

RESEARCH PAPER

Curcumin improved liposomal mitomycin-induced cell toxicity in bladder cancer cell

Farjad Amanolahi¹, Ali Mohammadi¹, Reza Kazemi Oskuee², Leila Gholami³, Bizhan Malaekheh-Nikouei^{4*}

¹Department of Drug and Food Control, School of Pharmacy, Tehran University of Medical Science, Tehran, Iran

²Targeted Drug Delivery Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

³Department of Modern Sciences and Technologies, School of Medicine, Mashhad University of Medical Science, Mashhad, Iran

⁴Nanotechnology Research Center, Institute of Pharmaceutical Technology, Mashhad University of Medical Sciences, Mashhad, Iran

ABSTRACT

Objective (s): This study aimed at investigation of preparation and stability study of Mitomycin and Mitomycin loaded nanoliposomes and efficacy of the combination of Mitomycin -loaded nanoliposomes and Curcumin versus Mitomycin in suppressing HTB-9 and L929 cell lines, in vitro.

Materials and Methods: An HPLC method was validated based on Q2 (R1) International Conference on Harmonization (ICH) guideline for determination of Mitomycin in pharmaceutical samples. Soybean phosphatidylcholine (SPC) or hydrogenated soybean phosphatidylcholine (HSPC) mixed with cholesterol at 2:1 molar ratio respectively in two different groups to prepare Mitomycin nanoliposomes. Cell toxicity of free Mitomycin, Mitomycin loaded liposomes and curcumin was measured using MTT assay in bladder cancer cell line for treatment group and Fibroblast-like cell as control group. Also, propidium iodide staining was done to determine the level of DNA fragmentation.

Results: The validated HPLC method showed stability based on the ICH guideline (n=3 and RSD<2%). Encapsulation efficiency values were 78 and 63 % for SPC and HSPC liposomes, respectively. Sizes of SPC and HSPC liposomes were 112 and 128 nm, respectively. Encapsulation efficiency of Mitomycin was 27 and 21 % for SPC and HSPC liposomes, respectively. Addition of Curcumin to the samples remarkably improved Mitomycin cytotoxicity. Concerning DNA fragmentation, Curcumin exhibited a protective effect when used in combination with Mitomycin.

Conclusion: Synergistic effects of Curcumin and Mitomycin were observed in terms of cell toxicity. Together, since Curcumin exerts anti-oxidative properties, its co-administration with a chemotherapeutic agent might protect normal cells from adverse effects of such drugs.

Keywords: Curcumin, Combination therapy, Cytotoxicity, Liposome, Mitomycin

How to cite this article

Amanolahi F, Mohammadi A, Kazemi Oskuee R, Gholami L, Malaekheh-Nikouei B. Curcumin improved liposomal mitomycin-induced cell toxicity in bladder cancer cell. *Nanomed J.* 2018; 5(4): 235-244. DOI: 10.22038/nmj.2018.05.00008

INTRODUCTION

Mitomycin is an important antitumor and anti-fungal molecule that was isolated from *Streptomyces caespitosus* and *Streptomyces lavendulae*. Mitomycin acts as an alkylating agent, markedly crosslinks DNA double helix and causes apoptosis in cancer cell [1].

Mitomycin is often administered intravesically as a chemotherapeutic agent in bladder cancer

management and was able to prevent cancer recurrence when used in long-term maintenance regimens [2], or in combination with other therapeutic agents [3]. It has previously been shown that intravesical delivery of Mitomycin to the bladder is affected by its dose and pharmacokinetics, as well as residual urine volume, urine production, dosing volume, urine pH and dwell time. Optimization of these parameters improves drug efficacy and oncologic outcomes [3-5]. Common side effects of Mitomycin are allergic reactions such as difficulty

* Corresponding Author Email: malaekheh@mums.ac.ir

Note. This manuscript was submitted on June 30, 2018; approved on August 1, 2018

in breathing, throat tightness, swelling of the lips, tongue, or face, decreased bone marrow function and blood problems such as extreme fatigue, easy bruising or bleeding, black, bloody or tarry stools. Mitomycin has a sensitive chemical structure as it is stable in the solid state but loses activity in solutions. It was shown that Mitomycin kept in protected solution with pH 7 at 5 °C was not stable for 4 days and it must be freshly prepared before injection [6].

Nanoencapsulation technique, as a promising delivery system is being used to improve the bioavailability of hydrophobic and hydrophilic agents and provide dispersible systems to protect sensitive molecules from degradation. Liposomes are small spherical vesicles composed of cholesterol and non-toxic phospholipids. Due to their small size, existence of a hydrophobic bilayer and hydrophilic core in their structure, and advantages such as their ability to enhance drugs' stability following encapsulation, flexibility, biocompatibility and biodegradability, liposomes are attractive and efficient drug delivery systems [7-9].

