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Green Synthesis of Bismuth Oxide Nanoparticle by cell-free extract of Spirulina Platensis and evaluation of its performance on expression of apoptosis regulator genes in MCF-7 breast cancer cell line

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A B S T R A C T

Bismuth and its related compounds such as bismuth oxide nanoparticles (Bi_2O_3 NPs) are widely used in the medical field of science for drug delivery, cancer therapy, bio imaging, and fighting against pathogenic microorganisms. Most of the chemical compounds used in the synthesis of nanoparticles are toxic to human health and the environment, for this reason, recently considerable interest has been focused on the green synthesis of non-toxic and environmentally friendly nanoparticles (NPs). In the biosynthesis procedure, chemicals reducing agents are replaced by cell extract of organisms that contain bio-reductive compounds as stabilizing and capping agent for NPs. In this study, bismuth oxide nanoparticles (Bi_2O_3 NPs) were prepared using green synthesis by cyanobacterial cell extract of *Spirulina platensis*. The physico-chemical properties of the Bi_2O_3 NPs characterized by X-ray diffraction (XRD), energy dispersive X–ray (EDX) analysis, Fourier-transform infrared (FT-IR) spectroscopy, scanning and transmission electron microscopy (SEM). The apoptotic properties of biosynthesized nanoparticles on MCF-7 and MCF-10a cell lines were determined using the effect of the Bi_2O_3 NPs on the expression of BAX and BCL2 genes by real-time polymerase chain reaction. The size, shape, and morphology of Bi_2O_3 NPs are suitable for penetrating into the cancer cells and affecting their vital activities. In the MCF-7 cell line, the ratio of BAX/BCL2 expression was significantly higher than that of in MCF-10a cell line treated with the Bi_2O_3 NPs. The results revealed the stimulation of apoptosis in breast cancer cell line by green synthesized Bi_2O_3 NPs through an increase in apoptosis regulator genes expression.

Introduction

Researchers have studied cancer for many years, and they have discovered valuable information about how cancer develops, grows, and spreads in the body. However, due to the lack of definitive treatments, many people around the world die each year from various types of cancer (Hanahan *et al.*, 2022). According *to* GLOBOCAN 2020 data (Sung *et al.*, 2020) the worldwide number of cancer new cases for both sexes and all ages has increased to 19.3 million new cases and 9.9 million deaths in 2020. In between, for the first

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time, breast cancer became the most common type of cancer with a diagnosis rate of 11.7% for both men and women. In 2020, breast cancer was the most common and leading cause of cancer deaths in women and the fourth deadliest cancer worldwide, with approximately 700 deaths. The information shows the importance of improving cost-effective early diagnostic methods, making available treatments more effective and safer, and reducing cancer mortality.

To date, many different methods and drugs have been used to treat cancer. However, these methods have some weaknesses. Among them, common chemotherapeutic drugs have less bioavailability, rapidly clearance by renal excretion, uneven distribution, and serious side effects. Nanotechnology is a multidisciplinary area of the science that has experienced a rapid rise in recent years and has various applications in biology and medicine. Nano biotechnology is the synthesis, application, and control of Nano sized materials (1–100 nm) in the field of biology. Currently, various nanoparticles designed and used for drug delivery, diagnosis of diseases in the early stages and their treatment.

Silver nanoparticles (Ag NPs) are one of the metallic nanoparticles that many researchers have studied for their anti-cancer and antimicrobial properties. Yesmin *et al.* (2017) investigated the in vitro anticancer activity of shape-controlled Ag NPs in different organ-specific cell lines. Their studies showed the antioxidant activity and antitumor properties of Ag NPs in a dose-dependent manner, which can be an indication for the potential use of these nanoparticles in anti-cancer treatment. The anti-tumor property of silver nanoparticles has been tested and confirmed in some other studies such as Salehzadeh *et al.* (2018), Al-Sheddi *et al.* (2018), Alhadrami *et al.* (2021). Furthermore, silver based nanoparticles are highly toxic for microorganisms. There are some reports about the antibacterial potential of Ag NPs against *Vibrio cholerae*, *Staphylococcus aureus*, *Salmonella typhi*, and *Salmonella paratyphi* (Thomas *et al.*, 2015). Zinc (Zn), copper (Cu), and gold (Au) are other metal nanoparticles reported to have anticancer (Andleeb *et al.*, 2021) and antibacterial (Slavin *et al.*, 2017) properties. It seems that these nanoparticles affect the target cells by activating of autophagy, apoptotic and necrotic death pathways based on the production of reactive oxygen species (ROS) (Kovacic and Somanathan; Canaparo *et al.*, 2020; Yu *et al.*, 2020).

