Folated-nanocarrier for curcumin drug delivery in breast cancer therapy

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ABSTRACT

Among the potent anticancer agents, curcumin (CU) has been found to be very efficacious against many different types of cancer cells. Chitosan-coated magnetic nanoparticles (CS MNPs) were prepared and utilized as a nano-carrier for loading of curcumin (FA-CS-CU-MNPs) through a reverse micro emulsion method. This nano formulation was evaluated against breast cancer cell lines in the in vitro conditions. Both shape and size properties were studied by zeta sizer, AFM and FESEM and the cell internalization ability of prepared nanoparticles was determined by fluorescence microscopy. It was found that the synthesized FA-CS-CU- MNPs were spherical in shape with an average size of 90±15 nm, low aggregation and good magnetic responsive properties. Meanwhile, the high drug loading efficiency (~73%) was remarkable. These FA-CS-CU- MNPs also demonstrated sustained release of CU at 37 ◦C in different buffer solutions. Afterwards the suitable dose and therapeutic effects of (FA-CS-CU- MNPs) for both breast cancer and normal cell lines were evaluated by MTT assay. The result showed that FA-CS-CU- MNPs retained significant antitumor activities, no adverse effects was detected for normal cells. Additionally, it was observed that the FITC-labeled FA-CS-CU- MNPs could effectively enter into the cancer cells and induced cell apoptosis.

Keywords: Cancer, Chitosan, Curcumin, Nanoparticle, Nanosystem.

حامل النانوي الفوليتي لتحويل داء الكوركومين في علاج سرطان الثدي

الخلاصة

من بين العوامل القوية المضادة للسرطان، قد أثبت بأن مادة الكركمين (CU) تكون فعالة جدا ضد العديد من أنواع مختلفة من الخلايا السرطانية. قد أعدت و استعملت مغلفة الكايتوزان النانوية المغناطيسية (CS MNPs) باعتبارها الناقل النانوي لتحميل الكوركمين (FA-CS-CU- MNPs) من خلال طريقة مايكرو امولشن (microemulsion) العكسي. تم تقييم هذا الفرمول النانوي ضد خلايا سرطان الثدي في الظروف المختبرية (in vitro conditions). وقد درس كلا من الشكل و خصائص الحجم باستخدام أجهزة تحليل الحجم الحبيبي و مجهر الإلكتروني الماسح و أيضا تم تحديد قدرة استيعاب الخلية لدخول جسيمات متناهية الصغر بإستخدام مجهر الفلورسنتي. قد تبين أن المركب النانوي المصنع، قد يكون كروي الشكل، ذات حجم متوسط 15± 90 نانومتر، قليل التجمع و يملك خصائص مغناطيسية جيدة وفي الوقت نفسه، يملك كفائة عالية و ملحوظة لتحميل الأدوية (73٪). هذا الفرمول النانوي أيضا قد يحرر الدواء في $37^{\circ C}$ في محاليل بافرية مختلفة. بعد ذلك تم تقييم الجرعة المناسبة التأثيرات العلاجية لدي الجزيئية المركبة بالدواء لكل من

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خلايا السرطانية للثدي و الخلايا العادية بإستخدام تقنية فحص MTT assay . و أظهرت النتائج بأن الجزيئية النانوية المركبة بالدواء تملک تأثير ملحوظ في مجال الأنشطة المضادة للورم، بحيث لم يتم الكشف عن أي آثار سلبية على الخلايا الطبيعية. بالإضافة إلى ذلك، لوحظ أن الجزيئية النانوية المركبة بالدواء و مادة FITC تدخل بشكل فعال في الخلايا السرطانية و تسبب موت المبرمج لها.
كلمات المرشدة: النظام النانوي، جزيئية النانوية، كركومين، كايتوسان، سرطان.

