Original Article

The Effect of Titanium Dioxide Nanoparticles Synthesized by *Bacillus tequilensis* on *clb* Gene Expression of Colorectal Cancer-causing *Escherichia coli*

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Abstract

Objective: The incidence of colorectal cancer has increased dramatically in recent years. One of the most important causes of colorectal cancer is infection with a specific type of *Escherichia coli* bacterium that has polyketide synthase (*pks*) gene, which produces a secreted toxin named colibactin that changes intestinal microflora condition for its pathogenicity and by interrupting the cell cycle triggers the development of colorectal cancer. Due to the drug resistance of this bacterium, it is necessary to produce new antimicrobial agents such as nanoparticles. Therefore, this study aimed to investigate the effect of titanium dioxide nanoparticles on the expression of *clb* genes of *E. coli* responsible for colorectal cancer. **Methods**: The synthesis of titanium dioxide nanoparticles was done by *Bacillus tequilensis* that was identified by sequencing 16S rRNA. Titanium dioxide nanoparticles were examined by ultraviolet spectroscopy, X-ray diffraction, infrared spectroscopy, and scanning electron microscopy Real-Time PCR was used to analyze the effect of nanoparticles on *clb* expression. Human embryonic kidney cells 293 (HEK 293) cell line was treated with different concentrations of titanium dioxide nanoparticles for 72 hours and then cell viability and cytotoxicity were determined MTT method. **Results:** The synthesized nanoparticles were often spherical between 35.76-78.17 nm with absorption at 350 nm. RT PCR showed a 20-fold reduction in the expression of *clbB* and *clbN* genes. MTT showed that cell viability, increased after 72 hours that depends on titanium dioxide concentration. **Conclusion:** The results obtained in this study indicate that the synthesized nanoparticles could reduce the expression of *clbB* and *clbN* genes of *E. coli* with the lowest cytotoxicity. Therefore, it can be considered for the treatment of colon cancer caused by *clb* gene-positive *E. coli*.

Keywords: *Bacillus tequilensis*, titanium dioxide nanoparticle, *clb* genes, *Escherichia coli*

Introduction

Despite significant advances in medical technology for the diagnosis and treatment of cancer, this disease is still considered a major threat to mortality [1]. Colon cancer is one of the most common malignancies in men and women, causing many deaths in the world every year [2]. Colorectal cancer is the third most common cancer (more than 9% of all cancers) and the fourth most common cause of death in the world. In Iran, this cancer after stomach, bladder, and prostate (in men) is the fourth most common cancer and is the second in women after breast cancer [3].

Investigations have shown that increasing the number of *E. coli* containing the *pks* gene can cause colorectal cancer by damaging the DNA of the intestinal lining cells [4]. *Phylogenetic* studies have shown that *E. coli* can be *divided into* four groups: A, B, B2, and D. Some commensal strains of *E. coli*, from phylogenetic B2 group, carry a pathogenic island of polyketide synthase (*PKS*) gene that codes for the enzyme required for the synthesis of a hybrid *peptide-polyketide genotoxin* called colibactin. Both types of *E. coli*, commensal and pathogenic, have been found in the colon. The colibactin toxin has been observed in *Enterobacteriaceae,* not only in *E. coli* strains but also in

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Klebsiella pneumoniae, *Enterobacteria aeruginosa* and *Citrobacter koseri* [5].

The enzymes required for the synthesis of colibactin are encoded and expressed by 54 kb gene, located in the *ansW* locus of tRNA. The ORFs of this region contains 3 *NRPS*, 3 *PKS*, 2 hybrid *NRPS*/*PKS* and 9 accessory, tailoring and editing enzymes including *clb n, clb k, clb j, clb i, clb h, clb c, clb b, clb a and clb d* [6]. The chemical properties of colibactin are due to the presence of three enzymes, *clb* N, *clb* B and *clb* P that contribute to the synthesis, prolongation and the folding degradation of colibactin. At first, *clb* N, along with *clb B* and *NRPS*/*PKS*, produces pre-colibactin in the cytoplasmic space of the bacterium, and then through the channels enters the periplasmic space, where is converted to colibactin by *clb* P [5, 6].

Colibactin can be secreted by passing through the outer membrane of the bacterium and has a cytotoxic effect on the host enterocytes. By activating the signaling pathway, the colibactin can damage the DNA by activating the ataxia telangiectasia mutated (ATM) protein, which causes cdk2 phosphorylation and then cdc25c phosphorylation. In the end, cdk1 (by playing the role in the G2 checkpoint of the myosis of cell cycle division) is phosphorylated and the large amount of inactive phosphorylated try15 appears from cdk1. Also, the ɣH2AX histone and the cell cycle stops at the G2 stage and this action causes cell cycle disorder, which results in colon cancer [7, 8]. Today, the use of nanoparticles is common in the treatment of tumor cancers.

By identifying the concentration and the shape of the nanoparticles, it can be used to inhibit the growth of *pKS* positive *E. coli* isolated from cancerous tumors. Researchers have shown that biological systems can synthesize some nanoscale metal particles. Physical and chemical methods of nanoparticle production have a special place in terms of environment and lower energy consumption and costs [8]. Among the various methods of biosynthesis of nanoparticles, the use of bacteria is of particular interest. For this reason, researchers have come up with biological systems for the production of nanoparticles, which have minimal environmental hazards and are biocompatible with a simple production method. The nanoparticles have good antimicrobial properties, which are due to their surface to high volume ratio [9].

Nanomaterials in the life cycle and ecosystems exhibit the lowest toxicity. Studies have shown that the smaller the nanoparticles are, the more distinctive properties and new activities they show [10, 11]. These features have made the use of nanomaterials widely recognized in all areas of life, such as the fight against germs, diagnosis, and treatment of diseases and packaging of food products [12, 13]. Today, due to the unique properties of nanoparticles, titanium dioxide got more attention [14]. Titanium is the fourth most common metal in the world after aluminum, iron, and magnesium, and accounting for about 0.6% of the Earth's crust. Titanium is commonly used in both metal and oxide form. The metal form due to the supply and purification problems is not used, but its oxide in the form of titanium dioxide is used widely in the industry [14, 15].

