# Comparison of anti-tumor properties of the cell walls of Saccharomyces cerevisiae and Saccharomyces boulardi probiotics, individually and in combination with iron nanoparticles on K562 cancer cell line

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Salar Mokriani[1], Amir Tokmachi[*,2], Majid Nojavan[1]

## ABSTRACT

**Background:** Chronic myeloid leukemia is a common cancer in human and chemical therapy is a routine method for treatment that has some side effects. So the goal of this study was the use of natural compound such as cell wall obtained from Saccharomyces cerevisiae (S. cerevisiae) and Saccharomyces boulardi (S. boulardi) and iron nanoparticles on the growth inhibition of K562 cell line.

**Methods:** For cell wall preparation both yeast were cultured in a basic medium at aerobic condition and 28 °C. Then the medium was centrifuged and precipitant washed with sterile buffer and the cells disrupted by sunicator. Also iron nanoparticles were prepared by biological method. Anti cancer property of different concentrations of the yeasts cell wall with iron nanoparticles were assayed by MTT and electrophoresis methods.

**Results:** The results showed that S. boulardi cell wall significantly ($p=0.029$) inhibits the growth of K562 cell line compared to S. cerevisiae. Also iron nanoparticles significantly ($p=0.021$) inhibit K562 cell line. Results revealed that combination the iron nanoparticles with both yeasts cell wall decreased anti cancer property but this was not significant at the level of $p<0.05$.

**Conclusion:** Based on this finding it should be concluded that combination of iron nanoparticles with Saccharomyces cell walls could inhibit the growth of K562 cell line in vitro. But these anti cancer properties would warrant further study on the clinical application of yeast cell wall.

**Key words: Saccharomyces cerevisiae, S. boulardi, iron nanoparticles, Growth inhibition, K562 cell line**



1. Department of Biology, Faculty of Basic Science, Urmia Islamic Azad University Iran.
2. Department of Pathobiology and Quality Control, Urmia Lake Research Institute, Urmia University.

***Corresponding Author:**
Amir Tokmachi
Department of Pathobiology and Quality Control, Urmia Lake Research Institute, Urmia University.

Email: a.tukmachi@urmia.ac.ir

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## Introduction

Probiotics are live non-pathogenic microorganisms found in some food stuff that leave a positive impact on host's health if taken in sufficient amounts[1]. Probiotic foods are a group of health promoting foods, known as functional foods[2] that provide nutrition-based health[3]. Because of their health promoting properties, microbes such as Bifidobacterium, Lactobacillus, Saccharomyces, and Propionibacterium are considered probiotics[4] and are able to inhibit growth of induced tumor cells in rodents. Probiotics produce various substances with inhibitory effects on gram-positive and gram-negative bacteria, which include: organic acids such as acetate, propionate, butyrate, H2O2, and bacteriocin compounds. Not only do these compounds reduce live pathogenic cells, but they may also affect metabolism of bacteria or toxins produced by them[5]. Another effective mechanism of probiotics is the competitive inhibition of bacterial binding sites on intestinal epithelial surfaces. To cause disease, many intestinal pathogens have to bind themselves to the intestinal walls. A number of probiotic strains have been selected because of their binding ability to epithelial cells[6]. Probiotics probably use nutrients used by pathogenic bacteria. Probiotics increase immunity by increasing levels of cytokines and immunoglobulins, and proliferation of mononuclear cells, activation of macrophages, and increasing natural killer's activity, modulating self-immunity and stimulating immunity against pathogenic bacteria and protozoa. Saccharomyces boulardii activates both complement and reticulo-endothelial systems[7-9]. Studies indicate that heat-killed Saccharomyces cerevisiae can induce apoptosis in breast cancer cell lines: MCF-7, ZR-75-1, and Hcc70[10]. Lactic acid bacteria can prevent colon cancer by mechanisms such as: changes in metabolic activity of intestinal microflora and changes in physical and chemical conditions of the colon, binding to carcinogens and destroying them, quantitative and qualitative changes in intestinal microflora, preventing production of carcinogens like ammonia, production of anti-cancer material, and strengthening immune responses of the host[11]. This has been demonstrated through use of 1, 2-dimethyl-hydrozine carcinogens in rat colon[12]. Probiotics produce high amounts of short-chained fatty acids, and thus, induce protective glutathione transferase II in rat colon[13]. Existing lactic acid bacteria in fermented milk can have inhibitory effects on spread of pre-cancerous wounds and tumors in animal models[14]. Streptococcus thermophilus and Lactobacillus bulgaricus in fermented milk can be highly effective in deactivating intestinal carcinogens and risk factors. Reducing harmful intestinal enzymes leads to reduction in carcinogenic compounds in intestines, urinary tract, and bladder[12]. Isolated beta-glucan from Saccharomyces cerevisiae is able to increase resistance of the host against various foreign antigens such as: viruses, bacteria, and parasitic infections, and may cause the host to act against tumors by stimulating the immune system[15]. Cytoplasmic extract from Lactobacillus casei and Bifidobacterium bacteria has a direct effect on inhibiting growth of cancer cells[16]. Direct intra-tumor injection of heat-killed Saccharomyces cerevisiae ferment can cause significant tumor regression, induce apoptosis, and regulate normal function of the immune system[10]. In-vitro culture of Saccharomyces cerevisiae ferment can reduce aflatoxins B1 and B2 in fish powder[17]. It can also eliminate lead from food stuff[12]. In-vitro use of Saccharomyces cerevisiae and Saccharomyces boulardii cell walls and extracts can inhibit growth of K562 cancer cell lines[18]. Over the past two decades, nanotechnology has made great advances in technology, equipment, and materials with tiny dimensions, and shall transform human civilization by the end of the century[19]. Among newly emerging and high technologies, nanotechnology is highly

