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# Folated-nanocarrier for paclitaxel drug delivery in leukemia cancer therapy

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***Abstract***

Among the robust anticancer agents, paclitaxel (PTX) has been observed to be very fantastic towards many kinds of cancer cells. Chitosan-covered magnetic nanoparticles (CS-SPION) were prepared and used as a nano-carrier for loading of paclitaxel (FA-CS-PTX-SPION) through a reverse microemulsion technique. This nanosystem was evaluated towards leukemia cancer cell lines. Each shape and size properties were studied by means of zeta sizer, AFM and FESEM and the cell internalization capability of prepared nanoparticles were determined by using fluorescence microscopy. It became discovered that the synthesized FA-CS-PTX-SPION were sphere-shaped with an average diameter size of 90 ± 15 nm, negligible aggregation, and appropriate magnetic responsive properties. Meanwhile, the great drug encapsulation efficiency (~77%) was notable. This FA-CS-PTX-SPION also exhibited controlled release of PTX at 37 ◦C in phosphate and citrate buffer solutions. Afterwards, the ideal dose and therapeutic effects of (FA-CS-PTX-SPION) for both leukemia cancer and normal cell lines were evaluated through MTT assay. Then the flow cytometry assay shows significant cells apoptosis induction by FA-CS-PTX-SPION. The results confirmed that FA-CS-PTX-SPION retained considerable antitumor effects; no adverse consequences were detected for normal cells. Moreover, it became determined that the FITC-labeled FA-CS-PTX-SPION should efficaciously enter the cancerous cell and triggered cellular apoptosis.

**Keywords:** Cancer, Chitosan, Nanoparticle, Nanosystem, Paclitaxel.

## INTRODUCTION

The cancer is a developing international fitness problem and one of the most essential causes of dying worldwide. even though several tactics were evolved for most cancers treatment, maximum of these strategies are not handiest deadly to most cancers cells however everyday cells are also affected, ensuing in death or excessive damage to them [1, 2]. Very widespread researches are in progress to find out approaches to overcome these effects and improving the therapeutic percentage. One of these strategies which have been interested and applied in recent years is tumor drug delivery by means of the drug loading in a biocompatible material and tumor site targeting and releases it especially in cancer cell line position [3, 4]. Paclitaxel is the main extracted substance from pacific yew L. Paclitaxel has illustrated notable activity in clinical trials against different types of tumors such as lung, cervical, breast and pancreatic cancer, head and neck carcinoma, advanced ovarian carcinoma, cancer, and acute leukemias when mainly administered systemically by injection into a vein [5–7]. However, the main challenges to efficacious chemotherapy with paclitaxel are drug resistance of tumors against paclitaxel, water insolubility and consequently its poor bioavailability in vivo. Various approaches have been improving the aqueous solubility of paclitaxel such as synthesis of prodrugs, use of co-solvents and dissolving in hydrophobic segments of biocompatible materials in drug delivery systems (DDS) [8-10]. Meanwhile some biodegradable and biocompatible polymers like chitosan (CS) have been widely studied in DDS as nanocarriers. CS is a natural linear bio poly amino saccharide obtained due to alkaline deacetylation of chitin, which has been received remarkable attention as a bio compatible/degradable polymeric material due to its chemical structure and has been extensively utilize in various fields such as protein and metal adsorption. Furthermore, CS has also been studied in the improvement of sustained release, since CS's mucoadhesive characteristic can improve drug transmucosal absorption and advance controlled release of a drug. [11-14]. With the accelerated evolution in nanotechnology, the magnetic nanoparticles, especially superparamagnetic iron oxide nanoparticles (SPION), are extensively investigated and have been discovered various capability such as cell apoptosis study, cell separation processes, and enzyme immobilization [15-17]. SPION has become an interesting targeted nanoparticle in the biomedical researches containing gene and drug delivery systems and magnetic resonance imaging (MRI) [18-21]. The DDS based on nano carriers of core-shell magnetic nanoformulations can be easily conducted magnetically to localize at the target region duo to externally applied magnetic field [22]. There are two important advantage make the SPION the appropriate nanocarrier for DDS. First, the capability of this nanoparticle in surface coating by different functional groups and various materials, can supply the water solubility and further modifications respectively [23-24]. Second, the covering of DDS by different materials, simplify the encapsulating of drugs as well as SPIONs simultaneously [25–27]. Additionally, the exterior shell (polymer layer) of the synthesized nanoformulation can efficiently decrease the rate of drug release. Accordingly, a (DDS) using core-shell polymer-coated SPION is recognized as an efficacious approach for passive tumor targeting, which can not only enhance drug circulation time but also decrease the pain in patients [28, 29]. Nevertheless, limited researches studied the direct application of folated CS-coated SPION as paclitaxel drug nanocarriers. The purpose of this study is a preparation of paclitaxel nanoformulation that is capable of remaining biocompatible and stable in the body [30, 31]. Accordingly, the first step was synthesizing a new formulation of FA-CS-PTX-SPION and after characterization of its properties such as, zeta potential, diameter size, shape and polydispersity, cell internalization and determining the suitable dosage for treatment, we study its effect in chronic myeloid leukemia K562 cell lines and human lymphoblastic GK-5 cell line as a normal cell line.

