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Evaluation of the anti-cancer effect of Dianthin-30 on MCF-7 breast cancer cell line in 3D cell cultures

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ABSTRACT

Objectives: Breast cancer is one of the most common types of cancer among women. One of these toxins that inhibits the growth of breast cancer cells in 2D cell culture and has significant anti-tumor effects is Dianthin-30. Therefore, in this manuscript, for the first time, the anti-cancer effect of Dianthin-30 toxin against breast cancer cells (MCF-7) in 3D culture has been studied.

Materials and Methods: In order to evaluate the anti-cancer effects and cytotoxicity of the toxin at concentrations of 1.25, 2.5, 5 and 10µg/ml, MTT methods were used and a Neutral red test was used to validate the results of this test. Nitric oxide, Catalase, GSH assays, cytochrome c, Caspase-3 and Comet assay tests were also used to determine the type of mortality in cancer cells.

Results: This toxin did not induce nitric oxide production, but at concentrations higher than 5µg/ml increased catalase production compared to the control. However, the level of GSH produced in all of the concentrations was significant compared to the control. In addition, Dianthin-30 increased cytochrome 30 and activation of caspase-3 in the above concentrations, but this effect was not significant compared to the control. The results of alkaline comet test also showed that the rate of induction of apoptosis by toxin was upward compared to the control.

Conclusion: The results of this study show that Dianthin-30 has anti-cancer effects and has caused death in breast cancer cells and this toxin probably induced apoptosis in cancer cells more than the non-mitochondrial pathway.

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Abbreviations

PBS, Phosphate-buffered saline; DMSO, Dimethyl sulfoxide; TCA, Trichloroacetic; HCl, Hydrochloric acid; DNTB, Dinitrothiocyanobenzene; OD, Optical density; GSH, This test measures levels of Glutathione; NMA, Normal melting agarose; LMA, Low melting agarose.

Introduction

Cancer is one of the most important diseases of the present century and the second leading cause of death after cardiovascular disease. Breast cancer is the second common cancer in the world. it is still the leading cause of cancer death among women, Despite many advances in early diagnosis and treatment of this disease (1). Surgery, radiotherapy, chemotherapy, and hormone therapy are common treatment methods methods for breast cancer (2).

Radiotherapy is one of the treatment steps of breast cancer that damages healthy cells in addition to tumor cells (3-5). Radiotherapy complications reduce by using Targeted therapy. In this method, sensitive points are determined and mortality is induced in them to cause losses in tumor cells (6). In addition, these combination therapies reduce the systemic toxicity of radiotherapy or chemotherapy because using this strategy the dose received by the patient will be reduced (5).

In recent years, various biological materials, such as Dianthin-30, which is an anti-cancer ingredient, have been investigated. Various studies have shown that many herbal toxins have been considered by many research and pharmaceutical centers due to their pharmacological importance and effectiveness in the treatment of some diseases such as cancers(1).

Although many synthetic chemical drugs have a specific and rapid effect, they have not been considered as ideal drugs by the medical community due to their side effects in human hosts.

Some of these plant toxins such as Ricin, Abrin, Viscumin, which are castor plants (Ricinus communis), Rooster's pea (Rosary pea) or Rosary pea (scientifically named Abrus precatorius) and Mistletoe (Viscum album) are extracted, they are very dangerous for humans (2).

Dianthin-30 is a family of ribosome inactivating proteins that was first extracted and reported in the early 1960s as a medicinal substance from the clove plant Dianthin-30 spp, including D. caryophyllus. The reason for the toxicity of clove plant is the presence of this toxin, which is present in its various organs (2). Reports indicate that Dianthin-30 has anti-cancer properties and has an inhibitory effect on the growth of viruses and fungi (7-8). Studies also show that Dianthin-30 inhibit protein synthesis in the reticulocytes of some animals, such as rabbits (7-8).

The aim of this study was to evaluate the anti-cancer effect of toxin Dianthin-30 to increase the efficacy of MCF-7 breast cancer treatment in 3D culture medium.

Materials and methods

This study is an experimental study.
Breast cancer cells (MCF-7 IBRC C10082) were obtained from the cell bank of Imam Hossein Comprehensive University.

Preparation of Dianthin-30 toxin

Dianthin-30 purified was prepared from Imam Hussein Comprehensive University.

Dissolve Dianthin-30 in a suitable buffer: 1 mg of crude venom was dissolved in 1 ml PBS. After vortex, it was transferred to minus 4 ° C.