important for it has more potential for advancement. By providing a new approach in various sciences and technologies, nanotechnology has been dubbed one of the most promising technologies of the current century. Cancer cells are inherently more vulnerable against chemotherapy than healthy cells, but drugs do not function selectively and may harm healthy cells, as well. Cancerous tissues obtain their nutrition through diffusion until they grow about 2nm[20]. After this time, blood vessels need to be formed. Angiogenesis is abnormal in cancerous tissues; producing perforated capillaries, thereby increasing permeability of blood from vessels to tissues, and lead to growth of cancer cells. The vascular perforations in cancerous tissues measure between 100nm and 780nm, with normal size between 5nm and 10nm[21] that is the principle difference between healthy capillaries and cancerous ones[22]. In treatment of cancer, size of nanoparticles is so chosen to pass through perforations in membrane of tumor capillaries, while they are unable to pass through normal capillaries. This creates high concentration of nanoparticles in the tumor. Smaller nanoparticles have longer half-life, and are identified later by opsonin proteins, and eliminated later by the immune system. One of the ways nanoparticles evade the immune system is by creating a polymer cover on their surface. Another way is to make them hydrophilic[23]. The effects of silver nanoparticles on pathogenic gram-negative bacilli resistant to beta-lactam antibiotics show that these particles can have inhibitory effects on all gram-negative bacilli, and growth inhibition zone diameter increases with increasing concentration of silver nanoparticles[24].

Blood cancer or leukemia is a progressive malignant disease of hematopoietic tissues of the body. This disease is caused by defective development of leukocyte and its precursors in blood and in bone marrow[25]. In leukemia, normal process is disrupted and blood cells grow out of control[26]. There are 4 types of leukemia, including: Acute Lymphoid Leukemia (ALL), Chronic Lymphoid Leukemia (CLL), Acute Myeloid Leukemia (AML), and Chronic Myeloid Leukemia (CML)[27]. Lymphoid leukemia affects lymphoma cells[28]. Diagnosis of CML is confirmed by demonstrating colonial development in these cells and translocation of ab1 gene on chromosome 9 and BCR gene on chromosome 22 in stem cells[29]. Thereby, bcr.ab1 fusion cell is created, which produces a 210 Kilo Dalton protein with tyrosine kinase activity called p210 bcr-ab1 protein. This protein causes uncontrolled proliferation independent of growth factor of myeloid progenitor cells, cell division and disruption in apoptosis[30]. The bcr-ab1 fusion gene is called Philadelphia Chromosome. The principle cause of this cancer is production of a protein called BCR-ABL, which is an active cytoplasmic tyrosine kinase and induces leukemia in hematopoietic stem cells[31].

## Methods

This study was conducted at the genetic and cellular/molecular biology laboratories of the Islamic Azad University, Urmia Division in the following stages:

**Preparation and culture of the yeast and preparation of their cytoplasmic extract and cell wall:**

Saccharomyces cerevisiae and Saccharomyces boulardii were procured from Persian Type Culture Collection (PTCC 5269) and American Type Culture Collection (ATCC 74012) respectively, and separately cultured in the basic culture medium containing: yeast extract (1%), glucose (5%), K2HPO4 (1%), with pH=5.8, and incubated at 28 °C in a shaker at 130 rpm for 72 hours. After growth, yeasts were centrifuged at 3000 rpm for 10 minutes and the sediment was rinsed out twice with sterile saline[32].