## MATERIALS AND METHODS

### *Preparation of FA-CS-PTX-SPION*

The SPION ($Fe_3O_4$) was obtained from Sigma–Aldrich Company. FA-CS-PTX-SPION was synthesized by reverse microemulsion method. Briefly, chitosan (CS) powder (50 mg) was dissolved in 1 ml of 1% (w/v) acetic acid comprising SPION solution and then a certain amount of paclitaxel drug (10 mg) was mixed with the CS-$Fe_3O_4$ solution. The acquired mixture was then added dropwise to

100 ml three-necked flask containing 50 ml of Tween-80 at 60°C. Consequently the resulted water-in-oil microemulsion transferred in a water bath and stirred at 1000 rpm/min for 8 h. Meanwhile, in the same condition 1 ml of 20% (w/w) sodium citrate solution was slowly poured into the flask and the reaction was still kept for 20 minutes in neutral pH. After the reaction, CS-PTX-SPION were collected with a strong magnet and washed frequently with isopropanol. Subsequently, the collected CS-PTX-SPION was subjected to 65°C for overnight. Drug free CS-SPION was synthesized in a same process as mentioned above. [32, 33].

***Folate decoration of PTX loaded FA-CS-SPION***

For this reason, FOL powder (25 mg) was dispersed in water (5 ml). NaOH solution (about 50 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 CS-PTX-SPION. The solution was continuously stirred (400 rpm) at ambient temperature for 45 minutes. The FOL-decorated PTX loaded FA-CS-SPION solution was centrifuged at 12000 rpm for 5 minutes. The supernatant containing free FOL was separated and precipitated material was washed with distilled deionized water.

***Characterization of nanoparticle size and morphology***

The polydispersity, size and surface charge of nanoparticles were obtained utilizing nano zeta sizer (Malvern Instruments Ltd., Malvern, UK). The nanoparticles morphology and size were estimated using atomic force microscopy (AFM) and field emission and also scanning electron microscopy (FESEM). Atomic force microscopy (AFM) images were obtained by (Nanoscopy Digital Instruments, USA) at room temperature using a drop of fresh solution. Also the morphological studies were completed using field emission scanning electron microscopy (Philips XL30) and gold coating the FA-CS-PTX-SPION.

***Determination of PTX encapsulation in the nanoparticles***

CS-PTX-SPION (5 mg) was dispersed in 40 ml of 1mol/L HCL by sonication. After 2 h, the supernatant was collected by centrifugation at 14000 rpm and magnetic separation. The concentration of PTX in the supernatant was measured by fluorescence spectroscopy (Hitachi, Japan) in 420 nm absorption and 430-600 nm emission and the gap width of 5 nm. The supernatant from CS-PTX-SPION was used as a contrast. The drug encapsulation efficiency of CS-PTX-SPION was taken utilizing the following equation:

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

***Paclitaxel release profile determination***

For determination of paclitaxel release from FA-CS-SPION, the phosphate (0.01 M and pH=7.4) and citrate (0.01 M and pH=5.4) buffers in 37˚ C were utilized. 1 ml of nano drug solution were placed in dialyze bag and putting up in 100 ml phosphate and citrate buffers separately. The tween 20 was used as an emulsifier for prevent sedimentation of released drug. Release profile was conducted applying shaking water bath. The sampling was done at 0, 4, 8, 12, 24, 48, 72 and 96 h. The 250 µl was aliquoted, froze and dried in each sampling and resolved in 2 ml methanol. Finally the paclitaxel release was evaluated using fluorescence spectroscopy.

