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RESEARCH PAPER

Anti-tumor activity of nanoliposomes containing crude extract of saffron in mice bearing C26 colon carcinoma

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ABSTRACT

Objective(s): Saffron, the dehydrated stigma of the *Crocus sativus L.* flower, has been reputed as an effective anticancer and chemopreventive agent in cancer therapy. This study aimed to design PEGylated nanoliposomes containing crude extract of saffron for the treatment of cancer.

Materials and Methods: Various PEGylated nanoliposomes containing 25 mg/ml aqueous extract of saffron were prepared using the thin lipid film method. The characterization of liposomes was indicated by their size, *in vitro* cytotoxicity, and *in vivo* therapeutic efficacy against C26 tumor-bearing mice.

Results: By increasing cholesterol levels, the IC_{50} values of the formulations increased. Liposome characterization illustrated the properties of formulation of choice, as follows: Z-average size: 73.7 ± 1.3 nm; PDI: 0.103 ± 0.035; zeta potential: -20.8 mV ± 3.7; % encapsulation: 91 ± 0.059, % release after 168 hours in 30% FBS: 16.26 ± 0.01.

Conclusion: Treating tumor-bearing mice with the selected saffron liposomes indicated that, for the first time, the i.v. injection of nano-liposomal saffron at a dose of 300 mg/kg significantly increased the anti-tumor property compared to the negative control group, while no significant difference was observed compared with aqueous extract of saffron. Hence, to achieve an optimal formulation for human use, the formulation merits further study.

Keywords: *Anti-tumor activity, C26 colon carcinoma, Experimental study, PEGylated nanoliposome, Saffron*

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INTRODUCTION

Saffron, the world's most expensive traditional spice is the dried stigma of the flower named *Crocus sativus L.* native to the Eastern Mediterranean and belongs to the Iridaceae family, the line of Liliaceae. Saffron is contributed to three crucial bioactive compounds including crocin, a carotenoid compound responsible mainly for the special color of saffron, picrocrocin, mono-terpene glycoside and precursor of safranal that induce the bitter taste of saffron as well as safranal which gives the special odor to saffron [1]. Beside its use as a natural food colorant, saffron bears health-promoting properties such as an antitumorigenic [2-8] antioxidant [9-12], antidepressant and anxiolytic [9, 13, 14],

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memory-enhancing [15-18], aphrodisiac [19-21], genoprotective [22, 23], cardioprotective [24, 25], neuroprotective [26], antitussive [27]. Investigations have contributed advantageous effects of saffron to its antioxidant and antitumor properties associated with its active constituents. For the first time in the 1990s, it was reported that saffron extract reach in carotenoids could suppress the growth of malignant cells *in vitro* and *in vivo* [28-30]. Further *in vitro* studies on human cervical carcinoma Hela cells proved this inhibitory effect exerted by crocin [2, 4, 28]. It has been reported that saffron may exert toxicity against cancer cells via various mechanisms including inhibition of RNA and DNA synthesis and increasing apoptosis. Animal studies have also demonstrated the anti-tumor effect of saffron which may attribute to its antioxidant and pro-apoptotic actions in cancer cells [31]. Long-term treatment of rats bearing colorectal tumors with crocin showed a significant increase in their survival and decreased tumor growth [5, 32].

Liposomes, as promising vehicles, could molecules to the targeted site and reduce off-target adverse effects contrasted to free drugs [33]. Long-circulating PEGylated nanoliposomes tend to accumulate passively in tumor tissue using enhanced permeation and retention (EPR) phenomenon. The foundation of this phenomenon is using the pathophysiological and anatomical differences between the healthy and tumorous tissues for selective targeting of chemotherapeutic drugs to the area of tumors due to the absence of efficient lymphatic drainage. Thus, by lessening MPS (mononuclear phagocyte system) uptake of particles, liposomes can improve the therapeutic effects of drug molecules and other anticancer agents [33].

The current investigation was developed to investigate the effect of long-circulating nanoliposome encapsulating crude extract of saffron in treating colon cancer in animal models and comparing its efficacy with saffron.

