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RESEARCH ARTICLE

Assessment of Antibacterial, Cytotoxicity and Wound Healing Influence of Copper Nanoparticles Synthesized Using Probiotic Bacteria

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Abstract

The present article targeted to biosynthesis and description of copper oxide nanoparticles (CuO NPs) using probiotic isolate and assessment the antibacterial, cytotoxicity of these NPs and its effect on wound heals. The CuO NPs are biosynthesized using probiotic isolate identified as *Bacillus cereus*. These CuO NPs are characterized using UV, FT-IR, Zeta and TEM. The antibacterial efficiency is assayed by agar plate diffusion protocol. Results assured that the CuO NPs can prevent *Staphylococcus aureus* jointly *Escherichia coli* growth. MTT procedure exercised to detect cytotoxicity and wound healing effects. The results revealed that CuO NPs can use as antibacterial agent with non-significant effects on host cells and wound healing.

Keyword: *Bacillus cereus*, Copper oxide nanoparticles, characterization, antibacterial impact, cytotoxicity, wound healing.

Introduction

The biosynthesis of nanoparticles (NPs) using microbial sources is an ecofriendly and very economic method, so, is look as creative method than physical and chemical procedures. Prokaryote act as a major producer for NPs biosynthesis, own properties like simple for cultivation, short incubation period, biosynthesis of NPs was extracellular, more stable and easy experimental condition. Several articles have revealed that different genus of microorganisms such as *Lactobacillus casei*, Actinomycetes and *Aspergillus* sp. can be used for reducing certain metal salts to NPs[1,2,3].

Probiotics covered human body in several parts like mouth, skin and gut. Probiotics has been extensively used for zinc and copper NPs, can be reduce metals in a fast process through their incubation period and producing many types of enzymes. Probiotics was recommended as mendicancy and clinically use for human health of different organs [4,5,6].

Nanoparticles own applications in biological fields like biotechnology and medicine, and non-biological science like microelectronics, information storage, optics and energy-conversion[7]. Copper nanoparticles (CuO NPs) have extensively uses as highly strong sensors, substance and antibacterial agents through their high potency and reaction with other substance. Several researchers confirmed that CuO NPs strong agent as antibacterial against several bacterial and fungal pathogens[8]. In rapprochement to NPs types, CuO NPs have received special notices through its special physical characters such as stability and conductivity, and chemical properties like catalytic, moreover biological activity, for example anticancer and antibacterial activity[2,9, 10].

In our research, we used probiotic bacteria from caw milk to installation CuO NPs. then some characterization analysis were achieved of NPs using UV, FT-IR, Zeta and TEM techniques. We used Agar Well-diffusion for assays the antibacterial effect of CuO NPs on *S. aureus* jointl *E coli*. We elucidate the antibacterial, cytotoxic efficiency of these NPs on vero cell lines and its effect on wound healing activity on cell line.

Materials and Methods

Probiotics source and growth conditions

Probiotics isolate in our study was isolated from Egyptian caw milk and propagated on MRS broth [11]. Turbidity of bacterial growth was calibrated using 0.5 McFarland standards where hundred microliter ($1x10^7$ cells/ml) of bacterial growth was used as standard inoculum and incubated for 48 h static at 35°C.

Molecular identification probiotic bacteria

The 16S ribosomal DNA gene of the chosen isolate was amplified by PCR. Direct sequencing was consumed to determine the 16S rDNA sequence. Utilizing the Wizard genomic DNA purification kit, total DNA was extracted (Promega, Madison, USA) primers for DNA sequencing and PCR. SPO/SP6, a universal primer for Grame positive bacillus bacteria, was used in

the PCR amplification to target specific 16S ribosomal DNA regions. Applications for 16S rDNA PCR were refined using the Microcon YM-100 kit product from (Bedford, MA, USA) and sequenced by the Big Dye Terminator V3.0 kit according to the instructions. The sequencing of PCR strands results was performed by the supplier with primers (ABI, Forster, USA). By aligning the sequence assent of the 16S rDNA genes found in database, the sequences acquired consequently employed for phylogenetic analysis (Gene bank). After that, the data were evident as a relation of similarity between the sequence that was submitted and those that came from the software [12].

