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CHARACTERIZATION AND ANTI-TUMOR ACTIVITY OF PEGYLATED NANOLIPOSOMES CONTAINING SAFRANAL IN MICE BEARING C26 COLON CARCINOMA

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ABSTRACT: The cytotoxic effect of safranal, a pharmacologically active component of saffron, has been established *in vitro*. The aim of this study was to develop safranal nanoliposomes with a higher therapeutic index for the treatment of cancer. Thus, various PEGylated safranal nanoliposomes were prepared using HSPC and cholesterol by solvent evaporation. The liposomes were characterized by their size, *in vitro* cytotoxicity and *in vivo* therapeutic efficacy against C26 tumor bearing mice. Liposome characterization illustrated the size range of 140-230 nm and PDI of 0.2-0.3. The entrapment efficiency was considerably low due to the high instability of safranal in liposomes, causing a substantial *in vitro* release. *In vitro* cytotoxicity indicated higher toxic effects of safranal liposomes compared to free form. Treatment of tumor-bearing mice with selected safranal liposomes (50 mg/kg) did not improve the tumor size and survival of animals compared to controls. These results were presumably due to the physicochemical properties and dose dependent effects of safranal molecules. In addition, the intensive hydrophobic molecular interaction between safranal and cholesterol within the bilayers of liposomes cause the low percentage of encapsulation, high instability while in the circulation and untoward site directed drug delivery. Results indicated that the current safranal liposomes could increase the *in vitro* cytotoxicity, however did not enhance the antitumor activity at a dose of 50 mg/kg; thus, to obtain an optimal formulation, it merit further investigation.

INTRODUCTION: Saffron scientifically known as "*Crocus Sativus,* Linn"(*Iridaceae* family), contains four crucial bioactive compounds namely, picrocrocin, crocin, crocetin and safranal [1, 2].

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Of the ingredients present in saffron, the low water-soluble safranal (2, 6, 6-trimethyl-1, 3-cyclohexadien-1-carboxaldehyde) has shown to possess various health-promoting properties including a high antioxidant potential as well as cytotoxicity towards certain cancer cells *in vitro* [3-8].

However, the low aqueous solubility of safranal prevents it's using as a therapeutic or preventive agent. The water solubility of safranal at 25 °C is about 134.2 mg/L [9] and the aqueous solubility in phosphate buffer (pH 7.4) according to Higuchi and

Connors method has been reported as 578.61 mg/L [10]. Liposome drug delivery systems have emerged as a promising technology in formulating hydrophilic and hydrophobic drugs to improve the therapeutic potential of the targeted molecules delivered to the site of actions [11, 12]. By reducing mononuclear phagocyte system (MPS) uptake, long-circulating PEGylated nanoliposomes can passively accumulate in the tumors area through enhanced permeation and retention (EPR) and improve the therapeutic effects of liposomal anticancer therapeutics versus free agents[11-14].

The purpose of this study was to investigate the effect of liposome as a vesicular vehicle for enhancing the anti-tumor activity of safranal and to compare with its free form. To this end, formulations of PEGylated nanoliposomes containing safranal were prepared by solvent evaporation plus extrusion. Then, safranal liposomes were characterized by their size, zeta potential, encapsulation efficiency and release properties and were subsequently tested for *in vitro* cytotoxicity. Those with the optimum characteristics were opted for further investigation regarding *in vivo* therapeutic efficacy against C26 colon carcinoma tumor bearing mice.

MATERIALS AND METHODS:

Liposome preparation: Liposomes encapsulating safranal were prepared by hydration of thin lipid film followed by sonication and extrusion [15]. To put it briefly, lipid mixtures (HSPC, cholesterol and $MPEG_{2000}$-DSPE) were dissolved in chloroform. Solvent removal using rotary evaporator (Heidolph, Germany) formed the thin lipid film. Traces of organic solvent were further removed by keeping the film under freeze-drier (Taitec, Japan) overnight. Liposomes were formed by hydrating lipid film with 10 mg/mL safranal solution in Histidine plus 10% sucrose buffer (pH 6.5) at 60 ºC. The safranal encapsulating liposomes were sonicated in a bath-type sonicator (Bransonic-Branson, U.S.A) for 15 min at 60 ^{o}C and were then extruded (Avestin, Canada) at 60 ^{o}C to produce uniformed size safranal liposomes.

