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Original Article

Cytotoxic Effects of Green Synthesis of Zirconium Nanoparticles of Aqueous Clover Extract on Breast Cancer

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Abstract

Background & Aims: Breast cancer is one of the major causes of death among women. In various studies, the effects of synthesized nanoparticles (NPs) on cancer cells have been measured. This investigation aimed to explore the cytotoxic effects of metal zirconium nanoparticles (ZrNPs) synthesized by aqueous extract of clover leaves (*Trifolium repens* L) on breast cancer cells.

Materials and Methods: metal ZrNPs were synthesized utilizing the aqueous extract of clover leaves. Physical and chemical evaluation of the synthesized NPs was done by UV-Vis, EDS, SEM, and DLS analyses. The cytotoxic effects of ZrNPs on MDA-MB-468 and HFF cell lines were investigated by the MTT assay and compared with those of Tamoxifen, a chemotherapy medication used to treat breast cancer.

Results: The aqueous extract of clover was utilized as a reducing agent in the synthesis of ZrNPs. The size of ZrNPs was 29-81 nm, in irregular polygonal form, and star-shaped, and flower-like colonies were observed homogeneously. MTT test showed that ZrNPs inhibited the growth of MDA-MB-468 cells in a concentration- and time-dependent manner. The IC50 value calculated from the dose-response curve for the MDA-MB-468 cell line was 264.3 µg/mL.

Conclusion: The aqueous clover extract showed a suitable ability with green synthesis of NPs. Considering the cytotoxic effects of ZrNPs on breast cancer cell lines, it can be used as an effective therapeutic agent to be examined and evaluated clinically to deal with cancers in future studies.

Keywords: Nanoparticles, Plant extracts, Zirconium, Breast neoplasms, Clover, Red, Trifolium repens

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1. Introduction

Breast cancer is the most common cancer in women and one of the most common causes of cancer-related deaths in women around the world [1,2]. Chemotherapy, radiation therapy, and surgery are the main treatment options for breast cancer. Drug therapy is one of the options for confrontation cancer. However, since drug products still do not penetrate the tumor site at sufficient levels, modified drug therapy increases systemic side effects and reduces pharmacokinetic effects. Chemotherapy, radiation therapy, and surgery are the main treatment options for breast cancer [3]. Different variables such as hereditary components, lifestyle, and living environment influence the incidence of this disease [4]. Breast cancer is caused by uncontrolled development and growth of cells in the breast [5]. Breast cancer is a complex disease caused by polygenic factors, and various pathogens differently contribute to the chance of expanding the cancer. [6]. Nanotechnology increases the success of chemotherapy and reduces the side effects of breast cancer treatment [7].

This technology includes synthesizing and applying materials with sizes ranging from 1 to 100 nm [8]. Nanoscale metals are broadly utilized in numerous fields such as environment, medicine, and engineering [9]. Nanoscale metals are basically synthesized by chemical methods, which have undesirable impacts such as environmental contamination, high energy consumption, and potential health issues. Green synthesis, which employs plant extracts, has been developed in response to these challenges. Green synthesis is more useful than conventional chemical synthesis since it costs less, decreases contamination, and improves environmental and human health safety [9].

However, the main concern regarding the biogenic regeneration (materials produced by living organisms) of metal forerunners to deliver the comparing nanoparticles (NPs) is related to their purity. Biogenic decrease could be a "bottom-up" approach comparable to chemical decrease in which a diminishing operator is supplanted with an extricate of a normal item with inalienable stabilizing, growth-terminating, and coating properties. Additionally, the nature of biological entities at distinctive concentrations in combination with organic reducing agents influences the size and shape of NPs [8]. Clover (*Trifolium repens* L) is an annual plant that grows well in the semi-arid conditions of the Mediterranean region [10]. *Trifolium* species, commonly known as clover species, have a worldwide distribution and are used by

