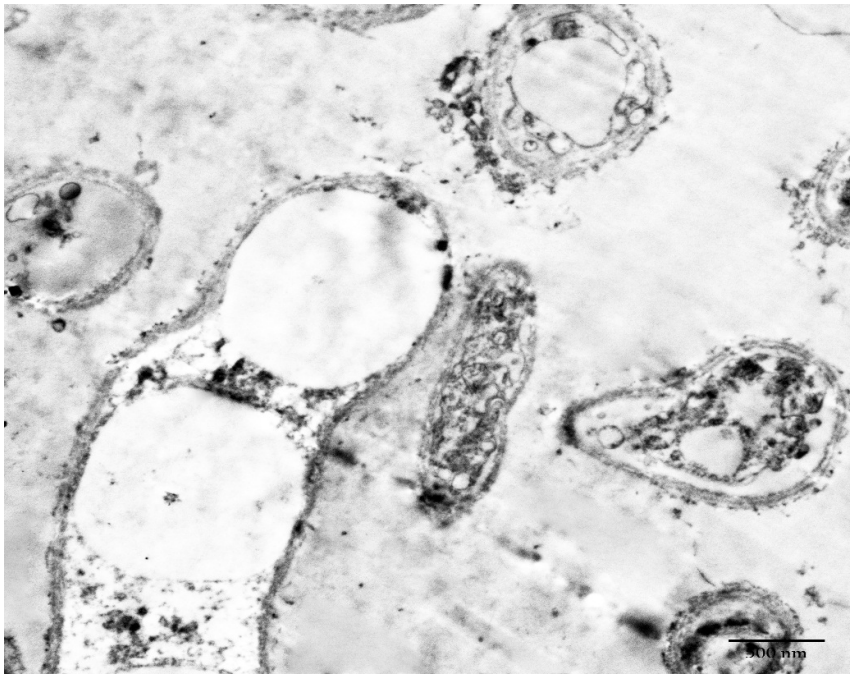

Fig. 9: Photomicrograph of TiO_2NPs treated cells of *Penecillium* spp. It shows debris of spores in the surrounding, ruptured cell wall and lysis of internal components. Abnormal feature of spores compared to the control ones. Hyphae show weak appearance of septa, lysis of internal components, aggregation of nanoparticles on the cell wall and inside hyphae.

The results of antibacterial activity in our study were similar to the results of Rajakumar *et al.,* (2012), Jesline *et al.,* (2015) and Mahdy *et al.,* (2017). The bactericidal and fungicidal activities of

TiO_2NPs were reported against *Staphylococcus aureus* (Razzaq *et al*., 2021), *Aspergillus niger* and *Penecillium* (Yu *et al*., 2013), these results similar to the results of our study. The toxicity mechanism of TiO_2 nanoparticles to microorganisms can be resulted from the generation of reactive oxygen species (ROS), attachment to the cell wall by electrostatic force owing to their large surface area and this resulted in cell wall damage and lipid peroxidation, cytoplasm flow out and TiO_2 nanoparticles attached to intracellular organelles and biological macromolecules following their cell membranes damage (Hou *et al*. 2018). Also the mechanism of action of TiO_2NPs on microbial cells was illustrated by Rajakumar *et al.* (2012) and Ziental *et al.* (2020).

3.2. Cytotoxicity assay of titanium dioxide nanoparticles

The effect of nanoparticles on human health has received many studies in vitro on various human cells (Niska *et al.*, 2015). The crystalline form, particle size, agglomeration of the nanoparticles, cells involved and concentration of the nanoparticles play an important role in determining their toxicity (Jin *et al*., 2008; Ziental *et al*., 2020). Magdolenova *et al.* (2012) suggested that the method of dispersing of the nanoparticles prior to treatment also may affect the resulting toxicity.

The present study evaluated the cytotoxicity of Oral Epithelial cell (OEC) line using WST-1 assay for TiO_2 nanoparticles at various doses of concentrations 0.01 mg/ml, 0.1 mg/ml, 0.3 mg/ml, 1 mg/ml and 10 mg/ml. The cell viability of the oral epithelial cell lines decreased with increasing the concentration of TiO_2 nanoparticles as presented in Figure 10 and Table 1. At concentration of 10 mg/ml of TiO_2NPs the viability decreased to 55.8 % so IC_{50} (the inhibition concentration at which half of the viable cells died) of biosynthesized TiO_2NPs was greater than 10 mg/ml concentration. This indicated the non-toxic nature of the nanoparticles even at high concentration (10 mg/ml).

Our results were similar to the results of Hashem and Al-Karagoly (2021) in which the treatment of L929 mouse fibroblast cell lines with different concentrations of TiO_2NPs ranging from 5:100 µg/mL showed no cytotoxicity.

