Journal of Kerman University of Medical Sciences
2022; 29(1): 31-38
http://jkmu.kmu.ac.ir/
10.22062/jkmu.2022.91861
Original Article

Study on the Cytotoxic Effects of Iron Oxide Nanoparticles Synthesized by Cytoplasmic Extract of Lactobacillus Fermentum

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ABSTRACT

Background: The science has focused on the development of nanoparticles based on the green chemistry methods. Iron nanoparticles, are of particular interest due to their fast reaction and high efficiency for the treatment of cancer cells without damage to healthy cells. In this research, iron oxide nanoparticles were synthesized using cytoplasmic extract of *Lactobacillus fermentum* and their cytotoxicity were investigated against MCF-7 cell and HEK293 normal cell.

Methods: Cytoplasmic extract of *Lactobacillus fermentum* was prepared using freeze thaw method. The achieved extract was added to an equal volume of ferrous sulfate III solution at a concentration of 10^{-3} molar and incubated for 3 weeks in the presence of 5% carbon dioxide XRD and TEM analyses were performed in order to determine the size and shape of the nanoparticles. The cytotoxic effects of the nanoparticles against cancer and normal cells were studied using MTT test.

Results: The change of solution color to black was a first sign of the production of Fe_3O_4 nanoparticles. XRD and TEM confirmed the production of the Fe_3O_4 nanoparticles and determined that the nanoparticles were spherical in shape and had the average particle size of 10-15 nm. A comparison of the toxicity of the synthesized nanoparticles on the two cell lines showed a significant decrease of the survival rate of MCF-7 cells compared to normal HEK 293cells with increasing Fe_3O_4 nanoparticles concentration.

Conclusion: The use of cytoplasmic extract of *Lactobacillus fermentum* for the production of iron oxide nanoparticles could be considered as an effective biological method in green synthesis of nanoparticles.

Keywords: Nanoparticles, *Lactobacillus fermentum*, Biosynthesis

Citation: Rezaee M, Ghandehari F, Fatemi M, Fani M, l Salehi D. Study on the cytotoxic effects of iron oxide nanoparticles synthesized by cytoplasmic extract of lactobacillus fermentum. Journal of Kerman University of Medical Sciences 2021; 29(6): 31-38. doi: 10.22062/jkmu.2022.91861

Received: 11.04. 2021
Accepted: 21.09. 2021
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Published by Kerman University of Medical Sciences

Introduction

One of the important areas of nanotechnology research is production of metallic nanoparticles and their different applications. Most of the procedures applied for nanoparticles production are not cost-effective in terms of materials and energy consumption and usually generate large amounts of hazardous waste. Hence, there is an increasing tendency toward the preparation of nanoparticles based on the green chemistry methods. For this purpose, various biological sources including plants, algae and microorganisms such as bacteria and yeasts are used (1).

Microorganisms are considered as small and live factories producing nanoparticles with the size of 1 to 200 nm using inexpensive and renewable reducing agents such as lactate and acetate at room temperature or higher temperatures (using thermophilic microorganisms). Among metallic nanoparticles, iron nanoparticles, due to their abundance, non-toxicity, fast reaction and high efficiency for the treatment of cancer cells without damage to healthy cells are of particular interest. These nanoparticles have also been synthesized in less than 20 nm in size, by some microorganisms (2). Iron oxide nanoparticles can cause cell death through lactate dehydrogenase depletion from cell membrane, mitochondrial dysfunction, chromosome compaction, production of reactive oxygen species, membrane lipid peroxidation and enzyme oxidation (3).

In this research, in line with the aims of green chemistry, iron oxide nanoparticles were synthesized using cytoplasmic extract of *Lactobacillus fermentum*, and their cytotoxic effects were assessed on MCF-7 breast cancer cell line and Human Embryonic Kidney (HEK293) normal cells.