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communities around the world as a food and medicine [11]. *Trifolium* species are rich in bioactive compounds that have medicinal value and this together with potential nutritional value can improve their dietary properties [12]. These plants contain a wide range of bioactive compounds called secondary metabolites such as phenolic acids, alkaloids, flavonoids, terpenoids, amino acids, alcoholic compounds, glutathione, polysaccharides, anti-cancer agents, organic acids (ascorbic, oxalic, malic, tartaric, and protocatechuic acids), and quinones. It is by and large known that these metabolites are included in redox response forms [13]. They are responsible for the lessening of metal particles to metal NPs. Even though the components included within the green blend of NPs and the fundamental instrument of particle bioreduction are still not fully understood [14]. The association and support of auxiliary metabolites (sugars, terpenoids, polyphenols, alkaloids, phenolic acids, and proteins) within the diminishment of metal particles that lead to the arrangement of NPs and in supporting their consequent soundness have moreover been expected [15]. Zirconium (Zr) is classified as a transition metal (d-block) in the titanium family (group IV) with atomic number 40 [16]. Zirconium dioxide (ZrO_2) or zirconia is one of crystalline oxides of zirconium [17]. Due to its very high steadiness and exceptionally harmfulness, ZrO_2 shows a wide range of applications for heat-resistant ceramic superalloys [18], dental restorations [19], fuel cells [20], heterogeneous catalysis [21], and cancer treatments [22,23]. Such promising applications make ZrO_2 an ideal nanomaterial, promoting a green technique for the synthesis of ZrO_2NPs [24].

This study aimed to measure the ability of metallic ZrNPs synthesized by the aqueous extract of clover leaves and investigate the cytotoxic effects of synthesized NPs on normal cell line (HFF) and breast cancer cell line (MDA-MB-468).

2. Materials and Methods

2.1. Production of aqueous extract of clover (Trifolium repens L) plant

The clover plant was collected in the middle of April (2023) from Sarieh Khatun village (Qom, Iran) and kept at a temperature of 4 °C. To prepare the aqueous extract from the clover plant, the leaves of the plant were separated from the root and stem. Then, the leaves were washed with distilled water and 70% ethanol was used to remove possible contamination. Afterwards, they were dried in the shade at room temperature. In the next step, the dried plant was crushed into powder by an electric grinder so that it can be used in the next steps. Then, 10 g of the prepared powder was dissolved in 150 mL of deionized water at a temperature of 80 °C and mixed for 40 minutes. After cooling the solution and removing suspended particles from it, the solution containing extract and plant residues was passed through Whatman filter paper No. 1 using a Buchner funnel under vacuum conditions. Then, the remaining particles were separated by centrifugation at 14000 rpm for 20 minutes until the final solution became completely clear [25,26]. Finally, the obtained solution was kept at 4 °C for use in the next steps.

2.2. Production of ZrNPs with clover extract

To synthesize and produce ZrNPs, 90 mL of 1 mM potassium hexafluorozirconate (K_2ZrF_6) solution was prepared. Then, 10 mL of the prepared clover extract was gradually added and incubated at 30 °C. Moreover, to examine the pH changes in the synthesis, the pH of the desired solutions was set to 10 [27]. The color change of the extract indicated the production of NPs.

2.3. Isolation of NPs

To separate the produced NPs, a sedimentation method with centrifugation at 11000 rpm for 10 minutes at 4 °C was used. To clean the surface of NPs, they were dispersed in deionized distilled water and separated again by sedimentation method. Then, the resultant sediment was dried in an oven at 80°C for 120 minutes. Finally, the obtained white powder was stored in a cryotube for biological analysis.

2.4. Ultraviolet-visible spectroscopy (UV-Vis)

The absorption intensity of the surface plasmon band in colloidal solutions containing NPs in the wavelength range of 200-800 nm was measured with the help of a Varian double-beam UV-Vis spectrometer (Agilent Cary 100, USA) [28].

2.5. Measuring the size distribution of NPs by dynamic light scattering (DLS)

The normal molecule size of ZrNPs was determined using a Zetasizer (model 3600, England) with a scattering angle of 90 degrees at Shahid Beheshti University of Medical Sciences, Iran. In this device, the default measurement range was between 0.1 nm and 10 μm. It seems necessary to state that the colloidal NP sample was prepared as a solution and for its preparation, polyethylene glycol 400 was used to disperse the particles properly. To do so, 0.001 g of dried powder of NPs was mixed with 5 mL of deionized water for 30 minutes and the desired samples were placed in a Sonicator (20 kHz) at 25 °C for 20 minutes to prevent the accumulation of particles.