Toxin protein assay: The Bradford method, invented in 1976, is used to estimate protein concentrations. This method is very fast and accurate. The assays were performed by adding Bradford reagent to the protein solution and measuring the OD of the solution at 595 nm. Protein content was obtained by comparing light absorption with a standard curve of a pure protein (1).

Removing toxin contamination

0.02 mg Dianthin-30 with sterile DMEM medium (buffer) containing 1% antibiotic-anti-mycotic volume of 1 mL is incubated overnight in a CO2 incubator (37 °C and 5% CO2) to remove biological contaminants.

3D cell culture, encapsulation in alginate and depolymerization of capsules

In order to achieve a 3D environment, we used encapsulation of cells in alginate hydrogel. At First, alginate solution was produced by dissolving 0.11 g of sodium alginate powder (Sigma-USA) in 10 ml of 0.9% sodium chloride solution. Alginate solution was filtered by 0.22 µm syringe filter and added to the cell pellet containing 2×10^6 MCF-7 cells and re-suspended. Its volume was increased to 1 ml with alginate solution. Cell isolation and suspension of cells in alginate solution was performed using G22 plastic syringe. Alginate capsules were produced by injecting a mixture of cell-alginate mixture through a G22 plastic syringe into a bath of 100 mM calcium chloride (drops were released into the bath from a distance of 5cm above the bath's surface). The capsules were allowed to be polymerized for 10 minutes. In the next step, after the removal of calcium chloride, the capsules were washed two times with PBS solution. The washing solution was replaced with 1 ml of DMEM (Gibco,usa) containing 10% FBS. Capsules containing cells were incubated at 37 ° C and humidity of 95% and 5% CO_2.

For depolymerization of capsules and releasing of cells in GSH, Catalase and Comet assay, after removing the media, the capsules were transferred to sterile tubes and washed 3 times with PBS. Then, 1ml of depolymerization solution (50 mM sodium citrate) was added to the cells(9).

MTT assay test

This method was performed according to the method performed by Mossman et al. (10). Briefly, 4×10^4 cells were encapsulated according to the previously described procedure and incubated for 24 h at 37 °C. The old media was discarded from each well and fresh media (serum-free) containing different concentrations of Dianthin-30 (1.25, 2.5, 5 and 10 µg/ml was added to each well containing the capsules and incubated for 24 h at 37 °C with 5% CO_2 and 95 % humiliation. then, 20 µl of MTT (SRL,india) solution with a concentration of 5 mg / ml was added to each well and incubated at 37 °C for 3 h under dark conditions. After transferring the plate to a CO_2 incubator and forming a dark blue crystal (Formazon), the contents of each well were removed and after washing with PBS, 200 µl of DMSO (Scharlau,spain) was added to it. To completely dissolve the precipitate, the plate was incubated for 2–4 hours in the dark at room temperature. In this test, culture medium was used as a control and culture medium containing cells was used as a control and its absorption was measured at 570 nm by a microplate reader (Biotech, USA).This experiment was repeated 3 times for each concentration of Dianthin-30. Cell viability was calculated after contact with different concentrations of Dianthin-30 by the following formula (9).

The vitality percentage of cells =100× (a/b)

a = Optical density (OD) of the test sample minus the blank's OD

b = OD of the control minus the blank's OD

Neutral red uptake assay test

This procedure was performed according to the method performed by Winckler et al. (11). NR uptake assay was used to verify the MTT assay results .The Neutral red test is based on the ability of live cells to combine and link a neutral red to lysosomes (10). The steps of the neutral red colorimetric test are similar to the MTT assay test, except that after incubating the adjacent cell with Dianthin-30 for 24 hours, instead of MTT solution, 1 µl of neutral red dys (5 µg/ml) was added to each well and incubated under dark conditions at 37 ° C, 5% CO_2 and a humidity of about 80% until a red crystal formed (neutral red binding to the cell lysosome surface). The solution in each well was then drained and after washing each well twice with PBS, 100 µl of fixing buffer (37% formaldehyde, $CaCl_2$ (10% (w/v) in water) was added.

After one minute, drain the buffer and add 100 µl of solvent buffer (0.5% acetic acid). The plate was then incubated on a shaker for 20 minutes in the dark at room temperature and finally the absorption at 540 nm nm by a microplate reader (Biotek, USA). Inhibitory percentage of different concentrations of Dianthin-30 on cell growth was calculated using the following formula.