Probiotic CuO NPs synthesis

Bacillus cereus was cultured in sugar tubes containing MRS broth for 24 h at 35°C. The liquid culture pH was then brought down to 9 by 1 N NaOH to stall the transformation reaction. The mixture was infused with a 1mM aqueous solution of $CuSO_4$ before being incubated for 48 hours at 37°C. At the bottom of the flask, the colour changed to a dark brown, signifying the creation of nanoparticles. The CuO NPs cleaned by deionized water, and then oven for four hours at 40°C[2].

Characterization of CuO NPs

UV–Vis spectrophotometry

The ultra violet dissection of CuO NPs utter by (T80+UV/VIS Spectrometer, PG Instrument Ltd., UK), then measured to determine the absorbance of particles over an absorbance range of 190 to 1000 nm until no more absorbance changes were detected.

Fourier transforms infrared spectroscopy (FTIR) spectrum

By combining with potassium bromide at a ratio of 1: 100 and compressing to a 2 mm disc for 2 min, an FTIR spectrum was produced. Utilizing a Nexus 670 FTIR spectrophotometer, spectra between the wave lengths (4000 and 400 cm-1) was captured (Iclet Co., USA).

Transmission electron microscopy (TEM)

TEM model Japan, (Electron probe micro-analyzer JEOL – JXA 840A) images for nanoparticles provide specialized information on morphological properties, such as shape and size. The sample was prepared using drop covered onto a grid that had been coated with carbon. After being vacuum dried, the samples were loaded into a specimen holder. Analysis of the ready grids resulted in the taking of TEM micrographs.

Zeta potential Measurements

Surface zeta possibilities were measured utilizing the laser zeta meter (Molecule Measuring Frameworks NICOMP, Inc. Santa Barbara, Calif., USA). Fluid tests of the NPs (5ml) were weakened with twofold refined water (50 mL). The tests were shaken for 3 minutes. After shaking, the harmony pH was recorded and the zeta potential of particles was measured. Zeta potential was utilized to decide the surface potential of NPs. In each case, an normal of three isolated estimations was detailed. The criteria of steadiness of NPs are measured when the values of zeta potential extended from +20 mV to -20 mV.

Antibiotic susceptibility test

The test pathogens *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739 sub cultured. The antibiotic resistance test against the test pathogens strains were evaluated using the disc plate diffusion method and were complete conformity with the roles of the Clinical and Laboratory Standards Institute (CLSI) [13]. The major four types of antibiotics examined, according to their mode of action, are bacterial wall, protein, membrane and DNA. Based on the inhibitory zone diameter (mm), test pathogens were incubated for 24 hours at 37°C, and the findings were seen and recorded as sensitive or resistant. [14].

Antibacterial activity and MIC of CuO NPs

The antibacterial potency of CuO NPs against test pathogens was determined by agar plate diffusion protocol [8]. A pure microbial colony of test pathogens was growing in nutrient broth. Spread the suspension of test pathogens on the medium plates with a sterile cotton swab, then make holes with a sterile cork drill and add CuO NPs concentrations (100, 110, 120, 130, and 140 µg/ml). Incubate the plate for 24 hours at 37 ^{o}C and finally measure the inhibitory zone.

MTT protocol method

Determination of CuO NPs cytotoxicity on normal vero CCL-81 cells, kidney epithelial tissue by MTT method was carried. The procedure is as follows: - To grow intact monolayers, 96-well tissue culture plates are seeded at 1 x 10^5 cells/ml (100 µl/well) and incubated for 24 hours at 37°C. After a confluent cell layer has formed, pour growth medium from the 96-well microtiter plate and wash the cell monolayer twice with wash medium. D dilutions of test samples were prepared in RPMI medium. Leave 3 wells as controls, receiving maintenance medium only, and measure 0.1 ml load to well for every dilution. Incubate the plate at 37 °C and test. Prepare a solution (5 mg/ml in PBS). Then, 20 µl of the solution was added to each well. Then place on a shaker at 150 rpm for 5 min. Then, in order for the MTT to be metabolized, incubate for 1-5 h at (37oC, 5% CO2). Drain the media (to remove any debris). Resuspend in 200 µl DMSO formazan (MTT metabolite). Then mix the formazan into the solvent and shake at 150 rpm for 5 min. At 560 nm, read optical density at 620 nm [15].

Results and Discussion

Probiotic identification

Probiotic isolate was identified after passing through the probiotic testes characterization, then 16S rDNA fragment used for gene sequence analysis. According to the sequence thesoft ware used to produce phylogenetic figure 1, our isolate identified

with similarity 100 %as *Bacillus cereus*. Also, some systemic classified strain as opportunistic bacteria can cause diseases, but others studies involving strain as probiotic [16].