Materials and Methods

Preparation of cytoplasmic extract of *Lactobacillus fermentum*

The strain of *Lactobacillus fermentum* PTCC (1638) was prepared from the Microbial Bank of Iran Scientific and Industrial Research Organization. It was cultured in MRS Broth medium and incubated at 37° C for 24 hours. Cytoplasmic extract of *Lactobacillus fermentum* was prepared using Freeze-thaw method.

Biosynthesis and characterizations of Fe_3O_4 nanoparticles

The prepared cytoplasmic extract of *Lactobacillus fermentum* was added to an equal volume of aqueous iron oxide solution (10^{-3} M) for the synthesis of nanoparticles. The pH of the mixture was adjusted at 5.6, and incubated at 37° C for 3 weeks in the dark in the presence of 5% carbon dioxide (4). After the incubation time, the Fe3O4 nanoparticles were synthesized and observed as sediment. The sediment was powdered by a dryer oven. The X-ray diffraction was performed to confirm production of nanoparticles. The powdered Fe_3O_4 nanoparticles was used to be characterized by X-ray diffractometer (Philips 1-1800) at a voltage of 40 kV and a current of 30 mA. The diffraction angle was varied in the range of 20–80°. The size of nanoparticles was calculated using Debye-Scherrer equation:

$D= 0.9\lambda/\beta\cos\theta$. The particle size and morphology of Fe_3O_4 nanoparticles was analyzed using (TEM) (Philips 208S 100Kv).

Cell culture

MCF-7 breast cancer cells and HEK293 normal cells were purchased from Pasteur Institute. The cell lines were grown in a RPMI1640 culture medium (Bio-Idea Co.) containing 10% FBS and 1% penicillin and streptomycin (Gibco) in the presence of 5% CO_2 at 37° C. The cell environment was changed every three days until the cells reached 80% concentration to be treated.

Cytotoxic assay

The cytotoxic effects of Fe_3O_4 nanoparticles synthesized by the cytoplasmic extract of Lactobacillus fermentum on MCF-7 cancer cells and HEK293 normal cells were studied using Microculture Tetrazolium Test (MTT) method. This method is based on the activity of mitochondrial dehydrogenases, which are important markers in cell survival. In this method, cell viability was evaluated by the classical method of mitochondrial reduction of 3,4,5-dimethylthiazol-2,5 biphenyl tetrazolium bromide (MTT) by viable cells to an insoluble purple formazan (5). To this purpose, 10^4 cells were cultured per well in a 96-well plate. 24 hours after incubation, the supernatants of cells were removed and they were treated with different concentrations of 10, 100, 1000 µg/ml of Fe_3O_4 nanoparticles for another 48 hours. Then, cells were washed twice with PBS and

incubated with 0.5 mg/mL MTT in culture medium for 2.5 h at 37 °C, 5 % CO2 and humidified atmosphere. Following incubation, the MTT solution was removed and formazan was extracted from cells with 200 µL of DMSO. The formazan-specific light absorption, was then measured at 595 nm with a spectrophotometer. The experiment was repeated three times.

Statistical analysis

The data for each group were expressed as mean ± standard deviation. The independent *t*-test was used for comparison of viability between the two cell lines, and one-way analysis of variance (ANOVA) was used to analyze the results of the cytotoxic studies of the nanoparticles on the cell lines. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 16.0 software. The statistical significance of all data was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

The first sign of formation of Fe_3O_4 nanoparticles after reduction of iron metal ions to nanoparticles by the bacterial cytoplasmic extract was the change of solution color to black (Figure 1).