INTRODUCTION

Today the cancer is one of the leading causes of death worldwide. Although several strategies have been considered for cancer therapy, but most of these methods are not only lethal for cancer cells but normal cells are also affected, resulting in death or high injury to them [1, 2]. Very extensive researches are ongoing to find ways to reduce these effects and enhancing the therapeutic ratio. One of these methods that have been interested and used in recent years is tumor drug delivery by the drug encapsulation in a biocompatible material and tumor site delivery and releases it in cancer cell line position [3, 4]. Curcumin is the main extracted substance from rhizome of curcuma longa L. That have numerous pharmaceutical properties such as antioxidant, anti-mutagenic, antitumor, anticancer, anti angiogenic, anti-cholesterol, antibacterial [5-13] But it can't be used widely in the treatment of diseases that's because its water insolubility and subsequently its poor bioavailability in vivo. To resolve this problem, much researches has been accomplished that in this, biocompatible materials are used in combination with curcumin. Polymeric nanoparticles are one of the advantageous compounds that can be placed capsule-like around the curcumin particles and injected the synthesized nano system in blood stream [14-18]. Among them, chitosan (CS) is a favorable type of drug delivery system. CS is a natural linear bio poly amino saccharide derived from alkaline deacetylation of chitin, which has been found to be the second most abundant natural biopolymer in nature behind only cellulose. Because of its chemical structure, CS has received increasing attention as a renewable polymeric material and has now been widely used in many fields such as protein adsorption and metal adsorption. Additionally, CS and its derivative have also been investigated in the development of controlled release drug delivery systems, since CS's muco adhesive property can enhance drug trans mucosal absorption and promote sustained release of drug [19, 20]. With the rapid development of nanotechnology, magnetic nanoparticles, especially super paramagnetic iron oxide nanoparticles, are currently being widely studied and have been found numerous applications in the fields of cell separation [14], cell apoptosis [15] and enzyme immobilization [16].Magnetic targeted nanoparticles can be used as drug carriers to provide targeted delivery and sustained release of chemotherapeutic agents to improve bioavailability. Drug based on carriers of core-shell magnetic nanoparticles can be easily guided to arrive at the interest position in the body by means of physical force from magnetic field [17]. Meanwhile, the outer shell (polymer layer) of the drug can effectively slow down the rate of release. Therefore, drug delivery system using polymer-coated magnetic nanoparticles is considered as an effective strategy for passive tumor targeting, which can not only increase drug circulation but also reduce pain in patients [18-21]. However, limited literatures reported the direct application of chitosan-coated magnetic nanoparticles as drug carriers. The purpose of this study is to preparation of curcumin

nanoformulation that is able to remain biocompatible and stable in the body [22, 23]. So first we synthesized a new formulation of FA-CS-CU-MNPs and after characterization of its properties such as size, shape, cell internalization and determining the appropriate concentration for treatment, we investigate its effect in human breast cell lines (MCF7) and breast normal cell lines (MCF10A).

Materials and methods

Preparation of FA-CS-CU- MNPs

The MNPs (Fe_3O_4) was purchased from Sigma–Aldrich Company. FA-CS-CU-MNPs were prepared by a reverse micro emulsion method. Briefly, chitosan (CS) powders (40 mg) were dissolved in 1 ml of 1% (w/v) acetic acid solution containing magnetite suspension and then a predetermined amount of curcumin (10-15 mg) was added to the CS solution. The solution was then drop wised into 50ml of fluid wax containing emulsifier (Tween-80) in a 100 ml three-necked flask at 60°C. A water-in-oil micro emulsion was formed by continuous stirring at 1000 rpm/min for 8 h in a water bath. Then, 1ml of 20% (w/w) sodium citrate solution was gradually added to the flask and the reaction was still kept the same condition for 30 minutes in neutral pH. After reaction, CS-CU- MNPs were collected with a permanent magnet and rinsed consecutively with light petroleum and isopropanol for three times. Finally, the obtained nanoparticles were dried overnight at 65°C. The preparation of CS-MNPs (not adding CU) was carried in a similar way mentioned above [19, 20].

Folate decoration of CUR loaded PU nanomicelles

The drug loaded NPs was surface modified by folic acid for site specific targeting of drug to cancer cells location [24]. For this purpose, FOL powder (20 mg) was dispersed in water (5 ml). NaOH solution (about 30 µl, 10 M) was added to the folate/water mixture and stirred magnetically. 0.5 ml of this solution was dropped into 5 ml (1 mg per ml) of drug loaded NPs. The mixture was stirred (400 rpm) at room temperature for 1 h. The FOL-decorated CU loaded FA-CS-MNPs solution was centrifuged at 10000 rpm for 7 minute. The supernatant containing free FOL was separated and precipitated material was washed by distilled deionized water.