**Preparation of yeast cytoplasmic extract and cell wall:**

Sonication method was used to break down the yeasts. First, a suspension was prepared from sediments of centrifuged yeasts by cold sodium phosphate buffer (pH=7.2, 0.1 Mole), and disintegrated by a sonicator with 60% range for 2 minutes. To prevent rising temperature in the solution and sonicator probe, the sonicator was switched off for 4 minutes. The process was repeated several times and breaking of yeasts was assessed by an optical microscope[26]. Once breakdown of cells was ensured, the above suspension was centrifuged at 500 × g at 4°C for 1 minute and the supernatant was presumed cytoplasmic extract and the precipitate as the cell wall. First, existing protein in cytoplasmic extract was measured by total protein measuring kit (Pars Azmon, Iran), and the precipitate was dissolved in 10 mL of sterile distilled water to form a suspension, which was then centrifuged at 1000 × g at 4 °C for 20 minutes. The cell wall was rinsed three times, and resulting precipitate was lyophilized and kept at -20 °C.

**Preparation of lysis buffer** (sodium phosphate, 0.1 M, pH=7.2):

First, 0.0716 gram of Na2HPO4.7H2O and 0.0285 gram of Na2HPO4.2H2O were weighed and dissolved in one liter of distilled water, and then autoclaved[33].

**Preparation of different concentrations of cytoplasmic extract and cell walls:**

Once concentration of existing protein in each cytoplasmic extract was determined with Biuret method using RPMI cell culture medium (Gibco, England), different dilutions were made. Cell walls obtained were freeze dried, and culture medium dilutions of 125, 250, 500, 1000, 2000, and 4000 µg/mL were prepared from cytoplasmic extract and cell wall[34].

**Biosynthesis of iron nanoparticles:**

In this study, feasibility of colloid biosynthesis of iron nanoparticles was evaluated. To that end, in the first stage, iron nanoparticles were prepared from aqueous manna extract and Acanthophyllum roots.

**Preparation and culture of cancer cells:**

Cancer cell line K562 was procured from cell bank of Pasteur Institute of Iran, and cultured in RPMI culture medium in presence of 10% fetal bovine serum (Gibco, England), 100 mg/mL streptomycin, and 100 IU/mL penicillin, and incubated at 37 °C with 5% CO2.

**Assessment of anti-tumor properties of cytoplasmic extract, cell wall, and iron nanoparticles using MTT method:**

In this study, Chang et al.[35] and Kim et al.[36] methods were used to assess anti-tumor properties of different concentrations of cytoplasmic extract, cell walls, and iron nanoparticles. Briefly, after centrifugation and K562 cell count using trypan blue method, 100 µL (with density of 20000 cells in RPMI culture medium and 15% FBS) was added to each well of a flat-bottom 96-well plate. Then, 100 µL of culture medium containing different dilutions of cytoplasmic extract, cell walls, and nanoparticles were added to the wells. Three wells were taken as control, and 100 µL cells with 90 µL culture medium and 10 µL lysis buffers were added to each. Next, plates were incubated at 37 °C with 5% CO2 for 12, 24, 48, and 72 hours. At the end of incubation, 20 µL of MTT solution (3-4 and 5 dimethyl thiazole, 2-5 diphenyle tetrazolium bromide, and 5 mg/mL buffered PBS) were added to all wells and microplate was incubated for 4 hours. During this period, bromine in MTT solution is reduced by existing succinate dehydrogenase in mitochondria of healthy live cells, and is transformed into insoluble purple formazan. Finally, purple formazan crystals formed in cytoplasm of cells are dissolved by addition of 100 µL of DMSO solution (dimethyl sulfoxide) to the wells, and plates were placed in a shaker incubator for 10-15 minutes and optical density was recorded at a wavelength of 492 nm using an ELISA reader (StatFax, USA).

Percentage of extracts' cells was calculated using the following formula:

Cell killing percentage= [(sample OD-control OD)/ control OD]×100

**Assessment of K562 cells apoptosis and necrosis by electrophoresis**

Apoptosis or necrosis was studied by DNA fragmentation test. Cells treated with cytoplasmic extract, yeast cell wall, and iron nanoparticles were placed under lysis buffer influence. After centrifugation, DNA was isolated using phenol-chloroform-isoamyl-alcohol. Isolated DNA was precipitated overnight with absolute ethanol. Precipitated DNA was dissolved in TE buffer (Tris-HCl, 10 EDTA 10 mM) and loaded onto 2% agarose gel at 80v in the presence of 1Kb marker for one hour[5,37].