Percentage of cell mortality =1-[100× (a/b)]

a = Optical density (OD) of the test sample minus the blank's OD

b = OD of the control minus the blank's OD

Nitric oxide assay (NO) test

This method was performed according to the method performed by Zargan et al. (12-13). The steps for the nitric oxide assay are similar to the MTT assay, except that after incubating the adjacent cells at concentrations of 1.25, 2.5, 5, and 10 µg/ml Dianthin-30 for 24 h, instead of MTT solution, 4 µl of neutral red dye (Merck,germany) (5 µg/mL) was added to each well and incubated for 1 h at 37 °C. Media from each well was transferred to sterile tubes and centrifuged at 500×*g* for 5 min at 4 °C. A total of 100 µl of the media were transferred to a 96-wells plate and mixed with the equal volume of Griess reagent (Sigma, USA) (0.04 g/mL in PBS, pH 7.4) and incubated for 10 min at room temperature. Absorbance at 520-550 nm was measured by a microplate reader (Biotech, USA). Nitric oxide concentration (µM/mL) in treated cells was calculated using sodium nitrite standard curve.

GSH test

This method was performed according to the method performed by Sedlak and Linsay (14). The steps of GSH test were similar to NO assay test, except that encapsulated cells were transferred to a 24-wells plate and incubated overnight in 5% CO_2 at 37 °C. Then, the old media were discarded, and fresh media containing 1.25, 2.5, 5, and 10 µg/ml Dianthin-30 were added to each well and incubated at 37 °C for 24 h. 200 µl of the lysis buffer was added to each well, and protein concentration was assessed by Bradford assay. 40 µl of the obtained solution was removed and transferred to new tubes. Tubes were added to 40 µl of 10% TCA solution (Merck,germany) and stored at 4 °C for 2 h. The centrifugation was performed at a speed of 1500 rpm for 15 min. The supernatant was transferred into a clean tube. Then, 75 µl of lysis buffer, 55 µl of Tris HCl (Merck,germany) buffer (pH=8.5), and 25 µl DNTB (Merck,germany) was added to 20 µl of the supernatant. OD of samples was measured at 412 nm.

Catalase activity test

This method was performed according to the method performed by Sinha et al. (15). The catalase test steps were similar to the GSH test, except that 50 µl of lysis buffer, 20 µl of distilled water and 25 µl of 15% hydrogen peroxide were added to each sample and the samples were shaken. Samples were incubated at 37 °C for 2 min and were mixed with 100 µl of potassium dichromate solution. At this stage, the soluble pink color with the blue color of the upper part of the solution was visible. Next, samples were incubated in boiling water for 10 to 15 minutes until the green color was formed. Then, the OD was measured at 570 nm using a plate reader (Biotech, USA).

Cytochrome c assay

The steps of cytochrome c test were similar to GSH test, except that 1×10^6 the capsulated cells were transferred to 6-wells plates and incubated overnight in 5% CO_2 and 37 °C. The cells were dissolved in 1 ml of cytosol purification buffer and incubated in ice for 10 minutes. The cells were homogenized in the Dounce tissue grinder. The homogenized samples were transferred to a new 1.5 ml tube and centrifuged at 700 g for 10 minutes at 4 ° C. The soluble samples were transferred to clean 1.5 mL tubes and centrifuged at 10,000 g for 30 min at 4 °C. The soluble solution, which was the cytosolic component, was collected. Protein concentration was measured using Bradford.

Alkaline comet test

This method was performed according to the method performed by Zimmermann et al. (16). The alkaline comet test steps were similar to the GSH method, except that the alginate capsules were dissolved according to the instructions and cell depositions was obtained. Then, 200 µl of PBS was added to the tubes, and the cells were separated from each other with a sampler and an insulin syringe. The slides required for the experiment were coated with agarose at normal melting point (1% NMA) and refrigerated 4°C for 10 minutes. The cell suspension with agarose with low melting point (1% LMA) was mixed (1:2 ratios) and were applied to the slides. To form a cell layer, a cover slip was applied to spread the preparation. To do cell lysis and nucleus distraction, all slides were incubated for 16–18 h in fresh and cold lysis buffer (NaCl 2.5M, EDTA 100 mM, Tris 10 mM, NaOH 0.2 M, and Triton X-100 %1; pH 10) at 4 °C. Then, slides were washed twice with electrophoresis buffer for 20 min at 4 °C and electrophoresed for 45 min at 4 °C (25 V and 300 mA). For neutralization, the slides were incubated for 10 min in the neutralizing buffer (Tris 0.04 M, pH 7.5). Then, the slides were incubated in 100 µl ethidium bromide (20 µg/mL) for 10 min at room temperature. Slides were washed two times (10 min each) with water and analyzed by an inverted fluorescent microscope (Nikon, Japan), and results were statistically analyzed.

