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Study of DNA sequence variation in *Streptococcus faecalis* bacteria by treatment with copper oxide nanoparticles

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ABSTRACT

Changes in the DNA sequence of Streptococcus faecalis by copper oxide nanoparticles were studied. Initially, concentrations of 30 and 60 micrograms per ml of copper oxide nanoparticles were added to the liquid medium of bacterial cells to penetrate nanoparticles into the cells of bacteria. After 24 hours, we carried out DNA extraction. Then, after 42 reactions RAPD-PCR and electrophoresis PCR, the results obtained were analyzed using EXCEL and NTSYS-PC programs. The results showed that the nanoparticles by changing the DNA sequence lead to inhibit the growth of bacteria.

Keywords: *copper oxide nanoparticles, DNA, Streptococcus faecalis, Rapid polymerase chain reaction (RAPD-PCR)*

Introduction

The role of antibiotics in preventing the growth of bacteria and control of bacterial infections, which threaten public health, has faded because different strains of bacteria (7, 10, 12). Current developments in the field of nano-biotechnology, especially its ability to provide metal oxide nanoparticles with specific size and shape are likely to lead to the creastion and the development of new antibacterial agents (11 and 17). Metal oxide nanoparticles, because of new features, including the large surface to volume ratio, and high reaction activity have attracted a lot of attention in recent decades (26, 1). The functional activity of metal oxide nanoparticles, penetrate into the cell and its influence is largely due to their small size (11, 13, 17 and 23). According to the scientific reports, it is proven that small-sized metal oxide nanoparticles show good antimicrobial activity (9). Recently, copper oxide nanoparticles among metal oxide nanoparticles have more attention more attention due to its lower price (6, 18). The ability of nano-particles to control pollution and infection control in vitro and living cell is discovered and proven. Metal oxide nanoparticles compared with antibiotics have greater stability and longer duration of action (25). Metal oxide nanoparticles can resist against unfavorable conditions, such as high temperature sterilization which disable conventional antibiotics (14 and 22). The antimicrobial activity of metal oxide nanoparticles depends on the size, stability and concentration of nanoparticles in bacteria culture media (17). Among the known reactions related to antimicrobial activity of metal nanoparticles is Reactive oxygen species (ROS), which prevents the process of DNA replication and synthesis of amino acids in microbes (2, 8). Metal oxide nanoparticles may change through mutations in the DNA sequences of bacteria, and lead to disable the bacterial cell. Due to the potential treatment of cancer cells by metal oxide nanoparticles, in this study, for the first time, changes have been studied in the DNA sequence of Streptococcus bacteria, which these changes have been caused by copper oxide nanoparticles, to be clear, these nanoparticles are effective in deactivating cancer cells, which is evaluated by studing the inhibitory effect of nanoparticles growth through changes in DNA sequences of bacteria. It is possible that these nanoparticles lead to mutation and abnormal function of the cell by altering the genome sequence of eukaryotic normal cells.

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2.Materials and methods

The method of this research is a survey-experimental, field method and the aim of this research is to study DNA sequence variation of Streptococcus faecalis in contact with copper

oxide nanoparticles. Respectively, the following steps were tested.

2.1.Bacterial culture and conditions associated with culture Streptococcus was cultured to ensure the viability and health of the target bacteria in blood and Eosin methyl-blue agar culture media. After 24 hours, the bacteria were taken in 5 mL of the BHI broth, and before adding copper oxide nanoparticles, placed for 24 hours in the incubator shaking at 37 ° C room and rpm 200, the growth of bacteria was controlled by measuring optical density (OD) at a wavelength of 600 nm (27).

2.2.Study of the growth inhibitory properties of copper oxide nanoparticles CuO was given us with a diameter of less than 20 nm, which was prepared by the Department of Chemistry, public university of Maragheh. Specifications and related analysis by electron microscopy are presented in the results section. Phosphate buffered saline with pH: 7.4, was used as a solvent for copper oxide nanoparticles. The concentrations of 30 and 60 micrograms/ml of Copper oxide nanoparticles were used to investigate the effects of nanoparticles on bacteria, so that the desired concentrations of nanoparticles were inoculated in each of the tubes. Test tubes were placed in the incubator shaking at 37 ° C and rpm 200, and the optical density of each of the pipes was measured at a wavelength of 600 nm, and at intervals of 2, 4 and 24 hours by a spectrophotometer to measure the growth of bacteria.