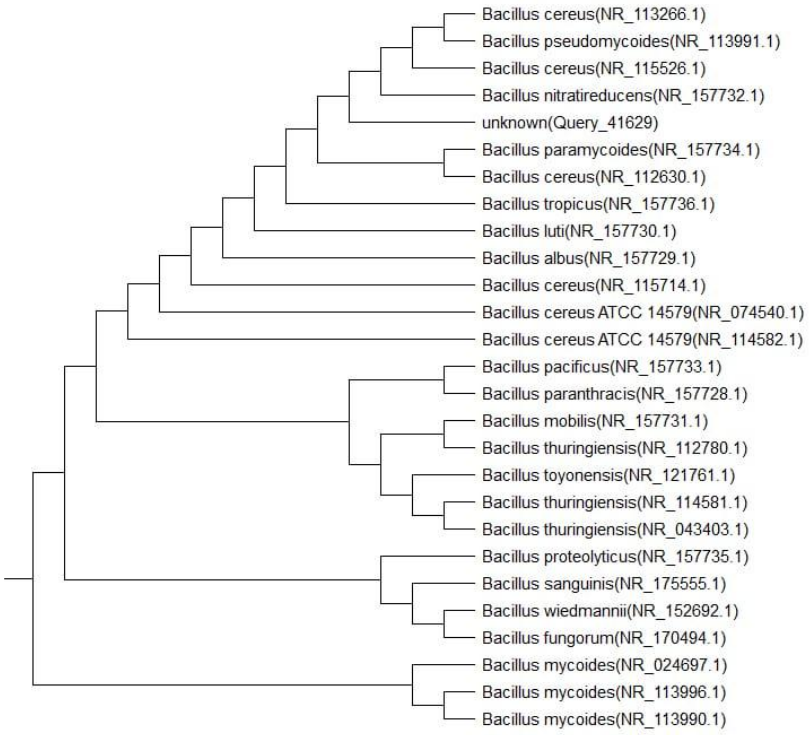

Figure 1: Phylogenetic tree of probiotic isolate identified as *Bacillus cereus*

Characterization of CuO NPs

Ultra Violet (UV) analysis

The biosynthesized CuO NPs from *Bacillus cereus* using $CuSO_4$ as a substrate was obtained at the bottom of flask. The UV spectrum peak was seen at 202 to 220 nm. The absorption peak was detected the confirmation of CuO NPs production in solution. Other green synthesis studies represented the peak in rang 200-270 nm, confirming the production of CuO NPs [17, 18].

FT-IR spectra

Characterization of bacterial synthesis using FT-IR was done to reveal functional groups in CuO NPs. The visible spectral range is 500-4000 cm^{-1} (Fig. 2). FT-IR spectra show signals at 3277-3729 cm^{-1} indicating N-H stretching vibrations of amine groups or amide signals in *Bacillus cereus* membrane proteins. The absence of signal at 2926 cm^{-1} is characteristic of the C-H stretch of the alkene group and is an indicator of purity. The bands at 1642 cm^{-1} and 1411 cm^{-1} correspond to the bond vibrations of the amide I and II signals of the Bacillus cereus protein molecule in the membrane, respectively. The observed signals at 1411 and 1236 cm^{-1} can be attributed to the C-N stretching vibrations of aromatic and aliphatic amines, respectively. The weaker signal at 1024 cm^{-1} corresponds to the C-O stretching vibrations induced by the -COOH and -OH groups, respectively [2, 17].

The zeta potential test of CuO NPs

The average zeta potential analysis of CuO NPs shows at Figure 3 revealed that NPs surface have negative charges -2.24 mV, similarly to other CuO NPs zeta potential produced from green synthesis [17].

TEM of biosynthesized CuO NPs

TEM images were taken to determine the particles shape and average size. TEM images at Figure 4 represent CuO NPs are cubic shape and seen size from 7 to 19 nm in with 10 nm average with 100 and 200 nm resolution powers. On other study, green synthesis of CuO-NPs TEM images was result spherical shape, overlap [17]. But, CuO-NPs produced from bacteria formed NPs were in spherical shape and size range was in 40-110 nm at 300 nm power [2].

