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ORIGINAL RESEARCH PAPER

Evaluation of the effect of crocetin on antitumor activity of doxorubicin encapsulated in PLGA nanoparticles

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ABSTRACT

Objective(s): The current study reports investigation of codelivery by PLGA nanoparticles (NPs) loaded with crocetin (Cro), a natural carotenoid dicarboxylic acid that is found in the crocus flower, and Doxorubicin (DOX).

Materials and Methods: Double emulsion/solvent evaporation method was used for preparation of PLGA nanoparticles containing Dox and Cro. Characterizations of prepared NPs were investigated by atomic force microscopy (AFM) and dynamic light scattering analysis. *In vitro* Cytotoxicity of DOX and Cro loaded PLGA NPs (PLGA-DOX-Cro) on MCF-7 cell line was evaluated using MTT test. Flow cytometry experiments were implemented to distinguish cells undergoing apoptosis from those undergoing necrosis. Furthermore the expression of caspase 3 was examined by western blot analysis.

Results: The prepared formulations had size of 150- 300 nm. Furthermore, PLGA-DOX-Cro nanoparticles inhibited MCF-7 tumor cells growth more efficiently than either DOX or Cro alone at the same concentrations, as quantified by MTT assay and flow cytometry. Studies on cellular uptake of DOX-Cro-NPs demonstrated that NPs were effectively taken up by MCF-7 tumor cells.

Conclusion: This study suggested that DOX-Cro-NPs may have promising applications in breast cancer therapy.

Keywords: *Breast cancer, Chemotherapy, Crocetin; Doxorubicin, PLGA*

INTRODUCTION

To improve therapeutic efficacy and reduce toxicity and frequency of cancer drug administration, various nanocarriers have been developed. Over the past few decades, there has been an increasing interest in the potential use of polymeric nanoparticles (NPs) as delivery vehicles for chemotherapeutic drugs and the studies have demonstrated that these nanocarriers can signiûcantly enhance the anti-tumor efficiency of various chemotherapeutic drugs [1-5].

Drug delivery systems which enable codelivery of drugs with different physiochemical properties to the same tumor cells *in vitro* and *in vivo* have been proposed to reduce the dosage of each drug and to achieve the synergistic effect in cancer chemotherapy [6, 7].

Recently, many multifunctional delivery systems have been designed for codelivery of different agents, including micelles [8-10], liposomes [11], and inorganic nanoparticles [12].

Although many efforts have been made on a single carrier for two or more therapeutic agents, such as chemicals, natural products, siRNA, plasmid DNA or peptide [13-15], codelivery of chemotherapeutic drugs with different water solubility characteristics is not easy to handle.Nevertheless, for the preparation of multifunctional drug delivery systems, PLGA (poly- D, L lactide-co glycolide) is the ideal candidate. PLGA-based nanoparticles have gained great interest in diagnostics and treatment applications such as

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preparation of sustained drug release systems [16]. On the other hand, PLGA have been approved by FDA for preparation of drug delivery systems.

In comparison with other carriers such as liposome, PLGA NPs is a sophisticated vehicle for drug delivery because of its unique characteristics comprising biocompatibility, bioavailability, high drug-loading capacity, stability and sustained drug release [17-20]. There are various PLGA based formulations in clinic including Trelstar Depot for prostate cancer, Sandostatin LAR for acromegaly and Nutropin Depot for growth deficiencies [21]. Numerous drugs using PLGA as carrier is also at the different pre-clinical stage.

Doxorubicin (DOX) is the chemotherapeutic drug which extensively used in clinical application, due to their good anti-tumor efficiency against various tumors.

Doxorubicin exhibits limited efficacy in extending time of therapy due to drug resistance and severe toxic side effects [22-26]. Some cancer patients use natural products derived from plants or nutrients, concurrently with routine chemotherapeutic regime and/or radiation therapy. Demand for new drugs has encouraged studies investigating feasible anti-cancer compounds in fruits, vegetables, herbs and spices. In this regards, saffron is a spice which derivate from the plant Crocus sativus L., has been implemented as a traditional ancient medicine against various disease including cancer [27]. It was previously reported that crocetin, an important active carotenoid of saffron, has significant anti-tumour effect *in vitro* and *in vivo* [28].

Crocetin inhibits the proliferation of cancer cells by preventing nucleic acid synthesis, enhancing apoptosis and interrupting growth factor signaling pathways [29].

Therefore, our strategy is to design biodegradable PLGA nanoparticles loaded with DOX and crocetin in order to improve therapeutic efficacy and reduce frequency of drug administration. In this study, MCF-7 was utilized as the *in vitro* tumor model to investigate the enhancement effect of DOX by crocetin.