Caspase-3 activity test

Caspase-3 activity was evaluated using the commercial Caspase-3/CPP32 laboratory kit according to the manufacturer's instructions (10).
The steps of the Caspase-3 assay were similar to the cytochrome c test, except that after dissolving the alginate capsules, the resulting cell precipitate and cell plate were suspended again in 100 ml of cold lubricating buffer and incubated in ice for 20 minutes. Samples were evaluated for protein content. The final volume of 50 µg of protein content of the samples was then increased to 100 µl using a lubricating buffer and combined with a reactive buffer (containing 10 mM DTT). In the next step, 5 µl of 4 mM DEVD-pNA (200 mM) was added to each sample and incubated for 1 hour at 37 ° C. In the last step, the absorbance of the samples was measured at 405 nm.

Statistical analysis

Comparison of the anti-cancer effect of Dianthin-30 against different controls and concentrations on MCF-7 breast cancer cells was evaluated using GraphPad InStat software and two-way analysis of variance (Data presented with SEM). $p < 0.05$ was considered significant for the results. This experiment was repeated three times for each concentration of Dianthin-30 in all methods.

Results

Results of MTT method

Results of the MTT method effect of cytotoxicity of Dianthin-30, at concentrations of 1.25, 2.5, 5, and 10 µg/ml on breast cancer cells (MCF-7) were investigated, and a culture medium containing MCF-7 cells was used as a control. MCF-7 cell viability in 3D culture in the concentrations of 1.25, 2.5, 5, and 10 µg/ml of Dianthin-30 was 74.3, 64, 59.3, and 61.6, respectively. The results showed that this toxin had a significant inhibitory effect on cells at all concentrations compared to control and it prevented cell growth (Figure 1).

Results of Neutral red uptake method

In this study a neutral red test was done to confirm the results of the MTT test and to evaluate the effect of cytotoxicity of Dianthin-30 in the 3D culture at concentrations of 1.25, 2.5, 5 and 10 µg/ml on breast cancer cell line growth (MCF-7). The culture medium containing MCF-7 cells was used as a control. The results show that the percentage of inhibition of MCF-7 cells in 1.25, 2.5, 5, and 10 µg/ml of Dianthin-30 was 39, 45.3, 48.7, and 51, respectively.

As can be seen in (Figure 2), Dianthin-30 had a significant inhibitory effect on cell growth at all concentrations compared to the control. Also, cell mortality at a concentration of 1.25 compared to 5 and 10 was not statistically significant and at other concentrations were not significant relative to each other.

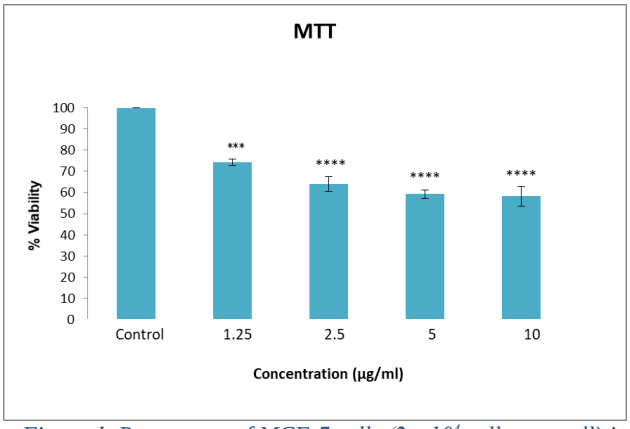

*Figure 1. Percentage of MCF-7 cells (2× 10^4 cells per well) in 3D culture after 24 hours of exposure to 1.25, 2.5, 5, and 10 µg / ml concentrations of Dianthin-30 based on the MTT test. Data were evaluated using GraphPad InStat software and a two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (*** p <0.001, **** p <0.0001).*