Liposome characterization: The particle diameter and zeta potential of each sample was measured using Particle Size Analyzer (Nano-300 HS; Malvern, UK). 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).

Percentage of encapsulation: The prepared liposomes were 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 safranal. To assay safranal concentration, 10 µL of liposome preparations before and after dialysis was lysed with 1990 µL Ethanol. The encapsulated safranal concentration was assayed by comparing the absorbance at 310 nm to a standard curve of a solution prepared from safranal with different concentrations using spectrophotometry (Jenway, England). The percentage of encapsulation was calculated as below.

$$\%Encapsulation = \left(\frac{amount\ of\ safranal\ in\ purified\ liposomes}{amount\ of\ safranal\ in\ unpurified\ liposomes}\right) \times 100$$

Release studies: The *in vitro* release profile of safranal form liposome formulations were studied in the presence of 10 mM Histidine buffer containing 10% sucrose (pH 6.5) at 25 °C. For this, 450 µL of safranal loaded liposomes were transferred into dialysis bags with 12-14 KD molecular weight cut-off, tightly closed and incubated with gentle magnetic stirring. At different points in time, aliquots were withdrawn and replaced with the same volume of buffer solution. Collected samples were then assayed for the safranal contents using spectrophotometry, as described earlier. Then liposomal safranal remained in the dialysis cassette was lysed with Ethanol and release percentage was assayed as follows.

$$\%Release = \left(\frac{amount\ of\ safranal\ in\ purified\ liposomes\ after\ release\ test}{amount\ of safranal\ in\ unpurified\ liposomes\ before\ release\ tast}\right) \times 100$$

***In vitro* cytotoxicity assay:** C26 murine colorectal cancer cells were preserved in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and were cultured in 5% CO_2 at 37°C**.** C26 cells were obtained from Cell Lines Service (Eppelheim, Germany). Cytotoxicity was assessed on C26 cells using MTT assay. C26 cells (2000 cells/100 µL) were seeded in 96-well plates and incubated for 24h at 37 ºC to allow for cell attachment. Safranal

at different concentrations (2, 1, 0.5, 0.25 and 0.125mM) were added triplicate in each well and the cells were incubated for 72h at 37ºC. Cytotoxicity was assessed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay [15].

Animals: The 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 Pharmaceutical Research Center in a colony room 12/12 h light/dark cycle at 21°C with free access to water and animal food. All procedures involving animals and the proposal were 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), based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran issued in 2005.

Liposomal safranal therapy in C26 mouse model: C26 murine colorectal cells (3×10^5) in 50 µL PBS were inoculated S.C in the right hind flank of BALB/c mice. On day 10 after tumor implantation, mice were classified into different treatment groups (n=5) including liposomal safranal (50 mg/kg), safranal (50 mg/kg in Histidine buffer), isotonic PBS (200µL), Caelyx® (15mg/kg) and doxorubicin® (10mg/kg)[15, 16]. Treatments were administered by the tail vein injection on alternate day in 5 doses for the first three groups and single dose for the other ones. Mouse tumor growth, weight and overall health were monitored. Tumor volume was calculated with the following formula: tumor volume=height×length×width [16].

Statistical analysis: The one-way ANOVA test was used to assess the significance of differences among the various groups. Results with $p< 0.05$ were considered to be statistically significant.

RESULTS: Liposomal formulations encapsulating safranal were developed using the distinct molar ratios of HSPC/Cholesterol/mPEG$_{2000}$-DSPE. The presence of MPEG$_{2000}$-DSPE effectively made the zeta potential of all formulations negative. Liposomes represent zeta average size ranging from 140 to 230nm and a PDI of around 0.3(**Table 1**). As shown in **Table 1**, by decreasing cholesterol contents of formulations, the size of liposomes was significantly decreased. Although the encapsulation efficiency of safranal in all liposome formulations is generally low, it is well affected by the cholesterol contents of bilayers. In other words, the compositions with higher cholesterol levels, as seen in F1 and F2, significantly decreased safranal entrapment within liposomes and conversely, lower cholesterol contents increased safranal encapsulation efficiency (F3, F4 and F5).