As the main bioactive compound found in the rhizomes of *Curcuma longa*, Curcumin exerts several pharmacological effects. In traditional Chinese and Indian medicine, *C. longa* has been used for treatment of several human diseases such as inflammatory conditions [10]. Many studies have reported several molecular targets and pathways such as Cyclin D1, TNF, IL 2, 6 and 8 and NFκB for Curcumin and indicated different therapeutics effects such as anti-inflammatory [11-14], anti-rheumatic, anti-carcinogenic, anti-microbial and hepatoprotective activities for this compound [15-20]. Generally, Curcumin is considered as safe compound even at high doses. Extensive clinical trials demonstrated that Curcumin at doses as high as 12 g/day, is well tolerated and without evidence of toxicity [21-23]. Therefore, acceptable safety and efficacy profiles along with considerable evidence for its pharmacological benefits, suggest that Curcumin should be regarded as a potential candidate for treatment and/or prevention of a wide variety of human diseases especially for prevention and treatment of several types of cancer. Administration of some chemotherapeutic agents such as Mitomycin increases the production of several free radicals. Curcumin with anti-oxidative properties, can be co-administered with chemotherapeutics to reduce their adverse effect

[24-25].

Therefore, the present study aimed at preparation and characterization of Mitomycin nanoliposomes and investigating the effect of prepared carriers on Mitomycin stability. In another part of this study, the possible synergistic effects of Curcumin and liposomal Mitomycin were evaluated in cancer bladder cell line.

MATERIALS AND METHODS

Methanol, chloroform, ammonium acetate, acetonitrile, cholesterol (Chol) and Mitomycin were purchased from Sigma-Aldrich (Germany). Egg phosphatidylcholine (SPC) and hydrogenated soy phosphatidylcholine (HSPC) were obtained from Lipoid (Germany). In this study, we used deionized and triple-distilled water. HTB-9 cell line (Bladder cancer) and L929 cell line (Fibroblast-like, as normal cell line) were used for cell cytotoxicity study and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C with 5% CO₂ in a humidified atmosphere.

Standard solution preparation

For this, 2 mg of Mitomycin was dissolved in 50 ml phosphate-buffered saline (PBS) to achieve a 40 µg/ml solution of Mitomycin and stored in dark at 4 °C. Calibration solutions were prepared by diluting the stock solution with PBS to obtain different concentrations (1, 2, 5, 10, 20 and 40 µg/ml) of Mitomycin.

HPLC instrument

The HPLC was performed using a Shimadzu (Kyoto, Japan), SCL-10A controller, LC-10ADVP pump and Shimadzu UV-SPD-10AVD spectrophotometric detector. A Varian C18 column (150 mm×4.6 mm×5 µm particle size) was used for separation. An isocratic system consisted of acetonitrile and 0.1 M phosphate buffer (20:80 % v/v; pH 7). Flow rate and detection wavelength were 1 ml/min flow rate and 365 nm, respectively. All solutions and samples were filtered through a 0.45-µm nylon membrane. The volume of injection was set at 20 µl.

HPLC validation

HPLC validation was done according to Q2 (R1) ICH guideline.

Precision and accuracy

Precision and accuracy of the method were

determined by three different concentrations of Mitomycin (5, 10, and 20 µg/ml). Intra-day precision and accuracy were evaluated by six replicates for each concentration. Inter-day precision was checked for each concentration in 3 days.

The precision was evaluated by percentage relative standard deviation (%RSD) and accuracy was calculated as the percentage of recovery by the following equation:

$$\text{Recovery}(\%) = (\text{Detection Concentration}) / (\text{Actual Concentration}) \times 100$$

Linearity and range

Six Mitomycin solutions with concentrations ranging from 1 to 40 µg/ml were analyzed in triplicate for each concentration. The linearity was evaluated by calculating the slope and y-intercept of calibration curve and coefficient of determination (r^2) was measured using least square regression.

Limit of detection (LOD) and quantification (LOQ)

Using the standard deviation (SD) of y-intercept and the slope (s), LOD and LOQ were calculated based on the following formula:

$$\text{LOD} = 10 \text{ SD}/s$$

$$\text{LOQ} = 3.3 \text{ SD}/s$$

Robustness

Flow rate, pH of mobile phase and mobile phase condition were changed to study the robustness of the method. pH of the buffer and flow rate was changed by ±0.3 unit and mobile phase varied for ±10%. Three different concentrations were injected in triplicates. The acceptance criteria were a %RSD of <2.