MATERIALS AND METHODS

Materials

Poly(d,l-lactic-co-glycolic acid) (PLGA) (Average M_w: 7000-17000; lactic acid : glycolic acid = 50:50) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich (Germany). Doxorubicin hydrochloride (DOX) was purchased from Euroasia Co., Ltd. (Delhi, India). Crocetin was extracted from plant Crocus sativus L. based on the method represented in the Iran Patent No. 84459.

Roswell Park Memorial Institute (RPMI) 1640 medium, foetal bovine serum (FBS) and trypsin were purchased from GIBCO (Germany). Polyvinyl alcohol (PVA, 87-89% hydrolyzed, average Mw=88,000-97,000) and other solvent and chemical reagents were procured from Merck (Germany) without further purification.

Preparation of PLGA NPs loaded with DOX

PLGA nanoparticles loaded with DOX were prepared using a water-in-oil-in-water (W/O/W) double emulsification and solvent evaporation technique [30]. Different parameters such as concentration of PVA as stabilizer (2 and 5%), drug/polymer ratios (1:10 and 1:20), organic solutions (Dichloromethane and Dichloromethane: acetone) and route of solvent removal (3 hours and overnight stirring) were considered. 25 mg PLGA was dissolved in 1 ml dichloromethane:acetone with 4:1 ratios, mixed with 0.2 mL deionized water containing 1.25 mg doxorubicin (drug/polymer ratio 1:20).

The mixture was sonicated in ice bath (pulse on 1 s, pulse off 1 s, amplitude: 90%) for 1 min using a probe sonicator (Fisons Instruments Ltd, Crawley, U.K).

The primary water-in-oil (W/O) emulsion was added to 4 mL of ice-cold 2% or 5% polyvinyl alcohol (PVA) solution (w/v) and emulsified for 10 min using a probe sonicator (Fisons Instruments Ltd, Crawley, U.K). The final emulsion was drop wise added to 5 ml of PVA 0.1% for diffusion under rapidly stirring. The organic solvent of emulsion was then removed under different conditions (overnight stirring, 3 hours stirring or vacuuming overnight).

The NPs were collected by centrifugation at 14000 rpm for 25 min and washed three times with deionized water. The final NPs were obtained as light orange powders after lyophilisation. The blank NPs (without drugs) were prepared by the same method.

Preparation of PLGA NPs loaded with DOX-Cro

25 mg PLGA was dissolved in dichloromethane: acetone with 4:1 ratio, mixed with 0.2 mL deionized water containing 1.25 mg doxorubicin with addition of crocetin solution (1.25 mg in pyridine).

The mixture was sonicated in ice bath (pulse on 1 s, pulse off 1 s, amplitude: 90%) for 1 min using a probe sonicator (Fisons Instruments Ltd, Crawley, U.K). The

primary water-in-oil (W/O) emulsion was added to 4 mL of ice-cold 5% polyvinyl alcohol (PVA) solution (w/v) and emulsified for 10 min using a probe sonicator (Fisons Instruments Ltd, Crawley, U.K).
The organic solvent of emulsion was then removed overnight stirring. The NPs were collected by centrifugation at 14000 rpm for 25 min and washed three times with deionized water in order to remove pyridine and PVA residues. The final NPs were obtained as light orange powders after lyophilisation.

Surface morphology of PLGA NPs

The surface morphology of PLGA NPs was studied by atomic force microscopy (AFM). An atomic force microscope (AFM, model: Nano Wizard II NanoScience AFM, JPK Instruments Inc., Germany) was implemented to investigate the structures of the prepared NPs.
The sample for AFM analysis was prepared as follows: Microscope glass slide was washed with acetone and ethanol, rinsed with ultrapure water and dried. After cleaning, the prepared PLGA NPs were diluted in deionized water (0.1 mg/mL), dispensed onto glass slide and dried at room temperature for 24 h [31].
The measurements were performed in the tapping mode and dehydrated state in the air, and ACT cantilevers (JPK Instruments Inc., Germany) were used.

Particle size analyzer

The measurement of NPs size and size distribution was carried out by dynamic light scattering. Briefly 1 mg lyophilized NPs was dissolved in 1000 µl of deionised water and then after sonication, analyzed with zetasizer (NANO-ZS, Malvern, UK) at a scattering angle of 90p equipped with a 4 mW He-Ne laser operating at 633 nm using back-scattering detection. All measurements were performed in triplicate at 25°C [32].