**Analysis of data:**

For analysis of data, ANOVA one-way variance and

## Results

**Cytocidal activity of cytoplasmic extract and yeasts cell wall**

The present study results showed cytocidal activity of S.cerevisiae and S.boulardii extracts, compared to the control group. Except for 125µg and 500µg, all concentrations of extracts were able to inhibit growth of K562 cancer cell line. Statistical study demonstrated that cytoplasmic extract concentration of 4000 µg caused the highest percentage of growth inhibition (table 1). Results also indicated that increased treatment duration of K562 cancer cell line with yeast cytoplasmic extract significantly reduces anti-tumor property ($P<0.05$). The highest rate of cancer cell destruction was observed by Saccharomyces boulardii cytoplasmic extract at concentration of 4000 µg/mL in the first 12 hours at 46.46%.

**Anti-cell property of yeast wall:**

Investigation showed that cancer cell death rate at 12, 24, 48, 72 hours increases with increasing concentration. The highest percentage of cytocidal activity of both yeasts was observed in the first 12 hours at concentration of 4000 µg (table 2). Similarly, by increasing duration of K562 cell exposure to yeast cell walls, their anti-tumor property was reduced. Comparison of yeast cell wall anti-tumor property showed that S.boulardii had significantly higher cytocidal property than S.cerevisiae, and S.boulardii at concentration of 4000 µg was able to kill 98.5±8.31 percent of cells in the first 12 hours.

**Iron nanoparticle cytovidal activity:**

Study results demonstrated antitumor property of iron nanoparticles against K562 cancer cell line compared to control group. The antitumor property is dependent on concentration, but has an inverse relationship with time (table 3). Considering that the highest cytocidal rate occurred with Saccharomyces boulardii cell wall at 4000 µg concentration in 12 hours, combined antitumor property of this yeast and iron nanoparticle at the same concentration was investigated. The results showed that although combination of these two substances causes growth inhibition of K562 line, it does not significantly differ from the yeast cell wall alone.

**Cell apoptosis and necrosis with S.boulardii cell wall:**

Treatment of K562 cells with S.boulardii cell wall shows a ladder pattern in gel electrophoresis, which is indicative of apoptosis in cells. Cytocidal rate increases with increasing concentration (Figure 1).

**Figure 1:** Status of cell death in K562 cells treated with S.boulardii cell walls after 12 hours. Well 1: 1Kb marker (positive control) is an indicator that contains specific DNA weights. Through electrophoresis, each DNA fragment stops in a specific region according to its weight. Well 2: negative control. Well 3: treatment with concentration of 1000 µg/mL. Well 4: treatment with concentration of 2000 µg/mL. Well 5: treatment with concentration of 4000 µg/mL.

**Cell apoptosis and necrosis with S.cerevisiae cell wall:**

**Table 1:** Cytocidal property of S. cerevisiae and S. boulardii extracts in K562 cells at different durations

| Concentration (µg/ml) | Saccharomyces boulardii | | | | Saccharomyces cerevisiae | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 | 24 | 48 | 72 | 12 | 24 | 48 | 72 |
| 125 | - | - | - | - | - | - | - | - |
| 250 | - | - | - | - | - | - | - | - |
| 500 | $18.21\pm1.12^{a}$ | $15.22\pm3.5^{b}$ | $9.4\pm2.98^{c}$ | $4.1\pm3^{d}$ | $14.09\pm0.2^{b}$ | $11.29\pm0.34^{c}$ | $7.88\pm0.52^{d}$ | $3.2\pm0.49^{d}$ |
| 1000 | $28.9\pm3.66^{a}$ | $25\pm5.12^{b}$ | $20.12\pm3.47^{c}$ | $13.7\pm4^{e}$ | $21.32\pm2.21^{c}$ | $17.44\pm2.89^{d}$ | $13.5\pm2.5^{e}$ | $8.95\pm1.95^{f}$ |
| 2000 | $36.43\pm4.99^{a}$ | $30.12\pm5.12^{b}$ | $25.73\pm6^{c}$ | $19.09\pm5.9^{d}$ | $30.22\pm4.09^{b}$ | $25.89\pm4.51^{c}$ | $19.11\pm3.43^{d}$ | $15.45\pm3.12^{f}$ |
| 4000 | $46.66\pm5.55^{a}$ | $41.09\pm6.09^{b}$ | $37.52\pm5.12^{c}$ | $29.11\pm4.34^{d}$ | $37.54\pm5.12^{c}$ | $32.11\pm3.21^{d}$ | $28.76\pm5^{d}$ | $22.33\pm4.29^{f}$ |

Cell death due to cell wall depends on dosage and duration, and cytocidal percentage increases with increasing concentration. Investigation shows that the created bands are in ladder shape, and cell death is of apoptosis type (picture 2).

