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Research Paper: Introduction of Dianthins: A New Promising Horizon Toward Continuous Research on Breast Cancer Bulldozing in Iran

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ABSTRACT

Background: The production and secretion of defense proteins are one of the protective mechanisms exploited by plants against pathogens. The production and secretion of defense proteins are one of the protective mechanisms exploited by plants against pathogens. Ribosome-Inactivating Proteins (RIPs), as the main class of these proteins, are considered to facilitate cancer therapy worldwide, because of the potential anticancer activity. Indeed, some of these proteins have cytotoxic and anticancer properties. Extracted from the carnation (Dianthus caryophyllus), Dianthin inhibits protein synthesis in many different cells.

Methods: In this research, the Dianthins was isolated and purified from the leaves of D. caryophyllus, using ion-exchange chromatography column (CM-Sephadex G-50). Subsequently, its cytotoxicity effect on MCF-7 cell line was investigated. The cell cytotoxicity assessment was performed, using 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), neutral red uptake, and alkaline comet assays at the concentrations of 1.25µg/mL to 10µg/mL of the protein applying the MCF-7 cell line.

Results: the toxin induces cell death, mostly via necrosis rather than apoptosis, but in the special range of concentrations.

Conclusion: because of the severe side effects of chemotherapy drugs, this toxin can undergo more research as a new drug candidate against breast cancer.

1. Introduction

Plants are a major source for the treatment of human diseases and, consequently, natural biomolecules are subject of great interests. They produce proteins that can inactivate Ribosome-Inactivating Proteins (RIPs), which were firstly extracted over a century ago. It has been shown that the catalytic domain of these toxins irreversibly inhibits protein synthesis by removing an adenine base from the large ribosomal RNA [1-3]. They can serve as the new light for the tunnel of can-

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cer. More than 100 RIPs with different levels of toxicity have been isolated from plants and bacteria. According to their structures, RIPs are divided mainly into 2 types; single-chain (type 1) and multi-chain (type 2) RIPs. Dianthins belong to type 1 RIPs [1, 4]. These are phytotoxins extracted from the caryophyllaceae plants [2, 3]. Up to now, 2 types of Dianthins have been introduced; Dianthin 30 and Dianthin 32, with a molecular weight of 29500 and 31700 Daltons, respectively. These toxins have been isolated and purified from the leaves of a carnation [5, 6].

Also, there are many reports on the anticancer activity of RIPs and they seem to be appropriate candidate compounds against cancerous cells [7, 8]. These findings are of great importance because, despite the progress in the understanding of the different aspects of cancer, the disease is still one of the leading causes of death worldwide. An important challenge of the disease treatment is the resistance of some types of cancers to chemotherapy drugs. The development of new therapies based on active biological materials, in particular peptides and proteins, is an attractive research field in cancer therapy [9].

The main goal of the present study was introducing Dianthin as potential candidates for cancer therapy, which was extracted from Dianthus caryophyllus carnation leaves. The anticancer properties of the protein were investigated by 3-(4, 5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), Neutral Red Uptake (NRU), and alkaline comet assays. This research was carried out from 2016 to 2018.

2. Materials and Methods

Plant collection

Dianthus caryophyllus plants were collected from a greenhouse located in Pakdasht, Tehran, Iran during April. Immediately after harvesting, the plants were washed with distilled water, kept in liquid nitrogen, and transferred to the lab. To avoid protein degradation, the plants were kept in -70°C until the protein extraction. The MCF-7 cell line was purchased from Razi Institute, Karaj, Iran. Chemical materials were mainly purchased from Sigma-Aldrich Company (The United States).