Liposome preparation

Mitomycin liposomes (SPC or HSPC) were prepared by the dehydration-rehydration method. Phospholipid and cholesterol at different molar ratios of 2:1 were mixed in 3 ml of methanol:chloroform (2:1 v/v). Solvent was removed and rehydration was done by adding Mitomycin solution (2 mg/ml) to phosphate-buffered solution (pH 7) at temperature above T_m of phospholipids. Samples were sonicated for 5 min to reduce the size of liposomes. Afterwards, liposomes passed through 1000, 600, 400, 200 and 100 nm filters. Liposomes were kept in vials wrapped in aluminium foil, at 4 °C.

Characterization of liposomes

Loading capacity was determined by an indirect method. Briefly, liposomes were centrifuged at 25000 rpm for 3 min. Supernatant containing unloaded Mitomycin was removed and the amount of Mitomycin was determined by the currently validated HPLC method. Loading capacity was calculated by the below-noted equation:

$$\% \text{ Entrapment Efficiency} = (\text{Initial drug added} - \text{Drug in supernatant}) / (\text{Initial drug added}) \times 100$$

Mean size of liposomes was determined using a dynamic light scattering method by Zetasizer 3000 HSA (Malvern, UK). Here, 0.1 mL of each liposome type was diluted in 0.9 mL phosphate-buffered solution, and particle size was measured three times every two weeks for 3 months. Data were reported as means ± standard deviations.

Mitomycin solution stability

To study the stability of Mitomycin in solution, accelerated stability study was done under ICH Q₂B guideline condition. For this purpose, 60 µg/ml Mitomycin solution (free Mitomycin as well as Mitomycin -loaded SPC and HSPC liposomes) were kept at 25±2 °C with 45±5 % humidity for 6 days. The amount of remaining Mitomycin was determined by HPLC.

In-vitro drug release

Dialysis bag (Sigma-D9652) method was used as previously described [23] to study the in-vitro release of Mitomycin from nanoliposomes. For this, 2 mL of liposomal and non-liposomal Mitomycin at the concentration of 4 µg/ml were placed in dialysis bag in phosphate-buffered solution (0.02 M; pH 7). All bags were kept at 37 °C and stirred at 100 rpm for 10 h. The amount of released Mitomycin was hourly determined for 10 hours by HPLC every one hour.

In-vitro cell viability

Viability of HTB-9 (1×10^5 cells) and L929 (1×10^8 cells) cells following incubation with free Mitomycin and Mitomycin -loaded nanoliposomes was examined using (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay [24]. Serial doses of Mitomycin -loaded nanoliposomes (15, 7.5, 3.75, 1.875, 0.987 and 0.459 µg/ml) and free Mitomycin (25, 12.5, 6.25, 3.125, 1.55 and 0.77 µg/ml) were added to the plates and incubated at 37 °C with 5% CO₂ for 24 and 48 h. Curcumin was used for combination treatment

with Mitomycin in MIC concentration of them. For this, serial doses of Curcumin were added to the plates of L929 (200, 100, 50, 25, 12.5 and 6.25 µg/ml) and HTB-9 (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) and incubated in similar condition. Then, cells were treated with 20 µL of MTT solution for 3 hr at 37 °C with 5% CO₂; next, the medium containing MTT solution was removed and 150 µl of DMSO was added to each well to dissolve MTT formazan crystals. Then, absorbance was measured by a plate reader at 545 nm.

$$\text{Cell Viability\%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}$$

After above procedures and calculated MIC concentration of each group, another MTT assay was performed to study combination treatment of Curcumin with free Mitomycin and Mitomycin loaded liposomes (SPC and HSPC) at MIC concentration in HTB-9 cell line. For this, 100 µl of Curcumin at MIC concentration was mixed with 100 µl of free Mitomycin and Mitomycin loaded liposomes (SPC and HSPC) and 50 µl of it added to the plate of HTB-9 cell line. Plate incubated at 37 °C with 5% CO₂ for 24 and absorbance of MTT formazan crystals was measured at 545 nm.

DNA fragmentation

DNA damage was calculated based on a previously described method [26]. Briefly, L929 and HTB-9 cells were treated with free Mitomycin as well as Mitomycin -loaded nanoliposomes (SPC and HSPC) at IC₅₀ concentration and incubated at 37°C with 5% CO₂ for 24 hr.

To study the possible synergistic, anti-oxidative and protective effect of Curcumin, cells (both L929 and HTB-9 cells) were exposed to free Mitomycin and Mitomycin -loaded liposomes with Curcumin at same time in separate groups

at IC₅₀ concentration and incubated at 37 °C with 5% CO₂ for 24 hr. Lysis buffer containing 20 mM EDTA 0.5 % and 5 mM Tris HCl (pH 8) was used for DNA extraction. Lysis buffer was added to the cell suspension in microtubes and kept at 4 °C for 30 min to complete cell lysis.