TABLE 1: PHYSICAL PROPERTIES, ENCAPSULATION EFFICIENCY, AND IC$_{50}$ OF LIPOSOMAL SAFRANAL FORMULATIONS.

Safranal Formulations	Molar Ratio	Zeta Average Size (Nm) ± SD	Polydispersity ± SD	Zeta Potential % (Mv) ± SD	Encapsulation	IC$_{50}$ (Mm) (Lower And Upper 95%)
F1-HSPC/mPEG$_{2000}$-DSPE/Chol	5.5/0.5/4	222.3 ± 2.601	0.321 ± 0.041	-19.6	0.99 ± 0.17	0.084 (0.06-0.12)
F2-HSPC/mPEG$_{2000}$-DSPE/Chol	6.5/0.5/3	228.1 ± 5.203	0.326 ± 0.01	-22.3	0.69 ± 0.254	0.092 (0.05-0.18)
F3-HSPC/mPEG$_{2000}$-DSPE/Chol	7.5/0.5/2	184.3 ± 0.416	0.223 ± 0.015	-18.9	1.33 ± 0.342	0.287 (0.23-0.35)
F4-HSPC/mPEG$_{2000}$-DSPE/Chol	8.5/0.5/1	158.5 ± 1.323	0.22 ± 0.014	-16.4	1.38 ± 0.282	0.375 (0.36-0.39)
F5-HSPC/mPEG$_{2000}$-DSPE/Chol	9.5/0.5/0	140.96 ± 0.7767	0.31 ± 0.007	-15.5	1.46 ± 0.639	0.524 (0.51-0.54)
Safranal in Histidin buffer	-	-	-	-	-	0.477 (0.32-0.71)

As is evident in **Fig.1**,there were no significant differences in the safranal release profiles of all liposomal formulations in the first 4h of incubation($p>0.05$). Safranal release from F1 and F2 was approximately 70% within the first 2 h, which almost reached 90% at the end of the incubation. Meanwhile, after 24 h, this experiment represents a release of almost 80% for F3 and F4 and 65% for F5 formulation. At this time point, the release of safranal from F5 liposome devoid of cholesterol, was significantly lower than F1 ($p<0.05$), liposomes, respectively. Apparently, there is a direct correlation between the cholesterol content of liposome and release kinetic which is completely compatible with the obtained results of encapsulation efficiency.

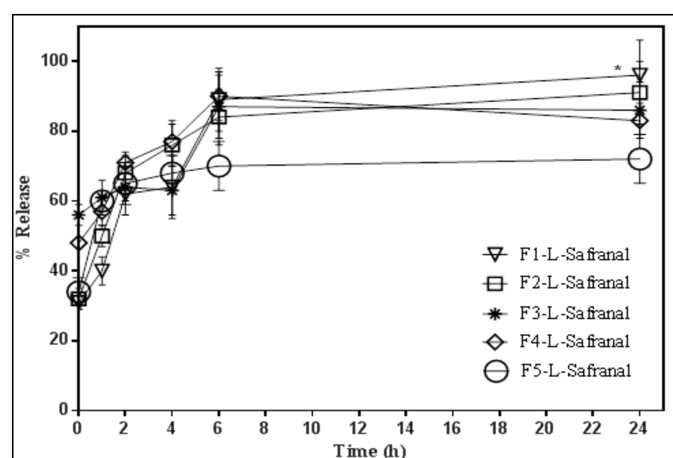

FIG. 1: RELEASE PROFILE OF SAFRANAL LIPOSOMES. F1 (HSPC/ $mPEG_{2000}$-DSPE/Chol-5.5/0.5/4), F2 (HSPC/ $mPEG_{2000}$-DSPE/Chol-6.5/0.5/3), F3 (HSPC/ $mPEG_{2000}$-DSPE/Chol-7.5/0.5/2), F4 (HSPC/ $mPEG_{2000}$-DSPE/Chol-8.5/0.5/1) and F5 (HSPC/ $mPEG_{2000}$-DSPE/Chol-9.5/0.5/0) in Histidin-10% sucrose buffer. *shows statistical significance ($p<0.05$) when compare F5 with F1 liposomes. Statistical analysis was done by One-way ANOVA and Tukey–Kramer multiple comparisons test.