Dianthins toxin extraction from carnation leaves

About 200g leaves were chopped up, using a blender and 320 mL of 0.14 M NaCl containing 0.005M sodium phosphate buffer (pH=7.2) was added to the chopped leaves. The mixture was agitated slightly in a shaker at 4°C for overnight. The extract was passed through cheesecloth and the filtrate (extract 1) was kept at -70°C; 150mL of the mentioned buffer was added to the remaining solid residue and stirred for an extra overnight at 4°C. Then, the lysate was passed through cheesecloth and the filtrate (extract 2) was collected. Extracts 1 and 2 were mixed and centrifuged at 40000g for 30min. The pellet was discarded and the supernatant was dialyzed against 1 liter 5 mM sodium phosphate buffer, pH=6.5, at 4°C for 24h (with the 3-time exchange of the buffer). Using a Whatman no. 2 filter paper, the supernatant was filtered and the filtrate was directly applied to a CM-Sephadex G-50 column (23cm x 1.6cm). The washing process was performed by the addition of 200mL of the previous buffer. The desorption of adsorbed material was carried out with 500 mL of the buffer and treated in a linear gradient of 0-0.3M NaCl to the column [10].

Cell culture

The MCF-7 cell line was cultured in 25mL flasks containing DMEM, (10%) FBS, and 10 µg/mL penicillin-streptomycin solutions or (1%) antibiotic-antimycotic; then, it was incubated in a CO^2 incubator (37°C, (5%) CO^2, and (80%) humidity). The refreshing of cells was achieved by replacing the media 3 times per week.

MTT reduction assay

To perform the MTT assay, 3×104 cells were seeded in a 96-well plate containing 100µL media with (10%) FBS and incubated overnight in a CO^2 incubator (37°C, (5%) CO^2, and (80%) humidity). Then, the medium was discarded and the same new medium without FBS containing different concentrations of Dianthins (1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL) was added and the plate was re-incubated for 24h in a CO^2 incubator [37°C, (5%) CO^2, and (80%) humidity]. Then, 5µL MTT dye (5 mg/mL) was added to each well. After appearing purple crystals (formazan), the medium was discarded and the wells were washed by PBS; 100µL DMSO was added to each well and incubated for 3-4h in a dark place to solubilize the purple crystals. The absorbance was measured at 570nm. The medium was used as a blank and the medium containing cells was used as the control. The assay was repeated 3 times for each concentration. Cells viability was measured by Equation 1 [11].

1.

$$Viability\ \% = \left(\frac{OD\ sample - OD\ blank}{OD\ negative\ control - OD\ blank}\right) \times$$

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Neutral red uptake assay

The Neutral Red Uptake (NRU) was performed to assess the viability of the cells following exposure to the toxin. To do this assessment, after 24h of Dianthins (1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL) treatment, 5µL of neutral red dye (5mg/mL) was added to each well and incubated for 1 h in a CO^2 incubator [(37 °C, (5%) CO^2, and (80%) humidity]. After the formation of red crystals, the supernatant was discarded and the wells were washed with PBS; 100µL fixation buffer [formaldehyde (37%), CaCl2 (10%)] was added to each well. After 1 min, 100µL solubilizing buffer (acetic acid 5%) was added and the plate was incubated for 20min in a shaker incubator in a dark place; finally, the absorbance was read at 540nm. The mortality rate was measured through Equation 2 [12].

2.

$$Metabolic\ activity\ \%=(1-\frac{OD\ sample-OD\ blank}{OD\ control-\ OD\ blank})\times 100$$

Alkaline comet assay

Alkaline comet assay or single cell gel electrophoresis is a DNA fragmentation assay test. This assay is used for the analysis of the apoptosis; 12×104 cells were seeded in each well and incubated overnight in a CO^2 incubator (37°C, 5% CO^2, and 80% humidity). Then, the medium was discarded and FBS-free media containing different concentrations of Dianthins (1.25 µg/mL, 2.5 µg/mL, 5 µg/mL, and 10 µg/mL) were added and incubated for 24h in a CO^2 incubator [37°C, (5%) CO^2, and (80%)humidity]. Then, the cells in each well were treated with trypsin, transferred to fresh micro-tubes, and centrifuged (1500x g for 5 min at 4°C). The supernatant was discarded and washed twice by 400µL PBS (pH=7.4). The slides were covered by agarose (1%) and incubated for 10min at 4°C.