Bismuth (Bi) is a relatively non-toxic and inexpensive heavy metal with remarkable properties and has many biomedical uses. Bismuth is commonly used in the form of nitrates, oxide, halides, and Oxo halides, derivatives. Bismuth-based compounds are widely used as pharmaceuticals to treat gastrointestinal disorders such as dyspepsia, stomach ulcers, and Helicobacter pylori infections. Recently, its medical applications have been expanded to include potential therapeutics for viral infections, multidrug-resistant microbial infections, cancer, as well as imaging, biosensing, and drug delivery.

Ionizing X-ray radiotherapy is a widely used method for treating cancer, and bismuth can be used as a radiosensitizer to enhance X-ray radiotherapy (Deng *et al.*, 2017). da Luz *et al.* (2020) reported no loss of membrane integrity or significant changes of RNS and ROS levels in the murine macrophage line RAW 264.7 exposed to Bi NPs. However, phagocytic activity was increased and DNA repair foci appeared in cells exposed to Bi NPs. Abudayyak *et al.* (2017) showed in vitro geno- and cytotoxic effects of Bi_2O_3 nanoparticles in A549 (lung carcinoma cell), Caco-2 (colorectal adenocarcinoma cell), NRK-52E (kidney epithelial cell), and HepG2 (hepatocarcinoma cell) cell lines. They observed that the predominant pathways of cell death were apoptosis in NRK-52E and HepG2 cells and necrosis in A549 and Caco-2 cells exposed to Bi_2O_3 NPs. Hernandez-Delgadillo *et al.* (2012) showed complete inhibition of biofilm formation and 69% antibacterial activity of stable colloidal bismuth nanoparticles against the growth of *Streptococcus mutans*. Furthermore, according to Qin *et al.* (2014) Bi_2O_3 porous Nano spheres showed highly efficient antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Chemical reducing agents are cytotoxic agents and the synthesis of nanoparticles by chemical and physical methods is usually expensive. Therefore, in recent years the biological synthesis of nanoparticles using living microorganisms or cell extracts obtained from them as a suitable alternative to the method chemical and physical methods have been developed (Ahmad *et al.*,

2019). The easy availability, nontoxic nature, various options available, the environmentally friendly protocols, and the quick synthesis procedure make green technique as the best choice for nanoparticles synthesis.

Although the toxicity of Bi-based compounds has been studied for years, information on toxicity effects on healthy human cells is very little and there is serious concern about the side effects of these nanoparticles. Therefore, the aim of this study is green synthesis and characterization of Bi(III) oxide (Bi_2O_3) NPs using *Spirulina platensis* and investigation of the in vitro toxic effects of these nanoparticles against human breast cancer cells (MCF-7) and human breast epithelial cell line (MCF-10a).

Materials and methods

Cyanobacteria extract preparation

The experimental cyanobacterium *Spirulina platensis* for this study was prepared from the Parsian Microalgae Company, Rasht, Iran. Forty grams of *Spirulina* powder were homogenized in 50 mL of deionized distilled water and maintained in a 55 °C water-bath for 20 minutes. The solution was centrifuged for 10 min at 6000 rpm (Eppendorf, Hamburg, Germany). The supernatant was *filtered through Whatman filter paper No. 1* and concentrated by a rotary evaporator vacuum pump (Rotavapor R-100 Rotary Evaporator, Buchi, Flawil, Switzerland). The supernatant was used and stored at 4 °C for further process, after the concentration was determined.