Drug loading and encapsulation efficiency

The amount of DOX in NPs was analyzed using a Jasco FP-6200 spectrofluorometer (Tokyo, Japan) at $\lambda_{excitatin}$=470 nm, $\lambda_{emission}$= 590 nm by dissolving 1 mg NPs in 1 mL dimethylsulfoxide (DMSO) and employing the calibration curve of 0–100 µg/mL DOX in the same solvent.
The amount of crocetin in NPs was analyzed using a Shimatzu UV–visible spectrophotometer (Tokyo, Japan) at 430 nm using the calibration curve of 2-20 µg/mL crocetin in the same solvent.
Each experiment was conducted in triplicate.
The encapsulation efficiency (EE%) and loading content (LC%) of PLGA NPs loaded with crocetin and/or DOX were calculated by the following equations
EE (%) = Amount of drug in NPs/amount of drug used for encapsulation×100
LC (%)= Mass of drug in NPs/ Mass of NPs ×100

Release study

NPs suspension were prepared by dissolving 10 mg of freeze-dried formulation powder in 10 mL Phosphate buffer saline (PBS 0.1 M, pH 7.4) containing 0.1% v/v of Tween 80 (to maintain a sink condition) [33]. The NP suspension was equally divided in ten tubes containing 1 mL and kept in a shaker incubator set at 90 rpm and 37p C. At specific intervals these tubes were taken out from shaker and centrifuged at 14000 rpm for 10 minutes. The supernatant was lyophilized and dissolved in 1 mL DMSO. The amount of DOX and Cro in the sample was determined as described previously. Each drug release experiment was repeated three times.

MTT assay

MCF-7 cells were cultured in the RPMI medium, supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), 1% penicillin-streptomycin at 37 °C and 5% CO_2.
MCF-7 cells were seeded into 96-well plates at 1×10^4 and incubated for 24 hours. DOX and Cro with different concentrations (DOX: 0.5, 1, 2, 4, 8 µM, Cro: 1, 2, 4, 8, 18 µM) were prepared in RPMI containing 10% FBS.
Blank PLGA NPs, DOX, DOX+Crocetin, , PLGA-Dox NPs, PLGA-Dox NPs+ free Crocetin, PLGA-Dox-Cro NPs (equivalent aforementioned concentrations of DOX and Cro either in solution or in PLGA NPs formulation) were also dispersed in RPMI containing 10% FBS.
The prepared drug concentration was added to each well, followed by incubation of the cells for 48 h.
20 µL MTT (5 mg/mL in PBS) solution was then added to each well. After 4 h, MTT solution was aspirated from the wells using vacuum and 100 µL DMSO was added to each well. The absorbance at 570 nm with a reference wavelength at 630 nm, was measured by a Infinite® 200 PRO multimode microplate reader (Tecan Group Ltd. Männedorf, Switzerland).

Apoptosis assay

MCF-7 cells were exposed to DOX, PLGA NPs containing equivalent amount of doxorubicin or

doxorubicin along with crocetin for 48 h at 37°C and then detached and collected.

The untreated and treated MCF-7 cells were washed twice with phosphate buffer, Propidium iodide was then added to a final concentration 50 µg/mL which can bind to double stranded DNA by intercalating between base pairs. Then the mixture was stored in the dark at 4°C for 2 h.

At final stage, the fluorescence of 10,000 fixed cells which stained with propidium iodide was assayed on a FACScaliber (Bector Dickinson, USA) using FL-2 channel [34]. The number of apoptotic cells was analyzed using the WinDMI 2.9 software.

Western blot analysis

The untreated and treated cells with different formulations or free drugs were harvested, lysed, and their protein concentrations were quantiûed by the Bradford assay [35].

The equal amounts of protein from each sample were separated by electrophoresis on a 12% denaturing SDS gel. Proteins were transferred onto 0.45 µm pore size PVDF membrane (BioRad, USA). Detection was conducted by immuno-staining using 1:1000 dilution speciûc primary antibodies of caspase-3 (9665s-Cell Signaling) and horseradish peroxidase-conjugated anti-IgG antibody (1:3000 dilution) (7074s-Cell Signaling). The protein bands were visualized by the enhanced chemiluminescence (ECL) assay according to the manufacturer's instructions.

Cellular uptake

MCF-7 cells were seeded in 12-well plates at 8×10^4 cells per well and cultured for 24 h.

The cells were then incubated at 37 °C for 2h, 24h and 48h with free drugs or prepared formulations (equivalent to 1 µM doxorubicin) in RPMI containing 10% FBS.