The cell suspensions were mixed with (1%) low melting agarose (1:2 ratios) and added to the slides. To form the 1 cell layer, a glass Lamel was placed to each slide. In order to lyse the cells and distract the nuclei, all slides were incubated for 16 to18h in both fresh and cold lysis buffers (NaCl 2.5 M, EDTA 100mM, Tris 10mM, NaOH 0.2M, Triton X-100 (%1), and pH=10) at 4°C. Then, the slides were washed twice with electrophoresis buffer for 20min and incubated in fresh electrophoresis buffer for 40min at 4°C. The electrophoresis was carried out, applying 25 V and 300mA for 45min at 4°C. For neutralization, the slides were incubated for 10 min in neutralizing buffer (Tris 0.04 M, pH=7.5). Then, the slides were incubated in 100µL Ethidium bromide (20 µg/mL) for 10min at room temperature [13]. The slides were washed twice (10min for each) with water and analyzed by a fluorescent microscope (Zeiss, Germany). For each sample, at least 100 pictures were captured and analyzed by One-Way Analysis of Variance (ANOVA) test.

Statistical analysis

The results were reported as Mean±SD and the data were analyzed, using GraphPad Prism software. The treated cells and controls were analyzed, using ANOVA and Tukey Kramer multiple comparison methods. Differences were considered significant at ($P<0.05$*), ($P<0.01$**), ($P<0.001$***), and ($P<0.0001$****). All experiments were conducted at least 3 times.

3. Results

Extraction and purification of dianthins

As can be seen in Figure 1, Dianthins 30 and 32 were purified by ion-exchange chromatography. It should be noted that Dianthin 30 was used for the subsequent studies.

Cells viability determination using MTT assay

Dialyzed crude extract has reduced the viability of MCF-7 cell line in a dose-dependent manner (Figure 2a). MCF-7 cell line viability was (59.83%), (38.87%), (34.2%), and (25.59%) for 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of Dianthin toxins, respectively. Fractions 1 and 2; Dianthin 30 and 32 significantly reduced the viability of MCF-7 cell line (Figure 2b). The other fractions had no toxicity effects on this given cell line (Figure 2b). The highest toxicity of the dialyzed crude extract was observed at 10µg/mL, and cell viability reduced up to (74.41%) compared to the controls. In addition, significant differences were revealed between samples treated with 1.25µg/mL of extract and those treated with 10µg/mL of extract.

Neutral red uptake assay

This method was used as a confirmatory test for the results of the MTT method. The MCF-7 cell line metabolic activity was (70.77%), (48.7%), (42.7%), and (30.9%) in 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of dialyzed crude extract, respectively (Figure 3a). Fractions 1 and 2 related to Dianthin 30 and 32 significantly caused the death of the MCF-7 cell line, but other fractions had no effect on MCF-7 cell line (Figure 3b). For NRU as-

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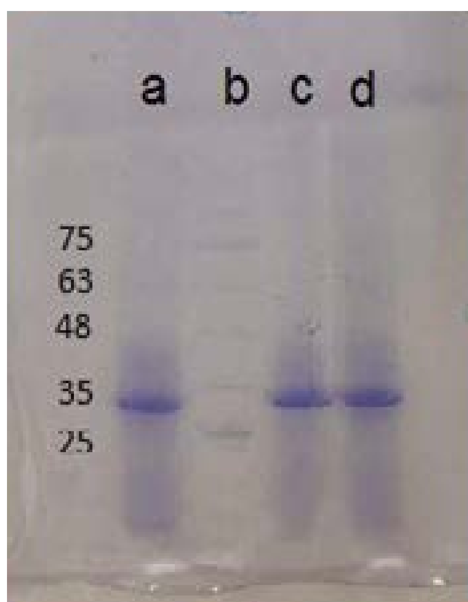

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Figure 1. SDS-PAGE electrophoresis (12%) of Dianthin 30 and 32 kDa

A: Dialyzed crude extract; B: protein marker; C: Dianthin 30; D: Dianthin 32

says, similar results were inferred. Cell viability reduced to 69.1 at the same concentration of dialyzed crude extract ($P<0.05$). By average, dialyzed crude extract (10 µg/mL) caused (71.75%) cell viability considering both MTT and NRU assays all in together.