Synthesis and characterization of Bi2O3 nanoparticles

To prepare bismuth oxide nanoparticles, 2 g of bismuth nitrate (Merck KGaA, Germany) was dissolved in the 10 mL of deionized distilled water and continuously stirred at 90 °C. In the next step, added 20 mL of aqueous cyanobacteria extract at 90 °C under constant stirring for 24 h. Bi_2O_3 NPs were prepared after 24 h at 90 °C . Then, the product washed several times with double distilled water, and dried in a vacuum. The product was heated at 550 °C in a furnace and static atmosphere of air for 5 h to ensure the removal of impurities (a yellow powder) .

The absorbance spectra of the reaction mixture were recorded with a Lambda 25 UV–visible spectrophotometer (Perkin-ELMER) in the range of 200–700 nm. Powder X-ray diffraction (PXRD) data were collected using a Philips X'Pert MPD diffractometer (Cu-Kα X-radiation, λ = 1.54 Å). Possible interactions between biosynthesized nanoparticles and proteins were determined by Fourier transform infrared (FTIR) spectroscopy with the KBr pellet method by a Shimadzu 8900 FT-IR spectrophotometer. Measurements were performed in the range of 500–4000 cm^{-1} at a resolution of 4 cm^{-1}. An energy dispersive X–ray (EDX) analyzer (MIRA3 FEG–SEM series) used for elemental analysis. The size and morphology of Bi_2O_3 NPs were characterized by scanning electron microscope (SEM) (KYKY-EM3200) and transmission electron microscope (TEM) (Philips EM208S 100KV).

RNA extraction and quantitative Real- Time PCR

BAX is a pro-apoptotic member of the BCL-2 family of genes which regulate programmed cell death. The BAX protein shares highly conserved domains with BCL-2, some of which are required for the formation of BAX -BCL-2 heterodimers. The ratio of BAX to BCL-2 also determines survival or death of a cell following an apoptotic stimulus. To evaluate the expression of BAX protein to BCL-2 in the presence of Bi_2O_3 NPs in MCF-7 cells, total RNA was extracted from the cultured cells in the absence and presence of bismuth oxide nanoparticles. According to Alamer *et al.* (2021), Bi_2O_3 NPs in concentrations greater than 5 µg/ml and based on the results of Ahamed *et al.* (2019) in concentrations greater than 50 µg/ml are toxic for human breast cancer (MCF-7) cells. Therefore, to investigate the effect of Bi_2O_3 NPs on the expression of BAX (BCL-2 Associated X-protein) and BCL2 genes, MCF-7 and MCF-10a cell lines were exposed to 100 µg/ml of green synthesized nanoparticles. Total RNA was extracted from the Bi_2O_3 NPs treated and the controls using RNX- Plus solution (sinaclon, Iran) following the manufacturer's instructions. To remove genomic DNA contamination, extracted RNA was treated with DNaseI (sinaclon, Iran) and subsequently, its quality and quantity determined by a Nano-Drop ND 1000 Spectrophotometer (Wilmington, USA). The first cDNA strand synthesized by 2-steps RT-PCR Kit (vivantis, Malaysia) using 1 µg RNA.

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Quantitative real-time PCR performed in triplicate using an Eppendorf Mastercycler® gradient thermal cycler (Germany.) Specific primers described in Table 1. The final reaction volume of 25 µL was prepared with SYBR Green master mix, 0.5 µL of the synthesized cDNA, 0.5 µL of forward and reverse primer, and DEPC treated water. PCR thermocycling conditions was as follows: initial denaturation (94°C/15 min), and 40 cycles of denaturation at 95 °C/20 s, annealing at 60 °C for 40 s, and extension at 72 °C for 15 s. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for normalization and quantitation of gene expression. BAX and BCL2 and GAPDH mRNA expression levels were analyzed as fold-change by the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001).

Table 1 - Forward and reverse primer sequences for the BAX, BCL2 and GAPDH.

Primer	sequence (5′ to 3′)	Target Size (bp)	Ref.
BCL2	F: TGAACTGGGGGAGGATTGTG R: CGTACAGTTCCACAAAGGCA	183	Vivarelli *et al.* (2020)
BAX	F: CCCAGAGGCGGGGTTTCA R: GGAAAAAGACCTCTCGGGGG	207	Jin *et al.* (2017)
GAPDH	F: AGAAGGCTGGGGCTCATTTG R: AGGGGCCATCCACAGTCTTC	258	Vivarelli *et al.* (2020)

Statistical Analysis

The data analysis performed using SPSS statistical software version 21.0 (SPSS, Inc., Chicago, IL, USA). All the experiments were done in triplicate and the results were expressed as mean ± SD. One-way analysis of variance test (University Park USA) used to compare means of control and treatment groups and P-value ≤ 0.05 was considered the minimal level of significant difference.