**Picture 2:** Apoptosis rate in cancer cells caused by cell walls of S.cerevisiae. Well 1: 1Kb marker (positive control). Well 2: negative control. Well 3: treatment with concentration of 1000 µg/ml. Well 4: treatment with concentration of 2000 µg/ml. Well 5: treatment with concentration of 4000 µg/ml.

**Cell apoptosis and necrosis with S.cerevisiae cytoplasmic extract**

Electrophoresis results show that cell death is of apoptosis type, and bands created are in ladder shape and a function of concentration and duration (picture 3). Well 1: 1Kb marker (positive control). Well 2: negative control. Well 3: treatment with concentration

**Table 2:** Cytocidal property of different concentrations of cell walls of S.cerevisiae and S.boulardii at different exposure durations with K562 cells

| Concentration (µg/ml) | Saccharomyces boulardii | | | | Saccharomyces cerevisiae | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 | 24 | 48 | 72 | 12 | 24 | 48 | 72 |
| 125 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 250 | a11.21±0.23 | 10.05±0.89 [a] | 8.12±0.43 [a] | 5.2±0.39 [a] | 8.7±0.45 [a] | 8.09±0.78 [a] | 7.2±0.66 [a] | 0 [a] |
| 500 | 33.3±2.11 [a] | 30.13±4.12 [a] | 26.3±3.81 [b] | 18.1±3.08 [c] | 24.2±3 [b] | 22.12±3.29 [b] | 19.88±3.09 [c] | 12±2.29 [d] |
| 1000 | 59.18±5.9 [a] | 51.5±6.11 [b] | 40.1±5.56 [c] | 33.3±5.09 [d] | 50.12±6.01 [b] | 47.23±5.09 [b] | 35.12±5.21 [d] | 22±4.88 [e] |
| 2000 | 74±6.12 [a] | 69.8±7.11 [b] | 62.6±6.77 [c] | 41±5.89 [e] | 67.5±5.54 [b] | 64.12±6.78 [c] | 54.2±5.78 [d] | 34.1±6.1 [f] |
| 4000 | 98.5±8.31 [a] | 81.15±7.01 [c] | 73±6 [d] | 62.9±5.5 [e] | 90.12±7.42 [b] | 73. 2±6.6 [d] | 66.13±4.45 [e] | 58.2±5.08 [f] |

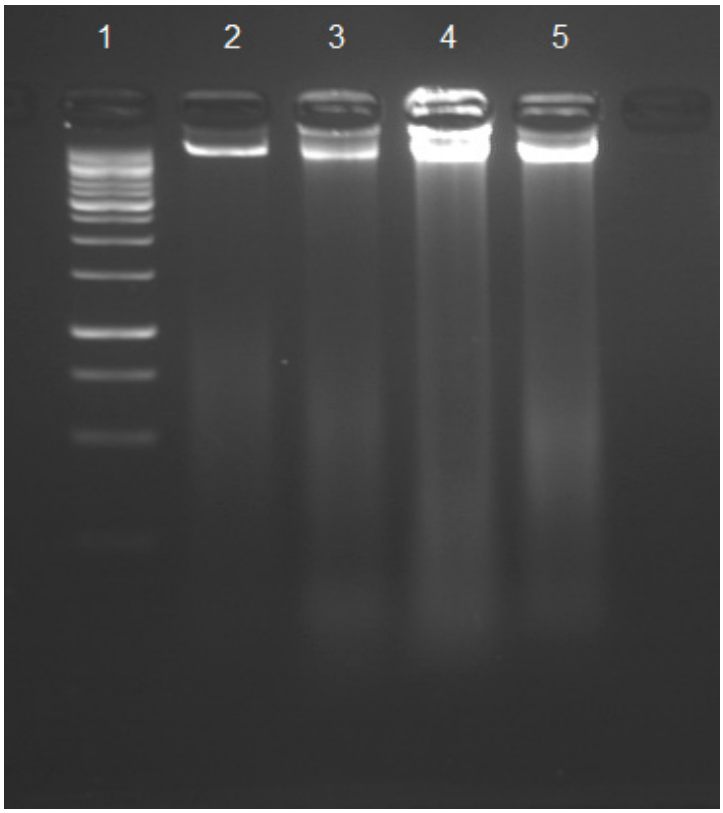


Fig. 1: Cytocidal rates of different concentrations of S.cerevisiae cell walls

of 1000 µg/ml. Well 4: treatment with concentration of 2000 µg/ml. Well 5: treatment with concentration of 4000 µg/ml