It has a very interesting physical and chemical properties that are stable against the light and is affordable, therefore, it is a good candidate for a variety of applications such as antimicrobial, antiviral, antifungal, anticancer, photocatalytic properties, water, and air purification properties, pharmaceutical use, antifogging, dental applications, and self-cleaning coatings [16]. Titanium nanoparticle production by microorganisms is superior to the physical and chemical methods in terms of cost, energy consumption, and safety. On the other hand, the production of nanoparticles varies in different bacterial genus and species. *Bacillus* is an aerobic bacterium with spores that can tolerate oxygen and survives in harsh and difficult conditions, making it an attractive candidate for the biosynthesis of metal nanoparticles such as *titanium dioxide*. The important function of the selected bacteria is the synthesis of metal nanoparticles and their oxides [17, 18].

In this survey, the effects of titanium dioxide nanoparticles synthesized by *Bacillus* isolated from soil were studied on the *clb* genes expression of *E. coli*, which causes colorectal cancer. Also, the effect of these nanoparticles was studied on the HEK293 cell line.

Materials and Methods:

Soil sampling and isolation of Bacillus bacteria

To isolate the bacteria producing titanium dioxide nanoparticles from the soil, samples from the surface and 5 centimeters below the soil were collected from metal mines of Kerman province, Kahnuj titanium mine, the abandoned lead mine of the Marvan aqueduct, Goharzamin iron mine and Sarcheshmeh copper mine. Soil samples were kept in sterile bags with the date and name of the place and transferred to the lab. Two methods of direct culture and thermal treatment were used to isolate the bacillus from the soil samples. Indirect culture, 10 g of each sample was mixed in 90 ml of ringer solution and after 10 minutes on the shaker at 150 xg, 10^{-10} to 10^{-3} dilutions were made from the suspension, and one ml of each dilution was cultured on the surface of Petri dishes containing trypticase soy agar and after 2 hr at 30 °C the growth was investigated.

In the thermal treatment, the above procedure was used, but after preparing the initial suspension from the soil, the samples were placed in the 100 ºC water bath for 10 minutes. After 72 hours' incubation, the purified colonies were isolated and cultured on the surface of Petri dishes containing trypticase soy agar by quadrant method and incubated for 72 hours at 30 °C, and then all the pure isolates and spores were Gram-stained [16].

Synthesis of titanium dioxide nanoparticles by Bacillus isolate

After growing the bacteria and ensuring the purity of the Bacillus colonies, the colonies were inoculated into the nutrient broth culture medium and incubated at 30 °C for 24 hours at 150 rpm. Then, the medium was centrifuged at 3000 rpm for 15 minutes and the supernatant was collected and 0.025 M titanium dioxide was added. After one hr on a shaker, the solution was placed into the 60 °C water bath for 30 minutes, and the precipitate was separated by centrifugation and then washed 3 times with distilled water. The dried precipitate was analyzed by ultraviolet spectroscopy, X-Ray Diffraction Analysis, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM), to confirm the titanium dioxide nanoparticles production.

UV-Vis spectrophotometry

The optical density of titanium dioxide nanoparticles was measured using a UV-Vis *spectrometer* (Biotech EPOCH, USA) at a wavelength of 300-700 nm.

X-Ray Diffraction Analysis

The X-ray diffraction analysis allows for the recognition of the crystallographic structure of the titanium dioxide nanoparticles. The nanoparticles were first lyophilized and then the resulting powder was studied by the Philips PW7730 XRD machine .at 2_{θ} angle from 30 ° to 80 °.

Fourier Transform Infrared Spectroscopy (FTIR)

The powder of titanium dioxide nanoparticles was used to determine the FT-IR spectrum by the Thermo AVATAR FTIR device.

Scanning Electron Microscopy (SEM)

The titanium dioxide nanoparticle powder was first bonded to the copper surfaces, and then a thin layer of gold-covered the surface by a sputter canter and finally was analyzed with the scanning electron microscope (FEI Quanta 200 ESEM model).

Molecular identification of the bacteria producing titanium dioxide nanoparticles

After confirmation of the presence of titanium dioxide nanoparticles, the synthesizing bacteria were identified. The genomic DNA was extracted by the CinnaGen kit (Iran, Tehran) using forward: 5/CAGGCCTAACACATGCAAGTC_3 / and rivers primers: 5 / _ACGGGCGGTGTGTACAA_3 /. The PCR mixtures (1 µl of each primer, 4 µl of the template DNA, and final volume with distilled water to 20 µL) were denatured at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s and then a final extension at 72°C for 10 min. To confirm the DNA replication, the PCR product was electrophoresed on 1% agarose gel (100 bp ladder was obtained from Sinaclon Co.), and then 1000 bp band was sequenced by Takapou Zist (Iran, Tehran). The similarity of 16S rRNA nucleotide sequences was compared with the BLAST database and then the corresponding phylogenic tree was mapped using the MEGA-7 software.

Preparation of E. *coli* containing *clb* B and *clb* N genes

A total of 12 samples of cancer-causing E***. coli*** were obtained from the Pasargad Research Center. To confirm the presence of both genes, Duplex PCR was used [17].

Duplex PCR reaction

To extract the DNA of E. coli bacteria, the CinnaGen kit (Iran, Tehran) was used. Forward primers including 5´GCGCATCCTCAAGAGTAAATA3´ and 5´TCGATATAGTCACGCCACCA3´, and reverse primers 5´GCGCTCTATGCTCATCAAACT3´and 3´GTCAAGCGAGCATACGAACA5´, were used for clbB and clbN respectively. (Arian Gen Teb Co. Iran). Primers were diluted to 25 picomols. Duplex PCR was performed based on Fig. 2B. The PCR mixtures (1 µl of each primer of clb B and clb N genes, 4 µl of the template DNA, 10 µl of Master mix 2X and final volume with 2 µl distilled water to 20 µl) were denatured at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s and 10 min respectively.

The expression of *clb* B and *clb* N genes of E. *coli* treated with titanium dioxide nanoparticles by Real-Time PCR:

Determination of the minimal inhibitory concentration (MIC) of the E. *coli* having *clb* gene

To investigate the expression of *clb B* and *ClbN* genes of *E. coli*, bacterial samples were treated with different concentration of titanium dioxide nanoparticles under UV light, and non-treated bacteria as the negative control. To determine the MIC, 100 µl of the Muller Hinton Broth and 100 µl of nanoparticle suspension with an initial concentration of 1024 µg/ml was added to the first well of the 96-well microplates and then serially diluted 12 more wells. Then, 100 µl of bacterial suspension of 0.5 McFarland (1×10^8 bacteria/ml) was added to each well. The plate was incubated at 37 °C on a rotary shaker (100 rpm) for 24 hours. The turbidities were observed due to the bacterial growth was considered the MIC dilution, and the previous well was considered the minimum inhibitory concentration. Sub MIC was considered as treated bacteria with titanium dioxide nanoparticles, which was used together with control bacteria for Real-Time PCR [18].