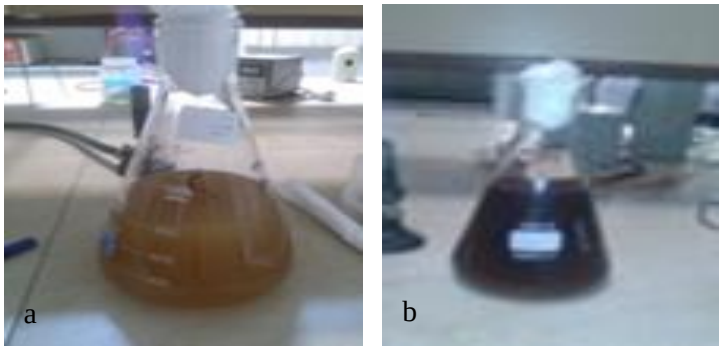

Figure 1. Color change of the reaction medium (Cytoplasmic extract of Lactobacillus fermentum and 1mM $FeSO_4$ solution) before (a) and after (b) the biosynthesis of nanoparticles

As shown in Figure 2, XRD analysis showed diffraction peaks at 2θ = 74°, 47°, 43° and 35° which can be indexed to (440), (400), (311) and (220), respectively. The size of the nanoparticles was calculated using the Debye-Scherrer equation and found to be 15 nm.

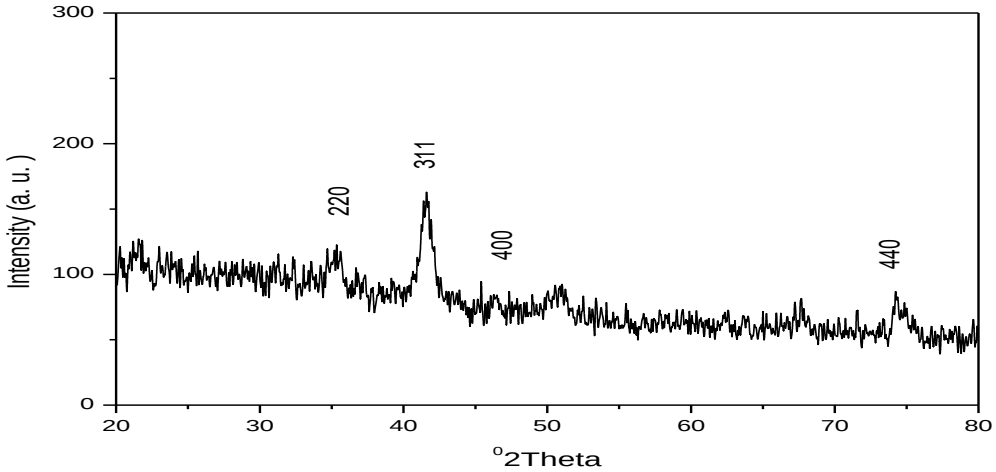

Figure 2. XRD pattern of the biosynthesized Fe_3O_4 nanoparticles. XRD analysis of iron oxide nanoparticles showed a nanoparticle size of 15 nm.

The average size of the biosynthesized Fe_3O_4 nanoparticles was observed in the range of 10-15 nm and they were spherical in shape as analyzed by TEM (Figure 3).

