Supporting Information

Anti-tumor Activity of PEGylated Nanoliposomes Containing Crocin in Mice Bearing C26 Colon Carcinoma
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Detailed experimental procedures

Plant extract and purification

*C. sativus* L. stigmata from Novin Saffron Co. were collected in November 2010 from Ghaen, Khorasan province, North-east of Iran. The plant was identified by Mazhari, and a voucher specimen (No. 11135), deposited at the Herbarium of the Mashhad School of Pharmacy, was analyzed in accordance to 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- E_{1cm}^{1\%} 257 nm, on dry basis, Min. (At this wave length it has a maximum absorbency of picrocrocin): 82.71- E_{1cm}^{1\%} 330 nm, on dry basis, Min-Max. (At this wave length it has a maximum absorbency of safranal): 38.70-E_{1cm}^{1\%} 440 nm, on dry basis, Min. (At this wave length it has a maximum absorbency of crocin): 225.67

Crocin was extracted and purified as defined by Hadizadeh and colleagues [9]. To determine the purity of crocin, a UV-visible spectrophotometer was used. For the maximum absorption, a
solution containing 5 µg/mL crocin in distilled water was prepared, and absorption was read in wavelength ranges 200-600 nm. Then the absorption of the same solution was read at 443nm.

**Cell culture**

C26 murine colorectal cancer cells were maintained in RPMI 1640 media supplemented with 10% FBS and were cultured in 5% CO2 at 37 ºC.

**Cytotoxicity assay**

Cytotoxicity was assessed on C26 cells using MTT assay. C26 cells were seeded in 96-well plates at the concentration of 2000 cells/100 µL and were incubated for 24h at 37 ºC to allow for cell attachment. Crocin at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mM) was added in triplicate in each well, and the cells were incubated for 72h at 37 ºC. Cytotoxicity was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, after the incubation period, 20 µL of MTT dye (5mg/ in PBS) was added to each well and incubated in darkness for 3 h. Cells were washed and the produced formazan was solubilized with 200 µL of DMSO and 20 µL of glycine buffer. Then the absorbance of each well was measured by an ELISA reader (enzyme-linked immunosorbent assay) (Statfax–2100, Awareness Technology, USA) at 570 nm [10].

**Liposome preparation**

Liposome encapsulating crocin was prepared by hydration of thin lipid film followed by sonication and extrusion [10]. Briefly, lipid components were weighted and dissolved in chloroform. The desired volumes of lipid components were mixed in a round-bottom flask
according to the molar ratio for each formulation presented in Table 1. The solvent removal using rotary evaporator (Heidolph, Germany) under reduced pressure resulted in a thin lipid film. Traces of organic solvent were removed by keeping the film under freeze-drier overnight (Taitec, Japan). Crocin was dissolved in histidine buffer (10 mM, pH 6.5) at 25 mg/mL. The lipid film was hydrated by adding the required amount of crocin solution at 60°C. The resulting liposome was sonicated in a bath-type sonicator (Decon, England) for 15 min at 60°C and was then extruded (Avestin, Canada) repeatedly through 200, 100, and 50 nm polycarbonate membranes at 60 ºC. Formulations were passed at least 11 times through the polycarbonate membrane to produce liposomes of uniform size. The preparation was then added to a dialysis cassette (Mwt cut off 12 kDa) and dialyzed three times against 10 mM histidine, 10% sucrose pH 6.5 to remove un-encapsulated crocin. To assay crocin concentration, an aliquot of liposome preparations before and after dialysis was lysed with acidified alcohol (90% isopropanol / 0.075 M HCl). The encapsulated crocin concentration was assayed by comparing the absorbance at 443nm to a standard curve of a solution prepared from crocin with different concentrations. The percentage of encapsulation was calculated as below.

\[
\% \text{ Encapsulation} = \frac{\text{amount of drug in purified liposomes}}{\text{amount of drug in unpurified liposomes}} \times 100
\]

**Liposome characterization**

The particle diameter of each sample was measured in triplicate using a dynamic light scattering instrument (Nano-ZS; Malvern, UK). The zeta potential of liposomes was determined on the same machine using the zeta potential mode as the average of 20 measurements. Particle sizes
were reported 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 the presence of 30% fetal bovine serum (FBS) at 37 °C [11]. At different time points, samples were removed and dialyzed against 10 mM histidine, 10% sucrose pH 6.5 to remove the released crocin. Then the liposomal crocin which remained in the dialysis cassette was lysed with acidified alcohol and assayed as described above.

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\text{% Release} = \frac{\text{amount of drug in purified liposomes after release test}}{\text{amount of drug in unpurified liposomes before release test}} \times 100
\]

In vitro cytotoxicity assay to determine IC\textsubscript{50} of crocin liposomal formulations

Cytotoxicity assay on C26 cells using MTT test was carried out as described above.

Animals

Female BALB/c mice, 6–8 weeks old (for C26 model) were purchased from the Pasteur Institute (Tehran, Iran). The mice were housed in an animal house of the Pharmaceutical Research Center in a colony room 12/12 h light/dark cycle at 21°C with free access to water and animal food. Experiments were carried out according to Mashhad University of Medical Sciences, Ethical Committee Acts (research project number: 86688, 11 May 2008).
**Liposomal crocin therapy in C26 mouse model**

C26 murine colorectal cells (3×10⁵) suspended in 50 µL PBS (phosphate buffer saline) were inoculated subcutaneously in the right hind flank of BALB/c mouse. On day 10 after tumor implantation, mice were classified into different treatment groups (n=6). Treatment groups were liposomal crocin (50 or 100 mg/kg), crocin (100 mg/kg in histidine buffer), isotonic PBS (200μL), Caelyx® (15mg/kg) and doxorubicin (Ebedoxo)® (10mg/kg) [11]. Treatments were administered by the tail vein injection on alternate day in 5 doses for the first four groups and single dose for the other ones. Mouse tumor growth, weight and overall health were monitored on alternate days for 60 days. Tumor volume was calculated by measuring the tumor in three dimensions with the following formula: tumor volume = height×length×width [11]. For ethical reasons, mice were sacrificed due to tumor enlargement (more than 2 cm in one dimension) or decrease in body weight (>15% loss). Mouse survival was analyzed with Prism software and Mantel- Cox test.

**Statistical analysis**

The one-way ANOVA test was used to assess the significance of differences among the various groups. In the case of a significant F value, multiple comparison Tukey test was used to compare the means of different treatment groups. Results with p < 0.05 were considered to be statistically significant. The IC₅₀ for each formulation was calculated by the CalcuSyn software Version 2.1 (Biosoft, Cambridge, UK). Statistical evaluation of survival was performed using Prism software and Mantel- Cox test.