Characterization of nanoparticle size and morphology

The dispersity, size and surface charge of nanoparticles were achieved using nano zeta sizer (Malvern Instruments Ltd., Malvern, UK). The surface morphology and size of nanoparticles were evaluated using atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM) and also dynamic light scattering (DLS). Atomic force microscopy (AFM) images were obtained by (Digital Instruments, Inc. Nanoscope III Santa Barbara, CA) in room temperature using a drop of fresh solution. Also the morphologic studies were performed using field emission scanning electron microscopy (Phillipis XL30) and gold coating the FA-CS-CU- MNPs.

Determination of CU encapsulation in the nanoparticles

CS–CU-MNPs (5 mg) were distributed in 40 ml of 1mol/L HCL by sonication. After 3 h, the supernatant was collected with centrifugation at 14000 rpm and magnetic separation. The concentration of CU in the supernatant was assayed by fluorescence spectroscopy (Perkin Elmer, Rockville, USA) in 420 nm absorption and 430-600 nm emission and gap width of 5 nm. The supernatant from CS MNPs was used as a contrast. The drug encapsulation efficiency of CS–CU-MNPs were obtained using the following equation:

Encapsulation efficiency (%) = [(drug fed – drug loss) / (drug fed)] × 100% … (1)

Curcumin release profile determination

For determination of curcumin release from FA-CS-MNPs nanoparticles, the phosphate buffer (0.01 M and pH=7.4) and citrate buffer (0.01 M and pH=5.4) in 37° C were utilized. 1 ml of micellar solution were puted in dialyze bag and placed in 100 ml phosphate buffer (0.01 M and pH=7.4) and citrate buffer (0.01 M and pH=5.4) separately. The tween 80 was used as an emulsifier for inhibition of released drug sedimentation. Release study was performed utilizing Shaking water bath. The sampling was accomplished at 0, 4, 8, 12, 24, 48, 72 and 96 h. The 300 µl was aliquoted, freezed and dryed in each sampling and resolved in 2 ml methanol. Then the fluorescence spectroscopy was utilized for evaluation of curcumin release quality determination.

The accumulated release was calculated using following equation:

$R= V \Sigma^{n-i} C_i + V_0 C_n / m_{drug}$

where, R is the accumulated release (%), V is the sampling volume, V0 is the initial volume, Ci and Cn are the paclitaxel concentrations, i and n are the sampling times, and mdrug is the mass of curcumin in nanoparticle.

Cell culture

MCF7 cell line and MCF10A normal cells were purchased from Iran Pasture institute cell bank. The cells were cultured in DMEM (Dulbecco's Modified Eagle) Medium medium containing 10 % FBS, 500 µg/ml ginitaicine, 300 µg/ml glutamine, 500 units/ml penicillin, 0.25 µg/ml fungizone 100 µg/ml streptomycine and were separated with 0.5 g/l tripsin and 0.2 g/l EDTA and passaged several time for achieve logarithmic phase. In all steps the cells should be incubated in 37℃ and 5% CO2 pressure. All cell culture materials were purchased from GIBCO Company, USA.

Cell internalization assay

For this purpose, the cells were treated with 25 µM curcumin nanoformulations at 4 h, then the drug contained medium were removed and after cell washing with PBS, the imaging performed by fluorescence microscope. In order to assessment the FA-CS - MNPs carrier efficiency for increasing the solubility and bioavailability of curcumin, one group of cells that were treated with same concentration of curcumin were imaged by fluorescence microscopy.

MTT assay

For preparation of MTT solution, 5 mg of MTT powder was dissolved in 1 ml PBS. 96 well plat was used for MTT test [25-28]. 10^4 cells were cultured in each well at 200 µl medium. Then allowed the cells to proliferate at 24 h. Afterward the different concentration of curcumin that solved in DMSO, in 2% V/V compare with medium (10-50 µM) and also the curcumin nanoformulations and FA-CS - MNPs nanoparticle with same concentration were injected into cells contained wells. Each experiment was carried out in pentaplicate wells and repeated at least three times. Then the MTT assay was performed after treatment at 24 and 48 h. After 4 h incubation, total solutions were removed and add 100 µl DMSO to each well. The plat pouted in shaker for 5 min then analyzed by elisa reader (BioTek Power Wave XS).

