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# The in vitro Effect of Doxorubicine-G2-FA Treatment on Breast Cancer

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## Abstract

**BACKGROUND:** To achieve delivery of a drug to tumors, folic acid (FA) was used as a targeting ligand to change nanocarriers. Since the folate receptor has more expression in several tumor types such as breast cancer.

**OBJECTIVES:** The present study aimed to evaluate the effect of Doxorubicine-G2-FA (Dox- G2-FA) with in vitro assays. The abbreviation of G2 represents the second generation of dendrimer synthesis.

**METHODS:** For this purpose, Dox-G2-FA was synthesized and mass spectroscopy was used to confirm the synthesized component. Also, MTT assay, flow cytometry, and gene expression assay by real-time PCR were used to evaluate cell viability, apoptosis, and necrosis.

**RESULTS:** In this study, the effect of Dox and Dox-G2-FA on the expression of Bax, Bcl2 genes showed that there was a significant decrease in the expression of the Bcl2 gene in the Dox-G2-FA group compared to Dox and control groups($P<0.05$). Also, the results of flow-cytometry showed that apoptosis in the presence of Dox-G2-FA was greater than in the Dox group ($P<0.05$).

**CONCLUSIONS:** Therefore, it seems that the effect of Dox-G2-FA on apoptosis is better than the effect of Dox usage alone.

**KEYWORDS:** Apoptosis, Bax, Bcl2, Breast cancer, Doxorubicine-G2-folate

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## Introduction

Breast Cancer is considered one of the most devastating diseases with a high mortality rate among the female population (AbuHammad and Zihlif, 2013). Therapeutic options of breast cancer are dependent on specific biological features of tumors. In low grade and estrogen-receptor-positive, hormone therapy is a good option for the treatment of cancer. Chemotherapy is another technique for the treatment of cancer and serves as the preferred treatment. Dox is the current therapy for the treatment of breast cancer. The main function of Dox is the intercalation of DNA, the prevention of topoisomerase II, and the formation of free radicals (Lovitt et al., 2018). A key mechanism in the answer of cancer cells to chemotherapeutic drugs is their apoptotic way of activation. Also, substantial toxic effects, side effects, and fast degradation have limited clinical use of chemotherapeutic drugs (Le et al., 2017). One study reported that the most harmful side effect of doxorubicine was cumulative dose-dependent cardiotoxicity. To minimize these side effects, and to limit drug dispersion into healthy tissues, chemotherapeutic drugs, including doxorubicine can be used with drug carriers (Tomankova et al., 2015). Nano-materials are considered noteworthy pharmacological carriers for drug delivery and cancer therapy. Targeted nano-materials selectively affect cancer cells via binding to them, with minor effects on healthy cells (Samad et al., 2009). Among several types of nano-materials, dendrimers are extensively ideal carriers (Senapati et al., 2018), because they are radially symmetrical, globular and Nanodimensionally compact structures with tree-like branches or arms (Singh et al., 2019). To achieve drug delivery to tumors, folic acid (FA) was used as a targeting ligand to change nanocarriers (Wang et al., 2015, Qin et al., 2013, Duan et al., 2012, Zwicke et al., 2012). Folic acid plays a major role in the synthesis of DNA and RNA as well as cellular growth and metabolism. Uptake of folate to a cell is mediated through cell membrane folate receptors which are considered heterogeneous proteins. Furthermore, folate receptor-alpha is a glycosylphosphatidylinositol (Sheikh et al., 2016) that has low expression in normal cells. However, it is over-expressed in several tumor types such as breast cancer and gynecological cancers of epithelial origin (Samad et al., 2009, Kalli et al., 2008). Studies have demonstrated that diverse folate conjugated nanomaterials as gold nanoparticles are highly important in targeted cancer therapy. Gold nanoparticles have been recognized as hopeful candidates for novel cancer therapy, owing to biocompatibility, chemo-physical stability, and easy functionalization (Samad et al., 2009). Therefore, this paper intends to evaluate the effect of Dox-G2-FA in vitro considering cytotoxicity, apoptosis, and gene expression assay on the MCF-7 breast cancer cell line.