2.3.DNA extraction and reaction RAPD_PCR DNA has been extracted from control bacteria and treated by using DNA extraction kit (Gene Mark Company) in accordance with the manufacturer's instructions, and the quantity and quality were analyzed using spectrophotometry and electrophoresis on agarose gel (1%).

2.4.Effect of copper nanoparticles on Bacterial DNA The molecular markers RAPD- PCR was used to investigate the effects of nanoparticles on Streptococcus DNA. 10 Rapid Primers, which were prepared by the Department of Biotechnology in State University Maragheh, were used in order to perform the procedure RAPD- PCR. Sequence and characteristics of the primers are given in Table 1. To perform the reaction PCR, the following ingredients were prepared at the following concentrations to replicate samples in the volume of 25 ml. DNA is extracted from 1 Micro liter primer, 2.5 ml (10x) PCR buffer, 3 Micro liter 2MgCl, 1 Micro liter mix dNTP, 1 Micro liter of sample, and 0.3 Micro liter of Taq DNA polymerase DNA polymerase was reached to volume 25 Micro liter with 16.2 Micro liter deionized distilled water. Mix these ingredients was placed in a thermocycler (Corbett research, Australia) with the following schedule: The initial denaturation of template DNA at 95 ° C for 5 minutes, followed by 40 cycles of reaction PCR, and were as follows: At 95 ° C for 35 seconds for denaturation of template DNA strands, at 30 ° C for 45 seconds to connect the primers to the template strand, at temperature of 72 ° C for 45 seconds for polymerization of the new strand of the template strand. 7 minutes was used to complete polymerization of incomplete strands. After optimizing the conditions of PCR, these compositions and temperature profile were used for all 14 primers.

Table 1- Nucleotide sequences of primers

Nucleotide sequences of primers	Primer Name
ACAGGTGCGT	OPR-12
GGGTAACGCC	OPA-09
CAGAGGTCCC	OPS-03
CCAACGTCGT	OPT-17
TCCTGGTCCC	OPS-09
GTCCACACGG	OPB-08
CAATCGCCGT	OPA-11
GGTGACGCAG	OPB-07
CTCACCGTCC	OPC-09
AATGCCGCAG	OPT-14
AGTCGGGTGG	OPS-11
GTGATCGCAG	OPA-10
ACCGCGAAGG	OPD-01
GGACGCTTCA	OPQ-14

2.5.Review the results of RAPD - PCR:

After the reaction PCR, to detect bands, 10 Micro liter of PCR product was electrophoresis. on agarose gel 2% (with a size of 14 × 26), which contains red safe, in buffer (1x) TBE, for 4 hours and 45 minutes, with voltage 120 V. And to determine the size of the product, Ladder DNA marker was used with the size of 100-bp, and the gel imaging system (Uvitec, France), were taken. The image was taken by using the camera. (Uvitec, France)

2.6.Analysis of data from the electrophoresis on the gel 2%

Bands obtained from analysis of RAPD, based on the presence or absence of them, was rated as one and zero, the data is given in the Tables 3 to 5. The data entered based on molecular in EXCEL software. Similarity matrix by Dic NTSYS-PC is calculated by the software, and dendrogram was drawn by using the UPGMA method in the NTSYS-PC software.

3.Research findings

3.1.Bacterial culture

The bacteria were grown on blood agar medium, and we ensure the viability of the bacterial cells. Gram-Positive bacteria Streptococcus did not grow on the Eosin-methyl blue agar medium, which is for gram-negative bacteria. 24 hours after the presence of bacteria cells in BHI broth, before adding copper oxide nanoparticles, the OD of each test tube was set in the amount of 0.7.

3.2.Copper oxide nanoparticles Profile

Electron microscope TEM and SEM has been used to study the morphology and estimate the size of the copper oxide nanoparticles.

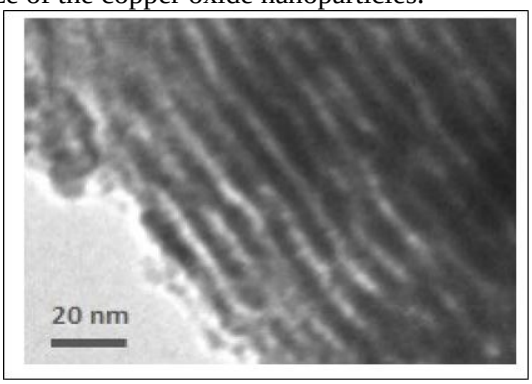

Figure 1- Microscope Photo of Transmission electron microscopy (TEM) of Cuo nanoparticles

Black dots indicate the Cuo nanoparticles which have been verified in Silica pores. Copper oxide nanoparticle size is less than 20 nm.