The final drug release was calculated applying following equation:

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

where, R is the final drug release (%), V is the volume of each sample, $V_0$ is the initial volume of drug, $C_i$ and $C_n$ are the paclitaxel concentrations, i and n are the sampling times, and $m_{drug}$ is the mass of paclitaxel in nanoparticle.

***Cell culture***

Chronic myeloid leukemia K562 cell lines and human lymphoblastic GK-5 cell line as a normal cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (1%), and streptomycin and were separated with 0.5 g/l trypsin and 0.2 g/l EDTA and passaged several time for achieving 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, first, the FA-CS-PTX-SPION was conjugated to FITC (fluorescein 5(6)-Isothiocyanate). Afterward, the cells were treated with 25 µM FITC-FA-CS-PTX-SPION at 4 hours, then the nanoformulation contained medium was removed and after cell washing with phosphate buffered saline (PBS), the imaging process performed by fluorescence microscope (Nikon Eclipse TE2000-U). In order to evaluate the FA-CS-SPION carrier efficiency for improving the solubility and bioavailability of paclitaxel, one group of cells considered to treating with the same concentration of void paclitaxel and imaged by fluorescence microscopy.

***MTT assay***

In order to the preparation of MTT solution, 5 mg of MTT powder was dissolved in 1 ml PBS. 96 well plates were applied for MTT analysis [25-28]. $10^4$ cells were cultured in each well at 200 µl medium. Then left the cells to proliferate at 24 h. afterward, the various concentration of void paclitaxel, nanoformulated paclitaxel and the bare nanoparticle (FA-CS-SPION) that solved in DMSO, in 2% V/V compares with medium (10-50 µM) was injected into cells contained wells. Each experiment was performed in pentaplicate wells and repeated at least three times. Then the MTT assay was accomplished after treatment at 24 and 48 h. After 4 h incubation, whole solutions were evacuated and then add 100 µl DMSO to each well. The plates were put in the shaker for 10 minutes then analyzed by ELISA reader (BioTek Power Wave XS).

***Apoptosis assay by flow cytometry***

$10^4$ cells per well were counted and then poured into 6-well culture plates. The cells were subjected to various treatments of FA-CS-PTX-SPION, void PTX, and FA-CS-SPION at 48 hours. Apoptosis profile was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Biotechnology Co., Ltd. Nantong, China). Briefly, after 48 hours the cells were trypsinized and centrifuged at 250 g for 5 min, the supernatant discarded and then resuspended the pellet with 500 µl of 1x binding buffer. The solution was treated with 5 µl of Annexin V–FITC and 5 µl PI and transferred to FACS tubes after mixing and incubation for 10 min at ambient temperature. Finally the Samples were evaluated by FACScan Flow Cytometer (Becton Dickinson, Oxford, UK).

***Statistical Analysis***

SPSS 22 software was applied for statistical studies. Statistical differences between control and treatment groups were estimated utilizing T-TEST method. The achieved data were considered statistically significant with the P <5%.

## RESULT

**Characterisation of nanoparticles size and morphology**

Field emission scanning electron microscope and atomic force microscope images confirmed that the obtained nanoparticles have a spherical shape with suitable dispersity without noticeable aggregation (Fig. 1). The average hydrodynamic diameter of FA-CS-SPION and its polydispersity in 25℃ was 90 ± 15 nm and 0.074 ± 1.5 respectively and the zeta potential was -31 ± 0.7 mV (Fig. 2). All findings showed 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-SPION was confirmed.

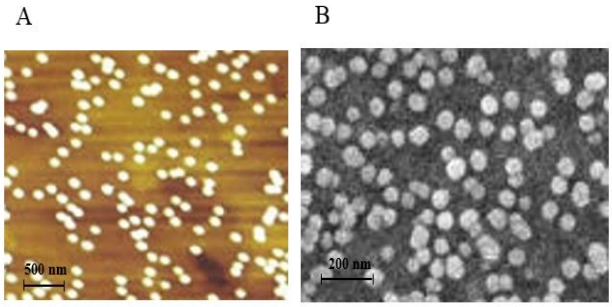


**Fig. 1**. Determination of morphology and size characteristic of FA-CS-SPION nanoparticles using atomic force microscope (A) and field emission scanning electron microscope (B).