Alkaline comet assay

The alkaline comet assay was used for analyzing potential apoptosis and necrosis induction effects of a dialyzed crude extract, as well as the fragmentation of DNA. In MCF-7 cells, apoptosis induction were (18.3%), (17.3%), (20.53%), and (19.7%) for 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of Dianthin toxins, respectively; necrosis was (32%), (44.3%), (46.63%), and (48.8%) for 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of Dianthin toxins, respectively (Figure 4a).

Dianthin 30 significantly induced necrosis and apoptosis in MCF-7 cell line (Figure 4b). Apoptosis induction was (8.46%), (10.46%), (12.75%), and (15.32%) in 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of fraction 1 of Dianthin 30 toxin, respectively; while, the necrosis was (17.53%), (36.5%), (56.13%), and (63.29%) for 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of fraction 1 of the toxin, respectively (Figure 4b).

Dianthin 32 significantly induced necrosis and apoptosis in MCF-7 cell line (Figure 4c). The apoptosis rate was (7.42%), (9.6%), (10.8%), and (13.1%) in 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of fraction 2 of Dianthin 32 toxin, respectively; while, the necrosis was (24.64%), (42.6%), (58.4%), and (61.7%) for 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of fraction 2 of Dianthin 32 toxin, respectively (Figure 4c).

The results of alkaline comet assay showed that both Dianthins 30 and 32 induced necrosis more than apoptosis in MCF-7 cell line but in a different range of concentrations. It was more dominant for Dianthin 32 in the range of concentrations from 1.25µg/mL to 10µg/mL. The aforementioned behavior was repeated for Dianthin 32 in the range of concentrations of more than 10µg/mL.

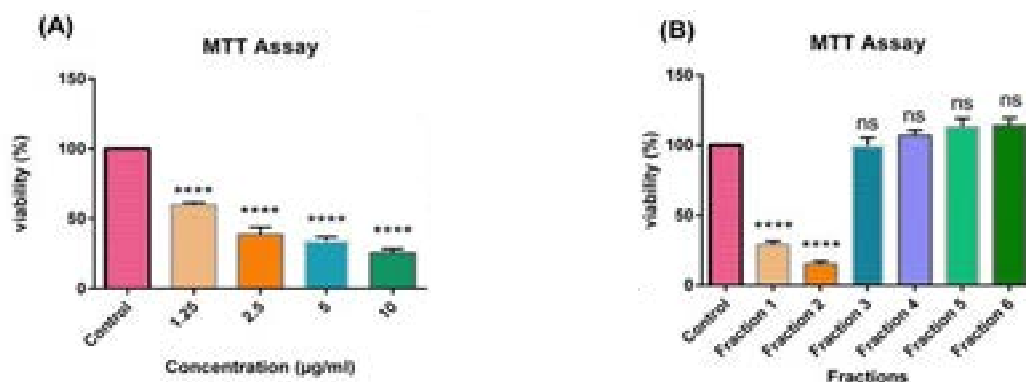

Figure 2. Determining cell validity using MTT Assay

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A: The MCF-7 cell line viability after exposure to 1.25µg/mL to 10µg/mL concentrations of dialyzed crude extract according to MTT assay (3×104 cells/well); B: Cytotoxicity effects of Dianthins (30 and 32) toxin fractions with 5µg/mL concentration using MTT assay; (NS: not significant), (****$P<0.0001$)