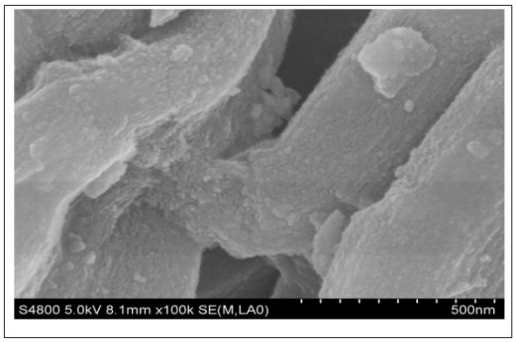

Figure 2- Microscope Photo which shows nanoparticles pores

Microscopy Photo of Scanning electron microscopy (SEM) that shows the pores in the nanoparticles of copper.

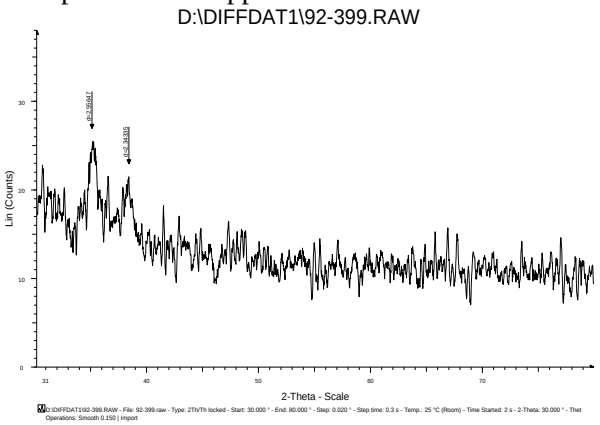

Figure 3- XRD analysis

XRD analysis which confirms the presence of CUO nanoparticles, 36 38 2-Theta are related to the Cuo particles.

3.3.Assessment of growth inhibitory properties of copper oxide nanoparticles

Summary results related to inhibitory activity of oxide nanoparticles growth against Streptococcus copper is given in Table 2, which was clearly demonstrated that bacterial growth was suspended after treatment with copper oxide nanoparticles, with different concentrations of 30 and 60 micrograms per ml, in 2 and 4 hour intervals, and only minor growth was observed in the cells after 24 hours.

Table 2- The antimicrobial activity of copper nanoparticles

Concentration	No. Tubes	OD Before treatment	OD after 2 hours	OD after 4 hours	OD after 24 hours
Control	1	0.7	0.75	0.8	0.85
30 µg / ml	2	0.7	0.7	0.7	0.72
	3	0.7	0.7	0.7	0.73
6 0 µg / ml	4	0.7	0.7	0.7	0.75
	5	0.7	0.7	0.7	0.75

3.4.RAPD- PCR products analysis

Electrophoretic bands of the amplification of 14 primers by RAPD- PCR are shown in figures 4, 5 and 6. In each photo, respectively, for each primer, from left to right, including the control sample, first treatment sample at a concentration of 30 micrograms per milliliter, the second treatment sample at a concentration of 60 micrograms per ml. Then, the size of bands obtained from analysis of RAPD was determined using the marker. Based on the presence or absence of them in samples, respectively, were scored as one and zero, which results are presented in Tables 3 to 5. Which conclusion is based on the difference in bands which each primer forms for control and treated samples.

OPA-11: Three bands were formed on the gel for this primer which the two bands were different between the control and treated samples.

OPB-07: A band was formed on the gel for this primer which was different between the control and treated samples.

OPC-09: Four bands were formed on the gel for this primer which the four bands were different between the control and treated samples.

OPT-014: Two pieces were amplified by the primer and both pieces were different between the control and treatment samples.

OPS-11: Eight pieces were amplified by the primer and six pieces were different between the control and treatment samples.

OPA-10: Five pieces were amplified by the primer and three pieces were different between the control and treatment samples.

OPR-12: Two pieces were amplified by the primer and two pieces were different between the control and treatment samples.

OPA-09: Three pieces were amplified by the primer and three pieces were different between the control and treatment samples.

OPS-03: Three pieces were amplified by the primer and three pieces were different between the control and treatment samples.