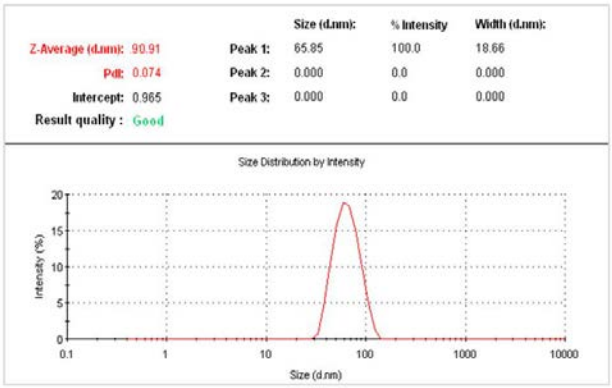


**Fig. 2.** Size measurement images. The FA-CS- PTX-SPION size using dynamic light scattering (DLS).

**Encapsulation efficiency of FA-CS-SPION loaded with PTX**

After preparing the FA-CS-PTX-SPION, this combination was centrifuged and the supernatant collected evaluation by spectrophotometer (Amersham, Uppsala, Sweden) in 432 nm. Paclitaxel encapsulation efficiency in FA-CS-SPION was detected 77 ± 0.3%. The nanoformulation showed high colloidal stability and suitable drug maintenance in this period.

**Release profile**

According to the release curves (Fig. 3), PTX releases from FA-CS-PTX-SPION 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 PTX, there are similar release profiles at pH 7.4 and 5.4., we observed a faster PTX liberation profile at pH 5.4 under the planned conditions compare with pH 7.4.

**Cell internalization of nanoformulation**

Estimation of paclitaxel internalization into cancer cells by its conjugation with FITC, performed by fluorescence imaging. As shown in Figure 4, the treated cells with fluorescence nanoformulation display green because of FITC-FA-CS-PTX-SPION internalization due to solubility rising of paclitaxel. While in cells treated with free paclitaxel this molecule is obvious from green and insoluble particles form in intercellular space due to its insolubility in aqueous.

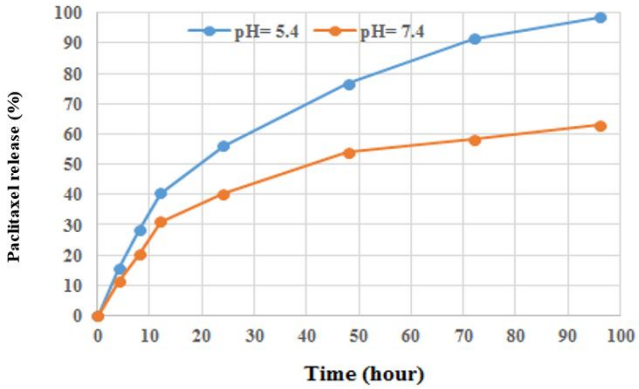


**Fig. 3.** Paclitaxel release profile in two different pH of 7.4 (phosphate buffer) and 5.4 (citrate buffer)

**Uptake kinetics of FA-CS-PTX-SPION**

The FA-CS-PTX-SPION handily internalized inside the cells and imagined clearly by fluorescence microscopy (Fig. 4), but free PTX accumulate as crystal mass with different scale. Additionally the cells without any treatment were applied as a reference value, setting the auto-fluorescence of the cells as "0" value.

***Cytotoxicity assay***

The cytotoxicity effect of void paclitaxel, bare FA-CS-SPION and FA-CS-PTX-SPION were estimated by MTT assay on K562 cancer and GK-5 normal cell lines. This test carried out in two 24 and 48 hours periods and was revealed in curve (fig. 5). Initially, the cells treated with different concentration of FA-CS-PTX-SPION (10-60 µM) for 24 and 48 hours. FA-CS-PTX-SPION significantly ($P<0.01$) prevented the cancer cells proliferation time and concentration dependent compared with bare FA-CS-SPION and void paclitaxel but didn't observe any significant difference in cell proliferation after treating normal GK-5 cell lines with three mentioned treatment. The calculated IC50 results exhibited that 34.83 and 27.11 µM are the IC50 concentration for 24 and 48 hours respectively. Moreover, both of bare FA-CS-SPION and void paclitaxel treatment didn't show any noticeable cytotoxic effect in all applied concentration.