The tubes were centrifuged at 2×10⁴ rpm for 20 min and the supernatant was removed.

Subsequently, 1 mL of trichloroacetic acid (TCA) was added to the pellet and supernatant, separately. After incubation at 4 °C for 24 hr, all tubes were centrifuged again and 1 ml of TCA was added to the pellet.

All tubes were kept in a water bath (at 100 °C) for 20 min and then, centrifuged (supernatant and pellet) at 3000 rpm for 15 min. Then, 1 ml of diphenylamine reagent was added to 0.5 mL of supernatant and the mixture was incubated at 37 °C for 5 hr. The absorbance was read by a spectrophotometer at 600 nm, and the level of damage DNA was determined using the following equation:

$$\% \text{ Fragmentation} = \frac{\text{absorbance of supernatant}}{\text{absorbance of (supernatant+pellet)}}$$

Statistical analysis

For each protocol, all experiments were performed in triplicate and data were expressed as mean± standard deviation (SD). Statistical analysis was performed by using Prism Software Ver.5. Comparison between differences of means was made by one-way ANOVA analysis and a p-value ≤ 0.05 was considered significant.

RESULTS

HPLC validation

Table 1 shows the precision and accuracy of the method. % RSD for all data in intra-day and

Table 1. Precision (% RSD) and accuracy (% recovery) of RP-HPLC for determination of Mitomycin levels

Test	Result	Stock concentration (µg/ml)	Amount found (µg/ml)	% RSD	% Recovery	
Repeatability (intra-day), n=9	Time 1	5	5.05	0.087	101	
		10	9.9	0.127	99	
		20	19.8	0.234	99	
	The First Day	5	5.09	0.099	101.8	
		10	10	0	100	
		20	20	0	100	
	Inter-day precision, 3 days, n=9	The Second Day	5	5.04	0.078	100.8
			10	9.9	0.118	99
			20	19.9	0.206	99.5
The Third Day		5	5	0	100	
		10	10	0	100	
		20	20.05	0.089	100.25	

inter-day precision was < 2 and recovery factor in ICH guideline range (100 ± 2 %).

So, it was concluded that this method was found precise and of high accuracy.

Correlation coefficient (R²=0.9999) and validation equation of method (Y= 8468.6 X –1301) showed linearity of this method at concentrations ranging from 1 to 40 µg/ml.

Table 2 showed robustness of the method. According to ICH validation guideline, any validated method must have a % RSD of <2 and recovery factor should be within 100±2 %. Based on Table 2, this method showed robustness at a concentration range of 1-40 µg/ml.

Table 2. Evaluation of robustness of HPLC method (n=3)

Parameter	Modification	Mitomycin	
		% RSD	% Recovery
pH	6.7	0.67	98.9
	7	0.99	99.6
	7.3	1.1	100.3
Buffer Composition (ratio)	75	1.4	100.1
	80	0.92	100.1
	85	1.1	99.3
Flow Rate (ml/min)	0.9	0.87	99.1
	1	0.89	98.3
	1.1	1.6	100.5

Physicochemical characterization of nanoliposomal curcumin

There was a significant difference in size distribution between the two types of nanoliposome (P< 0.0014). In SPC and HSPC liposomes, size of liposomes was increased during storage at 4 °C (P<0.001) (Fig 1). Mitomycin encapsulation rate was 27 % and 21 % for SPC and HSPC liposomes, respectively.

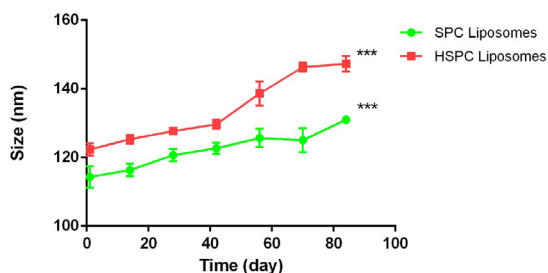


Fig 1. Size distribution during storage at 4 °C. Mean size of liposomes was analyzed by the DLS measurement method every 2 weeks. All groups had significant increase in particle size. Data is presented as mean ± SD of triplicates. *** P < 0.001 (Comparison between first and last points within each group)