OPD-01: Two pieces were amplified by the primer and one piece was different between the control and treatment samples.

OPQ-14: Five pieces were amplified by the primer and five pieces were different between the control and treatment samples.

OPT-17: Two pieces were amplified by the primer and two pieces were different between the control and treatment samples.

OPS-09: One piece was amplified by the primer and it was different between the control and treatment samples.

OPB-08: five pieces were amplified by the primer and all pieces were different between the control and treatment samples.

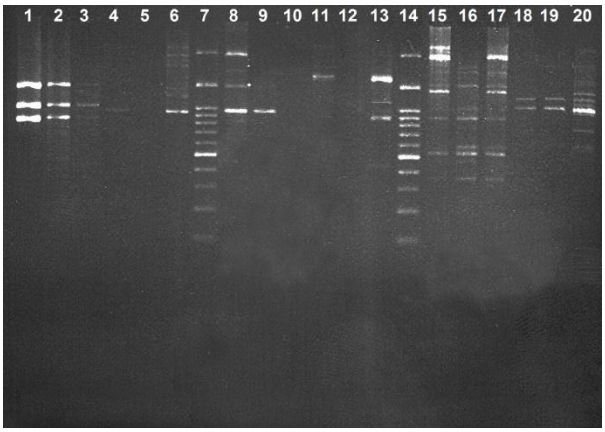

Figure 4- Electrophoresis of PCR products on agarose gel

Columns 1-3: primer OPA-11, Columns 4-6: primer OPB-07, Column 7: marker, columns 8-10: Primer OPC-09, Columns 11-13: primer OPT-14, column 14: marker, columns 15-17: primer OPS-11, columns 18-20: primer OPA-10.

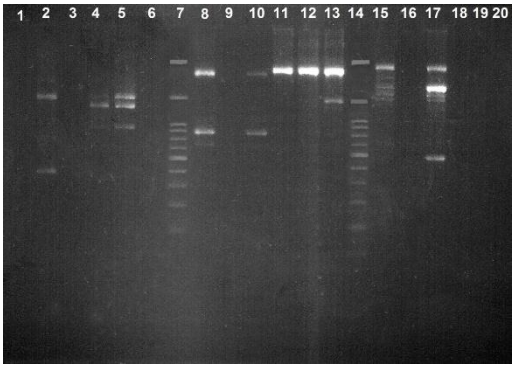

Figure 5- Electrophoresis of PCR products on agarose gel

Columns 1-3: Primer OPR-12, Columns 4-6: primer OPA-09, Column 7: marker, columns 8-10: Primer OPS-03, Columns 11-13: primer OPD-01, column 14 : marker, columns 15-17: primer OPQ-14, columns 18-20: empty.

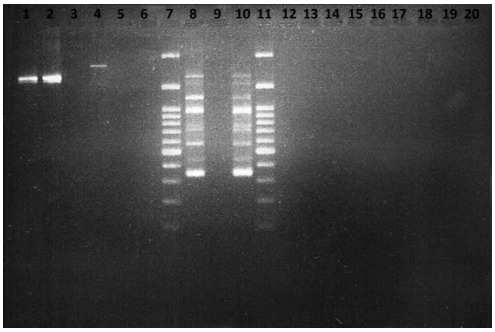

Figure 6- Electrophoresis of PCR products on agarose gel

Columns 1-3: primer OPT-17, Columns 4-6: Primer OPS-09, Column 7: marker, columns 8-10: primer OPB-08, column 11: marker, columns 12-20: Empty.

Table4- Results of primers band in figure 2

T 60	T 30	C	Band (pb)	Primer
0	1	1	1450	OPA-11
1	1	1	1000	
0	1	1	800	
1	0	0	950	OPB-07
0	0	1	3000	OPC-09
0	0	1	1950	
0	0	1	1500	
0	1	1	990	
1	0	1	1900	OPT-14
1	0	0	900	
0	0	1	3000>	OPS-11
1	0	1	2950	
1	1	0	1500	
1	0	1	1400	
1	1	1	900	
0	1	0	600	
1	1	1	540	
1	1	0	380	
1	0	0	1400	OPA-10
1	1	1	1300	
1	1	1	1100	
1	0	0	990	
1	0	0	800	