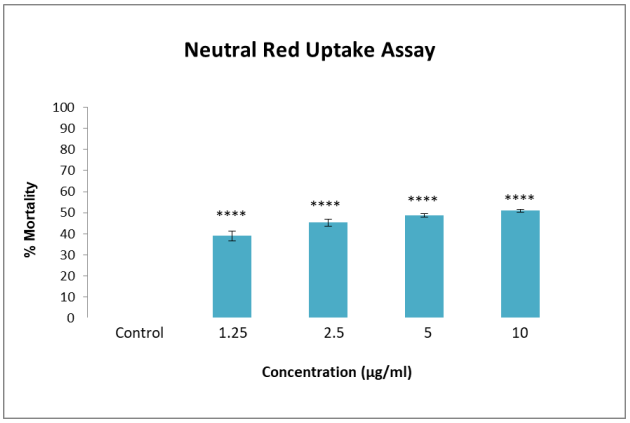

*Figure 2. Percentage of growth inhibition of MCF-7 cells (2 ×10^4cells per well) in 3D culture after 24 hours of exposure to concentrations of 1.25, 2.5, 5 and 10 µg / ml of Dianthin-30 by Neutral red test. Data were evaluated using GraphPad InStat software and a two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (**** p <0.0001).*

Results of Nitric oxide method

The amount of nitric oxide released from MCF-7 cells in 3D culture in the control sample and concentrations of 1.25, 2.5, 5 and 10 µg/ml of Dianthin-30 were 10.2, 9.02, 8.92, 9.36, 10.66 and As shown in (Figure 3), the amount of nitric oxide released from the cells due to the effect of Dianthin-30 in all concentrations was not significant compared to the control.

Also, the amount of nitric oxide production in cells at a concentration of 10 µg / ml was not statistically significant compared to 1.25, 2.5, and 5 µg/ml.

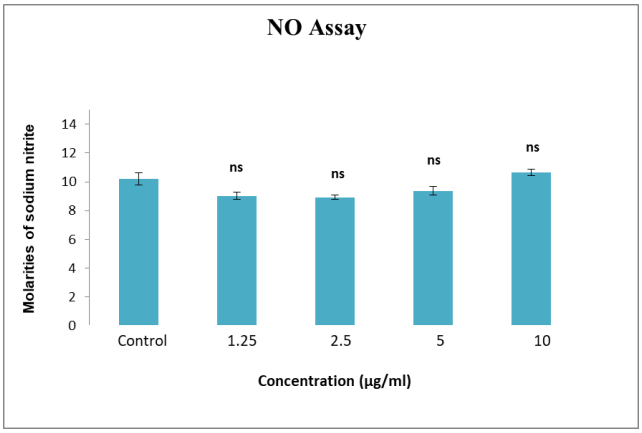

Figure 3. The amount of nitric oxide produced by Dianthin-30 in MCF-7 cells in 3D culture was evaluated compared to the control group. Data were evaluated using GraphPad InStat software and a two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (ns: not significant).

Results of GSH assay method

Results of this experiment showed that the level of cellular glutathione produced in 3D culture in the control sample and concentrations of 1.25, 2.5, 5 a, and 10 µg/ml of Dianthin-30, were 2.71, 2.63, 2.61, 2.58 and 2.55 respectively. The statistical study also showed that the level of cellular glutathione produced by Dianthin-30 was significant in all concentrations compared to the control (Figure 4). It is showed that the rate of induction of this effect at a concentration of 1.25 to 10 is not significant and at other concentrations is not significant relative to each other.

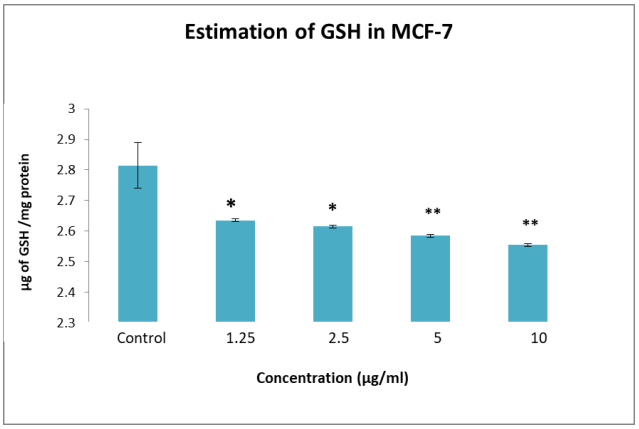

Figure 4. The amount of glutathione produced by Dianthin-30 in MCF-7 cells in 3D culture using the GSH assay. Concentrations were evaluated compared to the control group. Data was evaluated using GraphPad InStat software and a two-way analysis of varianc. This experiment was repeated three times for each concentration of Dianthin-30. (p <0.05, ** p <0.01).*

Results of catalase assay method

Results of this experiment showed that the amount of cellular catalase generated in 3D culture in control and samples treated with concentrations of 1.25, 2.5, 5, and, 10 µg/ml of Dianthin-30 were 6.13, 6.7, 5.97, 5.60, and, 5.54 respectively. Based on these results (Figure 5), the amount of catalase produced was only significant at concentrations higher than 5 µg / ml compared to the control. The amount of catalase produced in this test at concentrations of 1.25 and 5 µg / ml was significant.