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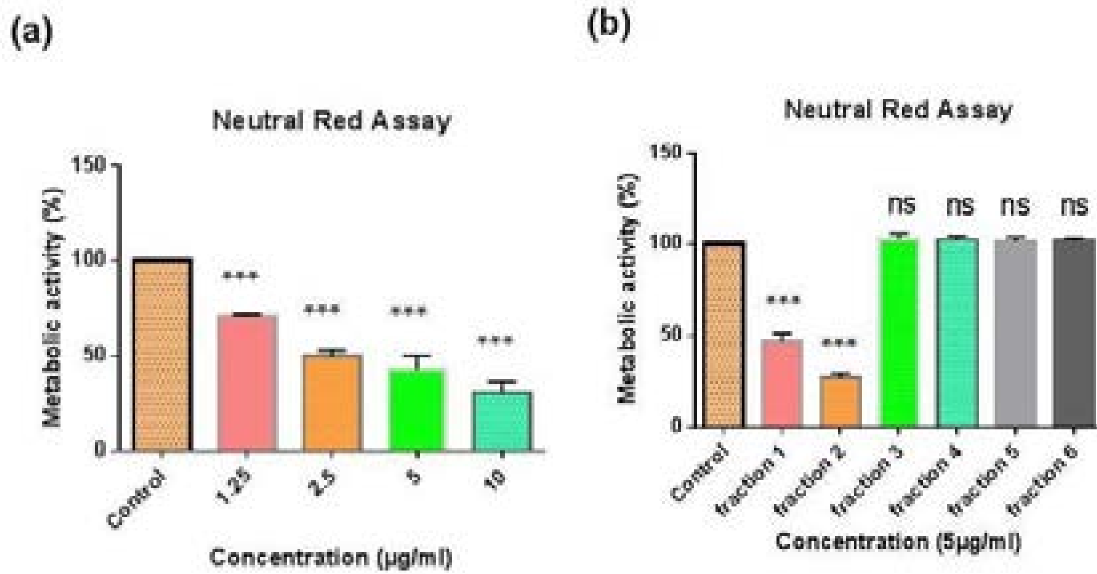

Figure 3. Cytotoxicity effects of Dianthin toxins on the MCF-7 cell line using neutral red uptake assay

A: MCF-7 cell line metabolic activity was (70.77%), (48.7%), (42.7%), and (30.9%) in 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL of dialyzed crude extract, respectively; B: Fractions 1 and 2, Dianthin 30 and 32 significantly caused the MCF-7 cell line's death; (NS: not significant), (****P<0.0001)