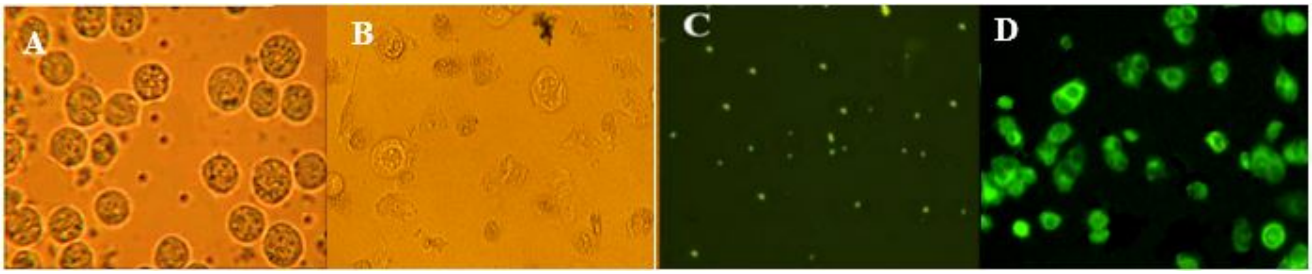


**Fig. 4.** Cell internalization study of FA-CS-PTX-SPION in K562 cell line using fluorescence microscope (400× magnification). Optic microscopy image of paclitaxel treated cells (A). Optic microscopy image of FA-CS-PTX-SPION treated cells (B). Fluorescence microscopy image of paclitaxel treated cells (C). Fluorescence microscopy image of FA-CS-PTX-SPION treated cells (D).

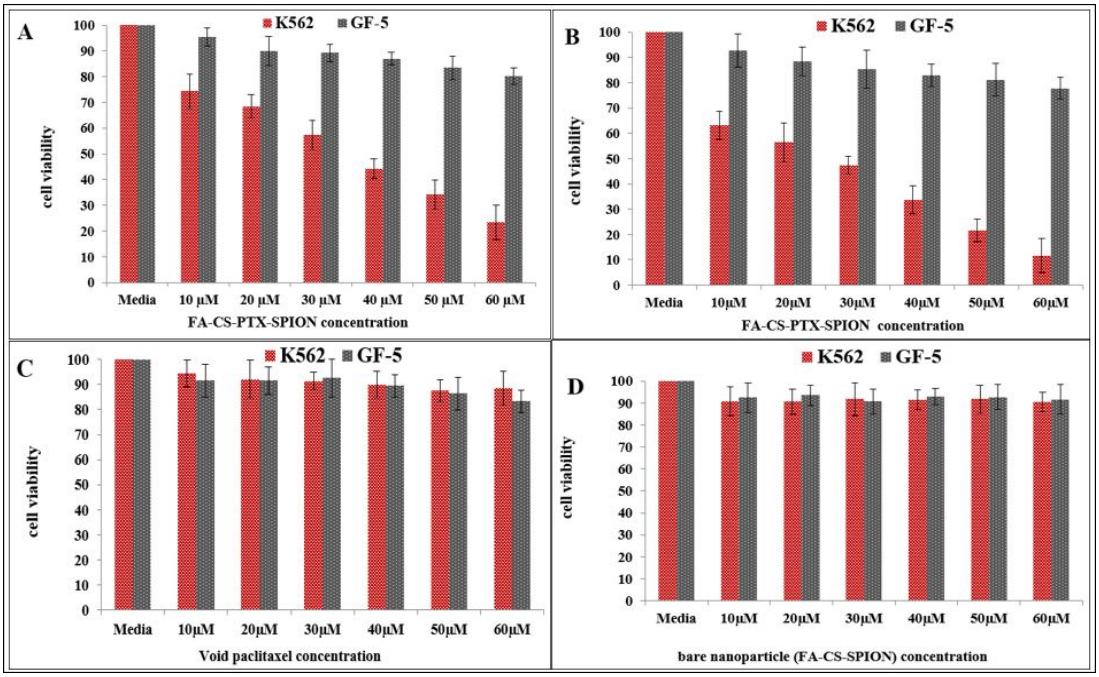


**Figure 5.** Study of different concentration (10-60 µM) of treated FA-CS-PTX-SPION after 24 (A) and 48 (B). Bare FA-CS-SPION (C) and void paclitaxel (D) after 48 h on K562 cancer cells and GK-5 normal cells.