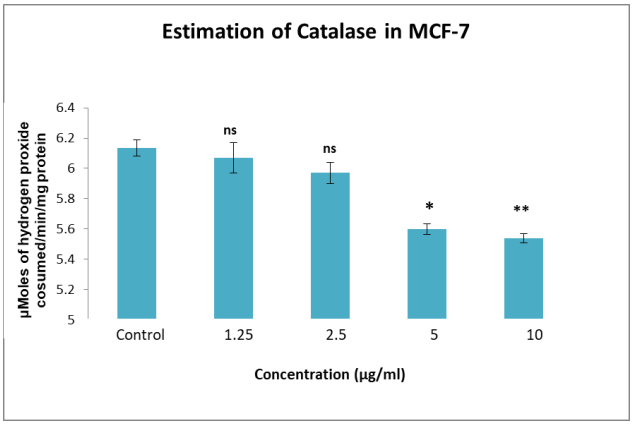

*Figure 5. The amount of catalase produced by Dianthin-30 in MCF-7 cells in 3D culture using catalase test. Concentrations were evaluated compared to the control group. Data evaluated using GraphPad InStat software and two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (ns: not significant, * p <0.05, ** p <0.01).*

Results of cytochrome C method

Results of the cytochrome C methods showed that the amount of free cytochrome released into the cytosol in MCF-7 cells in 3D culture in control and samples treated with concentrations of 1.25, 2.5, 5 and 10 µg/ml of Dianthin-30 were equal to 0.101, 0.103, . 0.104, 0.106 and 0.114 respectively. Although the results showed that the production of this enzyme in exposed cells with different concentrations of Dianthin-30 was not significant compared to the control. Also, the amount of enzyme released at two concentrations of 1.25 and 2.5 is significant compared to a concentration of 10 µg/ml. (Figure 6).

Results of Caspase-3 method

Results of the Caspase-3 method showed the activity of caspase-3 enzymes in MCF-7 cells in 3D culture in control and samples treated with 1.25, 2.5, 5 and 10 µg/ml concentrations of Dianthin-30 were equal to 0.0077, 0.0077. 0.007, 0.007, and 0.0,065 respectively. The results showed that the production of this enzyme in exposed cells with different concentrations of Dianthin-30 is not significant compared to the control and also to each other (Figure 7).

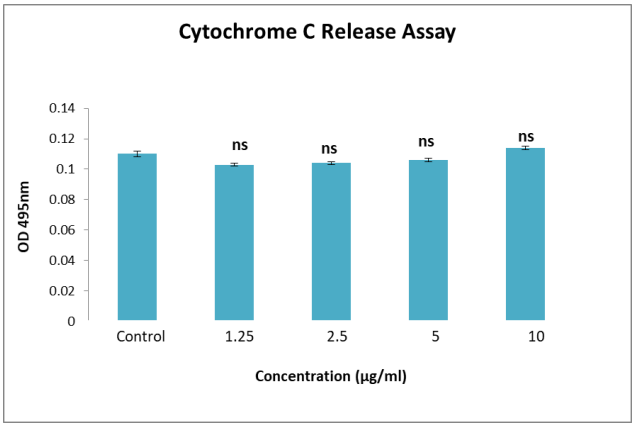

Figure 6. The amount of free cytochrome released into the cytosol in Dianthin-30 treated MCF-7 cells in 3D culture using Cytochrome c test. concentrations were evaluated compared to the control group. Data evaluated using GraphPad InStat software and two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (ns: not significant).

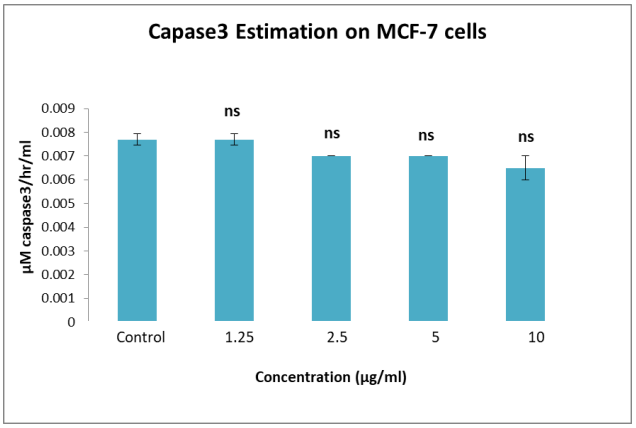

Figure 7. Caspase-3 activity in Dianthin-30 MCF-7 cells treated in 3D culture using Caspase-3 activity assay. Concentrations were evaluated compared to the control group. Data evaluation was d using GraphPad InStat software and a two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (ns: not significant).