2.6. Scanning electron microscope (SEM)

A scanning electron microscope (model VEGA3, TESCAN company, Czech Republic) was used to obtain the images of the prepared ZrNPs and determine their surface characteristics and morphology. To prepare the NPs for scanning electron microscope imaging, the

samples were sonicated for 20 minutes and a drop of the resulting colloid of ZrNPs was placed on carbon-coated copper grids. Then, it was dried at ambient temperature and to create an electric current, the surface of the sample was covered with a thin layer of gold and palladium alloy.

2.7. Energy dispersive spectroscopy (EDX)

To determine the elemental composition of the produced colloidal sample, a VEGA3 electron microscope was used. The sample preparation method was the same as the SEM sample.

2.8. Determination of the cytotoxic effect of ZrNPs on cancer cells

Human breast cancer cell lines (MDA-MB-468) and healthy cell lines (HFF) were obtained from the Iranian Biological Resource Center (IBRC) and used in this investigation. These cells were cultured in a mixture of Dulbecco's modified eagle's medium (DMEM) and Ham's F12 supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). Then, they were placed in an incubator with a temperature of 37 °C, 5% CO_2 concentration and 95% humidity. Flasks were examined daily by an inverted microscope in terms of cell growth and density, morphology, and contamination control. The complete culture medium containing 10% fetal bovine serum (FBS) was changed at the required time.

2.9. MTT Assay

The cytotoxic impact of ZrNPs was investigated by colorimetric method, using the dye 5-dimethylthiazol-2-y1]-2-5-diphenyltetrazolium bromide (MTT), 3-[4-tetrazolium bromide water-soluble salt. This method is based on succinate enzyme activity. The purple formazan crystals are produced by dehydrogenases in the mitochondria of living cells. This compound can be dissolved in DMSO. Since dead cells are incapable of changing over MTT to formazan, the level of formazan delivered is relative to the number of living cells. To conduct the experiment, MDA-MB-468 and HFF cells were cultured at a density of 5000 cells per well of a 96-well plate. The old culture medium was removed from the wells after 48 hours, and the cells were treated with ZrNPs (8, 32, 64, 128, 256, and 512 µg/mL) synthesized by clover aqueous extract and tamoxifen (1.25, 2.5, 5, 10, 20, 40 µg/mL). Four wells were considered for each treatment and 4 wells were assigned as controls in each experiment. The plates were kept in an incubator containing 5% CO2, 95% humidity, and 37 °C for 24 hours. They were incubated for 48 and 72 hours. After the incubation, the supernatant of each well was carefully removed, replaced with a complete culture medium containing MTT solution, and placed in the incubator for 4 hours. Then, the supernatant of each well was removed and 200 µL of DMSO solution was replaced with the previous solution to dissolve the formazan crystals. After 15 minutes, the optical density was read using an ELISA reader (US BioTek, Synergy/HTX) at a wavelength of 570 nm.

The amount of absorption corresponds to the number of living cells. The results are calculated in terms of the rate of live cells compared to the control using the following equation: 100x (average absorption of control cells/average absorption of treated cells) = percentage of living cells

2.10. Statistical analysis

Data analysis was done by Excel and GraphPad Prism 8.2.1 software. The concentration required to inhibit cell growth by 50% (IC50) was calculated and plotted by PRISM and non-linear regression analysis, utilizing dose-response curves.

Results

3.1. Evaluating the results of macroscopic observations and the Tyndall effect

The first step in the synthesis of ZrNPs is to observe the gradual color change of the response arrangement from green to yellow, which is the initial confirmation that takes place after the end of the incubation time. After the initial synthesis of ZrNPs and macroscopic observations such as the color change and examination of the Tyndall impact, all the syntheses carried out in unfavorable conditions were excluded from further experiments due to the lower intensity of color change compared to the NPs produced by aqueous clover extract. In fact, in terms of macroscopic observations, this color change indicates the production of ZrNPs (Figure 1, A and B). Finally, after the synthesis, the obtained NPs turned into a white powder.