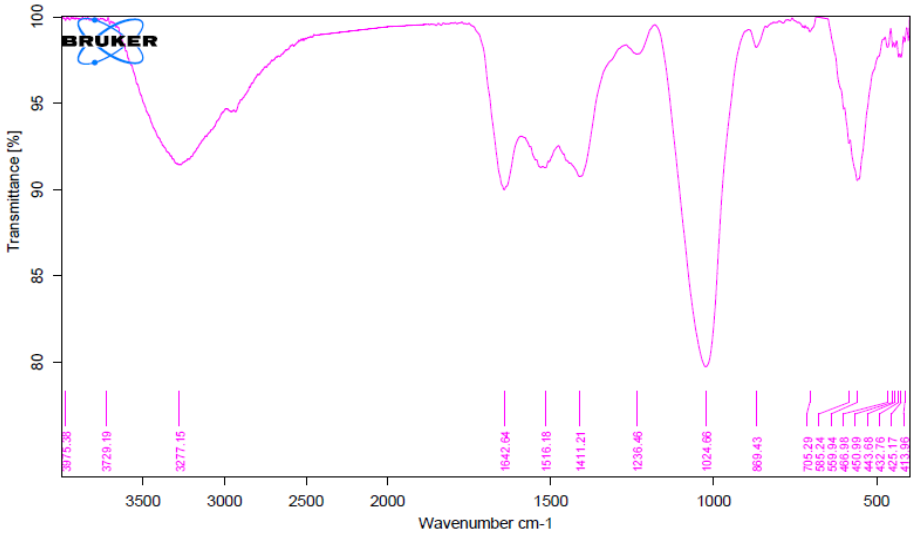

Figure 2: FT-IR spectra of CuO NPs present from probiotic *Bacillus cereus*

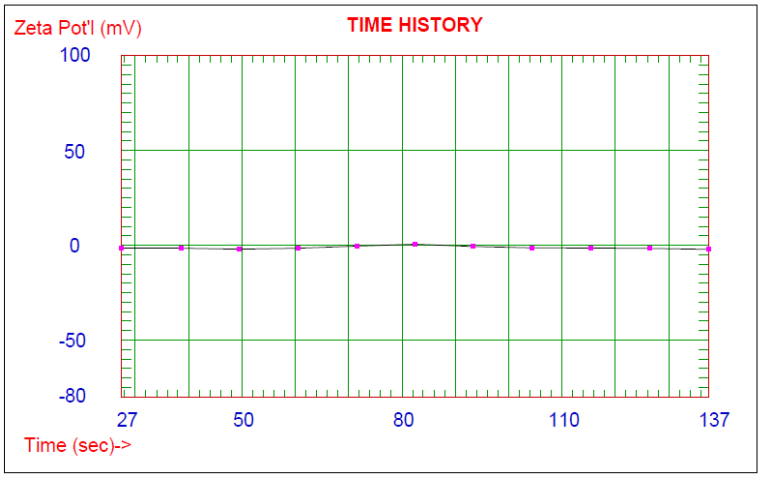

Figure 3: Zeta potential of surface charges of biosynthesized CuO NPs

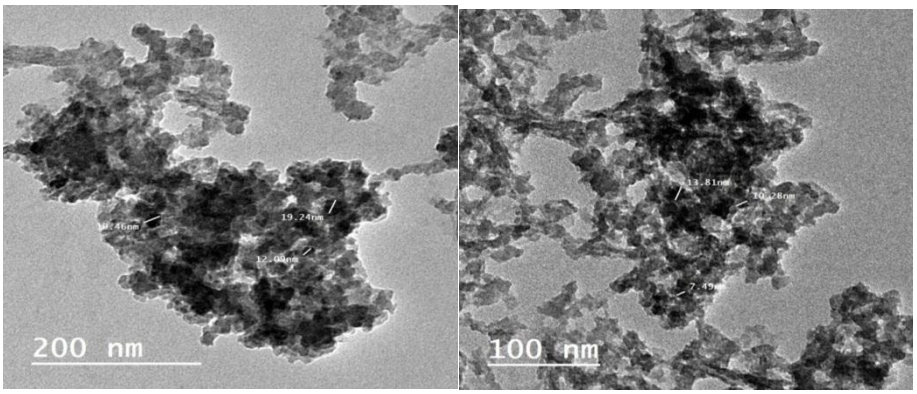

Figure 4: TEM images of biosynthesized CuO NPs with 100 and 200 nm magnification

Antibiotic sensitivity of tested microorganisms

By using several 11 types of antibiotics to test the sensitivity of *Staphylococcus aureus* and *Escherichia coli* to antibiotics, results in figure 5 explain the inhibition zone diameter of tested strains. Both tested strains were sensitive to six antibiotics from 11 and resist to five others. Both pathogens recorded sensitivity to Tobramycin, Azithromycin, Piperacillin/Tazobactam and Streptomycin. The most potent antibiotics recorded in case of *S. aureus* were Tobramycin with zone of inhibition 31 mm, and Streptomycin for *Escherichia coli* with inhibitory zone 28 mm.