RNA extraction and cDNA synthesis

Total RNA extraction was performed by the CinnaGen kit, and after agarose gel electrophoresis, cDNA was prepared by reverse transcriptase enzyme and used as a template in the polymerase chain reaction.

Real-Time PCR

The cDNAs of *E. coli* containing *the clb* gene and the control bacteria were used for Real-Time PCR. The Real-Time PCR mixture (1 µl of each primer, 1 µl of cDNA, 10 µl of SYBR Green master mix (2x), final volume with distilled water to 20 µL) were denatured at 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 56°C for 30 s, 72°C for 30 s, 95°C for 15 min and 60°C for 1 min. Finally, gene expression was studied.

Data analysis

For MTT data analysis, Image J and SPSS-16 software were used.

Titanium Dioxide Cytotoxicity Test by MTT

To investigate the effect of titanium dioxide nanoparticles synthesized by the *Bacillus* bacteria on the growth and proliferation of the cells, the MTT method was used. Human embryonic kidney cells 293 were cultured and 20,000 cells in 100 µl were added to each well of 96-well microplate and cultured for 24 hr. Then, different concentration (0, 0.01, 0.5, 0.1, 0.5, 1, 5, 10 , 50 and 100 µg/ml) of titanium dioxide nanoparticles was added to the wells and after 24 hrs of incubation, 100 microliter of 0.5 mg/ml MTT (3,5,4-dimethyl thiazolyl-2) (5,2-diphenyl tetrazolium bromide) (Sigma-Aldrich) was added to each well and the plate incubated at 37 °C for 4 hrs. Then, 100 µl of supernatant was removed from each well and 100 µl of dimethyl sulfoxide (DMSO) or isopropanol was added to dissolve the purple formazan crystals. The plate was placed on a shaker for 30 minutes at room temperature and then, OD was measured at 570 nm using ELISA reader. All samples were assayed in triplicate, and each experiment was conducted twice. The cytotoxicity was calculated by the following equations (19, 20). The results were analyzed using SPSS version 16 and $p<0.05$ was considered significant.

% viable cells= 100-% cytotoxicity

Results:

Out of 45 colonies that were isolated and purified from the direct culture and heat treatments used for the production of titanium dioxide nanoparticles, five *Bacillus* were identified that only one of them had more white sediment than other isolates. When the sequences of the 16S rRNA of the isolated bacteria (figure 1) were compared to the sequences of the genomic database using Blast software, the identified bacteria were *Bacillus tequilensis*.

The phylogenic tree in figure 2 shows the relationship between 16S rRNA sequences of the isolated bacteria and the reference sequences in the GenBank. The numbers in the branching node represent the bootstrap value (%). *Bacillus tequilensis* (LT986216) was used as an outgroup.

UV-vis spectrophotometer

showed the highest optical density of titanium dioxide nanoparticles at 350 nm that indicates biosynthesis of titanium dioxide (Fig. 3).

The X-ray diffraction analysis confirmed the formation of titanium dioxide nanoparticles crystals in the sample (Fig. 4). Strong and distinct peaks were seen at 2 thetas 25, 39, 40, 42, 45, 48, 55, 58, 63, 65, 70, 75, 78 and 83 degrees, that corresponding to the surface of the titanium dioxide with 101, 004, 221, 111, 210, 200, 211, 123, 002, 310, 301, 215, 212, and 224, respectively. No peaks of impurity were seen that indicates the relevant product is made with high purity. The average particle size was 48 nm that indicates the synthesized titanium dioxides are at nanoscale.

Fourier Transform Infrared Spectroscopy Analysis of Titanium Dioxide Nanoparticles

As Fig. 5 shows, the wide peak is seen in the cm-1 area between 3600-3200 is related to the O-H symmetric stretching vibration methods. The absorption peak in the cm-1area of 1670 is related to the bending vibration of the O-H. The peak observed in the cm-1 area of 683 is belongs to the tensile vibrational methods of the metal-oxygen. The peaks in the cm-1 area of 2900-2800 are also attributed to the tensile vibrations of the C-H.

SEM Analysis of Titanium Dioxide Nanoparticles

The image of SEM shows the spherical nanoparticles with 35.76 to 78.17 nm in diameter that confirms the morphology of titanium dioxide nanoparticles (Fig. 6).

X-ray Diffraction Spectroscopy (EDS)

To analyze the particles, randomly from two particles are selected from the sample and analyzed by the EDS. Figure 7 shows that in both patterns of X-rays, oxygen and titanium were detected. The titanium found in these two particles was about 6.72% by weight.

Identification of *clbB* and *clbN* genes in *E. coli* isolates by Duplex PCR

Out of 60 *E. coli* isolates, two bacteria had both *clbB* and *clbN* genes. Isolate No. 4 was considered for further investigation (fig.8).

The MIC of titanium dioxide nanoparticles for *E. coli*

Based on ocular opacity, the MIC of titanium dioxide nanoparticles for *E. coli* having both *clbB* and *clbN* genes was 256 µg/ml. We used a sub MIC concentration of 128 µg/ml to treat *E. coli* for analysis of gene expression by Real-Time PCR.

Analysis of *E. coli* clb gene expression treated with titanium dioxide nanoparticles by Real-Time PCR

In figure 9, S1 is the *clb* gene before treatment with titanium dioxide nanoparticles, S2 is the *clb* gene after treatment with nanoparticles. M1 housekeeping gene before treatment with

nanoparticles, and M2 housekeeping gene after treatment with nanoparticles. The NTCS (patternless controls) lacked the reaction progress chart, but the samples had exponential proliferation. A comparison of the duplication pattern, before and after exposure to the nanoparticle, showed that ΔCt was increased. The Ct of *16S rRNA* gene before and after treatment was 17.43 and 18.04, respectively. Since the 0.61 Ct difference is negligible, the *16S rRNA* gene was used as a housekeeping and internal standard gene. The main peak for this gene occurs at 86.79 ºC. The results of the ΔCt of *clb* gene in exposure to titanium dioxide nanoparticles are presented in Table 1.