MATERIALS AND METHODS

Chemicals

Hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and methoxy-polyethylene glycol (MW 2000)-di-stearoylphosphatidylcholine (mPEG2000-DSPE) were acquired from Avanti Polar Lipids (Birmingham, USA). Saffron was purchased from Novin Zaferan (I.R.I). Histidine and sucrose were obtained from Sigma (St. Louis. MO, USA). Caelyx™ (Liposome Doxorubicin HCl 2 mg/ml, Gilead Sciences, Inc., Foster City, CA) was purchased and reconstructed according to the manufacturer's instruction. Trypan blue, 3-(4, 5-dimethylthiaol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Dimethyl Sulfoxide (DMSO), isopropanol, and chloroform were procured from Merck (Darmstadt, Germany). C26 cells were purchased from Cell Lines Service (Eppelheim, Germany).

Saffron extraction

Crocus sativus L. stigmata from Novin Saffron CO. were accumulated from Ghaen city, Khorasan province, North-East of Iran. Mazhari identified the flora, and a specimen of receipt (No. 11135) entrusted at the Herbarium of Mashhad School of Pharmacy was examined by the ISO/TS 3632-2. The characteristics of the saffron sample according to the analysis report were as follows:

Total ash (Mass), on dry matter %Max: 4.41- E1cm 1% 257 nm, on a dry basis, Min. (At this wavelength it has a maximum absorbency of Picrocrocin): 82.71- E1cm 1% 330 nm, on a dry basis, Min-Max. (At this wavelength it has a maximum absorbency of Safranal): 38.70- E1cm 1% 440 nm, on a dry basis, Min. (At this wavelength it has a maximum absorbency of Crocin): 225.67 For saffron extraction, 8g of the dried stigmas of saffron were weighted and added to 300 ml of distilled water. The mixture was then incubated in the shaker incubator (at 40 °C) for a period of 72 hr. After liquid extraction, the samples were centrifuged and filtered through a paper filter membrane. The water-soluble extract was frozen and then dried in freeze-dried. The dark orange powder obtained was stocked at -20 ºC until use. A small amount of 5 µg/ml of saffron was dissolved in distilled water to obtain the maximum absorption, and absorption was observed in wavelength ranges 200-600 nm.

Liposome preparation

Liposome preparation was conducted by the hydration of thin lipid film, after that sonication and then extrusion [34]. In a word, a solution of

lipid components in chloroform was prepared as the desired volume of lipid components was mixed in a round-bottom flask following the molar ratio of each formulation represented in Table 1. Removal of solvent using a rotary evaporator (Heidolph, Germany) resulted in a thin lipid film formation. The film was freeze-drier overnight to remove traces of organic solvent (Taitec, Japan). Crude extract of saffron was dissolved in Histidine buffer (10 mM, pH 6.5) at 25 mg/mL. The hydrated lipid film was prepared by adding the required amount of aqueous extract of saffron solution at 60 ºC. Liposomes were then sonicated in a bath-type sonicator (Decon, England) for 15 min at 60 ºC and repeatedly extruded (Avastin, Canada) through 200, 100, and 50 nm polycarbonate membranes at 60 ºC to make uniformed size liposomes. Thereafter, a dialysis cassette (Mwt cut off 12 kDa) was filled by the preparations, and the dialysis was carried out three times against 10 mM Histidine, 10% sucrose pH 6.5 to detach the un-encapsulated saffron extract. To evaluate saffron encapsulation, an aliquot of liposome preparations before-after dialysis was lysed with acidified alcohol (90% isopropanol / 0.075 M HCl). The percentage of encapsulation was calculated as below:

amount of saffron in purified liposomes/ amount of saffron in unpurified liposomes ×100

Liposome characterization

The size of liposomes was assessed in triplicate using Dynamic Light Scattering Instrument (Nano-ZS; Malvern, UK). The zeta potential of particles was also evaluated on the same device using the zeta potential mode as the average of 20 measurements. Particle sizes were revealed as the means ± standard deviation and polydispersity index (PDI) (n=3). Zeta potentials were reported as the means ± zeta deviation (n=3).

Release studies

Liposome formulations were incubated in RPMI1640 medium (Roswell Park Memorial Institute1640) in the presence of 30% Fetal Bovine Serum (FBS) at 37 °C [35]. At different intervals, samples were removed and dialyzed against 10 mM Histidine, 10% sucrose pH 6.5, to eliminate the released saffron. Then, the remaining liposomes in the dialysis cassette were lysed with acidified alcohol and assayed as described above. The release percentage was calculated as below:

%Release=amount of saffron in purified liposomes after release test/amount of saffron in unpurified liposomes before release test×100

Cell culture and cytotoxicity assay

Mouse colon cancer cells, C26, were preserved in RPMI 1640 media complemented with 10% FBS and were cultured in 5% CO_2 at 37 ºC.