The *in vitro* system was also devised to analyze the release kinetics and the cytotoxicity of safranal modulated by liposomal formulation. The IC_{50} value of safranal was 0.477 mM (**Table 1**) and the cell proliferation inhibitory effects of F1 and F2 were significantly higher than safranal (much lower IC_{50} values). On the other hand, F3, F4 and F5 liposomes increased IC_{50} values which lie within the range of free safranal. Interestingly, formulations that showed premature release of safranal, as seen with F1 and F2, significantly enhanced *in vitro* cytotoxicity as well.

Although the plasma stability of the encapsulated content is required for delivering the therapeutic agents toward the target site, the increased cytotoxic effects may seem beneficial in *in vivo* studies. Thus, considering the rash release profiles and *in vitro* analysis of safranal liposomes, F1, F3 and F5 liposomal-safranal formulations (F1-L-safranal, F3-L-safranal and F5-L-safranal) were selected for further anti-tumor studies in animals.

To determine the therapeutic efficacy of liposomal safranal, anti-tumor activity was assessed in C26-tumor bearing mice. **Fig.2** illustrated that none of liposomal safranal formulations at 50 mg/kg showed significant tumor growth inhibition compared to other groups. Unexpectedly, compare to negative control, liposomal safranal did not significantly inhibit the tumor growth ($p>0.05$). There was also no substantial difference ($P>0.05$) comparing liposomal safranal with safranal at the same dose.

However, the intravenous administration of a single dose of Caelyx® resulted in significant tumor growth regression which lasted for more than 3 weeks ($p<0.01$).Despite, the treatment of tumor bearing mice with doxorubicin alone induce a significant tumor response compared to liposomal safranal ($p<0.05$),but did not last for a long period due to the adverse effect associated with free drug at the dose of 10 mg/kg [17].

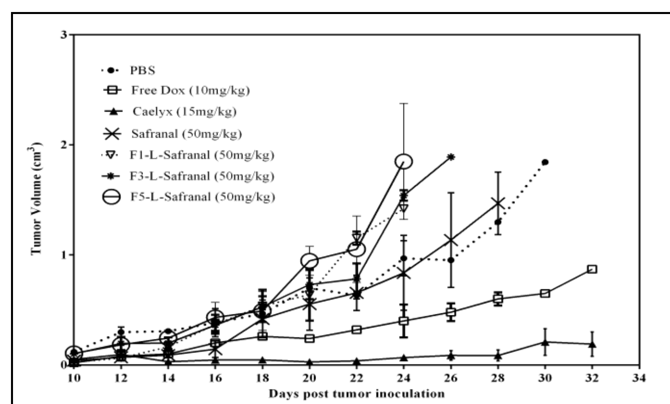

FIG.2: TUMOR GROWTH CURVE. BALB/c mice bearing C26 tumor (n=5) were i.v. administered on day 10 with PBS, free safranal (50 mg/kg), L-Safranal (50 mg safranal/kg): F1 (HSPC/ $mPEG_{2000}$-DSPE/Chol-5.5/0.5/4), F3 (HSPC/ $mPEG_{2000}$-DSPE/Chol-7.5/0.5/2), F5 (HSPC/ $mPEG_{2000}$-DSPE/Chol-9.5/0.5/0), for 5 consecutive doses every other day and single dose of free Dox: doxorubicin (Ebedoxo)® (10 mg/kg) and Caelyx®: liposomal doxorubicin (15mg/kg). Error bar represents SEM.

As is evident in **Fig.3,** in neither of the treatment groups, animals were excluded due to body weight loss (more than 15%).