Results of Comet assay method

In this study, the alkaline comet was used to evaluate the possibility of induction of cell death by Dianthin-30 on MCF-7 cells. The induction rate of Dianthin-30 apoptosis in control samples and concentrations of 1.25, 2.5, 5, and 10 µg/ml of Dianthin-30 on MCF-7 cells in 3D culture were 2, 11.3, 16.7, 19.7, and 21.7%, respectively. The results showed that the rate of induction of apoptosis by Dianthin-30 in all concentrations was significant compared to the control. As can be seen in Figure 8, the induction of programmed death of Dianthin-30 at concentrations of 2.5 µg/ml was not statistically significant compared to 5 µg/ml.

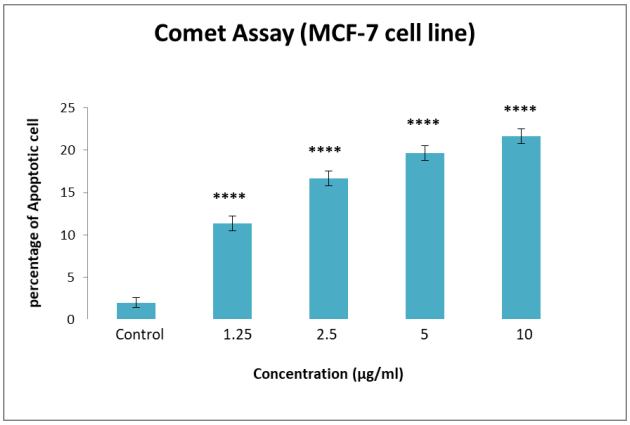

*Figure 8. The rate of Dianthin-30 induced apoptosis in MCF-7 cells in 3D culture using the Comet assay test. Concentrations were evaluated compared to the control group. Data evaluated was using GraphPad InStat software and a two-way analysis of variance. This experiment was repeated three times for each concentration of Dianthin-30. (**** p <0.0001).*

Discussion

Breast cancer is one of the most common types of cancer among women and is a heterogeneous disease due to the interaction of risk factors, heredity, and environment. It is the second leading cause of death in women after lung cancer (17-18).

Published research results show that some plant toxins belong to a group of phytotoxins that inhibit protein synthesis in eukaryotic and prokaryotic cells (2) and called ribosome-inactivating proteins (RIPs). Due to their antifungal, antiviral, and inhibitory effects on the growth of cancer cell proliferation, they have been considered by researchers for inducing anti-tumor effects (19-23).

One of these toxins is Dianthin-30, which has been extracted and purified from various organs of about 30 species of cloves (Dianthin-30) (2). This toxin is one of the first type of RIPs and has cytotoxic and anti-cancer effects (20-21). The gene encoding Dianthin-30 contains 1153 bp and is toxic to mammalian cells and causes the death of eukaryotic cells by inhibiting protein production (22).

So far, two types of Dianthin proteins with different molecular weights called Dianthin-30 and Dianthin-32 have been isolated and purified from the leaves of the clove plant Dianthin-30 cariophilus.

The molecular weight of Dianthin-30 is 29500 and Dianthin-32 is 31700 Daltons. The Dianthin-30 sequence showed that its original structure contained 293 amino acids. Its N-terminal length is 23 amino acids as a secretory signal sequence and its C-terminal length is 16 amino acids, which are responsible for the pre-peptide signal.

Cell culture is a very important part of laboratory processes, including drug discovery and development processes, and because there are limitations to two-dimensional culture, a new system called three-dimensional (3D) culture systems has been introduced as a new way to achieve correct results.

Alginate is a polysaccharide that due to its suitable properties can be used as a scaffold for three-dimensional culture.

Recent findings showed that in two-dimensional culture, cells cultured in on this model does not adequately simulate the microenvironment in-vivo (21). In this type of culture, the abnormal cell morphology affects many cellular functions, including cell proliferation, differentiation, apoptosis, and gene and protein expression (22).