Then, the culture medium was removed; the cells were then washed by PBS and were trypsinized and centrifuged at 1500 rpm for 10 min.

The supernatant were removed, and the pellet was washed three times with cold PBS. At the final stage, collected cells were suspended in 1 mL cold PBS and the intensity of the doxorubicin ûuorescence in the cells was determined using a FACScaliber (Bector Dickinson, USA) equipped with a 488 laser in the FL1 channel. The data were analysed using the WinDMI 2.9 analysis software.

Statistical analysis

The results were reported as means ± SD (n e" 3). Data were analysed by one-way analysis of variance (ANOVA). A probability value of less than 0.05 was considered signiûcant.

RESULTS AND DISCUSSION

Preparation of DOX or DOX/ Cro loaded PLGA NPs

In order to overcome the undesirable side effects of drugs, synergistic combination of two or more drugs is a versatile strategy [3].

In comparison with a single drug delivery system, codelivery of multiple drugs has numerous advantages including reducing administration dosage of each drug because of enhancement effect, suppressed drug resistance, reducing undesirable toxicity and accessing context-speciûc multiple targets.

Therefore, it is crucial to codeliver two or more drugs through a nanocarrier and convey them to the same cancer cell coincidentally [2, 5].

In the development of the codelivery systems, encapsulating drugs with different water solubility properties is a serious challenge.

The PLGA nanoparticles can be produced by various techniques. Usually emulsification solvent evaporation technique is used for PLGA NPs preparation because of its simplicity and high drug loading capacity.

The single emulsion method is a very useful method for encapsulation of hydrophobic drugs and leads to very low encapsulation efficiency of hydrophilic drugs.

The double emulsion method is a versatile technique for encapsulating both hydrophilic and hydrophobic drugs with high efficiency [36-38].

This technique allows preparation of NPs which encapsulated both hydrophobic and hydrophilic cargos.

Therefore, in this study, we developed a convenient W/O/W method for development of doxorubicin (hydrophilic drug) and crocetin (hydrophobic drug) co-encapsulated PLGA NPs.

Table 1 summarized the average particles size of Dox-loaded PLGA NPs prepared under different conditions. Four quantitative independent variables consisting of organic phase, concentration of PVA, concentration ratio of polymer/drug, route of solvent removal and dependent variables were diameter and polydispersity of NPs.

In PLGA formulations, the optimal condition was as follows: PVA 5%, 1:20 ratio of drug to polymer, dichloromethane:acetone (4:1) as organic solvent and overnight stirring for solvent removal. The particle size of the nanoparticles which prepared under aforementioned condition was about 178 nm. Obtained results demonstrate that the concentration of surfactant (PVA) and route of solvent removal was crucial for preparation of nanosized drug loaded particles (<300nm).

Both doxorubicin and crocetin were successfully entrapped in the PLGA NPs (F6 formulation) prepared by double emulsion method.

The drug loading efficiency was about 54% and 65% for DOX and Cro respectively. On the other hand, the loading content was also calculated to be 2.43% and 2.94% for DOX and Cro respectively. It seemed that the loading efficiency of Cro is higher than that of DOX. This was owing to that Cro is a hydrophobic compound, while DOX is a hydrophilic drug and may leak out to the external water phase during the emulsification. The real molar ratio of DOX/Cro in formulation was fixed.

Morphology of NPs

The morphology of PLGA-DOX-Cro NPs (F6) was examined by AFM (Fig. 1). It was revealed that the prepared NPs were homogeneously distributed as individual well-deûned spherical particles around a size of 200-300 nm.

In vitro drug release

The controllable release behaviour of drugs can be achieved by using polymeric nanocarriers for simultaneous encapsulation of two or more drugs in a vehicle.

Then prolonged release time, enhancement effects of encapsulated cargos and reduced administration frequency could be achieved for crocetin-doxorubicin loaded PLGA NPs.

The *in vitro* drug release experiment of F6 formulation has been conducted in a PBS solution at pH 7.4. As illustrated in Fig. 2, DOX released a little faster than Cro in the first few hours, and the release rates were similar after 20 hours.

This might be caused by some of the hydrophilic DOX was adhered to the surface of the PLGA NPs. Therefore, DOX released faster when the NPs were suspended in PBS. Approximately 80% of the drugs (DOX and Cro) were accumulatively released within 48 hours.

Doxorubicin and crocetin releases from PLGA NPs were nearly linear for the first 5 hours, with 60% and 40% DOX and Cro release respectively. Drug loaded PLGA nanoparticles often display burst release, which can be attributed to unencapsulated drug localized on the surface or near the surface of the nanoparticles [39, 40]. Also, the presence of suspended (undissolved) drug in the polymer matrix probably contributes to the linear drug release as reported previously [41]. It would appear that the suspended drug provides a constant driving force for diffusion of cargos (doxorubicin and crocetin) out of the nanoparticles.