Results

Characterization of Bi_2O_3 NPs

UV–Visible spectra of Bi_2O_3 NPs provided in Fig. 1. The maximum absorption of the synthesized Bi_2O_3 NPs was observed at ~ 290 nm, confirming the stability and good dispersion of the particles in the reaction medium.

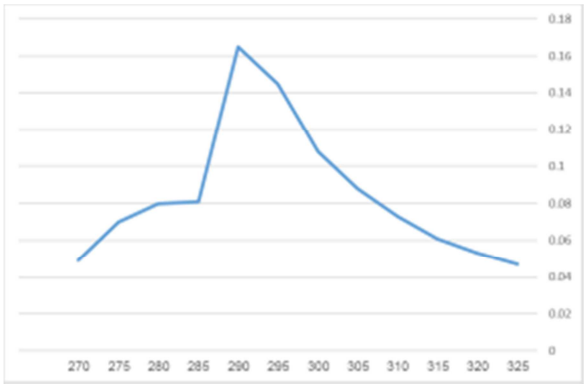
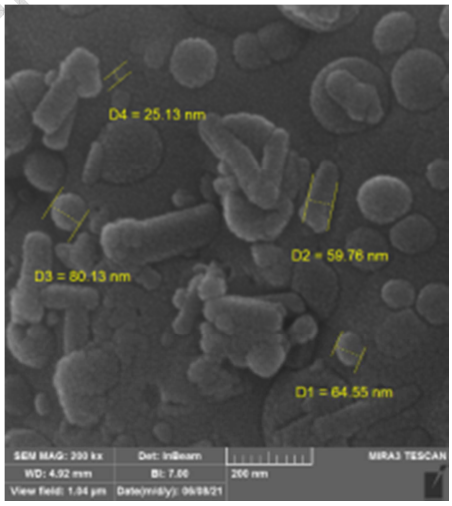


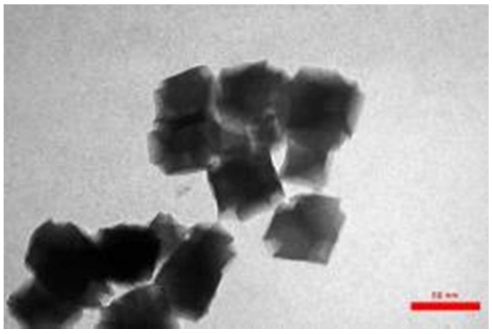
Fig. 1 - UV–Vis absorption spectra of Bi_2O_3 nanoparticles.

Fig. 2a and b display the SEM and TEM images of Bi_2O_3 NPs, respectively. Results of electron microscopy were showed the small nanoparticles with the average size of about 45 nm that are in good agreement with the particle sizes obtained from XRD results and suitable for affecting cancerous cells.

a)



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b)

Fig. 2 – (a) SEM image; (b) TEM images of Bi_2O_3 nanoparticles.

The X-ray diffraction (XRD) is a high-speed analysis method used to determine the crystalline structure of Bi_2O_3 NPs. The XRD patterns of the samples were recorded from 2θ= 0° to 90° and the results revealed Fig. 3. The diffraction peak at 2θ values of 28.51° indicate the face–centered cubic phase of Bi_2O_3 (J.C.P.D.S. file #41-1449). According to XRD data the average size of Bi_2O_3 nanoparticles was found to be 46 nm using the Debye-Scherer equation.