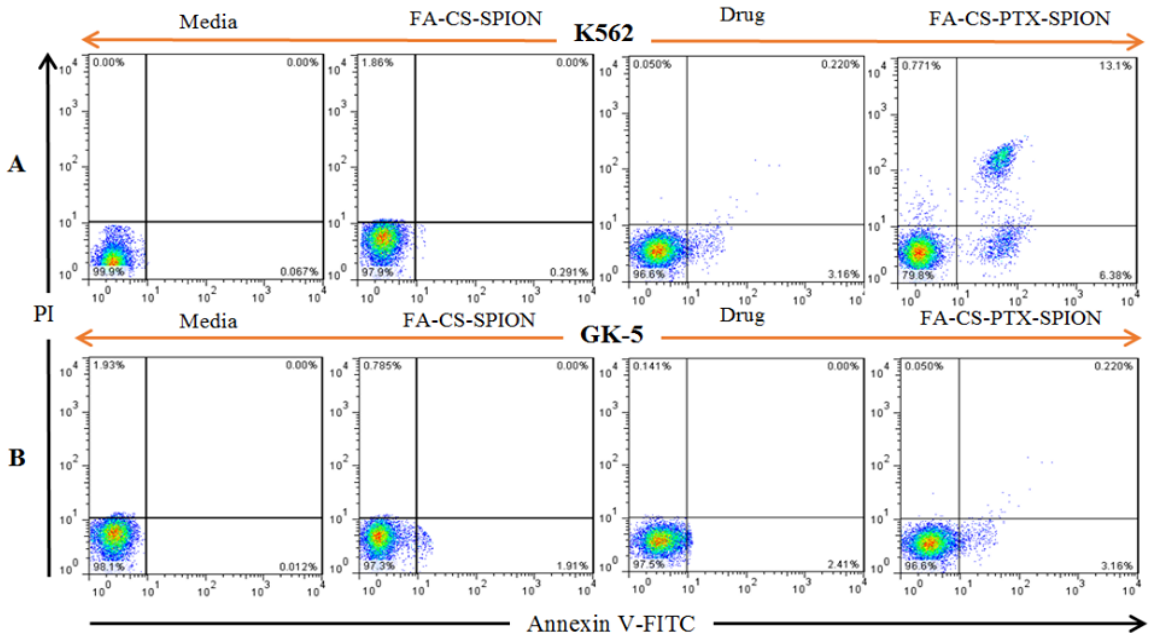


**Fig. 6.** Apoptosis induction by FA-CS-PTX-SPION on K562 and GK-5 cell lines.

***Flow cytometry***

Cytogram analysis was performed applying Flowjo 7.6.1 software. As shown in Fig. 6, the remarkable enhancement was achieved in the percentage of apoptotic cells in the case of cancerous cell lines compared to that of normal type. As seen in fig. 8 the FA-CS-PTX-SPION has revealed noticeable apoptosis in cancer cells whereas has no significant effect on normal cells. On the other hand the FA-CS-SPION didn't show any apoptotic effect in the given concentration as obvious from fig. 8 on leukemia cancer cell line (K562) as well as normal cell line (GK-5) which confirmed this fact that the FA-CS-SPION does not induce any apoptosis on both cancer and normal cell lines. The obtained results have also demonstrated that void PTX, does not show any apoptotic effect in both cancerous and normal cell lines.