In the present study, release profile clearly suggests a diffusion mode of release for both drugs in first 5 hours and then erosion being the main mechanism of release until complete release of drugs up to 48 hours.

This type of release pattern for DOX and Cro could be advantageous in cancer chemotherapy. The initial burst release over 5 h provides a primary dose, and the remaining dose is released during 48 hrs.

Such a controlled release system by which the drug will be available to the tumour tissue in an instantaneous manner is of significant importance.

Combinational effects of DOX and Cro on MCF-7 cell lines

Crocetin is a natural carotenoid and is one of the major active compounds of saffron. Crocetin consist of 20 carbon atoms including double bonds and a carboxylic acid group at each end of the chain (Fig. 3). Previously various pharmacological effects of crocetin were reported; comprising its antioxidant, anti-inûammatory, and anti-tumor effects [42, 43].

Recently, crocetin has attracted more attention as a potent natural product with anti-tumor activity.

Dehr *et al.* [44] demonstrated that crocetin can inhibit tumor growth in pancreatic cancer xenograft mouse model. Furthermore, crocetin prevents 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumors in mice [45] and shows protective effects against benzo (a)pyrene induced lung cancer [46].

Several studies have demonstrated that crocetin or crocetin containing combination regimens are very effective in the treatment of cancer.

Zhong *et al.* [47] demonstrated that crocetin caused cytotoxicity in cancerous cells by enhancing apoptosis in a time-dependent manner. Also they proved that the crocetin has synergistic effect on the cytotoxicity induced by vincristine.

Table 1. Preparation of drug loaded PLGA NPs using double emulsion method under different conditions

DOX encapsulation						
Formulation	DOX: Polymer	DCM (µl):Acetone(µl)	PVA	Solvent removal	Size (nm)	PDI
F1	1:10	1000:0	%5 4ml	Stirring overnight	230	0.1
F2	1:10	800:200	%5 4 ml	Stirring overnight	222.9	0.1
F3	1:10	800:200	%5 4 ml	3 hours stirring	364	0.4
F4	1:10	800:200	%2 4 ml	Stirring overnight	314	0.4
F5	1:20	800:200	%5 4 ml	Stirring overnight	178	0.06
Dual DOX-Cro encapsulation						
Formulation	DOX:Cro:Polymer	DCM (µl):Acetone(µl)	PVA	Solvent removal	Size (nm)	PDI
F6	1:1:20	800:200	%5 4 ml	Stirring overnight	256	0.2

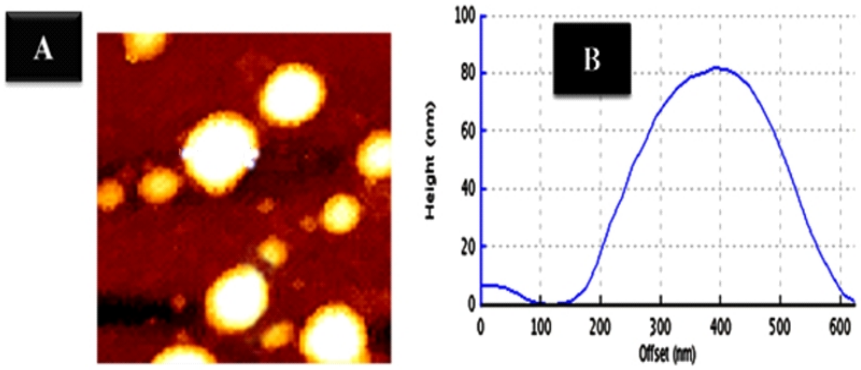

Fig. 1. AFM image of PLGA-DOX-Cro NPs (F8) obtained in the tapping mode (A). Height profile of PLGA-DOX-Cro NPs (B)

Li *et al*. [48] also evaluated the cytotoxicity of corcetin in colon cancer cell line and approved p-53-independent cellular toxicity of cocetin. Altogether these reports verified the potency of crocetin as a versatile anti-cancer agent. Herein, in order to investigate the enhancement antitumor effect of DOX in the PLGA NPs by crocetin, the cytotoxicity of PLGA-DOX NPs, PLGA-DOX-Cro NPs, PLGA-DOX NPs+ free Cro, free DOX and free DOX+ free Cro in MCF-7 cell line was evaluated. The Dox and Cro concentrations in these formulations were 0.5-10 µM and 1-16 µM respectively. In our study, Cro in 1-16 µM concentrations showed no cytotoxicity on MCF-7 cell line (Fig. 4A). Then, all prepared formulations contain nontoxic concentrations of Cro along with DOX.