3.2. Evaluation of the production of ZrNPs using aqueous clover extract

3.2.1. UV-Vis spectroscopy

The color change is attributed to surface plasmon resonance (SPR), which is related to the optical properties of ZrNPs. ZrNPs have shown the capacity to assimilate the resonance of electromagnetic waves in the visible light. The maximum absorption of ZrNPs usually occurs in the range of 200-400 nm. Therefore, to confirm the formation of ZrNPs, the presence of the SPR band and the

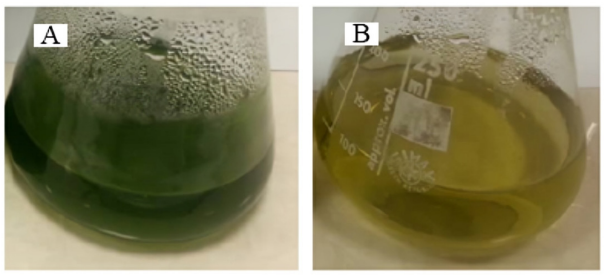

Figure 1. Change of the color from green (a: before the reaction) to yellow (b: after the formation of ZrNPs) using aqueous extract of the clover

absorption intensity of the produced NPs were checked with an UV-Vis light spectrometer. The wavelength of the maximum absorption band of the NPs formed using clover aqueous extract was 225-285 nm, which indicates the formation of ZrNPs.

3.3. Dynamic light scattering

DLS is a technique to confirm the formation of ZrNPs. Using the results of this analysis, the hydrodynamic diameter of the synthesized ZrNPs can be checked. It should be mentioned, based on the results obtained from macroscopic observations and DLS results of some aqueous extracts of clover, according to the difference in synthesis conditions such as extract concentration, metal salt concentration, temperature, and incubation rate It was not desirable for the ZrNPs synthesis and they were removed in the further work.The desired synthesis results based on the mentioned conditions can be seen as DLS results in Figure 2. Based on the results, the average size of NPs synthesized by clover at pH 10 and zirconium concentration of 1 mM was reported to be 64.33 nm.

3.4. Scanning electron microscope

SEM images of ZrNPs produced using clover aqueous extract are shown in Figure 3. In the SEM analysis of NPs, it can be seen the synthesized ZrNPs were irregular polygons, star-shaped and flower-like colonies,

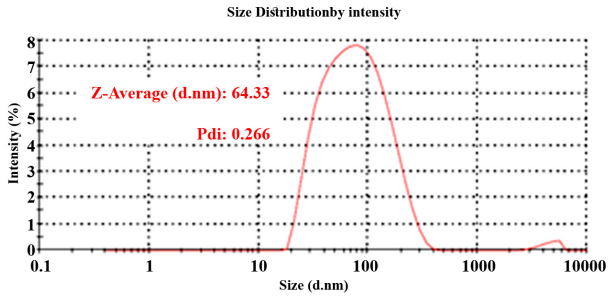

Figure 2. The results of DLS of ZrNPs synthesized by aqueous extract of clover plant at pH 8 and zirconium concentration of 1 mM

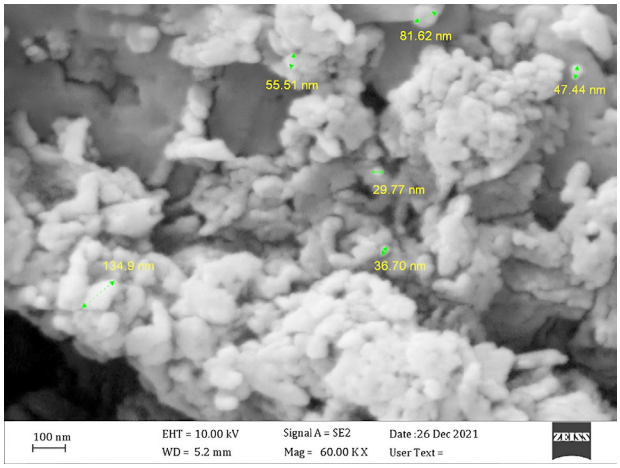

Figure 3. SEM image of nps synthesized by the aqueous extract of clover

homogeneous and with dimensions in the limited area of 29 to 81 nm. The accumulation of particles can also be seen in some areas; however, the particles in the accumulated areas are separate from each other and the border between them is quite clear (Figure 3).