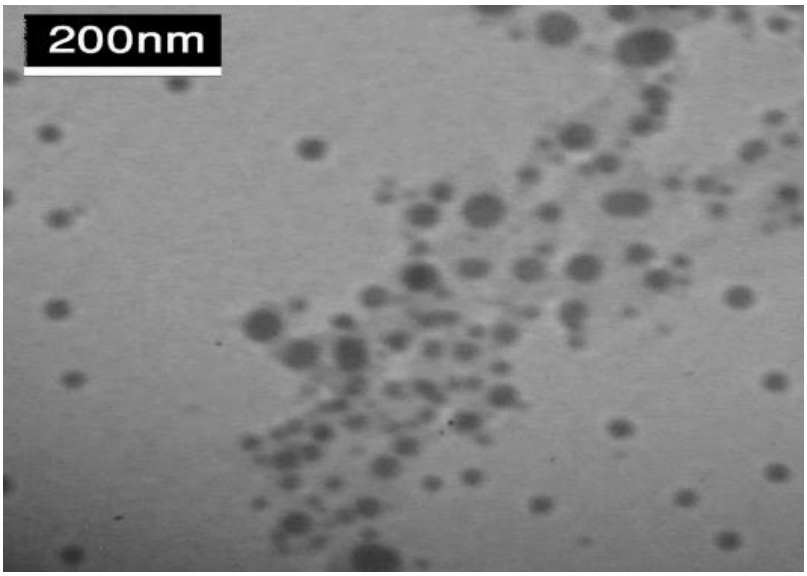

Figure 3. TEM micrographs of the biosynthesized Fe_3O_4 nanoparticles using cytoplasmic extract of *Lactobacillus fermentum* (Bar=200 nm)

The *in vitro* toxic effects of Fe_3O_4 nanoparticles at concentrations of 10, 100 and 1000 µg/ml were assessed on MCF-7 cancer cell and HEK293 normal cell lines. The cell viability percentages for MCF-7 cell line are presented in Figure (4-a). The MCF-7 cell viability was significantly ($p<0.001$) affected with a reduction in viable cells percentages to 45% and 50% for concentrations of 100 and 1000 µg/ml, respectively.

In Figure 4-b, the cell viability percentages for normal HEK293 cell line are represented. The concentration of 10 and 100 µg/ml did not significantly affect the viability of normal HEK293 cells. However, the effect of concentration of 100، µg/ml on cell viability of HEK 293 was significant ($P\leq0.001$) with a reduction in viable cells percentage to 40%.

Comparison of the cytotoxicity of the biosynthesized nanoparticles on the two cell lines shown in Figure 5 indicates that concentrations of 10 and 100 µg / ml of the synthesized nanoparticles show a more significant reduction in cancer cell survival compared to normal cells ($P\leq0.001$).

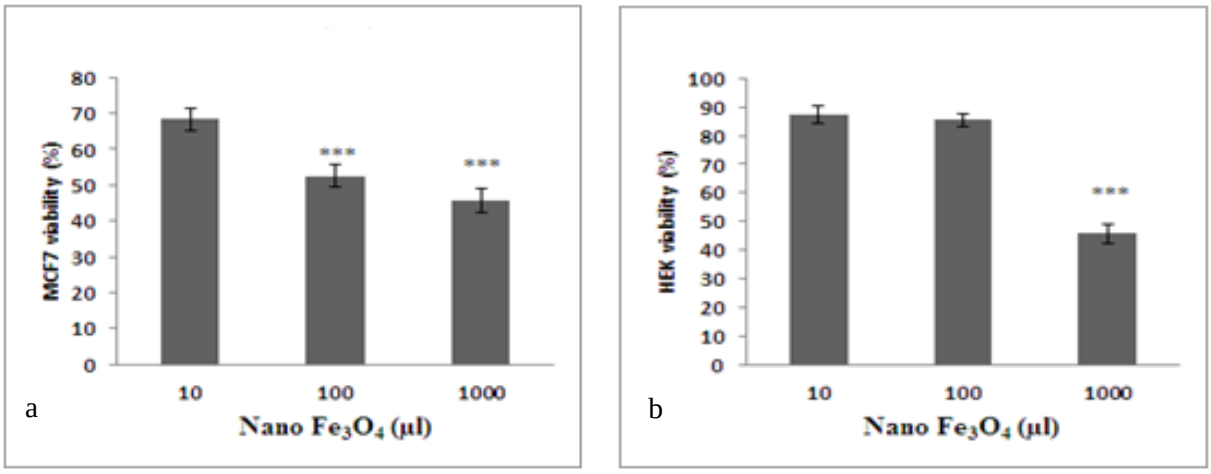

Figure 4. Cell viability of MCF-7 (a) and HEK293 normal (b) cell lines treated with Fe_3O_4 nanoparticles. Each point on the diagram is averaged over three days of testing and three replications per day. Error bar = Mean ± SD. Significant level was considered as * $P\leq0.05$, ** $P\leq0.01$ and *** $P\leq0.001$.