The cell viability of blank PLGA NPs was assayed and exhibited no pronounce cytotoxicity to MCF-7 cells. Obtained results demonstrated that crocetin could sensitize MCF-7 cells to doxorubicin-induced cell death.As illustrated in Fig. 4 B and table 2, DOX and crocetin in the PLGA-DOX-Cro NP exhibited significantly higher cytotoxicity compared to other formulations. However, PLGA-dox+cro and PLGA-Dox could also decrease IC50 compared to Doxorubicine (p valued"0.001). This may be due to the different release rates of DOX and crocetin in aqueous media or higher cellular uptake.Moreover, because of high cellular uptake of NPs via endocytosis, the co-encapsulation of DOX and

crocetin in PLGA NPs was demonstrated more cytotoxic acitivity against MCF-7 adenocarcinoma cell line in comparison with free DOX and crocetin. On the other hand, treatment with crocetin alone did not cause any cytotoxicity, it was suggested that the enhancement effect might result from the combination of individual anti-tumor mechanism for each drug. DOX binds to DNA by intercalation and promotes apoptosis in tumor cells, and crocetin can act as chemosentizer or MDR reverser. Codelivery of cytotoxic anti-cancer drugs with natural product is considered as a solution for better chemotherapeutic response *in vitro* and *in vivo*. As the key point for successful combination therapy is to design versatile codelivery systems.Previously, the combination therapy of routine anti-cancer drugs with different natural products which codeliver within PLGA nanoparticle has been reported. Among this, curcumin (active constituent of turmeric) is attracted much attention and obtained results demonstrated its synergistic effect along with doxorubicin, letrozol and gemcitabine for cancer chemotherapy [49-51]. Until now the combination therapy of DOX and crocetin was not investigated in detail. In our study, crocetin increased the cytotoxicity of DOX in MCF-7 cells *in vitro*. The reason to prefer DOX and crocetin for co-encapsulation was related to MDR reversibility of crocetin, transmitting and maintenance of DOX inside the nucleus. Then crocetin as a MDR reverser may help to enhance antiproliferative activity of DOX in MCF-7 cells.

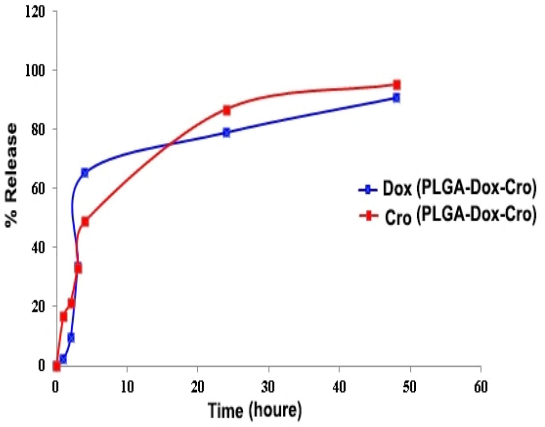

Fig. 2. *In vitro* release profile of DOX and Cro from PLGA NPs in pH 7.4 at 37°C up to 48 hours

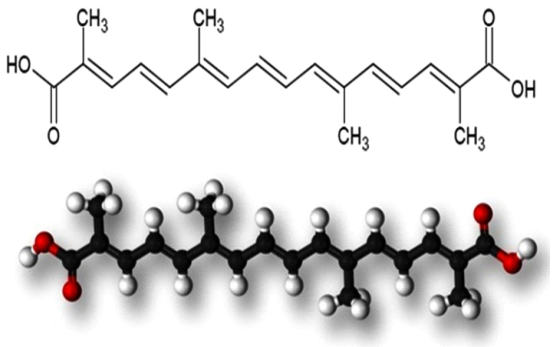

Fig. 3. 2-D and 3-D structures of crocetin

Appototic cell analysis by flow cytometry

A flow cytometeric analysis of propidium iodide (PI)-stained cells was also performed to assess the impact of crocetin on the generation of sub-diploid cells with Dox. Co-treatment of MCF-7 cells with crocetin in combination with doxorubicin increased the sub-diploid population (fig. 5). Treatment with NPs encapsulated both crocetin and doxorubicin significantly increased ($p<0.01$) the sub-diploid population for MCF-7 compared with free DOX+Cro. Since, treatment with crocetin alone did not cause increasing sub-diploid ($p> 0.05$) population for MCF-7 cells, suggesting crocetin is a qualified chemosentetizer agent.