Table4- Results of primers band in figure 3

T 60	T 30	C	Band (pb)	Primer
0	1	0	1500	OPR-12
0	1	0	450	
0	1	1	1500	OPA-09
0	1	1	1400	
0	1	0	900	
1	0	1	2500	OPS-03
1	0	1	900	
0	0	1	750	
1	1	1	2700	OPD-01
1	0	0	1500	
1	0	1	2900	OPQ-14
1	0	1	1800	
1	0	1	1600	
1	0	0	1500	
1	0	0	600	

Table5- Results of primers band in figure 3

T 60	T 30	C	Band (pb)	Primer
0	1	1	1800	OPT-17
0	1	1	1600	
0	0	1	2500	OPS-09
1	0	1	1800	OPB-08
1	0	1	1250	
0	0	1	950	
1	0	1	550	
1	0	1	350	

According to data from RAPD, similarity matrix for control and treated samples was calculated using Dic, which are presented in Table 6, and is interpreted as follows: There is similarity between the control and t 30 samples with a factor of 0.5098030. There is similarity between the control and t 60 samples with a factor of 0.6229508. There is similarity between the t 30 and t 60 samples with a factor of 0.3333333. Therefore, the similarity between 60t and control samples is logical and desirable, the similarity between 30t and control samples is in the middle, and the similarity between the treated samples is reverse. As a result, whatever the numbers are closer to number 1, genetic similarity is greater between samples.

Table 6- Similarity matrix for control and treated samples

Dice (Czekanowski or Sorenson) Measure			Case
3: t60	2: t30	1: ctr	
		1.000	1: ctr
	1.000	0.5098030	2: t30
1.000	0.3333333	0.6229508	3: t60

In Figure 4, Dendrogram was drawn by using the UPGMA method in NTYSYS-PC software to evaluate the similarity between data samples and compared genetic variation between the control, t30 and t60 samples. According to Figure 4, it can be seen that, there is similarity and close relationship between the control and t60 samples compared to t30 sample, and are in a group. Therefore, there are substantial genetic differences between T30 and control samples and the t60 samples.

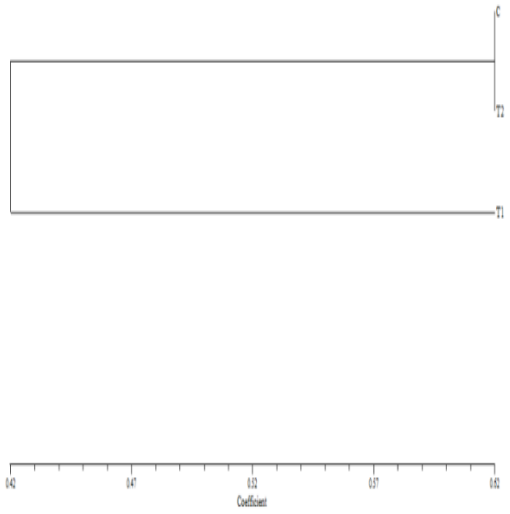

Figure 4- Dendrogram of analysis based on Rapid test by using UPGMA method by NTSYS-PC software

4.Discussion and conclusion

According to studies, the effect of nanoparticle size and the time can be justified, so that nanoparticle with small size and high exposure times will have more opportunities to penetrate into the cells and destroy cellular components (Yang et al, 2012). Bactericidal activity of copper oxide nanoparticles has been proven against Gram-positive (Listeria monocytogenes) and Gram-negative bacteria (*Escherichia coli*) which are a component of food pathogens (Shiv Shankar and jon-whan Rhim in 2014). In this study, a solution of copper oxide nanoparticles, with diameters less than 20 nm at concentrations of 30 and 60 qm/ml showed good growth inhibitory properties, and during the different time process affected the growth of bacteria. Therefore, according to the results, after two hours, the growth of Streptococcus bacteria was completely stopped, which can be concluded that, copper nanoparticles with the lowest concentrations can show most antimicrobial activity for short time. In addition to proven antimicrobial properties of metal oxide nanoparticles, there are reports based on the impact of nanoparticles on bacteria genome that the study was an attempt to prove it. The bacterial cell membrane has the proteins containing sulfur, and silver nanoparticles react not only with these proteins, but with phosphorus-containing compounds, including DNA. Also, nanoparticles by attacking the cells respiratory chain, which is involved in cell division, lead to cell death. Also, the ions released from the nanoparticles lead to increased antimicrobial property of these nanoparticles (5 and 16). Metal oxide nanoparticles have a similar mechanism. According to the research, stated that, metal oxide nanoparticles lead to disrupt in the processes of transcription and translation (20). Metal oxide nanoparticles can lead to fracture of single-stranded DNA, and be involved in gene expression (4). Because the antibacterial effect of copper oxide nanoparticles on DNA has not been investigated, this research to study the effects of nanoparticles on DNA Streptococcus, was performed as a model for Gram-positive bacteria.