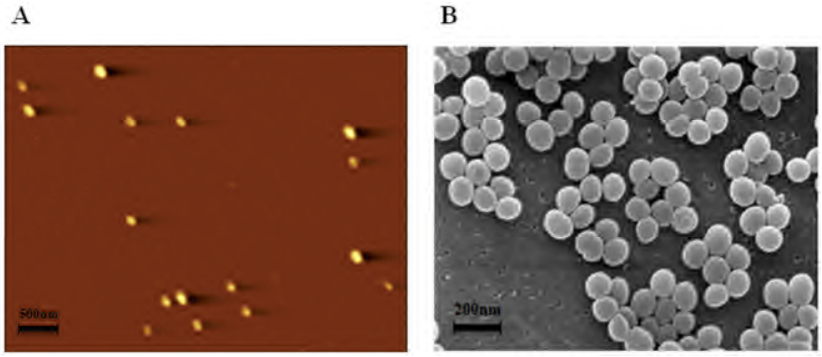
Statistical Analysis

SPSS 16 was used for statistical studies. Statistical differences between control and treatment groups, was assessed using T-TEST. The obtained data was considered statistically significant with the P <5%.

Result

Characterisation of nanoparticles size and morphology

Figure of field emission scanning electron microscope and atomic force microscope show that the obtained nanoparticles have a spherical shape with suitable dispersity without remarkable aggregation (Fig. 1). The average hydrodynamic diameter of curcumin nanoformulations and its polydispersity in 25˚C were 90 ± 15 nm and 0.074 ± 2.5 respectively and the zeta potential was -32 ±0.9 mV (Fig. 2). All findings indicated the similarity of DLS, FESEM and AFM data in morphology and size that in three experiments the size was 90 nm and in FESEM and AFM images the spherical morphology for FA-CS-CU- MNPs was confirmed.



Figure(1) Determination of morphology and size characteristic of FA-CS- MNPs nanoparticles using atomic force microscope (A) and field emission scanning electron microscope (B).

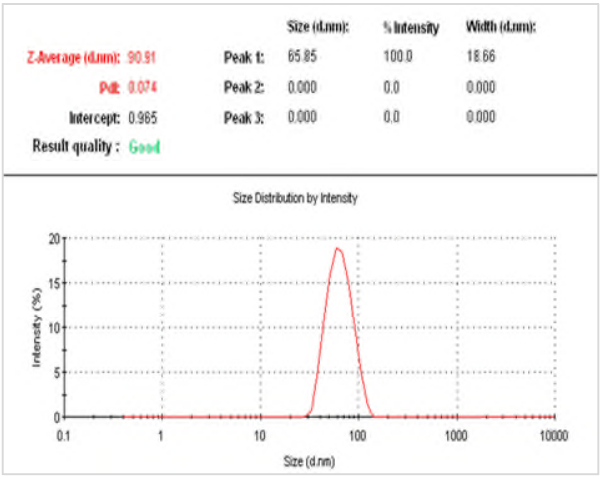


Figure (2) Size measurement images. size and polydispersity of FA-CS-CU- MNPs using dynamic light scattering (DLS)

FA-CS-MNPs nanoparticle loaded with curcumin

After preparing the curcumin nanoformulations, this combination was centrifuged and the supernatant calculated and evaluated in 432 nm by spectrophotometer (Amersham , Uppsala, Sweden). Curcumin encapsulation efficiency in FA-CS-MNPs nanoparticle was detected 73 ±0.2%. The nanoformulation indicated high colloidal stability and suitable drug maintenance in this period.

Release profile

According to the release curves (Fig. 3), CUR releases from polymeric micelles over a 96 h period and release time was slower at pH 7.4 as compared to pH 5.4. In Comparison with the release profiles of free CUR, there are similar release profiles at pH 7.4 and 5.4., we observed a faster CUR liberation profile at pH 5.4 under the studied conditions compare with pH 7.4.

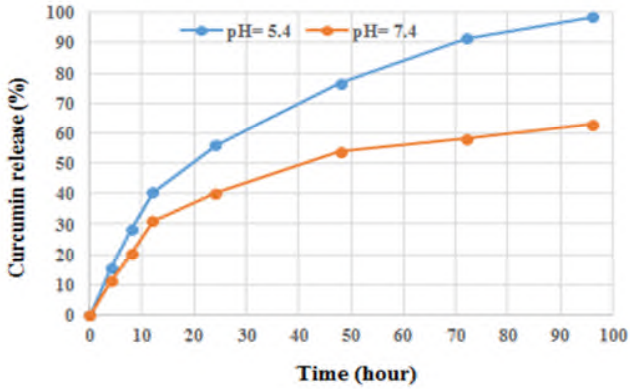


Figure (3) Curcumin release profile in two different pH of 7.4 (phosphate buffer) and 5.4 (citrate buffer)

Cell internalization of nanoformulation

Evaluation of curcumin internalization into cancer cells and by its intrinsic fluorescence property, were performed by fluorescence imaging. As shown in Figure 4, the treated cells with curcumin nanoformulations show green because of curcumin internalization due to solubility increasing of curcumin. Whereas, in cells treated with free curcumin this molecules is visible form green and insoluble particles form in intercellular space due to its insolubility in aqueous.