**Cell apoptosis and necrosis with S.cerevisiae cytoplasmic extract**

The results confirm apoptosis type cell death, which could be due to digestion of DNA by existing nuclease in cytoplasmic extract (picture 4). Well 1: 1Kb marker (positive control). Well 2: negative control. Well 3: treatment with concentration of 1000 µg/ml. Well 4: treatment with concentration of 2000 µg/ml. Well 5: treatment with concentration of 4000 µg/ml.

**Cell apoptosis and necrosis with iron nanoparticles treatment:**

Investigation of different iron nanoparticle treatments at 12 and 24 hour durations and different concentrations indicates necrosis-type cell death, and that DNA was not disintegrated (picture 5).

Figure A: 12 hour treatment

Figure B: 24 hour treatment

Well 1: 1Kb marker (positive control). Well 2: negative control. Well 3: treatment with concentration of 1000 µg/ml. Well 4: treatment with concentration of 2000 µg/ml. Well 5: treatment with concentration of 4000 µg/ml

## Discussion

Probiotics are microorganisms with beneficial effects on the host health, by affecting intestinal microbial flora[44], and they live by cohabiting with their

host[11]. Every person's microbial flora is predominantly constant, and use of these microorganisms changes intestinal microbial flora leading to reduced production of such enzymes as azoreductase and β-glucururonidase[27]. Probiotics have positive effects on the immune system and boost its effectiveness [15], and can reduce colitis symptoms caused by antibiotic use[1]. Unlike chemotherapy agents, probiotics destroy tumor cells without harming healthy one, with no side-effects. Saccharomyces cerevisiae reduces blood cholesterol in poultry[3]. Probiotics reduce risk of colon cancer. Seow reported that L. rhamnosus and L. casei inhibit growth of bladder cancer cells in MGH and RT112 cell lines. Heat-killed Saccharomyces cerevisiae induce apoptosis in breast cancer cell lines of HCC70 and MCF7ZR-15-1. Saccharomyces boulardii plays an important role in reducing diarrhea in children and travelers and intestinal lesions. Direct intra-tumor injection of heat-killed Saccharomyces cerevisiae can inhibit tumor growth, induce 84.5% apoptosis, and regulate normal function of the immune system [23]. Oxidized nanoparticles of chemically synthesized zinc have favorable anti-bacterial properties[2]. Nanoparticles can pass through blood brain barrier[32]. Identifying advantages and disadvantages of nanoparticles, can help human given their dimensions in treatment of diseases. Some nanoparticles are considered an innovation in advancing modern pharmaceutical science, and because of their high potential in specialized treatment processes, they have many applications in biological and pharmaceutical studies. For instance, they are able to kill as many as 650 cancer cells in 4 hours[18-26]. In life cycle and ecosystem, nanomaterials show the lowest toxicity, and many of them can be used given their antimicrobial properties[35]. Targeted drug delivery by nanoparticles can be performed in active and passive processes. In passive delivery, through low-effect infiltration or displacement, nanoparticles pass through perforated tumor blood capillaries and enter the tumor. Selective accumulation of nanoparticles and drug increases permeability and retention. In active delivery, drug is transferred to a specific location by molecular identification[33]. Liposomes are particles formed by layers of fat, which are classified into single and multi-layer groups according to number of layers of fat. Single-layer contains aqueous center for encapsulation of dissolved drugs in water, and in multi-layer, soluble drugs are stored in fat. The main problem in using liposomes is their rapid destruction and elimination from blood circulation by liver microphages. Liposomes can be so designed to target a specific tissue or limb, and thus drug acts totally specifically, and better drug safety is achieved because of reduced effect or ineffectiveness of drug on other tissues. Tumor blood vessels have a very weak structure, and passage of materials from blood to these tissues occurs with ease. Thus, drugs encapsulated in liposomes, spontaneously accumulate more in the tumor[7]. Nanoparticles produced by cells can especially refine uranium-contaminated water[19].

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