In this regard, of a total of 14 primers used in the reaction RAPD-PCR, the presence or absence of band in agarose gel image (Figs. 1, 2 and 3), indicate a change in the DNA sequence of Streptococcus by nanoparticles of oxide copper. The results showed that a considerable number of primers did not detect target sequences, and therefore did not reproduce the relevant parts, and these parts are on agarose gel without bond. The difference between the bands of T30 and t60 groups and the control group suggests that the target sequences of primers has changed on bacterial DNA in groups of t30 and t60, that makes a difference in binding primers, and amplification in PCR by them. The reason for this can be caused by mutations on the DNA directly or indirectly by copper nanoparticles, or according to the Paul et al studies in 2007, it can be caused by disrupting in the mechanism of replication by DNA polymerase. That in the event of disruption of the mechanisms of DNA polymerase replication, replication health is impaired, and during replication in DNA sequence, changes are created, which leads to differences in target sequences of RAPD primers (15). It can be noted this issue that the sequence of the gene coding for the enzyme DNA polymerase can also be changed under the influence of copper oxide nanoparticles, and in this way, the replication process is disrupted. Copper oxide nanoparticles can induce fractures in the single strand of DNA, and affect gene expression (3). Thus, copper oxide nanoparticles cause wide variations in the structure of bacterial chromosomal DNA such as fractures in the DNA molecules, since these components have been created at random, many of these parts have not been identified by the primers, and not reproduced. Copper nanoparticles, as well as lead to the disruption of genes that control replication and transcription. They also affect the activity and sequence of promoters, and the ability of RNA polymerase to open the spiral and the transcription process. Any factor such as copper oxide nano-particles that damage the DNA of living cells, could cause the death of living cells, which Duncan *et al* (2011), stated that in his research, nanoparticles of copper oxide during the oxidation reaction (I) Cu to (II) Cu, produce Ros, which causes the release of hydroxyl radicals, which damage essential proteins and DNA (3,19). Changes in the DNA sequence in this study because of mutations in genes associated with growth and cell cycle control can be a factor for cell growth inhibitory and disruption of cell cycle (24). According to Li et al (2006) studies, nanoparticles affect the enzymes involved in replication (21). Accordingly, given that we have treated bacteria growing and replication with copper oxide nanoparticles in this study, and evaluated growth in the periods 2, 4 and 24 hours, we can argue the following states:

Copper oxide nanoparticles have created disorder in sequences or promoter of genes involved in the growth and reproduction of bacteria. Copper oxide nanoparticles have created disorder in the cell replication process, and the integrity of this vital process is disrupted through mutations, as well as they have created disorder in the related mechanisms of healing which is done by enzymes.

All the above arguments are done by creating multiple mutations by copper oxide nanoparticles on the DNA sequence. Also, the reaction between copper and amine carboxyl groups on the surface of microbial cells, and the release of copper with amine and carboxyl groups formation on the surface of microbial cells, and the release of copper ions and the formation of ROS, which both of them prevent the DNA replication and synthesis of amino acids in microbes, lead to the anti-bacterial activity (Robert Y. et al., 2013). According to the results observed in this study which the growth of bacteria was significantly reduced by treatment with copper oxide nanoparticles, it can be concluded that, by creating mutations on the gene sequence involved in cell cycle control, expression of these genes has reduced, and finally it leads to stop the cell cycle (Rai *et al*, 2009).

Nanoparticles are used as a carrier of drugs for cancer treatment. Nano-particle is responsible for drug's protective role, and increases the effectiveness of the drug. The nanoparticles can carry medicinal compounds, and block a protein called Myc, Myc is active in many cancers, such as leukemia, the experimental results show that Myc inhibitor compound is very effective, but when is injected to flow blood, it quickly disappears, therefore, must be protected by other materials. According to the results obtained in this study, based on the fact that the copper oxide nanoparticles by creating mutations in genes controlling growth and cell cycle sequence, lead to decrease and stop the growth of bacterial cells, therefore this study provides the groundwork for

future research on cancer by disrupting the cell cycle of cancer cells and harnessing the uncontrolled growth of these cells by Metal oxide nanoparticles, so that we can find a way to cure cancer disease.

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