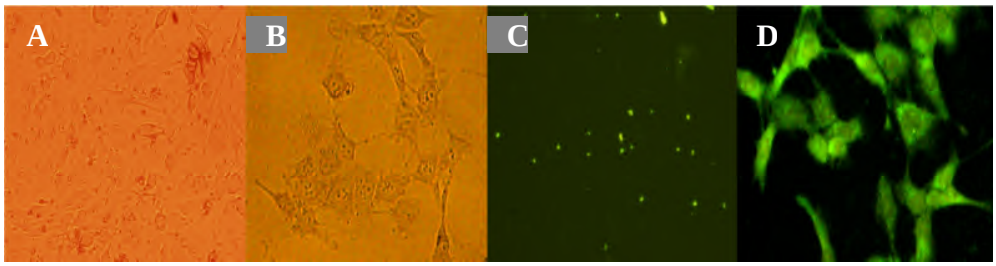


Figure (4) Cell internalization study of FA-CS-CU- MNPs in MCF7 cell line using fluorescence microscope (400× magnification). Optic microscopy image of curcumin treated cells (A). Optic microscopy image of FA-CS-CU- MNPs treated cells (B). fluorescence microscopy image of curcumin treated cells (C). fluorescence microscopy image of FA-CS-CU- MNPs treated cells (D).

Cytotoxicity assay

The cytotoxicity effect of void curcumin, bare FA-CS-MNPs nanoparticle and curcumin nanoformulation were evaluated by MTT assay on cancer MCF7 and normal MCF10A cell lines. This test performed in two 24 and 48 hours and was shown in curve (fig 5). First the cells treated with different concentration of curcumin nanoformulation (10-60 µM) for 24 and 48 hours. curcumin nanoformulation significantly ($P<0.01$) inhibited the cancer cells proliferation time and concentration dependent compared with bare nanoparticle and void curcumin but didn't show any significant difference in cell proliferation after treating normal MCF10A cell lines with three listed treatment. The IC50 calculated results indicate that 34.83 and 27.11 µM are the IC50 concentration for 24 and 48 hours respectively. Furthermore, both of bare nanoparticle and void curcumin treatment didn't show any remarkable cytotoxic effect in all applied concentration.

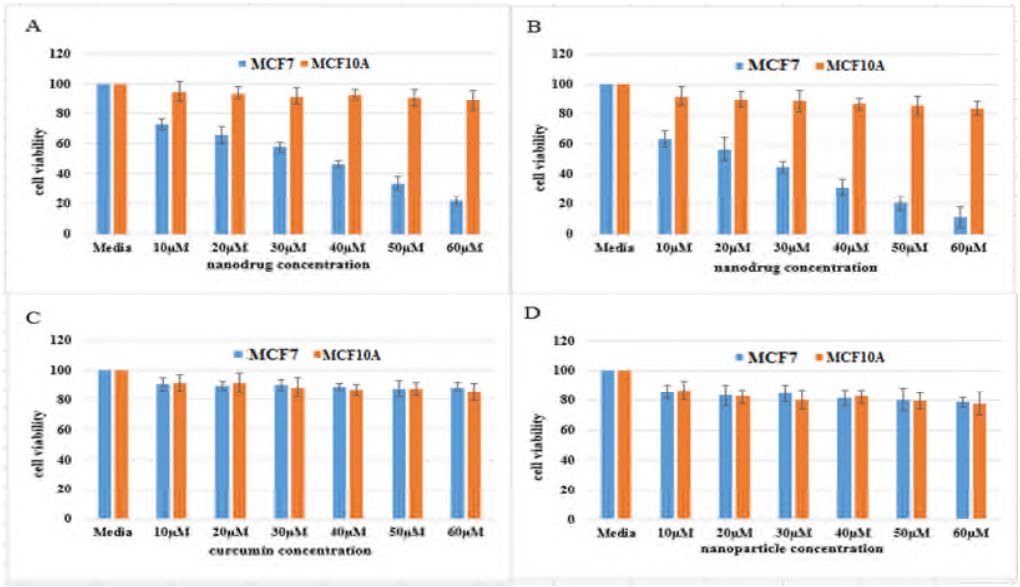


Figure (5) Study of different concentration (10-60 µM) of injected curcumin nanoformulation after 24 (A) and 48 (B). FA-CS-MNPs nanoparticle (C) and curcumin (D) after 48 h on MCF7 cancer cells and MCF10A normal cells.