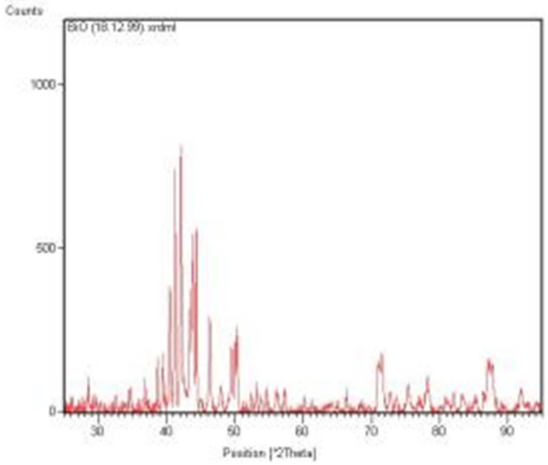


Fig. 3 - The XRD patterns of the synthesized Bi_2O_3 nanoparticles.

The FTIR (Fourier-transform infrared spectroscopy) absorption spectrum was recorded in the range of 500–4000 cm^{-1} to identify potential biomolecules in *Spirulina platensis* cell-free extracts involved in the reduction, diffusion and protection of Bi_2O_3 NPs (Fig. 4). The O–H stretching vibrations and the overlap of the tensile vibrations of the primary and aromatic amino groups of cyanobacterial cell extract molecules appeared at 3431 cm^{-1}. A peak is observed at 1631 cm^{-1}, which corresponds to the bending of the amino groups (N-H). This functional group is part of the amino acid side chains in the structure of proteins. Thus, proteins probably play an important role in the formation of nanoparticles in the environment. A sharp peak at 1421 cm-1 is observed due to the unusual deformation of C-H, which indicates the placement of cyanobacterial cell extract molecules on the surface of nanoparticles .This peak confirms the reaction between carbon compounds and oxygen from bismuth oxide nanoparticles. A peak is observed at 1039 cm^{-1} which is attributed to the Stretching vibration of the alkyl functional groups. The peak in the 874 cm^{-1} region corresponds to the Bi-O vibration and the peak in the 630 cm^{-1} region corresponds to the Bi-O non-bridging oxygen.

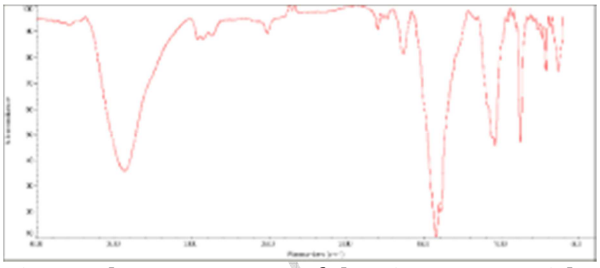


Fig. 4 - The FTIR spectra of the Bi_2O_3 nanoparticles synthesized using *Spirulina platensis*.

The results of energy-dispersive X-ray spectroscopy (EDS) analysis clearly confirmed the presence of Bi and O biosynthesized nanoparticles (Fig. 5).

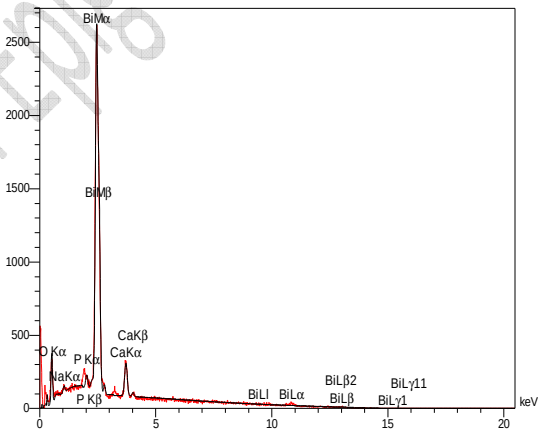


Fig. 5 - The EDS Image of Bi_2O_3 nanoparticles.

BAX and BCL2 expression

Changes in BAX and BCL2 levels are considered factors that may explain the favorable correlation between treatment with Bi2O3 nanoparticles and apoptosis. Increased levels of the pro-apoptotic BAX protein and decreased levels of the anti-apoptotic BCL2 protein permeabilize the mitochondrial outer membrane, allowing the

release of cytoplasmic apoptotic factors such as cytochrome c. This release leads to activation of the caspase cascade. The relative expression of the BAX and BCL2 genes in the MCF-7 and MCF-10a cells, evaluated in the presence of 100 µg/ml of green synthesized nanoparticles Bi_2O_3 NPs by qRT-PCR analysis. Based on the results (Fig. 6), BAX gene expression after treatment with nanoparticles increased by 3.73 units in the fold change diagram. The expression of BCL2 gene in this cell line also increased after treatment with nanoparticles and reached 1.25 units in the fold change diagram. GAPDH gene expression was unchanged in the presence of Bi2O3 NPs in both of MCF-7 and MCF-10a cells. Considering that a high ratio of BAX/BCL2 is a characteristic of cells sensitive to apoptosis, an increase in this ratio in MCF-7 and MCF-10a cells in the presence of Bi_2O_3 NPs indicates the effect of the nanoparticles in induction of apoptosis.