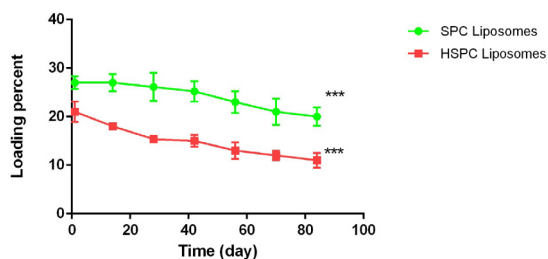


Fig 2. Mitomycin loading percent in SPC and HSPC liposomes. Mitomycin content was determined by RP-HPLC at 365 nm every 2 weeks. Data show significant decrease in Mitomycin encapsulation in liposomes during storage at 4 °C. Data is presented as mean ± SD of triplicates. *** P < 0.002 (Comparison between first and last points within each group)

Fig 2 shows significant decreases in encapsulation rate for SPC (P<0.001) and HSPC (P<0.001) liposomes during storage at 4 °C.

Stability of mitomycin solution

Fig 3 shows results of accelerated stability study of free Mitomycin and Mitomycin -loaded nanoliposomes (SPC and HSPC liposomes) in phosphate-buffered saline (PBS; pH7) at 27 °C. Mitomycin loaded in nanoliposomes was more stable compared to free Mitomycin (i.e. statistical analysis showed P-values of 0.0240 for HSPC and 0.0053 for SPC as compared to free Mitomycin). At the end of experiment, free Mitomycin was decomposed after 3 hr and only 4 % of it, was detected; On the other hand, SPC and HSPC liposomes protected Mitomycin against hydrolysis and over 60 % and 78 % of Mitomycin was detected in HSPC and SPC liposomes, respectively.

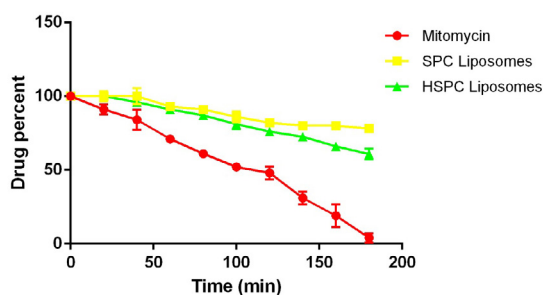


Fig 3. Accelerated stability study of Mitomycin and Mitomycin loaded liposomes (SPC and HSPC) in PBS (pH= 7). Mitomycin was determined by RP-HPLC at 365 nm every 20 min. Mitomycin loaded in both SPC and HSPC liposomes was more stable than free Mitomycin. (P value 0.0053 for SPC and 0.0240 for HSPC)

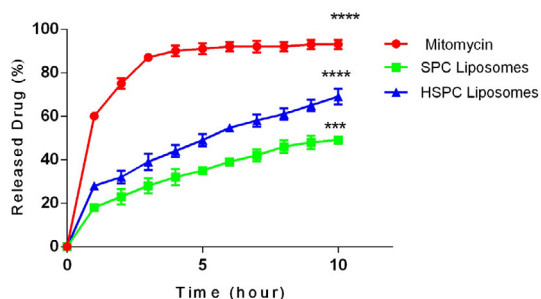


Fig 4. Percentage of drug release for free Mitomycin and Mitomycin-loaded nanoliposome, in vitro. Samples were placed in dialysis bag at 4 µg/ml for 10 h (PBS, pH 7; 0.02 M). *** P< 0.001 and **** P<0.0001

Study of in-vitro drug release

According to Fig 4, 60 % of free Mitomycin was detected outside the dialysis bag after 1 hour; but, in case of SPC and HSPC, 18 and 28 % of Mitomycin was released, respectively.

At the end of the experiment, 93 % of free Mitomycin was released while Mitomycin loaded in nanoliposomes, 69 % (for HSPC) and 49 % (for SPC) were released.

In vitro cell viability study

In the in vitro study, we used 6 concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.1 µg/ml) of free Mitomycin (MMC), Mitomycin nanoliposomes (SPC and HSPC) and Curcumin.

Fig 5a and 5b show the toxic effects of Mitomycin and liposomes containing Mitomycin and Fig E show toxicity effect of Curcumin in HTB-9 cell line, as reflected by percentage of cell viability.

For better comparison between groups, Table 3 show IC₅₀ concentration in all groups and makes a comparison among the groups in terms of minimum inhibitory concentration (MIC).