Discussion

Curcumin have many therapeutic properties such as antioxidant, antimutagenic, antitumor, anticancer, antiangiogenic, anticholesterol and antibacterial. Despite the strong therapeutic properties of this molecule, it can't be used widely in the treatment of diseases that is due to its water insolubility and poor bioavailability in the body system. Several reports suggest that curcumin solubility were increased when it solved or encapsulated in drug carriers [4]. In this study, we supposed that FA-CS-MNPs nanoparticles can increase

the stability and biocompatibility of curcumin and also the blood circulation time will be enhanced so utilize from the antitumoric effects. So in this study, we synthesized a formulation from combinate the FA-CS-MNPs nanoparticles and curcumin and then asses its effect on treatment of cancer and normal cell lines [31]. Different studies indicates that nanoparticles less than 10 nm will be excreted rapidly from clearance system and/or extravasation, while the more larger nanoparticles, maybe detected and excreted by mono nuclear phgocite system with higher probability [32]. So in the most studies, in order to remarkable enhancement of EPR effect and also effective scape from physiological barriers, the best and suitable size for drug delivery is 10 to 250 nm [33]. Accordingly the FA-CS-MNPs nanodrug that synthesized with 62.37 ±1.7 nm, has a suitable size for drug delivery purpose (fig 1, 2). After loading the curcumin in FA-CS-CU- MNPs nanoparticles, we survey the nanosystem characteristics. Encapsulation efficiency of curcumin in FA-CS-MNPs nanoparticle was 87 ±0.2 % that indicate excellent colloidal stability and good maintenance of drug in this time. Fluorescence spectroscopy system shows the 73% encapsulation efficiency for curcumin in the FA-CS-MNPs nanoparticles that indicate well and high (efficient) drug loading in nanoparticles. In addition, the nanoparticles show intelligent behavior in relation to drug release in acidic pH that subsequently the acidic pH of cancer cells lead to more drug release from nanosystem (fig 3). The results of fluorescence microscopy image for determination of cell internalization indicate that the curcumin internalized into the cells efficiently. So the resulted toxicity of the nano system injection into the cells, induced by internalization and permeation of curcumin into the cells and damage to sensitive parts and components within the cell (fig 4). Several studies, show the antitumoric effect of curcumin in relation to different kinds of cancers such as breast, lung, prostate, ovary, colon, pancreatic, brain, skin etc either in vitro and in vivo studies [30,34]. In this study, the in vitro data that resulted from MTT assay, indicated decreasing survival percent due to increasing the concentration of curcumin nanoformulations treatment on MCF7 cell lines after 24 and 48 hours respectively. The IC50 concentration for both 24 and 48 hours were happened in 34.83 and 27.11 µM respectively. While the nanoformulations toxicity in the normal cells, was negligible without any significant IC50 for treated concentration (fig 5). Study the effect of nanoparticles and curcumin with different concentration on both cancer and normal cell lines separately, indicated their inconsiderable toxicity. This data were in correspondence with the results of study the similar treatment effects of dendrosomal nanocurcumin [35]. Thus, the impact of nanodrug is specifically on cancer cells and has not any effects on normal cells that due to specific function of curcumin in effective targeting of cancer cells than normal cell. This is cause of the greater impact of curcumin on cancer cells. On the other hand the nanoparticle has not any toxicity effect and is perfectly biocompatible. Also the drug has not any cytotoxic effect on cell proliferation that is due to loss of drug efficacy because of the low half-life of curcumin (fig 5).

CONCLUSION

The MTT assay results on cancer (MCF7) and normal cells (MCF10A) indicate the specific effect of curcumin nanoformulation on cancer cells whereas has not significant cytotoxic effect on normal cells. Specific activity of curcumin drug in effective killing the cancer cells than normal cells is the major reason of this finding. Farthemore the nanoparticle has not any significant toxicity and have perfect biocompatibility that subsequently leads to less effect on reducing cell proliferation.

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