## Materials and methods

### Synthesis of DOX-G2-FA

Figure 1-A shows the synthesis of G2 dendrimer. Polyethylene glycol diacid was dissolved in Dimethyl sulfoxide (DMSO) by adding N, N'-dicyclohexylcarbodiimide (DCC) (0.75 gr, 0.0036 mol) to the solution and stirring for 30 min at room temperature, and carboxyl groups were activated for conjugation. G1 dendrimer was obtained by adding citric acid (0.71 gr, 0.0036 mol) to the solution and stirred for 1 h at room temperature. To the obtained product (2.25 gr, 0.01 mol) DCC and 5 mL DMSO were add-

ed and the reaction was carried out under the same-mentioned conditions for 15 min. Finally, citric acid (2.1 gr, 0.01 mol) was added and the mixture was stirred for 1 week at room temperature. After that, the G2 dendrimer was filtered twice and purified using a Sephadex G-50 fine column (GE Healthcare Life Sciences, UK) and dialysis (dialysis bag 500–1000 Da cut-off, Spectrumlab, USA) against double deionized water (1 × 1 L for 2 days). The purified product was freeze-dried (LyoTrap plus, LTE Scientific Ltd, Oldham, UK) and stored at −20 °C for future studies. To prepare a dendrimer-folate conjugate, 0.05 gr of dendrimer was dissolved in DMSO (15 ml) and mixed with DCC (2.25 gr, 0.01 mol). After stirring for 30 min, folic acid (0.001 gr, 2.26 μmol) was added and stirred continuously at room temperature for 4 days to react completely. The final conjugate was purified using a Sephadex G-50 fine column and dialysis (Namazi and Adeli, 2005; Alavidjeh et al., 2010). Figure 1-B shows the conjugation of folic acid to G2 dendrimer.