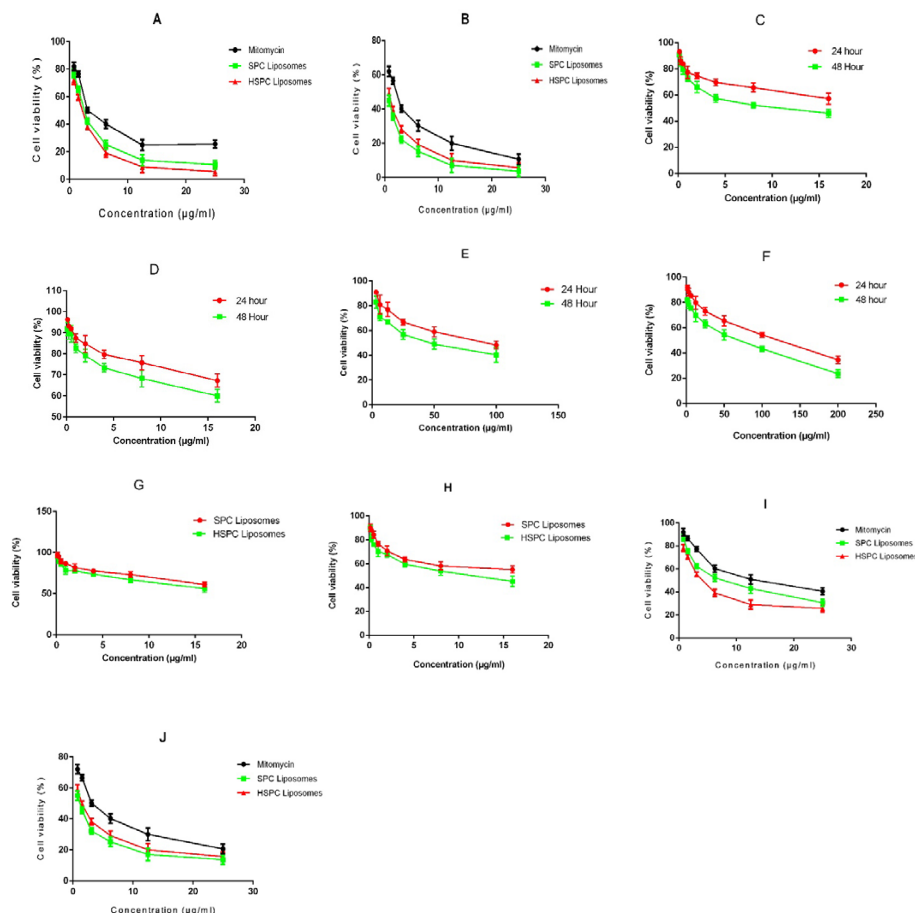


Fig 5. Viability of HTB-9 cells following 24- and 48-h treatment with free Mitomycin and Mitomycin-loaded liposomes (A and B); Viability of HTB-9 cells following 24- and 48-h treatment with Mitomycin-free HSPC and SPC phospholipids (C and D); Viability of HTB-9 cells following 24- and 48-h treatment with Curcumin (E); Viability of L929 cells following 24- and 48-h treatment with Curcumin (F); Viability of L929 cell following 24- and 48-h treatment with SPC and HSPC phospholipids (G and H); Viability of L929 cells following 24- and 48-h treatment with free Mitomycin and Mitomycin-loaded liposomes (I and J)

Table 3. MIC of different formulations against L929 and HTB-9 cell lines calculated following 24- and 48-hr treatments

Groups (µg/mL)	L929, 24 h	L929, 48 h	HTB-9, 24 h	HTB-9, 48 h
Free Mitomycin	13.5	3.509	4.241	1.844
SPC liposome	7.623	1.076	2.482	0.6561
HSPC liposome	4.235	1.445	1.976	0.8083
Void SPC liposome (µmol/mL)	42.68	19.40	78.61	42.26
Void HSPC liposome (µmol/mL)	32.42	10.39	35.28	9.393
Curcumin	106.8	50.32	45.53	26.35

In another part of this research, we examined possible synergistic effect of Curcumin and free Mitomycin and Mitomycin -loaded liposomes on percentage of cell viability. Curcumin at MIC (at 24 hr) was added to the samples along with either free Mitomycin or Mitomycin -loaded liposomes at MIC (at 24 hr).

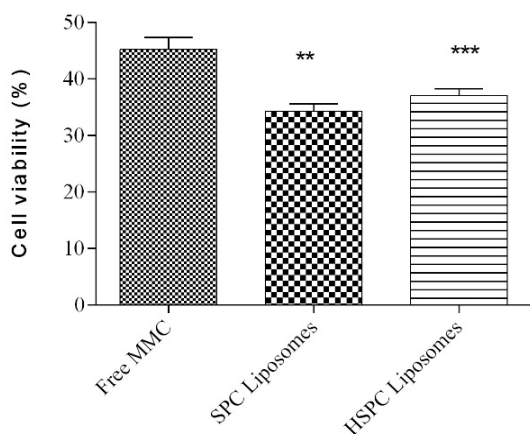


Fig 6. Synergic effect of Curcumin on cell viability of sample that affected by MIC concentration of free Mitomycin (MMC) and Mitomycin loaded liposomes. 100 µl curcumin solution at MIC concentration added to the plates containing free Mitomycin and Mitomycin loaded liposomes (** P value < 0.001, *** P value < 0.0001)

Fig 6 shows results of co-administration of Curcumin along with either free mitomycin or Mitomycin-loaded liposomes, on cell viability.