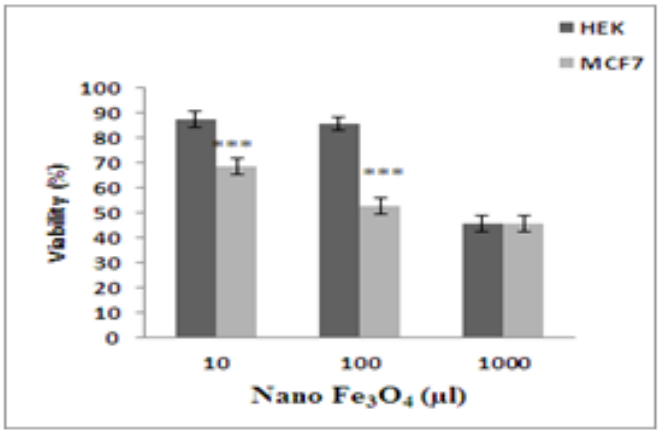

Figure 5. Comparison of cell viability between two cell lines of MCF-7 and HEK293 normal treated with Fe_3O_4 nanoparticles. Each point on the diagram is averaged over three days of testing and three replications per day. Error bar = Mean ± SD. Significant level was considered as ∗ P≤0.05, ∗∗ P≤0.01 and ∗∗∗ P≤0.001

Discussion

One of the most important challenges in the treatment of breast cancer is to find new drugs or methods with high impact on cancerous cells and minimum side effects. Microorganisms are able to reduce metal ions by a variety of extra-and intracellular polysaccharide enzymes and reducing agents. One of the reasons for the production of metal nanoparticles by microorganisms is to reduce the toxic effects of metal ions present in the growth medium of microorganisms (6). This reaction is performed through the biological reduction of toxic metal ions using nicotinamide adenine dinucleotide (NADH) reductase or nitrate reductase enzymes. In addition, the presence of some polysaccharides and biomolecules which microorganisms produce inside the cells or culture medium accounts for the production of metallic nanoparticles (6). Different researches have also confirmed that various nanoparticles can be synthesized using biological compounds, such as synthesis of iron nanoparticles using the extract of *Camellia sinensis* (7), silver nanoparticles using the cytoplasmic extract of *Cyanobacteria Desertifilum* and *lactobacillus* (8,9).

The use of microorganisms and plants to synthesize nanoparticles has many advantages including no need to stabilizer compounds in order to prevent the aggregation of nanoparticles to form macro-particles, the possibility of synthesis at low temperature (even at room temperature), being less expensive, and the ability of synthesis metal nanoparticles with very small particle sizes (9). However, in the biosynthesis of nanoparticles the time factor is of a particular importance because of the slower reactions carried out by the enzymes present in the biological compounds. It was reported that reaction time duration, reaction temperature and reactant concentrations could affect the size and distribution of nanoparticles (10). As shown in Figure 1, the formation of Fe_3O_4 nanoparticles in the reaction medium enables color change due to their specific optical properties. The color change is due to the excitation of Surface Plasmon Resonance (SPR) vibrations of Fe_3O_4 nanoparticles synthesized in the reaction medium.

The study of crystal structures of nanoparticles was carried out by X-ray diffraction (XRD). According to XRD analysis, the achieved diffraction peaks corresponded to the Miller indices and confirmed the formation of Fe_3O_4 nanoparticles during the biosynthesis process. These peaks were in accordance with the X-ray diffraction pattern of the iron oxide material, and no lateral phase was observed.

Similar results were reported in Hoag *et al.* study in which the synthesis of iron oxide nanoparticles with the size of 5-15 nm was carried out by leaf extract of *Camellia sinensis* (7). In order to determine the size and morphology of the synthesized nanoparticles, TEM images were prepared. As shown in Fig. 3 the particles were spherical in shape with a diameter of 10-15 nm, which is in accordance with the size calculated by the Debye-Scherrer equation.