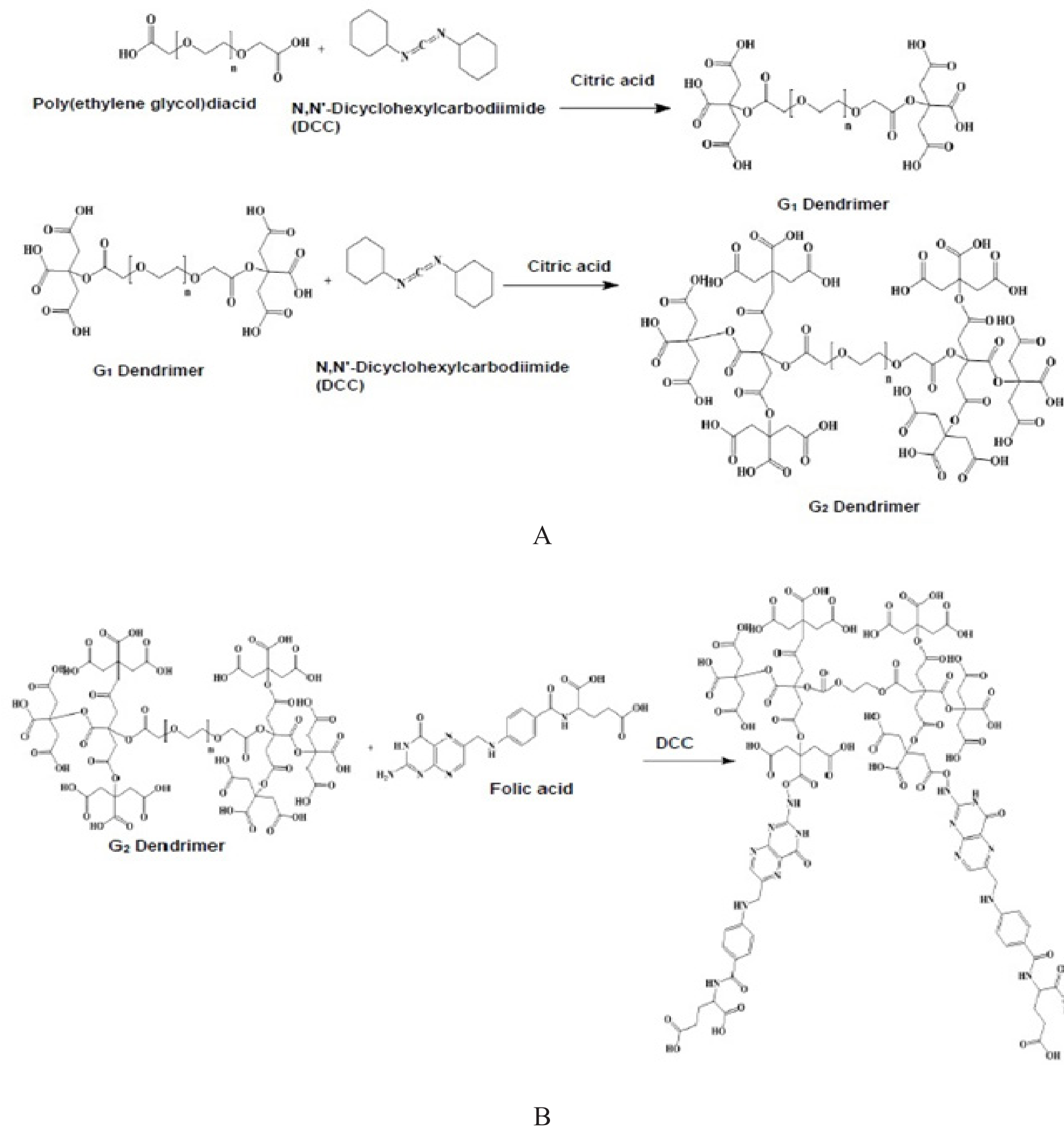


**Figure 1**. (A) Synthesis of G2 dendrimer; (B) Conjugation of folic acid to G2 dendrimer

Dox-G2-FA was obtained by mixing and incubating Dox (Santa Cruz Biotechnology Inc., Texas, USA) (5 mg, 9.2µmol) and 10 mg G2-FA for one day. Finally, excess Dox was removed by dialysis and the final product was centrifuged (13000rpm, 4 °C, 30 min) to remove any impurity. Therefore, an anionic linear globular dendrimer that is biocompatible and biodegradable (Le et al., 2017), conjugated to folic acid, and then doxorubicin, which acts nonspecifically against cancer cells, is loaded with this compound and the ultimately intelligent anti-cancer system was designed.

To evaluate and calculate the loading efficiency, a calibration curve was obtained by recording the absorbance value of different concentrations of the Dox solution at 480 nm wavelength firstly. Then, the absorbance value from the unloaded drug solved in the water (during dialysis) was used to calculate loading efficiency (LE) using the equation below (Khosravian et al., 2016):

$$LE\% = \frac{\text{Added drug - unloaded drug}}{\text{Added drug}} \times 100$$

The morphology, mean size, and zeta potential of the G2-FA conjugates are determined by atomic force microscopy (AFM) (ENTEGRA AFMNT–MDT, Russia) and dynamic light scattering (DLS)(Zetasizer Nano ZS; Malvern Instruments, Malvern, UK). The characterization of G2-FA was determined by mass spectroscopy (LC/MS). LC/MS analyses were provided by Triple Quadrupole LC/MS (Agilent 6410, USA). Fluorescence spectroscopy was used to investigate drug loading in dendrimer using a fluorescence spectrophotometer (Perkin Elmer, LS 45, USA).

**Cell culture**

MCF-7 human breast cancer cells were obtained from Pasteur Institute (Tehran, Iran) and were cultured in a low glucose Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 0.01 mg/ml insulin and 1% penicillin/streptomycin mix, supplemented with 10 % fetal calf serum (FCS)and need to be incubated at 37 °C in an atmosphere of 5% CO2(Mehrizi et al., 2018).