The PFAFFL method was used to determine the level of *clb* gene expression. In this method, it is assumed that the sample efficiency and the internal control were 100%, and to evaluate the gene expression, the 2 -ΔΔCT formula was used. According to Table 1, titanium dioxide nanoparticles reduced the expression of the *clb* gene by 20.44 fold but did not affect the *16S rRNA* gene expression.

The Cytotoxicity of Titanium Dioxide on the Cells

In the present study, our data show cell viability and % toxicity of different concentrations of titanium dioxide nanoparticles by time, as compared with the control group. The vital activity of the cells was minimal after 48 hours, but after 72 hours it increased significantly ($P > 0.05$). The lowest vital activity of the cells after 48 hours was 54.12% respectively when 0.1 µg/ml titanium dioxide was used. However, when 5 and 100 µg/ml nanoparticles were used the cell viability increased after 72 hours to 100%, respectively when 5 µg/ml titanium dioxide (fig.10).

Discussion:

In recent years, due to the prevalence of infections caused by antibiotic-resistant bacteria, many studies have been done to produce new antimicrobial compounds. Nanoparticles are very interesting for the development of new drugs, because of their small size that can cover more surface area and at the lowest concentration can react with bacterial cells. Currently, various physical, chemical and biological methods are known for the production of nanoparticles. In biotechnology, various biological compounds, such as microbes, are considered the best candidate for the synthesis of nanoparticles, because these methods are economical, simple and eco-friendly [19].

In this study, titanium dioxide-synthesizing bacteria were isolated and identified from soils of metal minerals of Kerman province. The microbicidal properties of these nanoparticles and the effect on colorectal cancer-causing *E. coli* containing the *clb* gene, and the level of expression of this gene were analyzed. Our data show that these nanoparticles at certain concentrations can have microbicidal effects and could reduce the expression of the *clb* gene that contributes to colorectal cancer.

The association of bacteria with cancer has long been studied [20], but recently an increased incidence of *E. coli* has been reported in colorectal cancer [21], similar to the role of *Helicobacter pylori* in stomach cancer [22]. Arlette Darfeuille-Michaud et al. (2004) studied on Crohn's disease and found that the number of *E. coli* in the colon mucous and ileum was increased and these bacteria can bind to the ileum and attacks the epithelial cells. In patients with ulcerative colitis or Crohn's disease, the risk of colorectal cancer increases 5 times. The colitis induces the establishment of intestinal pathogens such as *E. coli*, and if the epithelium, as the first defense layer of the intestine against antigens and bacteria, does not work properly, the probability of bacterial infection and inflammation will increase. Studies have shown that in some diseases, such as inflammatory bowel *disease* (IBD), the coexistence of the intestinal microbial flora and patient is lost. History of intestinal diseases such as IBD, ulcerative colitis and Crohn's disease increases the risk of colorectal cancer because in these diseases the intestine inflames for a long time [23]. Dastjani Farahani et al. (2014) for the first time isolated the *PKS* gene-positive *E. coli* from patients with colorectal cancer and used duplex PCR to confirm the *clbB* and *clbN* genes (colibactin producers). They showed that 12.2% of the patients with colorectal cancer had *clb B* and *clbN* genes that isolated from *E. coli* [24].

Our results are similar to the data of Dastjani Farahani et al. however, to reduce the expression of the *clb* gene, we used titanium dioxide nanoparticle. Johnson et al. (2008), studied the association between phylogeny and epidemiology of *PKS* positive *E coli* and evaluated the expression of *clbB* and *clbN* genes with the strains belonging to the phylogenetic group of extraintestinal pathogenic *E. coli*, and found out that 44 out of 58 strains are positive for these genes, which support our data [25]. Sosa I O, et al., (2012) isolated *E. coli* NC101 strain from the intestine to investigate the association between PKS islands, inflammatory diseases, and colorectal cancer. The data showed that among 35 IBD samples, 21 cases of colorectal cancer and 24 healthy samples, in terms of inflammatory bowel disease and colorectal cancer, the limit of genomic *PKS* was about 20.8% for control samples, 40% for IBD cases and 66.7% for colorectal cancer cases. Comparing these data to our study, out of 60 bacterial samples tested only two had both *clbB* and *clbN* genes. A high number of positive strains were observed in the European countries, because of the high incidence of colorectal cancer and intestinal diseases and also various *bacterial flora* of the *gastrointestinal* tract between Iranian and European societies [26].

Nowadays, nanoparticles with antimicrobial properties have a special place in the health and the industry. Since the last three decades, titanium dioxide due to the numerous photocatalytic, low toxicity, and no allergic reaction (contact dermatitis) has been considered by various researchers in various health and industrial settings. The properties of titanium dioxide are dependent on the particle size, its synthesis method, and ultimately the crystalline structure.

Colon used the sol-gel method on the carbon-activated surface for the synthesis of titanium dioxide nanoparticles [27]; Yung et al. used a hydrothermal method under heat and pressure [28]. Murugan et al. utilized the micro hydrothermal method [29] with the help of acoustic waves to synthesize titanium dioxide nanoparticles.

In this research, we used *Bacillus tequilensis* to synthesize titanium dioxide, because this biological method is important due to the high environmental compatibility, reduced energy consumption, and the low costs. Therefore, our nanoparticle synthesis is different from the procedures carried out by other researchers. Mina Saadat et al. evaluated the antibacterial activity of titanium dioxide nanoparticles and showed that its inhibitory effect against *Pseudomonas aeruginosa* was 2.2 µg, also the XRD and SEM data show that titanium dioxide nanoparticles were spheroid with a diameter of 40-65 nanometers, confirming the structure of titanium dioxide nanoparticles. In our study, the MIC of nanoparticles against *E. coli* was 256 µg/ml, which has a significant difference with the results obtained by other investigators, merely due to the method used for the production of the nanoparticles. Mina Saadat et al. used a chemical method for nanoparticle production rather than the biological method that we used.

In our study, the results of XRD and SEM showed that spherical nanoparticles have a diameter between 35.76 to 78.77 nm, which confirms the structure of the titanium dioxide nanoparticles [30]. Movargarnia et al. (2018) evaluated the toxicity of silver nanoparticles synthesized by green method against human embryonic kidney cell line (HEK293) using MTT assay. They showed that cytotoxicity of the silver nanoparticles is dose- and time-dependent and significantly reduced the cell survival rate, with an IC50 value of 61.38 µg/ml during 24hr [31].