Cytotoxicity was assessed by MTT assay. C26 cells were seeded in 96-well plates (2000 cells/100 µl) and incubated for 24 hr at 37 ºC to admit cell attachment. Distinct concentrations were loaded triplicate in each well, and the cells were incubated for 72 hr at 37 ºC. The cytotoxicity evaluation was achieved using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance of each well was measured by an ELISA reader (Enzyme-linked immunosorbent assay) (Statfax–2100, Awareness Technology, USA) at 570 nm (34).

Table 1. The properties of saffron nanoliposomes

Saffron formulations	Molar ratio	Zeta average Size (nm) ± SD	Polydispersity ± SD	Zeta potential % (mv) ± SD	Encapsulation (%)
F1-HSPC/ $mPEG_{2000}$-DSPE/Chol	5.5/0.5/4	73.7 ± 1.3	0.103 ± 0.035	-20.8 ± 3.7	91 ± 0.059
F2-HSPC/ $mPEG_{2000}$-DSPE/Chol	5/0.5/4.5	111.4 ± 2.88	0.118 ± 0.025	-19.1 ± 4.9	64 ± 0.132
F3-HSPC/ $mPEG_{2000}$-DSPE/Chol	7.5/0.5/2	129.86 ± 1.5	0.216 ± 0.028	-17.4 ± 3.8	49 ± 0.107
F4-HSPC/ $mPEG_{2000}$-DSPE/Chol	9.5/0.5/0	117.73 ± .93	0.206 ± 0.003	-14.5 ± 4.1	41 ± 0.09

Animals

The 6 to 8 week-old female BALB/c mice were obtained as Colon-26 carcinoma models from the Pasteur Institute (Tehran, Iran). The mice were stored in an animal house of Pharmaceutical Research Center in a colony room 12/12 hr light/dark cycle at 21°C with free access to water and animal food. All courses of action dealing with animals approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences (Education Office, dated Feb. 26, 2008; proposal code 87848) established on the Specific National Ethical Guidelines for Biomedical Research proceeded by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran in 2005.

Anti-tumor efficacy of liposomal saffron in the mouse model

Colon-26 murine cells (3×105/50 µl PBS/mouse) were subcutaneously inoculated in the right hind flank of BALB/c mice. When tumors became palpable, mice were classified into various treatment groups (n=6), including liposomal saffron (L-saffron) (75, 150, and 300 mg/kg), aqueous extract of saffron (75 and 300 mg/kg in Histidine buffer), isotonic PBS (200 µl), Caelyx® (15 mg/kg) and sterile NaCl (0.9 %). All treatment groups received tail vein injections on alternate days for five doses except for Caelyx®, administered as a single dose. Tumor growth, weight, and overall health of mice were monitored every second day for 30 days. Tumor volume was calculated by measuring the tumor in three dimensions with the formula below: tumor volume=height×length×width [33].

According to the ethical consideration, mice were sacrificed in case of tumor enlargement (more than 2cm in one dimension) or a decrease in bodyweight (>15% loss). Mouse survival was interpreted using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) and the Mantel-Cox test.

Statistical analysis

The one-way ANOVA test was applied to assess the considerable differences between various groups. In case of a significant F value, multiple comparison Tukey test was used to compare means of different treatment groups. Results with $P<0.05$ were statistically considered significant. The IC_{50} for each formulation was determined by the CalcuSyn software Version 2.1 (Biosoft, Cambridge, UK). Statistical assessment of survival was accomplished using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) and Mantel-Cox test.

RESULTS AND DISCUSSION

Liposome characterization

The properties of liposomes, including zeta average size, zeta potential, and PDI are shown in Table 1. Hemogenous-sized liposomes had the PDI range of 0.1- 0.2 and mean diameter ranging from 73 to 117 nm. Particles were negatively charged due to the presence of mPEG2000-DSPE in their formulations.

Percentage of entrapment

Encapsulation efficiencies of liposome preparations are shown in Table 1. The percentage of saffron entrapment of F1 was considerably higher compared to F2 ($P<0.05$), F3, and F4 ($P<0.001$).