Obtained results are in agreement with the cell viability data and verify the potency of the prepared PLGA NPs in delivering the drugs to the cells, and codelivery of DOX and crocetin through encapsulation in PLGA NPs exhibited more anti-cancer activity.

Activation of cell death-associated caspases

In our study, increase in expression of caspase-3 as an executioner of cell apoptosis pathway, might play a pivotal role in sensitizing MCF-7 cells to DOX-induced apoptosis.Compared to the untreated cells, the activity of caspase-3 in the MCF-7 cells treated with nanoparticles containing Dox (DOX=1 µM) was increased (Fig. 6). In comparison with free DOX+Cro, PLGA NPs encapsulated DOX and Cro caused higher expression of caspase-3 in treated cells.

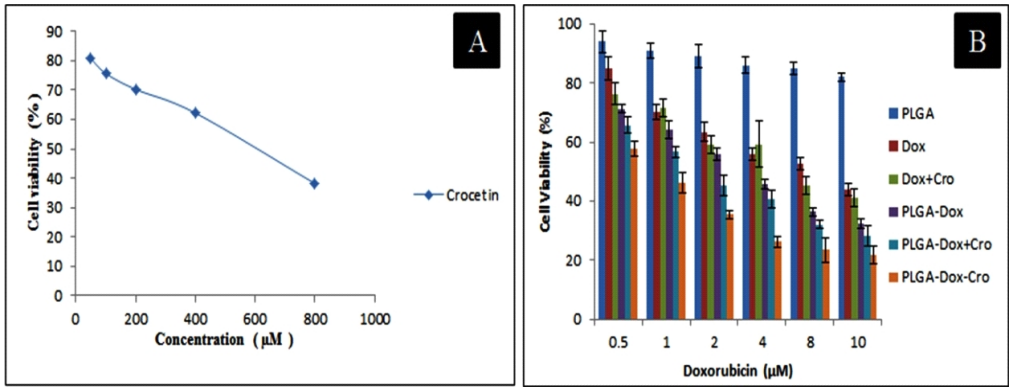

Fig. 4. Cytotoxicity of different concentrations of Cro (A); free DOX, blank PLGA NPs, free DOX+Cro, PLGA-DOX, PLGA-DOX+Cro and PLGA-DOX- Cro (B) in MCF-7 cell line for 48 hours. The Dox and Cro concentrations in these formulations were 0.5-10 µM and 1-16 µM respectively

Table 2. IC50 of DOX in Solution or in formulations in the presence or absence of Cro

Formulations	IC50 (µM)
DOX	7.81 µM ***
DOX+Cro	6.43 µM ***
PLGA-DOX	3.21 µM **
PLGA-DOX NPs + free Cro	2.19 µM *
PLGA-DOX-Cro	0.82 µM

*** p<0.001; ** p<0.01, *p<0.05

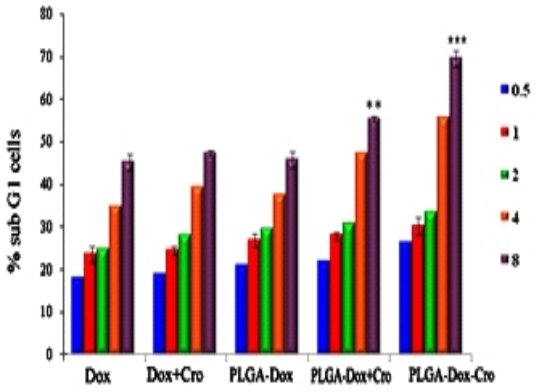

Fig. 5. % of sub G1 cells in MCF-7 cells after exposure to free DOX, PLGA-DOX, PLGA-DOX-Croc, PLGA-DOX+Cro, DOX+Cro (DOX concentrations were equivalent to 0.5, 1, 2, 4 and 8µM and Crocetin in terms of the different concentration existed in PLGA-DOX-Cro NPs) *** p<0.001; ** p<0.01

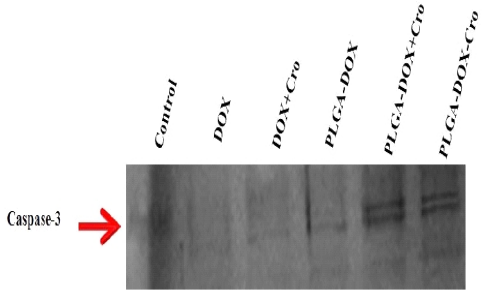

Fig. 6. Western blot analysis of caspase-3 following 48h exposure of MCF-7 cells with DOX, DOX+Cro, PLGA-DOX, PLGA-DOX+Cro and PLGA-DOX-Cro