**Cytotoxicity analysis (MTT assay)**

MTT assay is a rapid colorimetric test to determine the cell viability and cytotoxicity for screening new drugs (Mosmann, 1983). In this assay, NAD(P)H-dependent cellular oxidoreductase enzyme converts the yellow tetrazolium MTT 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan(formazan). The insoluble purple formazan can be dissolved with DMSO, and purple color intensity indicates the cell viability. In the current study, the MTT assay was performed as described by Bahuguma et al (2017). In brief, MCF-7 cells were plated in 96- well flat-bottom plates at a density of 1 x 104 cells/well for 24 h at 37 ◦C with 5% CO2 for stabilization. Then, after 24 h, the culture medium was removed and fresh medium was added. Afterward, the cells were treated with Dox and Dox-G2-FA independently of each other at different concentrations (5, 10, 50, and 100µg/ml). A negative control (untreated cells) was also used. Again, after 24 and 48 h treatment, the culture medium was replaced. Then, 10 µl of MTT (Sigma, Germany) was added and after 4 h at 37 °C in a CO2 incubator, the medium was removed. The formazan crystals were solubilized by adding 50 µL of DMSO and allowed to be incubated for 30 min at 37 °C in a CO2 incubator. Ultimately, the color intensity (absorbance) was recorded at 540 nm using the ELISA plate reader

(ELX 808, Biotek, USA). The mean absorbance of each reaction was converted to cell viability (%) through the following equation:

(Mean absorbance treatment/mean absorbance control) ×100

A blocking test was performed to determine the selectivity and importance of folate in binding to cancerous tissue. For this purpose, different values of folic acid (0.5, 1, 5, and 10 µg/ml) were added to the cell line, and then the same MTT assay was examined.

**Evaluation of apoptosis and necrosis by the flow- cytometry technique**

Briefly, the principle of Annexin V- Fluorescein isothiocyanate (FITC) staining assay for the flow- cytometry technique (Cy Flow Space, Partec, Germany) is presented as follows.

Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane. Phosphatidylserine (PS) is located in the inner leaflet of normal cells. Apoptotic cells exposed PS on the outer leaflet of the plasma membrane, while the membrane integrity remains unchallenged in the early phases of apoptosis. However, the translocated PS can be detected by staining with a FITC conjugate of Annexin V. Annexin V is a calcium-dependent phospholipid-binding protein that specifically binds to PS. Changes in PS asymmetry can be detected by Annexin V–FITC staining for the flow-cytometry method before the occurrence of morphological changes associated with apoptosis and before a loss of membrane integrity. To differentiate between apoptotic and necrotic cells, a membrane-impermeable DNA stain, like propidium iodide (PI), can be added concurrently to the cell suspension (Van Engeland et al., 1998).

Thus, 4 labeling patterns in the mentioned assay may be observed on 4 regions or quarters: Damaged cells during the isolation procedure (Q1) - Annexin V negative and PI-positive; Apoptotic cells (Q2) - Annexin V positive and PI negative; Vital cells (Q3) - negative for both Annexin V and PI; Necrotic cells (Q4) - positive for both Annexin V and PI.

In this technique, we placed 5×104 cells/ml in each plate, and Dox and Dox-G2-FA were added to each plate. The cells were separated from the plate after washing with phosphate buffer solution (PBS) and trypsin enzyme and were put through centrifuges. The cells were washed with a buffer solution and centrifuged to be isolated. FITC solution (Sigma, USA) was incubated with Annexin (Sigma, USA) (5 µl) for 15 min at room temperature in a dark environment. PI (Sigma, USA) (5 µl) was added, which was attached to the DNA in necrotic cells. Thus, for this purpose, the measurement of induced apoptosis by Dox and Dox-G2-FA was assessed by a FACScan flow cytometer (BD FACS-Calibur, USA).