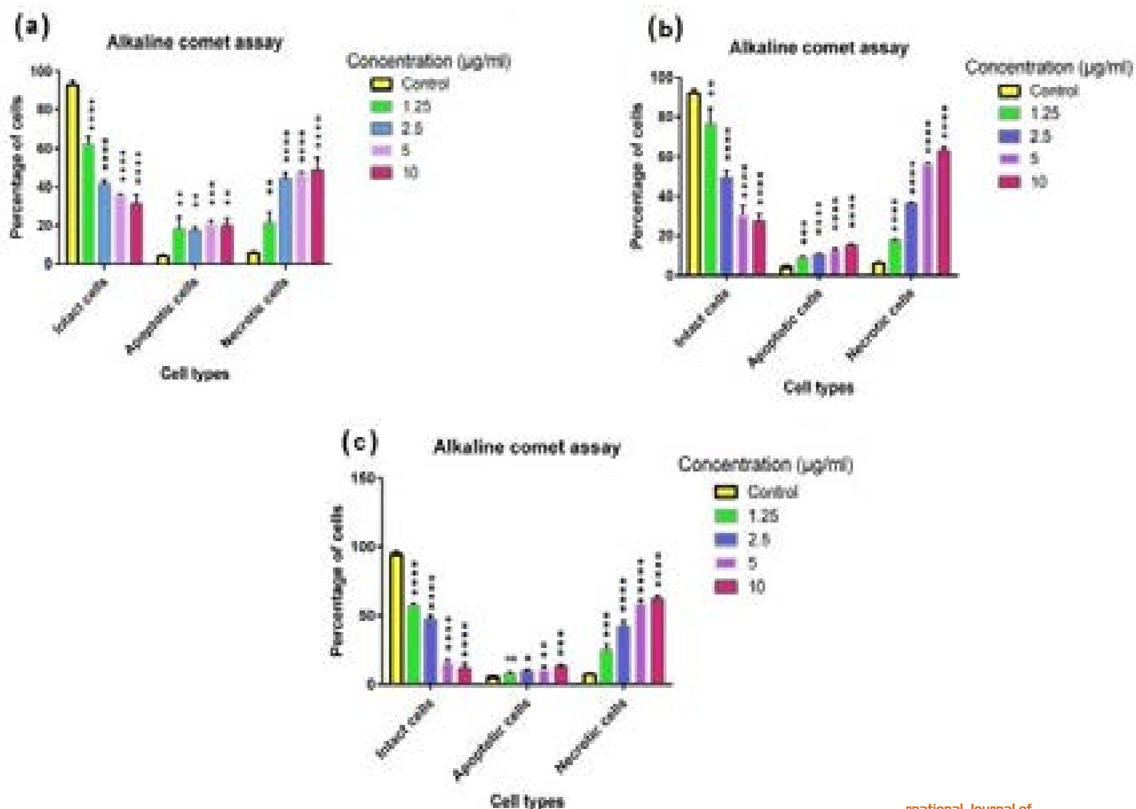

Figure 4. Analyzing the potential of apoptosis and necrosis induction of Dianthin toxins, using alkaline comet assay

A: Dialyzed crude extract; B: Dianthin 30; and C: Dianthin 32; (NS: not significant,*P<0.05,**P<0.01, ***P<0.001, ****P<0.0001)

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4. Discussion

Breast, lung, and colorectal cancers are the main 3 disease types and they are categorized in the main 5 disease types regarding mortality (first, fifth, and second, separately). Together, these 3 malignancy types constitute (33%) of the disease rate and mortality load around the world. Lung and breast cancers are the main cancers worldwide regarding the number of new cases; for every one of these sorts, 2.1 million determinations are roughly evaluated in 2018, contributing to about (11.6%) of the all-out disease rate trouble. Breast cancer is the most normally analyzed malignant growth in women (24.2%); for example, 1 out of every 4 of all new malignancy cases analyzed in women worldwide are breast cancer [14]. Breast cancer is the fifth driving reason for disease demise in Iranian women. This investigation set out to examine 3010 breast cancer cases (all women) that had alluded to a Cancer Research Center in Tehran in the range of 1998 to 2014 [15].

In order to determine the cytotoxic effects of each of the toxins, a number of concentrations were tested on the MCF-7 cell line, and their cytotoxic effects were determined, using both MTT and NRU. Taking into account the MTT assay, it was found that applying the concentrations of 1.25µg/mL to 10µg/mL, the toxin had cytotoxic effects. The highest concentration of the extract-tested for the two aforementioned RIP types namely Dianthin 30 and 32 was 10µg/mL, and much higher concentrations may be of interest to be applied on the selected cell line to observe and develop the horizon of concepts behind toxin's cytotoxic effects.

Cell viability was also evaluated by NRU assay in the same range. It was shown that employing the concentrations of 1.25µg/mL to 10µg/mL, the toxin has a close behavior upon cytotoxicity on the cell line. Further analysis using comet assay revealed that the toxin induced cell necrosis rather than apoptosis in the range of 1.25µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL.