DNA fragmentation

As shown in Fig 7, treatment of HTB-9 cells with free Mitomycin, Mitomycin -loaded liposomes (SPC and HSPC) caused 81.5, 84.6 and 88.7% DNA damage, respectively. Similar results were seen when L929 were treated with the above-mentioned formulations. Nonetheless, no significant differences between the two cell lines were observed in terms of percentage of DNA

damage; but, co-treatment of cells with Curcumin along with either free Mitomycin and Mitomycin -loaded liposomes caused significant decreases in %DNA damage that were attributed to the antioxidant effects of Curcumin.

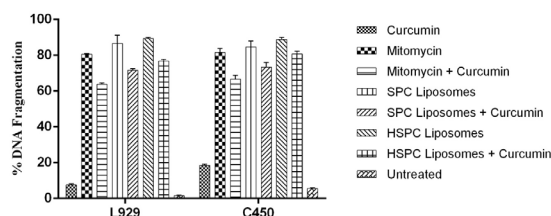


Fig 7. DNA fragmentation percentage in L929 and HTB-9 cell lines as determined based on Burton assay. Cells were treated with different formulations at MICs

DISCUSSION

Bladder cancer involves epithelial cells of urinary bladder. According to the report published by World Health Organization (WHO) in 2014, bladder cancer was recognized as the 9th cause of death. Several approaches such as immunotherapy, radiotherapy and chemotherapy are used for treatment of bladder cancer. Mitomycin, as an antibiotic with chemotherapeutic activity is a natural product with aziridine moiety which was isolated from *Streptomyces caespitosus* [25]. Mitomycin acts as a DNA cross-linking molecule and inhibits RNAase activity in cancer cells causing cell death. This activity is not only exerted in cancer cells but all cells exposed to Mitomycin undergo apoptosis which contributes to several side effects of this agent. Mitomycin is sensitive to hydrolysis and photodegradation. Mitomycin has been prepared in the form of a lyophilized powder for injection and this formulation must be kept in dark at 4 °C to reduce the rate of degradation. In the present study, we reported a new RP-HPLC method for detection and determination Mitomycin in pharmaceutical finished product with minimum retention time (3.83 min) and

lower chemical analyst compound (phosphate-buffered:acetonitrile in 80:20 ratio). In this research, we prepared two types of liposomes using SPC and HSPC phospholipid which resulted in protection of Mitomycin from hydrolysis. Fig 1 and 2 show the size of liposomes and rate of drug, loading respectively. Results showed significant increases in the size of particle (P value<0.001) and significant decreases in drug loading (P value<0.001) in both SPC and HSPC groups. Also, liposomes (SPC and HSPC) induced a delay in Mitomycin release for. Moreover, *in vitro* study demonstrated that liposomes acted as delayed drug delivery system where 49% percent of Mitomycin -loaded SPC and HSPC liposomes released 49 and 69% of their load, respectively.

This delivery system increased Mitomycin -induced cytotoxicity. HTB-9 cell line used as bladder cancer cell line. This cell line was developed from a primary tumor of bladder obtained from a 68-year-old Caucasian Male with Epithelial-like morphology. As normal cell line and control group, L929 cell line was used. This cell line has Fibroblast-like morphology and is a subclone of parental strain which is one of the first lines to be established in continuous culture. In all groups, cell viability significantly decreased with increasing concentrations (P<0.05). In groups treated for 48 hours, higher toxicity and significantly lower cell viability were observed in comparison to groups treated for 24 hr, indicating the effect of time on Mitomycin efficacy. Moreover, Mitomycin loaded in liposomes was more effective than free Mitomycin at equal concentrations. Liposomes protected Mitomycin from hydrolysis and induced a delay in drug release leading to higher levels of cell toxicity observed at lower concentrations. Also, probable cytotoxicity of SPC and HSPC phospholipids on HTB-9 cell line, was evaluated (Fig 5c and 5d). Results showed that nanoliposomes prepared from HSPC phospholipid, were more toxic than those comprising SPC phospholipid at equal molarity. Fig 5e and 5f show the effect of Curcumin in HTB-9 and L929 cell lines, respectively. In both cell lines, Curcumin significantly decreased cell viability with increasing Curcumin concentrations in media (P<0.0012). Mitomycin -free SPC and HSPC liposomes were also incubated with normal cell line (Fig 5g). It was observed that Mitomycin -free liposomes do not exert major toxicity at standard doses. Furthermore, toxicities of 24- and 48 hr treatment with free Mitomycin and