In this report, we evaluated the toxicity of the titanium dioxide nanoparticles, synthesized biologically, against HEK293 cell line by MTT assay. Our data show that the vital activity of the cells was minimal after 48 hours, but after 72 hours it increased significantly ($P > 0.05$). The lowest vital activity of the cells after 48 and 72 hours was 54.12% and 63.43%, respectively, when 0.1 µg/ml titanium dioxide was used. However, when 5 and 100 µg/ml nanoparticles were used the cell viability increased 72 hours to 100% when 5 µg/ml titanium dioxide was used. Therefore, our results contradict the data obtained from TiO2 nanoparticle's use. Real-time PCR analysis showed that titanium dioxide could reduce the expression of the *E coli clb* gene by 20.44%. These data indicate that titanium dioxide nanoparticles with the lowest cytotoxicity could be used for the treatment of microbial diseases and cancers. In conclusion, the results obtained in this study indicate that the synthesized nanoparticles could reduce the expression of *clbB* and *clbN* genes of *E. coli* with the lowest cytotoxicity. Therefore, it can be considered for the treatment of colon cancer caused by *clb* gene-positive *E. coli*.

Figures and Tables legends

Table 1. The ΔCt of *clb* gene exposed to titanium dioxide nanoparticles

ΔCT value (Experimental)	ΔCT value (control)	Delta Delta Ct value	Expression Fold change
ΔCTE	ΔCTC	ΔΔCt	2- ΔΔCt
7.65	7.32	0.33	0.7955

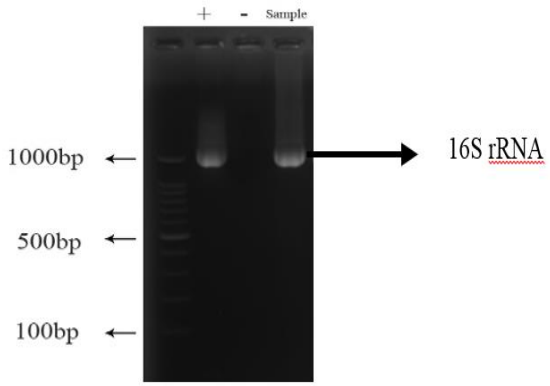

Figure 1. PCR of 16S-rRNA of the *Bacillus tequilensis* using specific primers

From left to right, DNA markers, positive control, negative control and *16S-rRNA* of the *Bacillus tequilensis*.

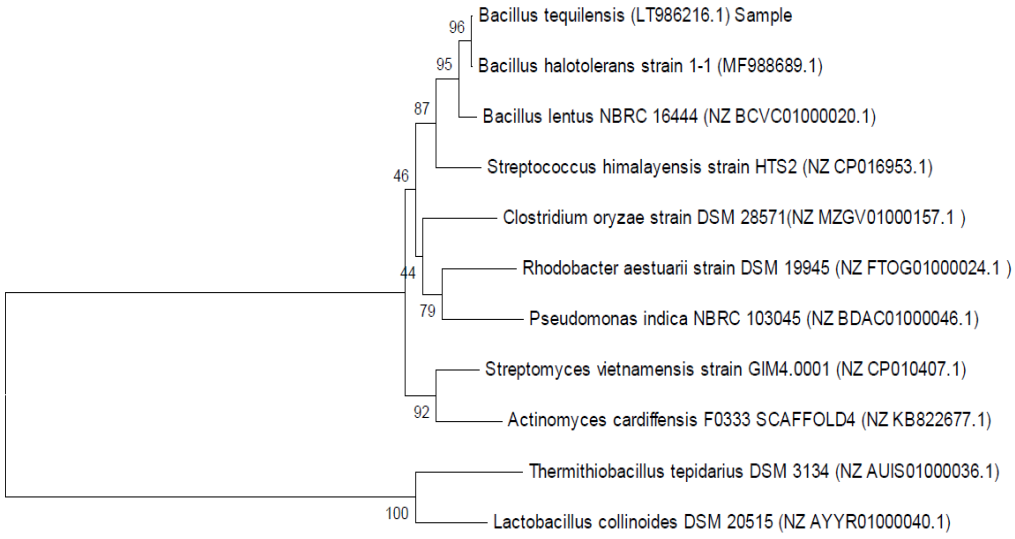

Figure 2. The phylogenic tree representing the relationship between the *16S rRNA* sequences of the *Bacillus tequilensis* (LT986216) with other bacteria using GenBank. The numbers in the branching node represent the bootstrap value (%).

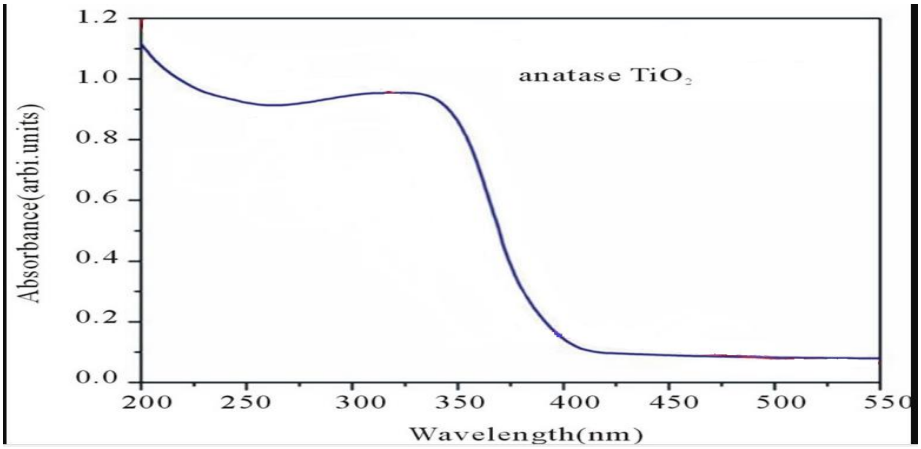

Figure 3. UV-vis spectrophotometer of the titanium dioxide nanoparticles.

The highest optical density of the titanium dioxide nanoparticles was seen at 350 nm.