Release kinetics

The release kinetic of saffron from liposomal formulation was assessed during 168 h. There was a remarkable similarity in release profiles of F1, F2 ($P>0.05$) and F3, F4 ($P>0.05$) in the period of 7-day observation, as shown in Fig 1, while, a significant difference ($P<0.001$) was observed in release profiles of F1 and F2 compared to that of F3 and F4 formulations,

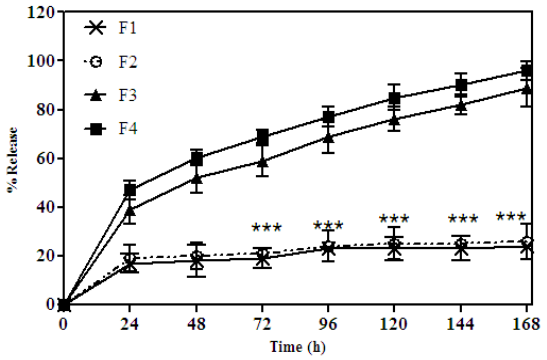

Fig 1. Saffron release profile. F1 (HSPC/ mPEG2000-DSPE/Chol-5.5/0.5/4), F2 (HSPC/ mPEG2000-DSPE/Chol-5/0.5/4.5), F3 (HSPC/ mPEG2000-DSPE/Chol-7.5/0.5/2) and F4 (HSPC/ mPEG2000-DSPE/Chol-9.5/0.5/0). *In vitro* distribution from liposome formulation during 168 h incubated in 30% Fetal Bovine Serum (FBS) at 37 °C. *** Statistical significance ($P<0.001$) between F1 and F2 and F3 and F4 calculated by One-way ANOVA and Tukey–Kramer multiple comparisons test. Error bar represents SEM

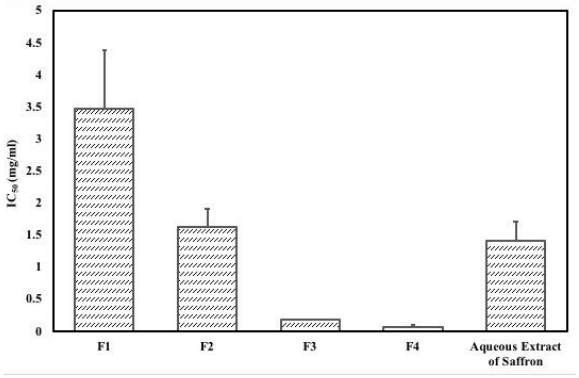

Fig 2. *In vitro* cytotoxicity (IC_{50}). F1 (HSPC/ mPEG2000-DSPE/ Chol-5.5/0.5/4), F2 (HSPC/ mPEG2000-DSPE/Chol-5/0.5/4.5), F3 (HSPC/ mPEG2000-DSPE/Chol-7.5/0.5/2) and F4 (HSPC/ mPEG2000-DSPE/Chol-9.5/0.5/0). C26 cell line at the density of 2000 cells/well were seeded in 96-well and then exposed to saffron extract at different concentrations. Cytotoxicity was assessed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Results are presented as mean mg/ml ± SD (n=3). Error bar indicates SEM

which is probably owing to the higher amount of cholesterol in the first two formulations.

MTT assay

In order to determine the cytotoxicity of aqueous extract of saffron and to compare it with liposomal saffron, an MTT assay was done, and IC_{50} values were calculated using Calcusyn software. The IC_{50} of aqueous extract of saffron was 1.41 mg/ml. The inhibitory effect of F3 and F4 formulations was notably more significant when compared with the aqueous extract of saffron, as shown in Fig 2, while cytotoxic effects of F1 and F2 were lower as apposed to saffron aqueous extract. In other words, IC_{50} of F1 and F2 was higher than saffron extract.

Therapeutic efficacy of saffron liposomal form in the animal model

According to *in vitro* test results, F1 liposomal-saffron (F1-L-saffron) was opted to assess its anti-tumoral effect in Colon-26 tumor-bearing mice. As mentioned previously, bodyweight, tumor size, and survival were observed on alternate days for 30 days. None of the animals were counted out because of bodyweight loss (more than 15%), as described earlier (Fig 3). Fig 4, demonstrated that intravenous injection of a single dose of Caelyx® resulted in tumor growth suppression. Liposomal form of saffron at 75, 150, and 300 mg/kg (P<0.01) had more