**Gene expression assay by real-time PCR**

RNA was extracted from the breast cancer cell line (MCF-7) according to the manufacturer's instructions (Qiagen, USA). The final RNA was detected by the spectroscopy method. Primers were designed by the computer program Primer Express. Real-time PCR includes 0.35 ml cDNA template and 50–900 nM of oligonucleotide primer, 1X SYBR RT-PCR buffer, 0.2 mM each of dATP, dCTP, dGTP, 3 mM MgCl2, 0.4 mM dUTP, 0.005 U Ampli Taq Gold, 0.002 U AmpErase UNGerase enzyme, primer and forward primer in 10 ml volume. PCR reactions were performed following thermal cycling profile: 95 °C for 10 min, followed by 40 steps of 95 °C for 30 s and 58 °C for 1 min and 72 °C for 1 min. After 40 cycles, the samples were run

for the dissociation protocol. Then, the PCR products were run on a 2% agarose gel to detect the presence of a band with the expected size (Hashempour Alamdari et al., 2017).

Forward and reverse primer used in the current study are listed below:

| | |
|---|---|
| Bcl-2 | Forward: 5'-TGTGGATGACTGAGTACCTGAACC-3' |
| | Revers: 5'-CAGCCAGGAGAAATCAAACAGAG-3' |
| Bax | Forward: 5'-TTGCTTCAGGGTTTCATCCAG-3' |
| | Revers: 5'-AGCTTCTTGGTGGACGCATC-3' |
| GAPDH | Forward: 5'-CGTCTGCCCTATCAACTTTCG-3 |
| | Revers: 5'-CGTTTCTCAGGCTCCCTCT-3' |

**Data analysis**

Statistical data analysis was conducted by one-way ANOVA. All the analyses were conducted by SAS (SAS Institute Inc., Cary, NC, USA). Data are presented as mean ± SEM. Differences with P-value<0.05 were considered significant.

## Results

The size of the G2-dendrimer and the G2-FA were 80 nm and 140 nm respectively. Besides, the results of zeta potential analysis indicated that G2-FA(-52 mV) was negative compared to G2-dendrimer (-22 mV) confirming the conjugation folic acid to the G2-dendrimer.

The AFM analysis results showed that the G2-dendrimer has a semi-spherical shape with an average size of 70 nm (Figure 2-A), while the G2-FA had a stretched shape and a size of 125 nm (Figure 2-B). Furthermore, these results are in good agreement with the DLS analysis results.

The results of the Dox loading showed that in the first 14 h of the study, 58% of the drug was loaded into the dendrimer. After that, there was no significant loading (Figure 2 – C). Also, it was illustrated that the drug was released slowly, and the highest percentage of release occurred at the acidic pH (81%; pH= 5.5) (Figure 2- D).

The results of the MTT assay showed that the viability of the cells after treatment with different concentrations of Dox-G2-FA (except 5 µg/ml) in comparison to Dox was significantly decreased ($P$<0.05) (Figure 3-A). Also, the results of the MTT assay indicated that the viability of the cells after treatment with 50 and 100 µg/ml of Dox-G2-FA compared to the Dox group was significantly decreased(in the presence of the blocking test) ($P$<0.05)(Figure.3-B).

As Figure 3-C showed, there is a significant decrease in the Dox-G2-FA group compared to the Dox and the control groups in terms of the Bcl2 gene ($P$<0.05).

As Figure 4 shows, 95.05 % of the cells existed in normal status in the control group. In the Dox group, 52.16 % of the cells showed apoptosis and 30.88 % of cells necrosis in the presence of Dox; While 61.69 % of cells showed apoptosis, and 24.11 % of the cells necrosis in the presence of Dox-G2-FA ($P$<0.05).