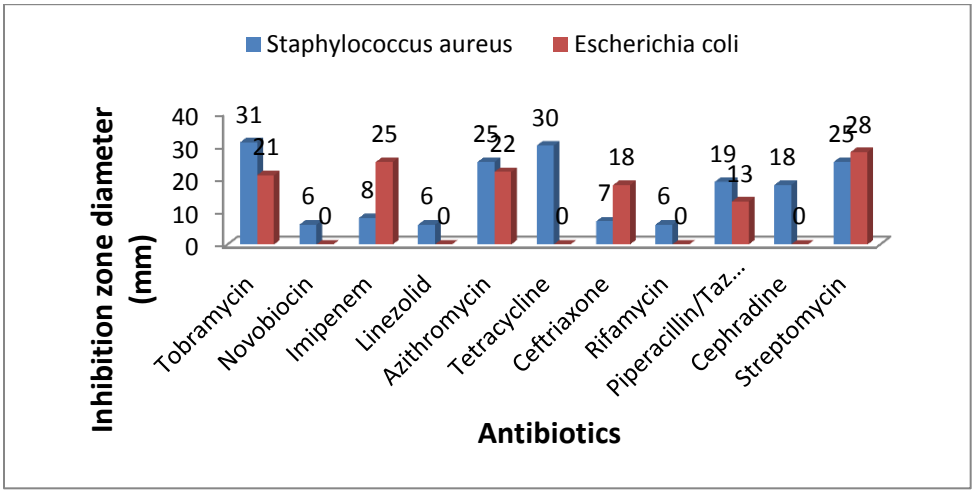

Figure 5: Antibiotics sensitivity for tested microorganisms

Antibacterial activity and MIC of CuO NPs

The antibacterial impact at figure 6 of CuO NPs biosynthesized using *Bacillus cereus* against test pathogens indicated that; CuO NPs strong inhibitor of bacteria in a concentration dependent manner. The results of CuO NPs in MICs test were shows 110 and 140 µg/ml in cases of *Staphylococcus aureus* and *Escherichia coli* respectively.

In another study shows the antibacterial impact of bacterial synthesized CuO NPs using *L. casei* against *Staphylococcus aureus* and *Pseudomonas aeruginosa* resulted zone of inhibition 12 mm and 10 mm respectively. The results of MICs were obtained the same MIC average 0.5 ± 0 mg/ml [2]. But, the chemically synthesized CuNPs was assayed against *Staphylococcus aureus* showed high antibacterial activity with MIC 40 µg/ml and inhibition zone 9 mm [8].

Subsequently, copper particles are released into bacterial cells and damage DNA structure and biochemical reactions that inhibit microbial growth [19,20] . Another explanation for antibacterial effect of NPs the disrupt of cytoplasmic membrane shape of tested pathogens when are exposure to CuO NPs. These changes in permeability cause increase of lead and cell death [21]. The effect of CuO NPs on *Staphylococcus aureus* was greater than that on E. coli. The difference in susceptibility of Gram-positive and Gram-negative bacteria can be attributed to the cell wall structure of the bacteria. The cell wall of E. coli is composed of lipopolysaccharides that are resistant to biocides. However, the cell wall of S. aureus lacks lipopolysaccharide or plasma membrane structure, physiology, metabolism, and its interaction with charged CuO NPs [22, 23]. Our findings are consistent with previous findings on the antibacterial activity of CuO NPs, although the shape and size of NPs can affect their antibacterial activity, leading to differences in these experimental results [24].