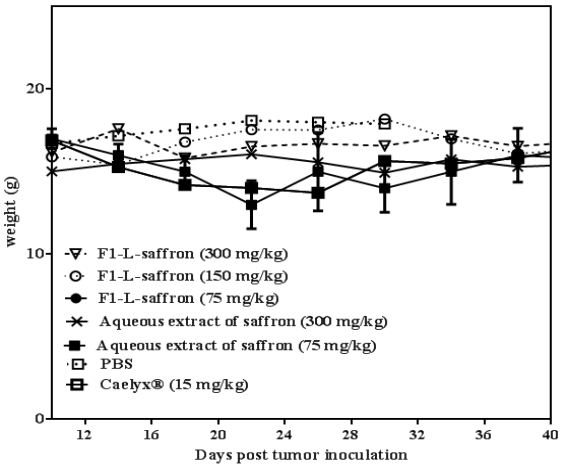

Fig 3. Effect of saffron liposomes treatment on weight of mice. BALB/c mice bearing C26 tumor (n=5) treated on day 10 for 5 consecutive doses on alternate days with i.v. injection of liposomal saffron (L-saffron) (75, 150 and 300 mg/kg), aqueous extract of saffron (75 and 300 mg/kg in Histidine buffer), isotonic PBS (200 µL), sterile NaCl (0.9 %). and a single dose of Caelyx®: liposomal doxorubicin (15 mg/kg). Error bar indicates SEM

significant tumor growth deterrence compared to the negative control on days 20 to 30 after treatment. There was no difference (P>0.05) between liposomal saffron at three doses up to day 22; however, at 300 mg/kg, liposomal saffron showed better control on tumor size

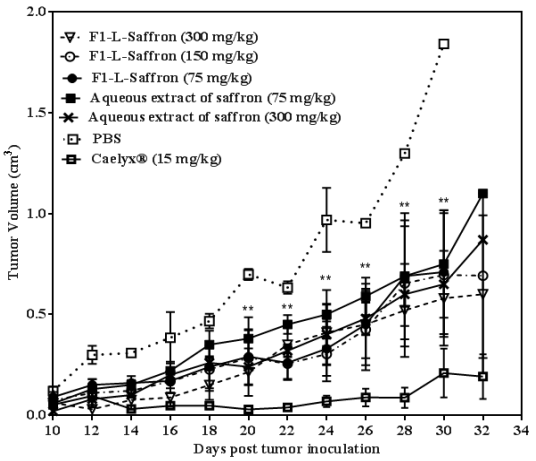

Fig 4. Tumor growth curve. BALB/c mice bearing C26 tumor (n=5) were treated on day 10 for five sequential doses on alternate days with i.v. injection of liposomal saffron (L-saffron) (75, 150 and 300 mg/kg), aqueous extract of saffron (75 and 300 mg/kg in Histidine buffer), isotonic PBS (200 µL), sterile NaCl (0.9 %). and a single dose of Caelyx®: liposomal doxorubicin (15 mg/kg). PBS and Caelyx® curves are identical as in Ref 7; these two experiments were conducted simultaneously. Error bar indicates SEM

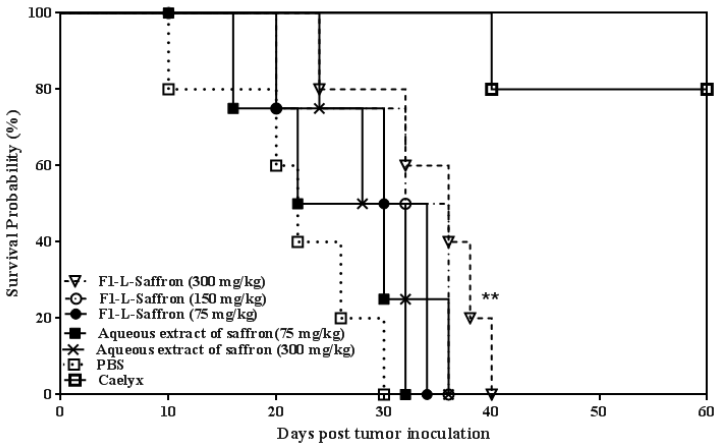

Fig 5. Survival curve. BALB/c mice bearing C26 tumor (n=5) were treated on day 10 for five sequential doses on alternate days with i.v. injection of liposomal saffron (L-saffron) (75, 150 and 300 mg/kg), aqueous extract of saffron (75 and 300 mg/kg in Histidine buffer), isotonic PBS (200µL), sterile NaCl (0.9 %) and a single dose of Caelyx®: liposomal doxorubicin (15 mg/kg). PBS and Caelyx® curves are identical as in Ref 7; these two experiments were conducted simultaneously

than other groups. Moreover, no significant differences (P>0.05) were observed between aqueous saffron extract and liposomal form throughout the monitoring period. Fig 5. clearly illustrated that the survival of mice treated with Caelyx® was significantly greater than other groups. Mice medicated with both aqueous saffron extract and liposomal form had similar survival times (P>0.05); however, the survival of animals treated with F1 at 300 mg/kg increased significantly compared with PBS (P<0.01).