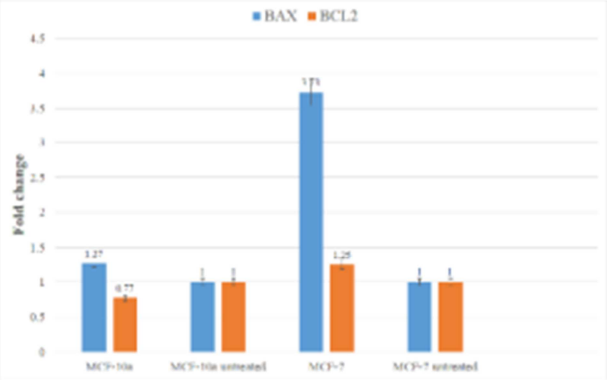


Fig. 6 - The fold relative transcription level of BAX and BCL2 genes in the MCF-7 and MCF-10a cell lines in the presence of Bi_2O_3 nanoparticles (n=3, P<0.05).

Discussion

Despite great advances in cancer treatment, breast cancer is now the leading cause of cancer-related death in women worldwide. In Iran, breast cancer accounts for 76% of the most common cancers in women (Jazayeri et al., 2015). Therefore, researchers are constantly looking for new methods as well as novel and more effective anticancer agents with reduced toxicity and adverse side effects on healthy cells. In between, metallic nanoparticles with antitumor properties have been receiving considerable attention in recent years. However, there have always been concerns about the side effects of these nanoparticles on normal tissues. In this study, we investigated the effects of Bi_2O_3 NPs, which have many applications in the medical field, including cancer tissue diagnosis and therapy, on the expression of BAX and BCL2 genes in MCF-7 and MCF-10a cell lines.

In this study, to prevent the effects of toxic substances used in the chemical methods of nanoparticle synthesis on the tested cells, Bi_2O_3 NPs were synthesized by the green method using the cyanobacterial cell extracts of *Spirulina platensis*. The formation of fabricated Bi_2O_3 NPs was characterized and verified by UV-Vis spectroscopy analysis. The FTIR spectrum results clearly showed the existence of active biomolecules like hydroxyl and carbonyl in the extract of *Spirulina platensis*, which may act towards the bio reduction and stabilizing of Bi^{3+} to Bismuth oxide nanoparticles. Scanning and transmission electron microscopy and *XRD* analysis showed biosynthesized Bi_2O_3 NPs have a spherical shape with the average size of ~45 nm. The size of Bi_2O_3 NPs synthesized chemically by Varshney et al. using NaBH4 was 14 nm. Motakefu Kazemi et al. (2020) reported that the size of Bi_2O_3 NPs synthesized using Mentha plegium extract was about 200 nm and, Ascencio Aguirre et al. (2015) reported an average size of 10.99 ± 0.27 nm for particles synthesized using tannic acid. The size of the nanoparticles synthesized by Kusuma et al. (2022) using Probe Sonication method was 60 nm. Although the size of the biosynthesized nanoparticles may be larger than of chemically synthesized, the absence of toxic chemicals on the surface of these nanoparticles is a great advantage that enables the investigation of the toxicity of the nanoparticles themselves. Therefor the size of nanoparticles synthesized using *Spirulina platensis* extract is acceptable compared to other cases of Bi_2O_3 NPs preparation techniques.