Our results in agreement with the results of Browning *et al.* (2015) that used human skin fibroblast cell line which treated with 10, 50 and 100 µg/cm^2 of TiO_2NPs, the resulting cell viability after 24 hour exposure were 97.8 %, 88.8 % and 84.7 % respectively. The treatment of HCT116 cell line with TiO_2NPs resulted in survival and growth, which was agreed with the study that found that TiO_2NPs promoted cell viability and proliferation in NIH 3T3 cell line in a dose- and time-dependent manner (Bhattacharya *et al*., 2009; Huang *et al*., 2009).

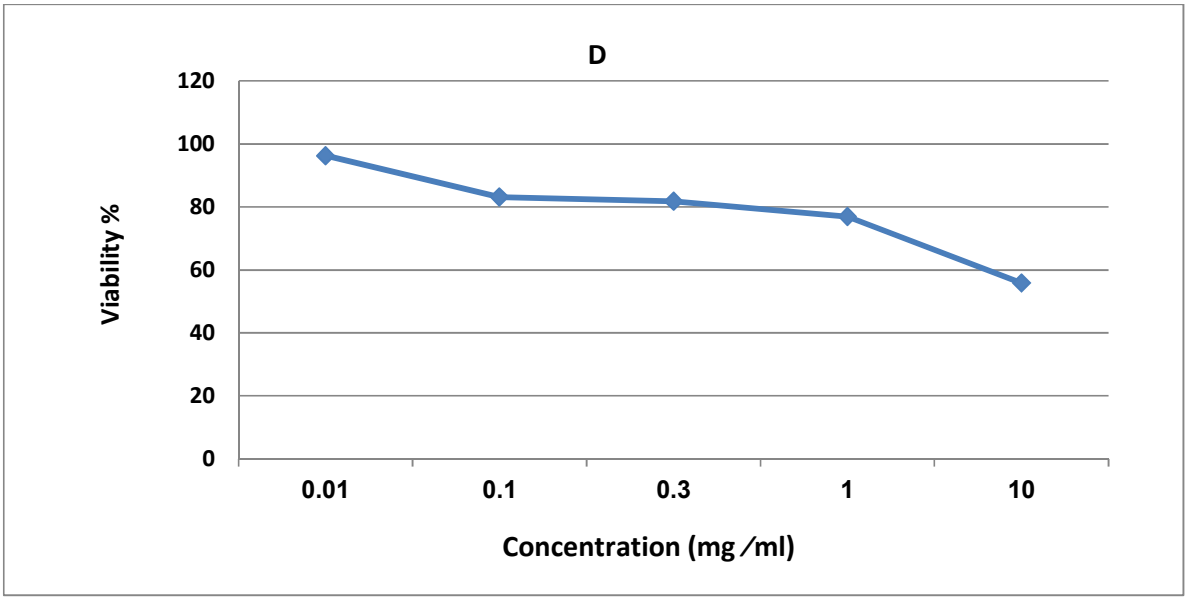

Fig. 10: The cell viability of OEC line when treated with different concentrations of TiO_2NPs.

Table 1: The mean of cell viability percentage with TiO_2NPs concentrations

Concentration (mg/ ml)	Mean Viability % ± SD
0.01	96.2666 ± 0.0451
0.1	83.1607 ± 0.7062
0.3	81.8217 ± 0.8369
1	76.9449 ± 0.7286
10	55.8053 ± 0.5507

Mean viability % ± standard deviation.

Conclusion

Our study demonstrated that Tio_2NPs significantly affect the cellular ultrastructure of the pathogenic food-borne microorganisms (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. and *Penecillium* spp.). Meanwhile, the cytotoxicity assay of Tio_2NPs on oral epithelial cell line revealed the non-toxic effect of Tio_2NPs on human OEC at concentration 10 mg/ml. So Tio_2NPs with concentration not more than 10 mg/ ml, can be safe used in various applications including; food industries, food preservation due to their antimicrobial activity, drugs and personal care products.

Reference

Bhattacharya, K., M. Davoren, J. Boertz, R.P. Schins and E. Hoffmann *et al.,* 2009. Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not DNA-breakage in human lung cells. Part Fibre Toxicol., 6:17.

Browning, C.L., T. The, M.D. Mason and J.P. Wise, 2015. Titanium dioxide nanoparticles are not cytotoxic or clastogenic in human skin cells. Journal Environ Anal Toxicol., 4(6): 10.

Feliciano, L., J. Lee and M.A. Pascall, 2012. Transmission electron microscopic analysis showing structural changes to bacterial cells treated with electrolyzed water and an acidic sanitizer. Journal of Food Science and Technology, 77(4): 182-187.

Hashem, M., and H. Al-Karagoly, 2021. Synthesis, characterization, and cytotoxicity of titanium dioxide nanoparticles and *in vitro* study of its impact on lead concentrations in bovine blood and milk. Journal of Biotechnology Research, 12: 93-105.