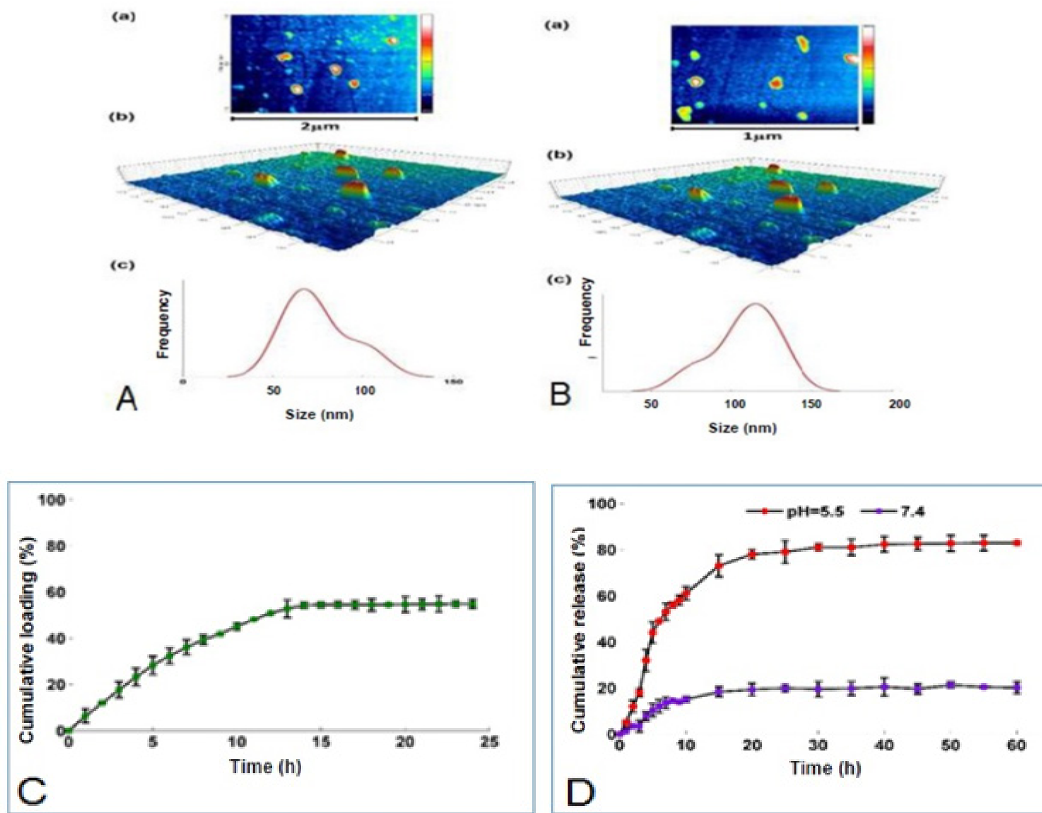


**Figure 2**. (A)- (a)2D, (b)3D and (c) size distribution AFM micrograph of G2-dendrimer (B)- (a) 2D, (b)3D and (c) size distribution AFM micrograph of G2-FA (C)- Drug loading profile; (D) Cumulative release curve