Referring to previous studies, it is well established that Dianthin 30 has cytotoxic activity [16-18]. The cytotoxicity of the toxin has been evaluated on different cell lines as Hodgkins' Lymphoma cells (infusion with anti-CD30, IC50: 162 pg-50ng), human pancreatic carcinoma cell lines BxPC-3 (IC50: 10µg), MIA PaCa-2 (IC50: 100ng), NIH-3T3 mouse embryonic fibroblast cells (IC50: 22ng), and Jurkat cells (IC50: 21ng) [16]. All aforementioned studies indicate that the toxin has significant cytotoxicity and can be applied as an anticancer drug. Many anti-tumor studies of various RIPs were conducted by researchers based on the MCF-7 cell line [19-26]. However, to the best of our knowledge, it is the first study that evaluates the toxicity of this toxin on MCF-7 cells.

The IC50 of the extracted toxin on MCF-7 cells was 190nM. The higher IC50 of the toxin may be related to the type of the examined cell line as well as the purity of the extracted toxin. As a matter of fact, this is an extremely normal and common circumstance and a similar medication to have distinctive IC50 in various cells lines because of so-called "cell-specific response". Every cell line is totally one of the framework kinds with its individual natural attributes. Notwithstanding, it is more important when cell lines were set up from a similar tissue, animal categories, and histological sorts of tumor they contrast. The causes are different including singular qualities of the material from, which the cell lines were built up, heterogeneity of tumor cells (high mutational capacity of tumor cells, the constant appearance, and choice of new cell populaces in the tumor; subsequently, every tumor cell line is totally special), and the progressions happening in the cells over the span of their cultivation in research facility conditions. Additionally, IC50 can be distinctive when you assess the impact of one and a similar medication as well as a similar cell line but utilizing diverse cytotoxicity assays (NRU cytotoxicity assay, trypan blue dye exclusion technique, crystal violet staining, LDH assays, etc. have different molecular/cellular targets and mechanisms of action).

Then again, impurities in the test mixes can deliver false positive outcomes and in this manner complicate the understanding of information and cause the unseemly elimination of good target candidate at a beginning time [27]. The findings of this study indicated that Dianthins mostly induce necrosis in eukaryotic cells. While in the case of other RIPs, such as saporin, ricin, and abrin, apoptosis is the frequent response [4]. The reason for the event is under question and demands a distinct investigation. From the practical view, for anticancer drugs, herbal- or plant-based molecules may be of great interest and desire for cancer therapy, but it needs to be used cautiously.

Necrosis can directly induce tumor cell death. Based on the comet assay, from concentrations of 1.25µg/mL to 10µg/mL, Dianthin 30 showed apoptosis more than necrosis and in a concentration of higher than 10µg/mL, it revealed necrosis more than apoptosis. The probable reason for these phenomena is laid on this issue that the high concentration of cytotoxic materials can induce massive physical damage to most cell components, including cell membranes leading to membrane leakage and death by necrosis. However, it depends on the kind

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of the employed substance and the presumed mechanism, by which they induce apoptosis.

In general, it could be possible that the given toxin at high concentrations inhibits some specific molecules, and the process needed to apoptosis induction. Conversely, Dianthin 32 showed necrosis more than apoptosis and in a concentration of higher than 10μg/mL, it revealed apoptosis more than necrosis. The main cause for this event may be attributed to diverse processes, which mediates cell death. Hence, in lower concentrations, necrosis is more prevalent than apoptosis, but in higher concentrations, the metabolic route may change because of the aforementioned reason.

5. Conclusion

Cell-toxicity evaluation of MCF-7 cells was established in vitro. Notably, owing to the cytotoxic effect of Dianthin 30, the toxin is a potential candidate to be exploited as an anticancer drug, alone or as an immunotoxin; but, it needs more considerations to achieve this goal. The targeted form of toxin delivery as well as applying nanotechnology capacities is another route, which can be pursued in the following research to solve toxin capture in the endosomal region.

Ethical Considerations

Compliance with ethical guidelines

Ethical standards were received for this study from the Ethics Committee of the Biology Department at IHU University. Also, ethical approval and written consent of all participants (donor of blood) were taken according to the local Ethics Committee of Biology Department at Imam Hossein Comprehensive University and Declaration of Helsinki (1983 Revision).

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Author's contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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