Hou, J., L. Wang, C. Wang, S. Zhang, H. Liu, S. Li and X. Wang, 2018. Toxicity and mechanisms of action of titanium dioxide nanoparticles in living organisms. Journal of environmental Science, 6-10.

Huang, S., P.J. Chueh, Y.W. Lin, T.S. Shih and S.M. Chuang, 2009. Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO_2 long-term exposure. Toxicology Applied Pharmacology, 241(2): 182-194.

Huth, K.C., F.M. Jakob, B. Saugel, C. Cappello, E. Paschos, R. Hollweck, and K. Brand, 2006. Effect of ozone on oral cells compared with established antimicrobials. European Journal of Oral Sciences, 114 (5): 435-440.

Jesline, A., P.N. John, M.P. Narayanan, C. Vani, and S. Murugan, 2015. Antimicrobial activity of zinc and titanium dioxide nanoparticles against biofilm- producing methicillin- resistant *Staphylococcus aureus*. Applied Nanoscience, 5:157-162.

Jin, C.Y., B.S. Zhu, X.F. Wang, and Q.H. Lu, 2008. Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. Chem. Res. Toxicol. 21(9): 1871-1877.

Magdolenova, Z., D. Bilaničová, G. Pojana, L.M. Fjellsb, A. Hudecova, *et al.,* 2012. Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. J. Environ. Monit., 14: 455–464.

Mahdy, A.S., H.W. Mohammed, H. Emad, A.H. Kareem, R. Shamel, and S. Mahdi, 2017. The antibacterial activity of TiO_2 nanoparticles. Journal of Babylon University, Pure and Applied Sciences, 25(3): 955-961.

Marimuthu, S., A.A. Rahuman, C. Jayaseelan, A.V. Kirthi, T. Santhoshkumar, K. Velayutham, A. Bagavan, C. Kamaraj, G. Elango, and M. Iyappan, 2013. Acaricidal activity of synthesized titanium dioxide nanoparticles using *Calotropis Gigantea* against *Rhipicephalus microplus* and *Haemaphysalis bispinosa*. Asian Pacific J. Trop. Med., 6 (9): 682–688.

Nasrollahzadeh, M.,and S.M. Sajadi, 2019. Risks of nanotechnology to human life. International Journal of Interface Science and Technology, 28: 323-336.

Niska, K., K. Pyszka, C. Tukaj, M. Wozniak, M.W. Radomski, and I. Inkielewicz-Stepniak, 2015. Titanium dioxide nanoparticles enhance production of superoxide anion and alter the antioxidant system in human osteoblast cells. International Journal of Nanomedicine, 10: 1095-1107.

Rajakumar, G., A. Abdul Rahuman, S.M. Mohana Roopan, V.G. Khanna, G. Elango, C. Kamaraj, A. Abduz Zahir, and K. Velayutham, 2012. Fungus-mediated biosynthesis and characterization of TiO_2 nanoparticles and their activity against pathogenic bacteria. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, Part A, 91:23– 29.

Rashid, M. M., P.F. Tavcer, and B. Tomsic, 2021. Influence of titanium dioxide nanoparticles on human health and the Environment. Nanomaterials, 11: 2354.

Razzaq, Z., A. Khalid, P. Ahmad, M. Farooq, M.U. Khandaker, A. Sulieman, I.U. Rehman, S. Shakeel, and A. Khan, 2021. Photocatalytic and Antibacterial Potency of titanium dioxide nanoparticles: A cost-effective and environmentally friendly media for treatment of air and wastewater. Catalysts, (11): 709.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology, 17: 208-212.*

Rosli, N.S.B., A.A. Rahman, A.A. Aziz, and S. Shamsuddin, 2015. Determining the size and concentration dependence of gold nanoparticles in vitro cytotoxicity (IC_{50}) test using WST-1 assay. *In AIP Conference Proceedings*, 1657(1).

Tu, M.G., W.M. Liang, T.C. Wu, and S.Y. Chen, 2009. Evaluation of cytotoxicity of resin bonding materials toward human oral epithelial cells using three assay systems. Elsevier, Journal of Dental Sciences, 4(4): 178-186.

Warheit, D.B., R.A. Hoke, C. Finlay, E.M. Donner, K.L. Reed, and C.M. Sayes, 2007. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. Toxicology Letters, 171(3): 99-110.

Yu, K.P., Y.T. Huang, and S.C. Yang, 2013. The antifungal efficacy of nano-metals supported TiO2 and ozone on the resistant *Aspergillus niger* spore. Journal of Hazards Materials, 261: 155–162.

Ziental, D., B. Czarczynska-Goslinska, D.T. Mlynarczyk, A. Glowacka-Sobotta, B. Stanisz, T. Goslinski, and L. Sobotta, 2020. Titanium dioxide nanoparticles: prospects and applications in medicine. Nanomaterials, 10: 387-390.