Today, three-dimensional cell culture systems are more similar to the in-vivo conditions of living organisms due to their environmental simulation (23). They have greater advantages in providing more physiological information and predictable data compared to 2D cell culture systems for use in in-vivo experiments and have been considered for the discovery of new drugs (24-25).

In this study, for the first time, the anti-cancer effect of toxin Dianthin-30 against breast cancer cells (MCF-7) in three-dimensional culture conditions has been experiment.

In this study, after preparing purified Dianthin-30 toxin, the method of encapsulating cells in alginate hydrogel was used to achieve a three-dimensional environment (4).

To evaluate the anti-cancer effects and cytotoxicity of Dianthin-30 at concentrations of 1.25, 2.5, 5 and 10 µg/ml in 3D culture medium by MTT, Neutral red uptake assay (to determine the type of mortality in cells), Nitric oxide (NO assay), catalase, glutathione (GSH), cytochrome c, Caspase-3 and Comet assay tests were used for cancer, mitochondrial-dependent apoptosis and the effect of the toxin on the cellular stress.

This study examine the cytotoxic effect of Dianthin-30 at concentrations of 1.25, 2.5, 5 and 10 µg/ml on breast cancer cell growth (MCF-7) in 3D culture and in-vitro by the MTT method and to validate the results of this test Neutral red was used. The results showed that the percentage of cell viability in related concentrations was 74.3, 64, 59.3, and 61.6 respectively.

Studies by Hosseinpour et al. in 2019 showed that this toxin induced mortality in 2D culture cells of breast cancer cells at a concentration of 5 µg/ml and reduced the survival rate of cells to 71% (1).

Also, the results of the neutral red test in this study showed that the inhibitory effect of this toxin on cell growth in the above concentrations was dose-dependent and increased. However, in 2D culture conditions at a concentration of 5 µg/ml, this effect was previously reported to be 53.27 (1).

In this study, for the first time, the effect of Dianthin-30 on the induction of nitric oxide, catalase, glutathione, cytochrome c, and caspase 3 production was investigated. The results of this study showed that this toxin did not induce nitric oxide production in the studied concentrations but increased the catalase production in concentrations higher than 5 µg/ml compared to the control.

However, the level of cellular glutathione produced by the toxin was significant in all concentrations compared to the control.

In addition, the statistical study showed that Dianthin-30 caused the release of cytochrome c and also the activation of caspase-3 in the above concentrations, but this effect was not significantly compared to the control.

In this study, an alkaline comet assay was also used to evaluate the possibility of induction of cell death by Dianthin-30 on MCF-7 cells. Induction of Dianthin-30 apoptosis in control samples and concentrations of 1.25, 2.5, 5 and 10 µg/ml on cells in 3D culture increased with increasing dose of toxin were 2, 11.3, 16.7, 19.7 and 7 respectively. However, in 2D culture conditions and the above concentration, this effect was previously reported 6.33, 11.3, 8.3 and 9.6, respectively (1).

These results show that Dianthin-30 in 2D culture medium caused death in cancer cells mainly through necrosis, while in 3D culture conditions the rate of necrosis decreased and the rate of apoptosis increased.

In summary, the results of this study showed that Dianthin-30 has anti-cancer effects and has caused death in breast cancer cells.

Comparison results of MTT and Neutral red tests with the results of alkaline comet indicates that this toxin has mainly reduced the survival of cancer cells by inducing necrosis.

On the other hand, although the mortality of tumor cells due to the effect of this toxin in 2D culture reported by other researchers (1,25), but in 3D culture firstly, this effect was less than in 2D culture and Due to further simulation of 3D culture with natural conditions of living organisms, the effect of necrosis is reduced and the effect of apoptosis is increased.

In addition, due to the insignificant release of cytochrome c from the mitochondria of cells treated with different concentrations of Dianthin-30 in this study, this toxin probably induced apoptosis in cancer cells more than the non-mitochondrial pathway.

For mechanism study of this toxin, biotechnology and nanobiotechnology technologies studies suggested.

Declaration

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Conflicts of interest/Competing interests

There is no conflict of interest declared by any author.

Authors' contributions

MF, SZK, JZ, and SZ designed the study concept; JZ, MF, and SZK analyzed and interpreted the data; SZ, MF, and JZ made a statistical analysis; SZ and MF collected the data and drafted the manuscript. JZ, MF, SZK, AHNM, MM, and HKA edited and revised the manuscript.

Ethics approval

Not applicable.

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