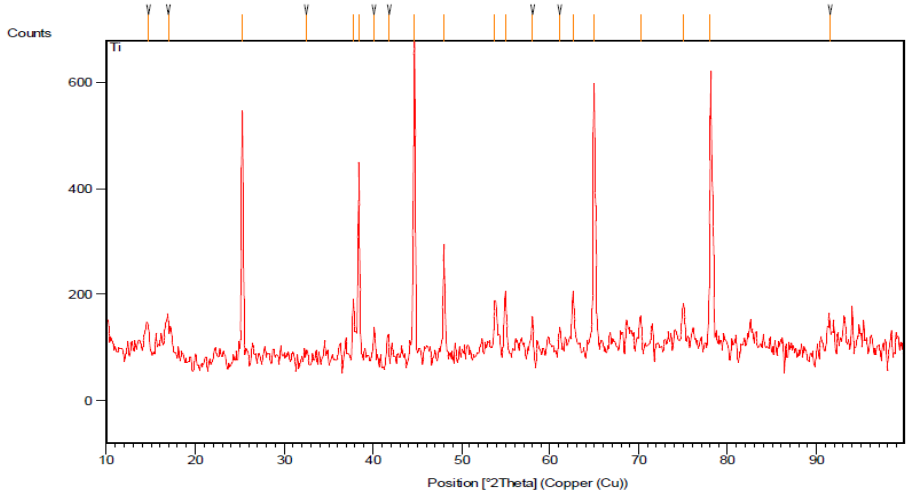

Figure 4. XRD pattern of titanium dioxide nanoparticles.

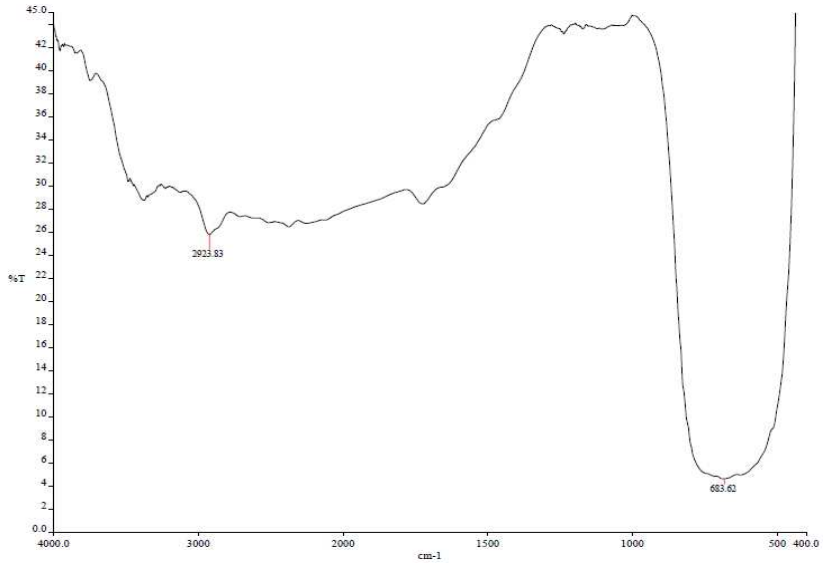

Figure 5. Fourier transform infrared spectroscopy of titanium dioxide nanoparticles

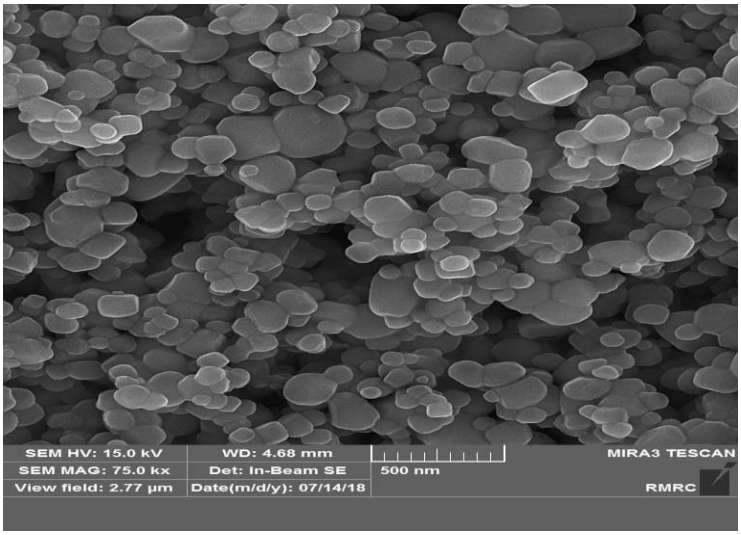

Figure 6. SEM image of titanium dioxide nanoparticles synthesized by the *Bacillus tequilensis*

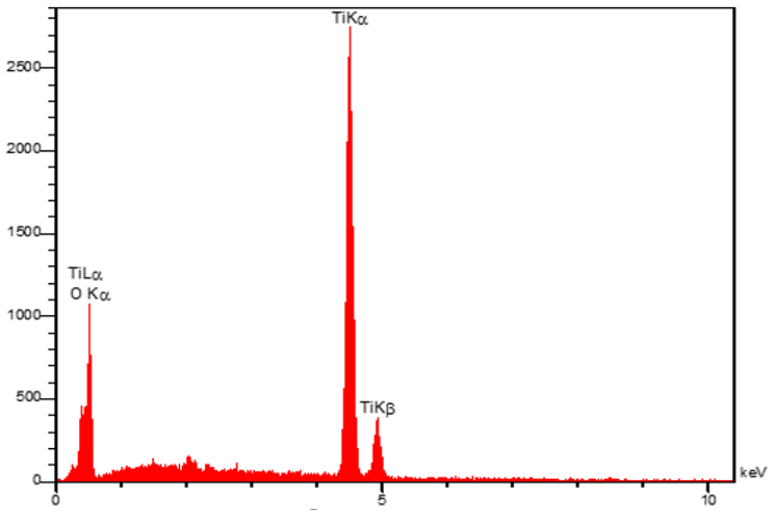

Figure 7. X-ray diffraction spectroscopy of nanoparticles produced by *Bacillus tequilensis*