A large body of studies has focused on the effects of saffron and its active components for cancer treatment. Recent scientific evidence has proposed the antitumorigenic contribution of saffron and its potential as the most promising cancer chemopreventive agent [31, 36].

Over almost five decades, liposomal drug delivery has developed critical technical advances such as remote drug loading, extrusion for homogeneous size, long-circulation (PEGylated), triggered release, and active targeting [37].

This study was designed investigate whether saffron liposomes would enhance the anti-tumor efficacy compared to its aqueous extract. Liposomes consisted of various HSPC/cholesterol molar ratios were developed by the thin lipid film method. Size, *in vitro* cytotoxicity, and *in vivo* therapeutic efficacy against C26 tumor-bearing mice were characterized. The IC_{50} of aqueous extract of saffron using MTT assay was 1.41 mg/ml, comparable to the previous report on lung cancer cells [28]. Cytotoxicity assay indicated that IC_{50} values of F1 and F2 were much higher than saffron alone, while IC_{50} values of F3 and F4 were significantly lower compared to aqueous saffron extract. It was well established that cholesterol is an essential ingredient in liposome formation, abolishing phospholipids' phase transition and reducing membrane permeability [38-40]. Therefore, the correlation between cholesterol contents and structural stability allows for the release of a large amount of aqueous extract of saffron from formulations with lower cholesterol contents which subsequently promote massive *in vitro* cell death. Fig 1 showed that saffron was well maintained in F1 and F2 compared to F3 and F4 preparations throughout the 168-h period. Indeed, PEG coating and the rigid vesicle bilayers of F1 and F2 liposomes due to the high level of cholesterol minimize their interaction with the cell membrane, thus resulting in a higher IC_{50} value compared to the aqueous saffron extract.

The lowest entrapment efficiency was obtained with F4 due to the absence of cholesterol content in this formulation. Similarly, F3 had a slightly greater encapsulation efficiency than F4, probably owing to the small cholesterol concentration. By contrast, the higher inhibitory activity of F3 and F4 compared to aqueous extract of saffron was likely because of the more fluid bilayers and the subsequent release

of saffron in cell culture media. The presence of mPEG2000-DSPE in all formulations made approximately similar negative surface charges improving extracellular matrix interaction.

Based on the properties of nanoliposomes (Table 1) and the release studies (Fig 1), F1 with the most favorable PDI, the highest encapsulation efficiencies, and the unique release profile was selected for further investigation in the animal model.

In vitro and *in vivo* studies represented saffron and its active constituents as promising anti-tumor agents against various cancer cells [31]. In the present investigation, analysis of tumor volume (Fig 4) indicated that F1-L-saffron at a dose of 300 mg/kg could exert the best control on tumor growth compared to the negative control group. Previously, it was reported that ethanolic saffron extract at a dose of 200 mg/kg could increase the life span in S-180 (Swiss albino mice bearing sarcoma-180), EAC (Ehrlich ascites carcinoma), or DLA (Dalton's lymphoma ascites) tumor models following oral administration [41]. It was earlier shown that parenterally administered saffron was more effective than oral route and that liposome encapsulation could improve its effectiveness. In another study, in mice, liposomal saffron could effectively increase the tumoricidal activity against S-180 and EAC solid tumors [42]. Thus, results suggested that the enhancement of anti-tumor activity by liposome encapsulation is likely due to the site-directed drug delivery [43] and the increase in the stability of bioactive ingredients [7].

CONCLUSION

In conclusion, this investigation, for the first time, indicated that i.v. injection of nano-liposomal saffron significantly increased the anti-tumor property compared to the negative control group while, no significant difference was observed compared with the aqueous extract of saffron. Thus, further modification of liposomal formulation is required to get better results regarding animal studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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