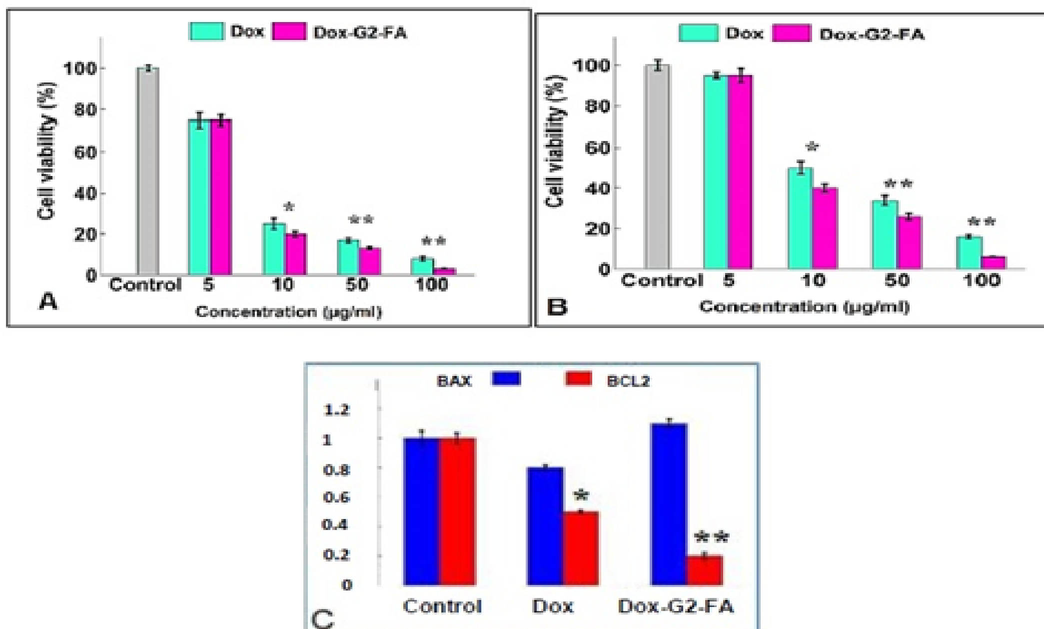


**Figure 3**. (A)- Results of the MTT assay on MCF-7 cells with different concentrations of Dox and Dox-G2-FA;(B)- Results of the MTT assay on MCF-7 cells with different concentrations of Dox and Dox-G2-FA (in the presence of the blocking test); (C)-Bax and Bcl2 gene expression of drug and nano-drug in MCF-7 cell line(*=P<0.05, **= *P*<0.01).

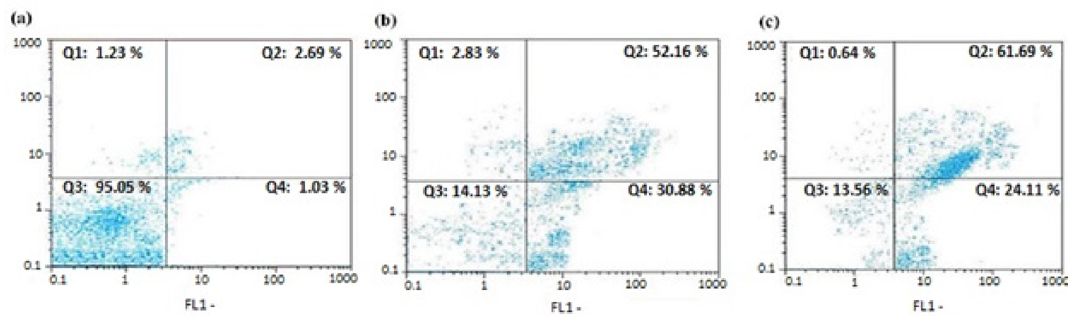


**Figure 4**. Flow- cytometry results - (a) control, (b) Dox and (c) Dox-G2-FA groups

## Discussion

The use of chemotherapeutic drugs is one of the several ways to treat cancer and tumors. These drugs can also be applied to radiation or surgery methods. However, they may have undesirable or unwanted effects (Senapati et al., 2018).

Dox is used to treat various cancers. Moreover, it is one of the chemotherapy agents used in the treatment of breast cancer. It acts by blocking the replication and transcription processes (Lovitt et al., 2018). Although an acceptable outcome has been seen in the chemotherapy of some cancers, they also show undesirable toxicity and severe complication (Le et al., 2017). The targeted drug delivery using polymer nanoparticles with their unique characteristics such as biocompatibility, monodispersity index, and three-dimensional globular shapes can overcome these problems (Lovitt et al., 2018). In the current study, we synthesized G2-Folic acid having the ability to carry Dox for targeted release (delivery) to the breast cancer cell. The amounts of drug loading and release from this nano-carrier were 58% and 81% respectively. Atomic force microscopy, mass spectroscopy, fluorescent spectroscopy, dynamic light scattering, and zeta potential, confirmed the successful synthesis and drug loading. In this carrier, folic acid conjugated via covalent binding. Targeted delivery of anticancer drugs may be more advantageous than chemotherapy (Mehrizi et al., 2018). Some polymers such as polyethylene glycol and chitosan have been used for tumor-targeted delivery (Duan et al., 2012). Moreover, drug carriers may be modified with a ligand to increase their ability (Yoo et al., 2019). In the current study, we synthesized G2-Dox and confirmed it by mass spectroscopy, then attached it to folic acid. Folic acid as a group of water-soluble vitamin B is one of the main elements involved in DNA synthesis or cell division (Yilmaz and Walhout, 2014). Folic acid transports drugs to cancer cells via folate receptors on the cell surface. Parveen et al (2010) reported that the folate receptor was over-expressed in many cancers and emerged as a targeting ligand for selective delivery. Compared to some commonly used dendrimers like Poly amidoamine (PAMAM) having a positive charge, the synthesized nano-carriers are more biocompatible owing to its negative charge. This feature led to the use of second-generation dendrimer for drug delivery. The positive charge of PAMAM dendrimer was instigated to use fourth and fifth-generation which leads to increase in the cost and time of synthesis and low drug-loading efficiency (Javani et al., 2017, Luong et al., 2016, Sowinska and Urbanczyk-Lipkowska, 2014, Alavidjeh et al., 2010).