3.5. Energy dispersive spectroscopy

In addition to UV spectra and images related to SEM analysis, to investigate the constituent elements of ZrNPs, the purity of the product, and the energy distribution spectrum of ZrNPs produced by aqueous clover extract are also shown in Figure 4. In this Figure, the peak related to the zirconium element can be seen. Given that ZrNPs have SPR properties, they have a specific optical absorption peak at around 2.2 keV, indicating the formation of ZrNPs. In addition, the distribution of ZrNPs produced by the aqueous extract of clover along with other elements is shown in Figure 4.

3.6. Investigating the cytotoxic effect of ZrNPs

The cytotoxic effect of ZrNPs synthesized by aqueous extract of clover on MDA-MB-468 and HFF cells was estimated using the MTT method. These cells were treated

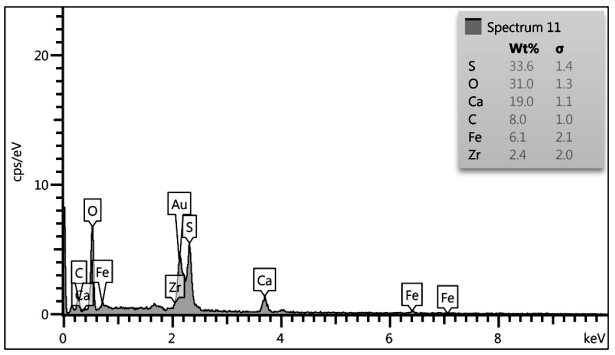

Figure 4. EDX Spectrum of ZrNPs synthesized by the aqueous extract of clover to check the constituent elements of NPs and product purity

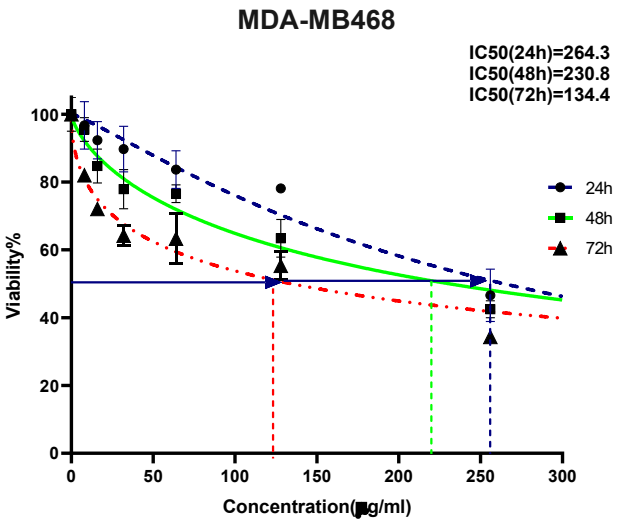

Figure 5. Cytotoxic effect of ZrNPs obtained from aqueous extract of clover plant at different concentrations on MDA-MB-468 cells after 24, 48, and 72 hours

with six different concentrations of NPs (8, 32, 64, 128, 256, and 512 µg/mL) for 24, 48, and 72 hours. As displayed in Figure 5, ZrNPs could inhibit the growth of MDA-MB-468 cells in a concentration- and time-dependent manner. By examining the results of the MTT test, it was observed that with increasing NP concentration and treatment time, the survival rate of cancer cells decreased significantly compared to the control cells. The IC50 values calculated from the dose-response curves for the cell line MDA-MB-468 are shown in Figure 5. IC50 values after 24, 48, and 72 hours were 264.3, 230.3, and 134.4 µg/mL, respectively.

Figure 6 shows the toxicity of different concentrations of ZrNPs on the HFF cell line, which was compared with MDA-MB-468 cells.

By examining the results of the MTT test, it was observed that the effect of ZrNPs on normal HFF cells was much lower compared to cancer cells. The IC50 of ZrNPs in the case of normal HFF cells was calculated using GraphPad Prism 8.0.2 at 420.0, 331.9, and 181.7 µg/mL after 24, 48, and 72 hours, separately.