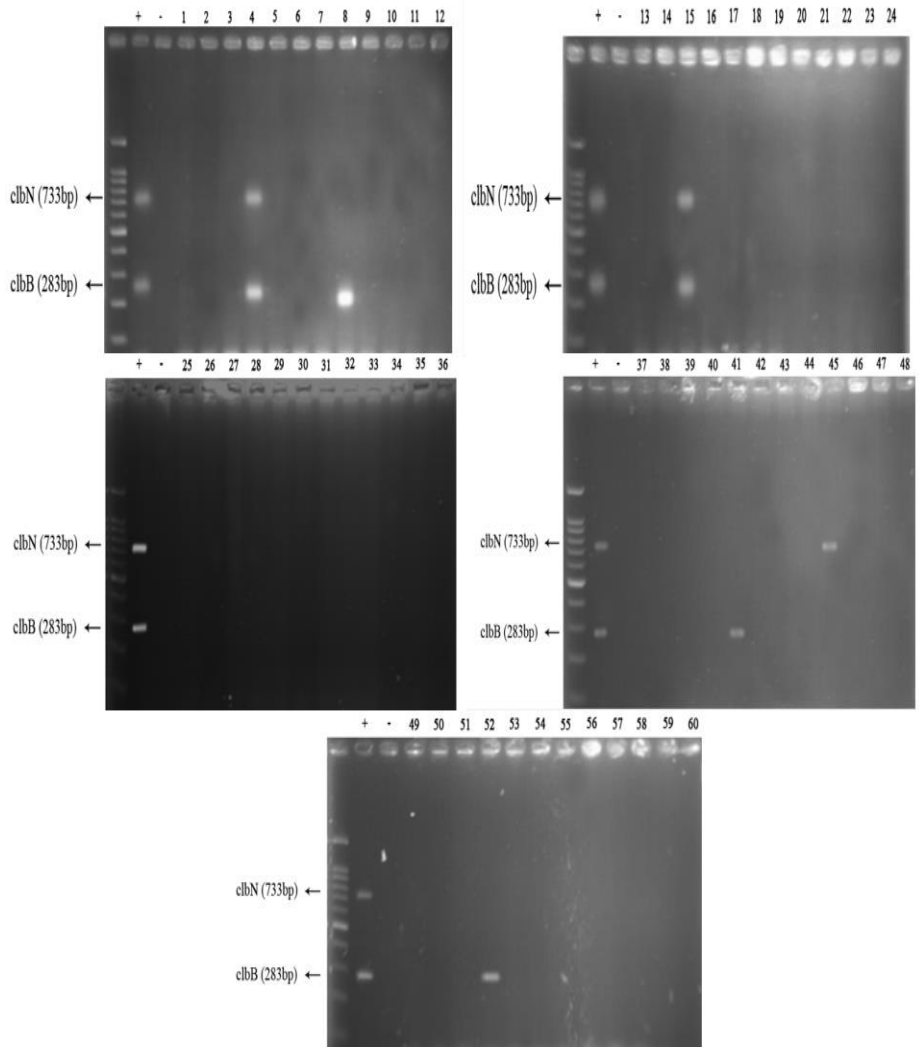

Figure 8. The frequency of *clbB* and *clbN* genes in *E. coli* strains. From left to right, M; markers, (+); positive control, (-); negative control and lanes 1 to 60 isolates of *E. coli*

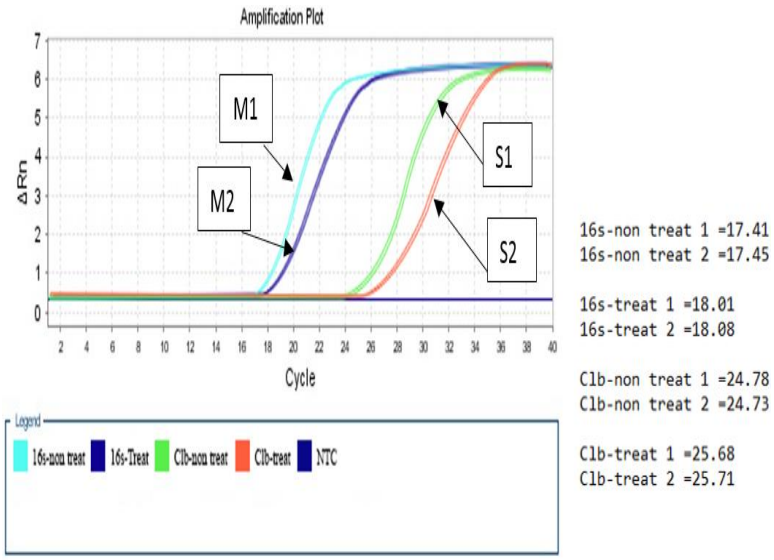

Figure 9. The *clb* gene expression pattern before and after treatment with titanium dioxide nanoparticles and the progression curve of *16S rRNA* gene response as a Housekeeping gene together with Ct was calculated.

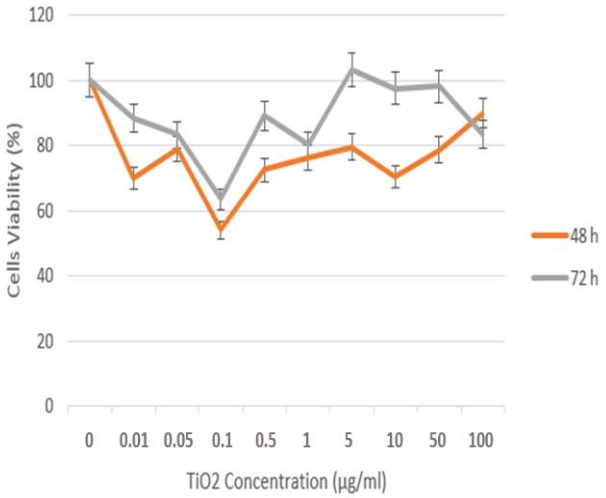

Figure 10. Percent of HEK293 cells viability after 48 and 72 hr treatment with titanium dioxide nanoparticles.