Flow cytometer analysis showed that apoptosis and necrosis were observed in 52.16% and 30.88% of cells in the presence of doxorubicin. In the presence of Dox-G2-FA, 61.6% and 24.11% of the cells showed apoptosis and necrosis, respectively. It seems that Dox-G2-FA causes more apoptosis than Dox. The mechanism of the action of doxorubicin on apoptosis is not completely clear. Probably, it causes loss of the mitochondrial membrane potential and activates the intrinsic mitochondrial/caspase-9 way(Debatin, 1997). Compared to the result of Myc et al (2010) (Myc et al. 2010), the synthesized particle has shown an acceptable and better result that can be derived from the small size, the low-generation, ability to load the wizard, the adaptability of the structure. Also, compared with the control and Dox groups, reduction of the antiapoptotic Bcl-2 expression could be attributed to an increased sensitivity of the MCF-7 cells to induce apoptosis when exposed to synthesis structure (Javani et al., 2017), indeed the reduction of Bcl2 can restore the apoptotic process in tumor cells (Czabotar et al., 2014).

According to the present study results, there was a significant decrease in the expression of the Bcl2 gene in the Dox-G2-FA group compared to Dox group. Moreover, in flowcytometry, apoptosis in the presence of Dox-G2-FA was greater than in the Dox group. Therefore, it seems that the effect of Dox-G2-FA on apoptosis is better than free Dox. In this regard, more investigations are needed on the effect of Dox-G2-FA on different cell lines as well as in vivo assay.

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## Conflict of Interest

The authors declared that there is no conflict of interest.

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# تاثیر in vitro دوکسوروبیسین -FA-G2 بر سرطان سینه

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## چکیده

**زمینه مطالعه:** برای رسیدن دارو به تومورها، از اسید فولیک (FA) به عنوان لیگاند هدفمند جهت تغییر حاملین نانو استفاده می شود. زیرا که گیرنده فولات به میزان زیادی در چندین نوع تومور همچون سرطان سینه بیان می شود.

**هدف:** هدف مطالعه حاضر، ارزیابی in vitro اثر دوکسوروبیسین FA-G۲-(FA-Dox- G۲) بر روی سرطان سینه می باشد و اختصار G۲ نسل دوم سنتز دندریمر را نشان می دهد.

**روش کار:** به همین منظور، FA-Dox-G۲ تولید و از طیف سنجی جرمی برای تایید مؤلفه های سنتزی استفاده شد. همچنین آزمایش MTT ، فلوسایتومتری و تعیین بیان ژن جهت ارزیابی زنده مانی سلول، آپوپتوز و نکروز نیز انجام شد.

**نتایــج:** در این مطالعه، اثر Dox و FA-Dox-G۲ در بیان ژن های Bax و Bcl۲ نشان داد که در بیان ژن Bcl۲ در گروه FA-Dox-G۲ در مقایسه با گروه های کنترل و Dox کاهش معنی داری وجود دارد ($P<۰/۰۵$). همچنین، نتایج فلوسایتومتری نشان داد که نرخ آپوپتوز در حضور FA-Dox-G۲ بیشتر از گروه Dox می باشد ($P<۰/۰۵$).

**نتیجه گیری نهایی:** براساس نتایج مطالعه حاضر ، اثر FA-Dox-G۲ بر آپوپتوز ، بهتر از اثر Dox به تنهایی می باشد.

واژه‌های کلیدی:

آپیتوزیس، Bax،Bcl2 سرطان سینه، دوکسوروبیسین -FA-G2.

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