In the present study, HFF cells were less sensitive to ZrNPs. In other words, the IC50 value in HFF cells after 48 hours was approximately 1.43 times higher than in MDA-MB-468 breast cancer cell lines. In addition, the comparative effects of ZrNPs obtained from the aqueous extract of clover and tamoxifen were investigated in this research. The IC50 value for tamoxifen was determined to be 13.1 µg/mL and 23.84 µg/mL after 48 hours of treatment in MDA-MB-468 and HFF cells, respectively (Figure 7).

4. Discussion

In the present research, clover plant (*Trifolium repens* L) collected from Sarieh Khatun village (Qom, Iran) was used for the first time to synthesize ZrNPs. The Synthesized NPs with dimensions in the limited area of 29-81 nm were observed in the SEM analysis as irregular polygons, star-shaped and flower-like colonies, and homogeneous.

Biological synthesis methods have paved the way for the green synthesis of NPs. The green synthesis is undoubtedly a better method due to slower kinetics, better manipulation, control of crystal growth, and stabilization [29]. In a study conducted by Sai Saraswathi and Santhakumar, tetrahedral ZrO_2NPs produced by the extract of *Lagerstroemia speciosa* had good cytotoxic activity against breast cancer cell line (MCF-7), and increasing the concentration of NPs up to 500 µg/mL reduced cancer cells. Moreover, the cell membrane of 30 to 40% of the cells was swollen. In addition, apoptosis or cell death occurred due to the exposure of these cells to ZrO_2NPs [30]. The evidence of this issue is the increase in the size of the vacuole (a part of the cell organelle located in the cytoplasm) of some cells. In other words, the change in vacuole size occurs when the cell death happens. In addition to the mentioned features, the synthesized NPs have the property of photocatalytic degradation (methyl orange). The existence of this property is probably due to their contact surface, tetrahedral structure, and active sites.

In the study conducted by Shanthi et al, zirconium dioxide NPs were produced using *Acalypha Indica* extract. The average size of the produced NPs was found to be 20-100 nm. According to the EDX analysis, the synthesized NPs are of high purity [31]. In the research of Golnaraghi Ghomi et al, ZrNPs were produced by the extracellular method using three different strains of Penicillium fungus. The size of NPs produced by three strains of *Penicillium purpurogenum*, *Penicillium aquiolithium*, and *Penicillium notatum* was reported to be 53.60, 39.32, and 62.27 nm, respectively. In addition, the produced NPs have antibacterial properties and good resistance against the growth of *Escherichia coli* and *Pseudomonas aeruginosa* organisms with a minimum inhibitory concentration of 0.75 and 0.375 mM [32].

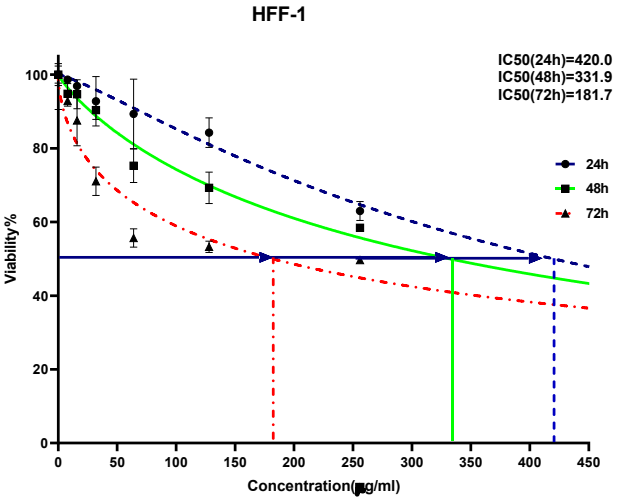

Figure 6. Cytotoxic effect of ZrNPs obtained from aqueous extract of clover plant at different concentrations on HFF-1 Cells after 24, 48, and 72 hours