References

1. Wu S, Powers S, Zhu W, Hannun YA. Substantial contribution of extrinsic risk factors to cancer development. Nature. 2016 Jan;529(7584):43-7.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016 Jan;66(1):7-30.
3. Moradi A, Khayamzadeh M, Guya MM, Mirzaei HR, Salmanian R, Rakhsha A, Akbari ME. Survival of colorectal cancer in Iran. Asian Pac J Cancer Prev. 2009 Jan 1;10(4):583-6.
4. Wassenaar TM. E. coli and colorectal cancer: a complex relationship that deserves a critical mindset. Critical reviews in microbiology. 2018 Sep 3;44(5):619-32.
5. Bakthavatchalu V, Wert KJ, Feng Y, Mannion A, Ge Z, Garcia A, Scott KE, Caron TJ, Madden CM, Jacobsen JT, Victora G. Cytotoxic Escherichia coli strains encoding colibactin isolated from immunocompromised mice with urosepsis and meningitis. PloS one. 2018;13(3).
6. Bossuet-Greif N, Vignard J, Taieb F, Mirey G, Dubois D, Petit C, Oswald E, Nougayrède JP. The colibactin genotoxin generates DNA interstrand cross-links in infected cells. MBio. 2018 May 2;9(2):e02393-17.
7. Faïs T, Delmas J, Barnich N, Bonnet R, Dalmasso G. Colibactin: more than a new bacterial toxin. Toxins. 2018 Apr;10(4):151.
8. Lee E, Lee Y. Prevalence of Escherichia coli carrying pks islands in bacteremia patients. Annals of laboratory medicine. 2018 May 1;38(3):271-3.
9. Wang, X., and Xi Chen, eds. Novel Nanomaterials for Biomedical, Environmental and Energy Applications. Elsevier, 2018..
10. Hair BB, Conley ME, Wienclaw TM, Conley MJ, Berges BK. Synergistic Activity of Silver Nanoparticles and Vancomycin Against a Spectrum of Staphylococcus aureus Biofilm Types. BioRxiv. 2018:337436.
11. Singh R, Vora J, Nadhe SB, Wadhwani SA, Shedbalkar UU, Chopade BA. Antibacterial activities of bacteriagenic silver nanoparticles against nosocomial Acinetobacter baumannii. Journal of nanoscience and nanotechnology. 2018 Jun 1;18(6):3806-15.
12. Liu J, Li S, Bhethanabotla VR. Integrating metal-enhanced fluorescence and surface acoustic waves for sensitive and rapid quantification of cancer biomarkers from real matrices. ACS sensors. 2018 Jan 26;3(1):222-9.
13. Arora A, Padua GW. Nanocomposites in food packaging. Journal of Food science. 2010 Jan;75(1):R43-9.
14. El-Wakil NA, Hassan EA, Abou-Zeid RE, Dufresne A. Development of wheat gluten/nanocellulose/titanium dioxide nanocomposites for active food packaging. Carbohydrate polymers. 2015 Jun 25;124:337-46.
15. Chaudhry Q, Castle L. Food applications of nanotechnologies: an overview of opportunities and challenges for developing countries. Trends in Food Science & Technology. 2011 Nov 1;22(11):595-603.
16. Weir A, Westerhoff P, Fabricius L, Hristovski K, Von Goetz N. Titanium dioxide nanoparticles in food and personal care products. Environmental science & technology. 2012 Feb 21;46(4):2242-50.
17. Peng T, Zhao D, Dai K, Shi W, Hirao K. Synthesis of titanium dioxide nanoparticles with mesoporous anatase wall and high photocatalytic activity. The journal of physical chemistry B. 2005 Mar 24;109(11):4947-52.
18. Kirthi AV, Rahuman AA, Rajakumar G, Marimuthu S, Santhoshkumar T, Jayaseelan C, Elango G, Zahir AA, Kamaraj C, Bagavan A. Biosynthesis of titanium dioxide nanoparticles using bacterium Bacillus subtilis. Materials Letters. 2011 Sep 1;65(17-18):2745-7.
19. Djerdj I, Arčon D, Jagličić Z, Niederberger M. Nonaqueous synthesis of metal oxide nanoparticles: short review and doped titanium dioxide as case study for the preparation of transition metal-doped oxide nanoparticles. Journal of Solid State Chemistry. 2008 Jul 1;181(7):1571-81.
20. El-Batal AI, Amin MA, Shehata MM, Hallol MM. Synthesis of silver nanoparticles by Bacillus stearothermophilus using gamma radiation and their antimicrobial activity. World Appl Sci J. 2013;22(1):01-16.
21. Iravani S, Korbekandi H, Mirmohammadi SV, Zolfaghari B. Synthesis of silver nanoparticles: chemical, physical and biological methods. Research in pharmaceutical sciences. 2014 Nov;9(6):385.
22. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM. Intestinal inflammation targets cancer-inducing activity of the microbiota. science. 2012 Oct 5;338(6103):120-3.
23. Darfeuille-Michaud A, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. Gastroenterology. 2004 Aug 1;127(2):412-21.
24. Mohammad Ganji S, Sohrabi M. Study of relationship between a strain of E. coli and colorectal cancer. Iranian Journal of Medical Microbiology. 2015 Jul 15;9(2):26-31.
25. Johnson JR, Russo TA. Extraintestinal pathogenic Escherichia coli:"the other bad E coli". Journal of Laboratory and Clinical Medicine. 2002 Mar 1;139(3):155-62.
26. Sosa IO, Noguez C, Barrera RG. Optical properties of metal nanoparticles with arbitrary shapes. The Journal of Physical Chemistry B. 2003 Jul 3;107(26):6269-75.
27. Colón G, Hidalgo MC, Munuera G, Ferino I, Cutrufello MG, Navio JA. Structural and surface approach to the enhanced photocatalytic activity of sulfated TiO2 photocatalyst. Applied Catalysis B: Environmental. 2006 Mar 22;63(1-2):45-59.
28. Lee J, Lee YH, Choi JS, Park KS, Chang KS, Yoon M. Hydrothermal synthesis of defective TiO 2 nanoparticles for long-wavelength visible light-photocatalytic killing of cancer cells. RSC Advances. 2015;5(121):99789-96.
29. Murugan K, Dinesh D, Kavithaa K, Paulpandi M, Ponraj T, Alsalhi MS, Devanesan S, Subramaniam J, Rajaganesh R, Wei H, Kumar S. Hydrothermal synthesis of titanium dioxide nanoparticles: mosquitocidal potential and anticancer activity on human breast cancer cells (MCF-7). Parasitology research. 2016 Mar 1;115(3):1085-96.
30. Saadat M, Roudbar Ms, Khavarinejad R, Taghavi E. The evaluation of the antibacterial activity of TiO2 nanoparticles, EDTA and garlic on the standard strain of Pseudomonas aeroginusa by microdilution method. 2011.
31. Movagharnia R, Baghbani-Arani F, Sadat Shandiz SA. Cytotoxicity effects of green synthesized silver nanoparticles on human colon cancer (HT29) cells. KAUMS Journal (FEYZ). 2018 Apr 10;22(1):31-8.