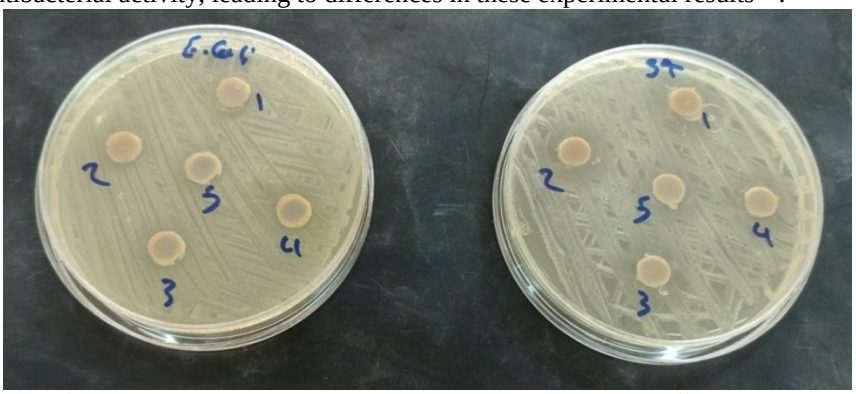

Figure 6: Antibacterial assay of CuO NPs against *Staphylococcus aureus* and *Escherichia coli* at different concentration (1=110, 2=120, 3=130, 4=140 and 5= 150 µg/ml).

Cytotoxicity of CuO NPs on vero cell line

The biosynthesized CuO NPs cytotoxicity (figure 7) was measured *in vitro* against the viability of epithelial vero cells using MTT protocol. Different concentrations of CuO NPs was assayed (31.25, 62.5, 125, 250, 500 to 1000) µg/ml. The lowest cytotoxicity presence at CuO NPs 250 $\mu g ml^{-1}$ was 27.7 % with IC50 value 404.9 $\mu g ml^{-1}$. Results showed that viability decreases when CuO NPs concentration increases. Our results show that CuO NPs may be cytotoxic above IC50 404.9 µg ml^{-1}.

In another study copper NPs synthesized using chemical method was tested on WI-38 cell line using MTT at the same concentrations observed that cytotoxic effect increase when NPs increases. These results suggest copper NPs cause toxicity over than 62.5 µg/ml [8]. Finally the biosynthesized CuO NPs in our research results confirmed less toxic than chemical synthesis.

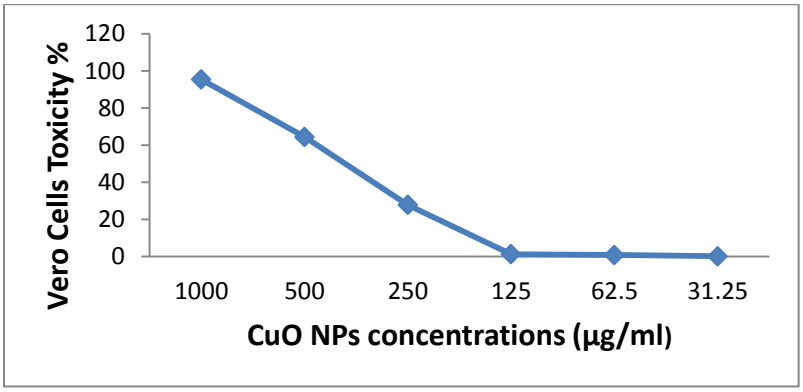

Figure 7: Cytotoxicity of CuO NPs on vero cells.

Effect of probiotic synthesis CuO NPs on wound healing activity

The wounds performed (table 1) in control (CuO NPs negative) and CuO NPs treated cells healed at the same time. The wound closure percentage (μm^2) of wound healing treated with CuO NPs was compared with the control. No significant changes in case of control (CuO NPs negative) and CuO NPs treated at 24 and 48h. This is considers no toxicity copper at the concentration used and this amount appears to be safe.

CuO NPs act as major factor in angiogenesis [25,26,27]. In another research carried on copper ion in nano form synthesized ecofriendly by *Pseudomonas aeruginosa* and testing their activity in enhancing the time of wound healing. Study was found wound healing pace was enhanced by copper NPs compared with control [28,29].

Table 1: Effect of CuO NPs on Area difference wound healing

Samples	Mean Effect (µm) with time (h)							
	at 0h		at 24h		at 48 h		Wound closure % μm^2	Area difference %
	area	width	area	width	area	width		
Control	789.3	788.3	698.3	697.4	281.1	280.1	64.37	508
CUONPs	872.5	871.5	752.8	751.8	368.5	367.5	57.76	504

Conclusion

This study confirmed that probiotic *Bacillus cereus* is a favorable producer for CuO NPs biosynthesis. CuO NPs has action as antibacterial on *S. aureus* and *Escherichia coli.* The concentration dependent cytotoxic of CuO NPs against epithelial vero cells was suggest these CuO NPs can use as antibacterial agent with non-significant effects on host cells and wound healing.

Conflict of interest:

The authors state that they have no conflicts of interest.

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