## DISCUSSION

Paclitaxel has several therapeutic properties such as antioxidant, antimutagenic, antitumor, anticancer, antiangiogenic, anticholesterol and antibacterial. In spite of powerful therapeutic properties of this molecule, it can't be utilized extensively in the treatment of diseases that are because of its water insolubility and poor bioavailability in the body system. Numerous reports suggest that paclitaxel solubility was improved when it solved or encapsulated in drug carriers [34]. In this study, we assumed that FA-CS-SPION can enhance the stability and biocompatibility of paclitaxel and also the blood circulation time or durability will be

improved so apply for the antitumoral effects. So in this research, we synthesized a nanoformulation by combination the FA-CS-SPION nanoparticles and paclitaxel and then assess its effect on the treatment of leukemia cancer and normal cell lines Various studies show that nanoparticles smaller than 10 nm will be evacuated immediately from clearance system and/or extravasation, while the larger nanoparticles, may be recognized and eliminated by the mononuclear phagocyte system with higher probability [35]. Therefore in the most studies, in order to the noticeable improvement of enhanced permeability and retention (EPR) effect and also efficient escape from physiological barriers, the excellent and more suitable size for drug delivery is 10 to 250 nm [36]. Consequently, the FA-CS-PTX-SPION nanoformulation that synthesized with 90 ± 15 nm, has a proper size for drug delivery purpose (fig 1, 2). After loading the paclitaxel in FA-CS-SPION, we studied the nanosystem characteristics. Encapsulation efficiency of paclitaxel in FA-CS-SPION nanoparticle was 77 ± 0.3 % that exhibit excellent colloidal stability and good drug sustenance. Fluorescence spectroscopy system displays the 77% encapsulation efficiency for paclitaxel in the FA-CS-SPION that exhibit high and efficient drug encapsulation in nanoparticles. Moreover, the nanoparticles show intelligent behavior in relation to drug release in acidic pH that consequently the acidic pH of cancer cells leads to further drug release from FA-CS-PTX-SPION nanosystem (fig 3). So the resulted toxicity of the FA-CS-PTX-SPION injection into the cells, induced by internalization and permeation of paclitaxel into the cells and damage to sensitive parts and components within the cell (fig 4). So the resulted toxicity of the nano system injection into the cells, induced by internalization and permeation of paclitaxel into the cells and damage to sensitive parts and components within the cell (fig 4). Numerous studies, show the antitumoral effect of paclitaxel in relation to the different type of cancers such as colon, breast, ovary, prostate, brain, pancreatic, lung, skin etc either in vitro and in vivo studies [37,38]. In this study, the MTT assay result indicated decreasing in survival per cent of K562 cancer cell lines due to increasing the concentration of FA-CS-PTX-SPION treatment on after 24 and 48 hours respectively. The IC50 concentration for both 24 and 48 hours has happened in 34.83 and 27.11 µM respectively while the FA-CS-PTX-SPION toxicity in the normal cells was negligible without any significant IC50 for treated concentration (fig 5). Study the effect of FA-CS-PTX-SPION and void paclitaxel with the different concentration on both cancer and normal cell lines individually, showed their negligible toxicity. These results were in agreement with the outcomes of the study the similar treatment effects of FA-CS-SPION nano paclitaxel [39]. The apoptosis induction assay indicated that there is different sensitivity in relation to the cancerous and normal cell lines (Fig. 6). The apoptosis induction of FA-CS-PTX-SPION nanoformulation as well as toxicity induction was not detected in normal human lymphoblastic (GK-5). The apoptosis induction assay confirmed that the cell death is affected vigorously by drug nanoformulation of FA-CS-PTX-SPION and there is differential sensitivity to the two cancerous (chronic myeloid leukemia K562) and normal (human lymphoblastic GK-5) cell lines (Fig. 6). That way, the apoptosis induction of FA-CS-PTX-SPION as well as toxicity result was not detected in normal human lymphoblastic (GK-5). Consequently, the impact of FA-CS-PTX-SPION is specifically in cancer cells and has not any effects on normal cells that due to the specific function of paclitaxel in the effective targeting of cancer cells than normal cell. This is the cause of the higher impact of paclitaxel on cancer cells. On the other hand, the bare FA-CS-SPION has not any toxicity effect and is perfectly biocompatible. Also the paclitaxel drug has not any cytotoxic effect on cell proliferation because of drug efficiency losing due to the low half-life of paclitaxel (fig 5).

## CONCLUSION

The MTT assay results on cancer (K562) and normal (GK-5) cells indicate the specific effect of FA-CS-PTX-SPION on cancer cells whereas has not significant cytotoxic effect on normal cells. The specific activity of paclitaxel drug in efficiently killing the cancer cells than normal cells is the main reason for this finding. Moreover, the bare FA-CS-SPION has not any meaningful toxicity and has excellent biocompatibility that subsequently leads to less effect on reducing cell proliferation.

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