The results of cytotoxicity assay of different concentrations of Fe_3O_4 nanoparticles

biosynthesized on MCF-7 breast cancer and HEK293 normal cell lines indicated that there was a direct relationship between the nanoparticle concentrations and the toxicity effects.

Comparison of the toxicity effect of green nanoparticles synthesized on cell lines indicates that at a concentration of 1000 µg / ml there is no significant difference between the viability of MCF-7 and normal HEK cells, but at both 10 and 100 µg / ml, the viability of MCF-7 cells in green synthesis decreased significantly ($P \leq 0.001$) compared to normal HEK cells. In a general view, the results of our research showed that the best cytotoxic effect of iron oxide nanoparticles by green and chemical methods on MCF-7 cell line is 1000 µg/ml. However, since this concentration also has toxic effects on normal cells, it is better to use a concentration of 100 µg / ml of green nanoparticles to inhibit cancer cells, because this concentration has the least inhibitory effect on normal cells and has the greatest toxic effect on MCF-7 cells. Other studies have shown that the cytotoxic effects of nanoparticles are concentration- dependent and bioparticles show more inhibitory effects on cancer cells and there is a direct relationship between nanoparticle concentration and nanoparticle toxicity on the cell lines tested (11-12). It is completely in line with the findings of the present research.

The mechanism of nanoparticle toxicity has not been well understood yet; however, various *in vitro* and *in vivo* studies suggested that the nanoparticles cause cell death by producing reactive oxygen species and subsequently DNA damage, gene transcription alteration, membrane lipid and enzymes peroxidation and interference with signaling pathways (13,14). The comparison of cytotoxicity effects of the biosynthesized Fe_3O_4 nanoparticles on the two cell lines represented that the toxic effects of nanoparticles on cancerous cells were more than those on the normal cells. It was reported that morphological differences between cancerous and normal cell membranes, as well as differences in shape, size, and surface charge of nanoparticles are among the factors affecting the toxicity of nanoparticles on cancerous and normal cells (15). Positively charged nanoparticles are attracted to cancer cells that have a high percentage of anionic phospholipids and certain groups of charged proteins and carbohydrates on their outer surface. Also, due to the high activity of mitochondria in the process of respiration of cancer cells compared to normal cells, a suitable substrate for nanoparticles is provided to destroy cancer cells (16). The smaller size of the nanoparticles causes their longer existence in the body, since their detection and elimination by the immune system will be delayed (17).

Conclusions

The biosynthesis of Fe_3O_4 nanoparticles was successfully performed using cytoplasmic extract of *Lactobacillus fermentum.* This type of synthesis of metal nanoparticles seems to be cost-effective, environment-friendly and an easy alternative approach compared to usual physical and chemical methods. The bioactive compounds in the extract caused reduction of iron oxide nanoparticles. These nanoparticles showed potential anticancer effects on MCF-7 breast cancer cell line. It is required to conduct further studies on the mechanism of construction and enhancing biomedical properties of iron oxide nanoparticles.

Authors' contributions

All authors participated in the design and coordination of the study - conducting experiments and interpreting the data, and the article was read and approved by them.

Acknowledgment

The authors are grateful for the cooperation of the Research Laboratory of the Islamic Azad University, Falavarjan Branch, for conducting the experiments.

Funding

This article was extracted from a Master's thesis project in Microbiology, conducted by Ms. Mina fani, and supervised by Dr Ghandhari, Dr Rezaee, Dr Fatemi and Denial Salehi at the Flavarjan campus of the Islamic Azad University, Flavarjan, Isfahan. We gratefully acknowledge the assistance of the University and the Department of Microbiology for providing the laboratories and equipment to conduct this study.

Conflict of interest

There is no conflict of interest.

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