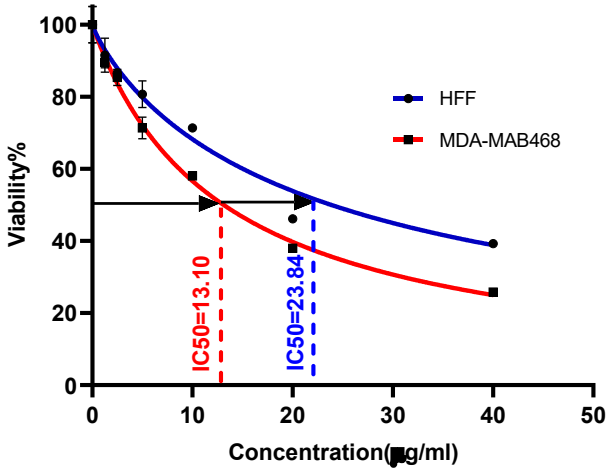

Figure 7. The Cytotoxic effect of tamoxifen at different concentrations on HFF-1 and MDA-MB-468 cells after 48 hours

The use of the leaf extract of Lul tree or Macarzan for the biosynthesis of ZrO_2NPs was reported by Shinde et al. Based on the results of the research, the synthesized NPs had a tetrahedral shape, particle size of 15 nm, and very high cross-sectional area (around 88 m^2/g). Besides, the photocatalytic effect of NPs in the presence of ultraviolet rays on methylene blue and methyl orange was examined, and the results indicated that the photodegradation of methylene blue and methyl orange was up to 91% and 69% within 4 hours. According to the results obtained from this research, the NPs synthesized by the leaf extract of the Lul tree are suitable alternatives to the NPs obtained by conventional methods [33].

In the current study, the cytotoxic effect of ZrNPs synthesized by aqueous extract of clover plant was investigated on the MDA-MB-468 breast cancer cell line and normal HFF. The results indicated that this NP had a very low toxicity effect on the breast cancer cell line and little very low toxicity effect on the normal cell line. This feature should be one of the main characteristics of antitumor drugs; in other words, these drugs should only affect tumor cells and have no adverse effects on normal cells. In other words, the results show more inhibitory effects of NPs on breast cancer cells compared to normal cells at a certain dose.

In the current study, the value of IC50 of ZrNPs after 24 hours on the MDA-MB-468 cell line was lower than the calculated value of IC50 on the HFF cell line, which indicates more inhibitory effects of ZrNPs on cancer cells than on normal cells.

The results of the current study showed that NPs synthesized in this study reduced the survival of cancer cells, but their toxicity on normal cells was lower. Due to severe glycolysis in cancer cells, the environment of cancer cells is acidic, and the acidity of the environment causes the activation of ZrNPs and the death of cancer cells.

Therefore, the greater effect of NPs on cancer cells than on normal ones was predictable. The difference in pore size of the membranes can be another factor in the significant difference in the toxicity of NPs between cancerous and normal cells in this study. The results of the current study showed that the aqueous extract of clover, a native plant of Iran, can be used in the synthesis of ZrNPs due to its secondary metabolites and antioxidant properties. Findings showed that synthesized ZrNPs have toxic effects on breast cancer cells, and since ZrNPs had very low toxicity effect on the death of the HFF cell line, it can be said that its side effects were minimal. Given that many drugs induce necrosis in cells and have side effects such as inflammation, they cannot be considered suitable drugs. While the ZrNPs synthesized in the present study caused toxicity in cancer cells, they do not have a toxic effect on normal cells. Therefore, they can be used as an effective drug for the treatment of breast cancer.

5. Conclusion

The results of the current study showed that the aqueous extract of clover had a significant role in the green synthesis of ZrNPs. On the other hand, these NPs had considerable cytotoxic effects on human breast cancer cells (MDA-MB-468) and could inhibit the growth of cancer cells. Therefore, it can be studied in future studies as an anti-cancer agent.

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Supervision: Mahdieh Ameri Shah Reza.
Validation: Mahdieh Ameri Shah Reza.
Visualization: Mahdieh Ameri Shah Reza.
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Writing–review & editing: Mahdieh Ameri Shah Reza, Alireza Rasouli.

Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

Ethical issues have been completely observed by the authors.

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