Mitomycin -loaded SPC and HSPC liposomes were studied on L929 cell line; results were comparable to those observed for HTB-9 cell line. Percentage of cell viability in L929 cell line was decreased by increasing Mitomycin concentrations (Fig 5h and 5i). According Table 3, a significant difference was observed between SPC and HSPC liposomes in terms of MIC. In this regard, Mitomycin loaded in nanoliposomes prepared from SPC phospholipid more effectively reduced the proliferation of cancer cells at lower concentrations of Mitomycin. Following 48-h incubation in all groups significant decreases in MIC and cell viability were observed.

Based on the literature, Curcumin (diferuloylmethane) possesses chemotherapeutic potential. This nontoxic plant-derived polyphenol is an active component of the perennial herb, *C. longa* (commonly known as turmeric) [26]. It has exhibited anti-tumorigenic effects in various types of cancer. Cyclin D1 is a protein which is required for progression through G1 phase of the cell cycle and is degraded when the cell enters S phase. In mutagenic cells, over expression of this protein causes an increase in the number of cancer cells and metastasis. Curcumin downregulates this protein and inhibits apoptosis-mediated cells death. Nuclear Factor Kappa-light-chain (NFkB) is another molecular target of Curcumin. This protein controls the transcription of DNA and many cytokines. NFkB protects the cell from physiological death and apoptosis. Cancer tissues present increased expression of NFkB gene. This protein regulates the expression of anti-apoptotic genes specially TRAF1 and TRAF2, leading to down regulation of caspase enzymes that are responsible for apoptosis. Curcumin significantly decreases NFkB gene expression resulting in induction of apoptosis [27]. Several studies have examined the effects of co-administration of Curcumin and chemotherapeutics. In 2015, a research done on breast cancer, showed that Curcumin increases anticancer/tumoricidal effects of Mitomycin by suppressing ABCG2 gene expression [28]. In another study, Curcumin reduced Mitomycin resistance in breast cancer by regulating the expression of Bcl-2 gene which has an important role in apoptosis [29]. In the present study, we used Curcumin along with Mitomycin in bladder cancer cell lines, to study the synergistic effect of Curcumin on the cytotoxicity of Mitomycin. Fig 6 shows synergistic effect of Curcumin and free Mitomycin and Mitomycin -loaded liposomes used

at their MICs. Data showed remarkable decreases in cell viability in groups treated with Curcumin along with Mitomycin formulations compared to groups that were not treated with Curcumin. Following treatment with Mitomycin at MIC, 50 % cell viability was observed, but this rate decreased to 43.5 % when Curcumin was added to the wells. Under similar conditions, treatment with SPC and HSPC liposomes resulted in 35.7 % and 36.8% cell viability, respectively. This data demonstrated that Curcumin has additive effect and it could improve tumoricidal effects of Mitomycin on bladder cell lines. Beside its anticancer effects, Curcumin has anti-oxidant and anti-inflammatory effects [30, 31]. Free-radical-mediated peroxidation of membrane lipids and oxidative damage of DNA and proteins are believed to be associated with a variety of chronic pathological complications such as cancer, atherosclerosis, and neurodegenerative diseases.

The anti-inflammatory effect of Curcumin is most likely mediated through its ability to inhibit cyclooxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) which are important players of inflammatory and oxidative processes [32].

In the present study, Curcumin improved Mitomycin cytotoxicity and helped to reach maximum effect at lower concentrations. As presented in Fig 7, percent of DNA fragmentation following treatment with free Mitomycin as well as SPC and HSPC liposomes decreased about 15 percent that also demonstrated high anti-oxidant potency of Curcumin.

CONCLUSION

in this study, we validated a new RP-HPLC method for detection and determination of Mitomycin in pharmaceutical finished products and stability studies. Preparation of Mitomycin liposomes caused significant increases in Mitomycin stability and decreased the doses of drug required to induce cytotoxic effects. In another part of this research, we demonstrated that co-treatment with Curcumin and Mitomycin was more effective than treatment with Mitomycin alone. Anti-oxidative effect of Curcumin could protect cells from Mitomycin side effects; but, more studies should be done to discover the effect and underlying mechanism of cytotoxic effects of Curcumin with and without Mitomycin in treatment of bladder cancer.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by Tehran University of Medical Sciences, to perform this study.

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