BCL-2 protein family consist of pro- and, anti-apoptotic molecules (Antonsson et al., 2000). For instance, BCL-XL and BCL-2 inhibit apoptosis, whereas BAX, BAD, and BAK, enhance apoptosis (Kausch et al., 2002; McDonnell et al., 1996). The pro- or anti-apoptotic properties of these proteins depend on the structure of the heterodimers (Adams et al., 1998). In addition, the ratio of anti- to pro-apoptotic proteins have been shown to determine, how a cell reacts to survival or apoptotic signals (Farrow et al., 1996).

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It has been reported that in some human cancer cell lines, the levels of BCL-2 and BCL-XL expressions have a positive correlation with the prevention of induction of apoptosis by various cytotoxic drugs as well as metallic nanoparticles (Schmitt et al., 1996). On the other hand, over-expression of BAX protein enhanced apoptosis in the cancer cells exposed to several chemotherapeutic agents by increasing the outer membrane permeability of the mitochondria in MCF-7 cells and releasing of the cytochrome C as cytosolic apoptogenic factors.

Real time PCR used to study expression of the BAX and BCL2 genes in MCF-7 and MCF-10a cell lines after treatment by Bi_2O_3 nanoparticles. The results showed that the expression level of BAX as a pro-apoptosis gene increased greatly in cancer cells. Although the expression level of the BAX gene also increased up to 1.25, but considering that the ratio of pro-apoptotic BAX protein to anti-apoptotic BCL-2 protein increased significantly, the intrinsic and extrinsic pathway induces apoptosis via activating caspases. Ahamed et al. (2019) showed the higher expression of apoptotic genes BAX and caspase-3 and lower expression of anti-apoptotic gene BCL2 after Bi_2O_3 NPs exposure. There are reports about the effect of some metal nanoparticles on the expression of these apoptotic genes. Selim and Hendi in 2012 showed that gold nanoparticles at a concentration of about 200 µg/ml increased the mRNA expression of p53, BAX, caspase-3 and caspase-9 genes and significantly decreased the expression of the anti-apoptotic gene BCL2. Bharara et al. (2015) reported a significant decrease in BCL-2 gene expression as well as a remarkable increase in BAX gene expression after treatment with Ag-NPs. Sadoughi (2017) evaluated BAX/BCL-2 Ratio in treated liver tissue of chick embryo with silver nanoparticles and reported a decrease in the levels of BCL-2 and antioxidant enzymes and increase in BAX levels. In the both human gastric adenocarcinoma (AGS) and MCF-7 breast cancer cell lines treated by $CuFe_2O_4$@Ag nanocomposite, the mean expression of BAX was significantly higher than that of BCL2 (Garfami et al., 2019). Based on the Jalili et al. (2016), the combination therapy of cold atmospheric plasma and iron nanoparticles on MCF-7 cells, resulted in downregulation of BCL2 gene expression level and upregulation of the BAX gene.

Known as a heavy metal with low toxicity to humans, bismuth is widely used in medicine due to its excellent antibacterial properties that eradicate *Helicobacter pylori*, the cause of diseases such as gastritis, stomach ulcers, and even stomach cancer. Other medical uses of it include using the compound to treat stomach ulcers, eye infections, and syphilis. Furthermore, as shown in previous studies, BAX and BCL-2 protein expression can affect clinical outcome, patient survival and response to chemotherapy agents in cancer and may also be useful in predicting cancer incidence. For this reason, we analyzed the relative expression of these two proteins at the mRNA level in normal and tumor breast cell line tissues after treatment with Bi_2O_3 NPs. The results of the present study show that bismuth oxide nanoparticles in relatively low concentrations can increase the expression of BAX/BCL2 ratio genes in both normal and tumoral tissue, and therefore, considering the wide applications of bismuth-containing compounds in the medical and health fields, it seems that more studies are needed to investigate the toxicity of this element for normal cells as well as its effects on different organs.

Conclusions

As a conclusion, in this in vitro study, Bi_2O_3 NPs was successfully synthesized by green method using cyanobacterium *Spirulina platensis* aqueous extract as a reducing agent. The results of the characterization tests performed on Bi_2O_3 NPs showed that the size, shape, and morphology of the green synthesized nanoparticles are suitable for penetrating into the studied cells and affecting their vital activities. Furthermore, based on the results of current research and reports of other studies, the ratio of BAX/BCL-2 proteins can be used as a marker to investigate the effect of nanoparticles on cancer cells.

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