Cellular uptake

To investigate the delivery of cargos via PLGA NPs, we evaluated the cellular uptake of drugs inMCF-7 using flow cytometry.While the uptake of drugs was determined at 2 hrs, 24 hrs and 48 hrs, our studies show that there was continued uptake of PLGA NPs for at least up to 48 hrs in MCF-7 cells (Fig. 7). This suggests the potential for even further improvement in intracellular delivery of drugs with longer incubation times. As illustrated in Fig. 6 PLGA NPs in the presence of crocetin enhances doxorubicin delivery to the cells after 48 hrs. Fluorescent intensity of MCF-7 cells incubated with PLGA-Dox-Cro was higer than PLGA-DOX+ free Cro. Free DOX or Cro enters to the cells through passive diffusion whereas NPs encapsulated Cro/DOX enter into the cells by endocytosis that results in higher uptake of drugs through NPs in comparison to free drug. Presence of crocetin as a carotenoid along with DOX in formulation prevents

the efflux of Dox in MDR MCF-7 cells overexpressing ABCB1 [52, 53]. Due to this reason the fluorescence intensity of the cells treated with DOX along with crocetin increased. In consistent with other reports, our studies demonstrate that PLGA nanoparticle encapsulation of hydrophilic drugs like doxorubicin can significantly improve its therapeutic effect [54-57]. Additionally, codelivery of a potent anti-cancer natural product such as crocetin along with doxorubicin could result in further enhanced therapeutic efficacy.

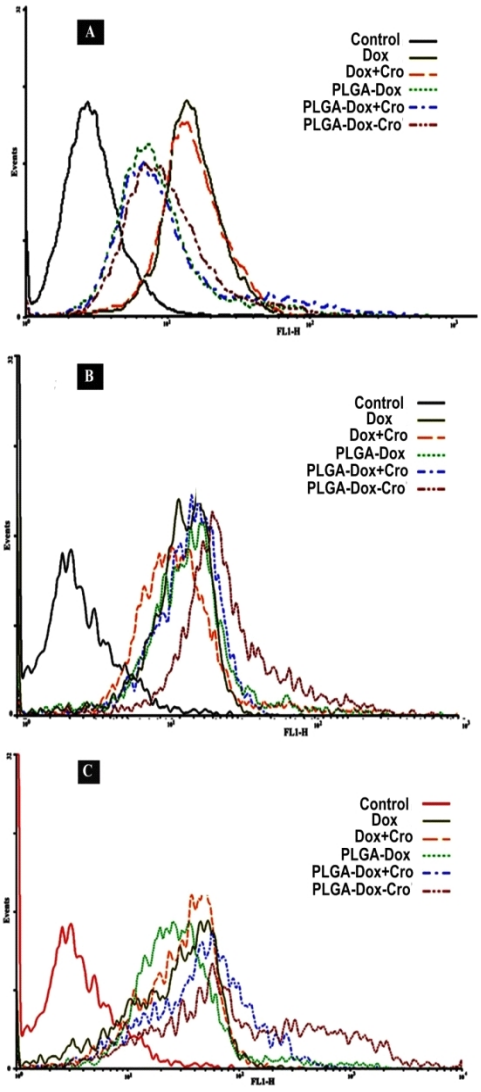

Fig. 7. Flow cytometry detection of drugs uptake using fluorescence intensity of the cells after 2h (A) , 24h (B) and 48h(C) incubation with free DOX, DOX+Cro, PLGA-DOX, PLGA-Dox+Cro and PLGA-DOX-Cro NPs in MCF-7 cells

CONCLUSION

The effect of crocetin on antitumor activity of doxorubicin encapsulated in PLGA nanpoparticles was investigated against MCF-7 cells. Experiments on *in vitro* drug release and cellular uptake of the PLGA-DOX-Cro formulation indicated that DOX and Cro were efficiently taken up by the cells and released simultaneously. Furthermore, the codelivery of DOX/Cro through PLGA NPs inhibits MCF-7 growth more effectively than the delivery of either DOX or Cro at the same concentrationsIt was proved that the codelivery of DOX and Cro cause apoptosis induction and also increase expression of caspase-3 protein. We also suggested a possible mechanism enhancing the tumor regression rate for the synergistic therapeutic effect of DOX and Cro. In conclusion, the PLGA NPs containing DOX and Cro have the potential for future clinical application.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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