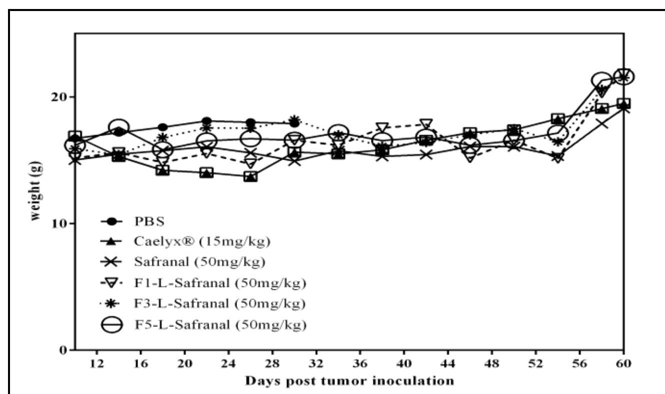

FIG. 3: EFFECT OF LIPOSOMAL SAFRANAL THERAPY ON WEIGHT OF MICE. BALB/c mice bearing C26 tumor were treated on day 10 for 5 consecutive doses every other day with i.v. injection of PBS, pure safranal (50 mg/kg), L-Safranal (50 mg safranal/kg): F1 (HSPC/ $mPEG_{2000}$-DSPE/Chol-5.5/0.5/4), F3 (HSPC/ $mPEG_{2000}$-DSPE/Chol-7.5/0.5/2), F5 (HSPC/ $mPEG_{2000}$-DSPE/Chol-9.5/0.5/0) and single dose of free Dox: doxorubicin (Ebedoxo)® (10 mg/kg) and Caelyx®: liposomal doxorubicin (15mg/kg).

Furthermore, the survival analysis verified the achieved results from tumor growth curve. As is clarified in **Fig.4**, survival of animals treated with Caelyx® was significantly different than other groups ($p<0.01$). Mice treated with either safranal or doxorubicin had similar survival as compared to PBS ($P>0.05$). The survival of mice received liposomal safranal at 50 mg/kg was very close and comparable to that of safranal, PBS and doxorubicin, respectively ($P>0.05$).

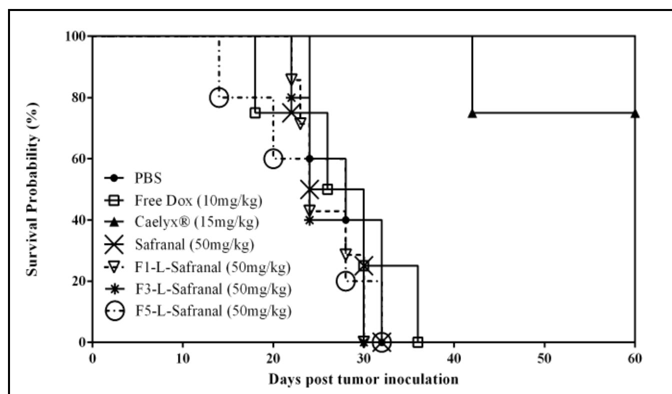

FIG. 4: SURVIVAL CURVE. BALB/c mice bearing C26 tumor were treated on day 10 for 5 consecutive doses every other day with i.v. injection of PBS, pure safranal (50 mg/kg), L-Safranal (50 mg safranal/kg): F1 (HSPC/ $mPEG_{2000}$-DSPE/Chol-5.5/0.5/4), F3 (HSPC/ $mPEG_{2000}$-DSPE/Chol-7.5/0.5/2), F5 (HSPC/ $mPEG_{2000}$-DSPE/Chol-9.5/0.5/0) and single dose of free Dox: doxorubicin (Ebedox)® (10 mg/kg) and Caelyx®: liposomal doxorubicin (15mg/kg).

DISCUSSION: Safranal, one of the major pharmacologically active component of saffron (*C. Sativus,* Linn), subscribes the health-promoting properties and high antioxidant potentials of saffron[1]. This low-water soluble cyclical terpenic aldehyde, accounts for the bitter taste and the actual color of saffron [9]. The promising properties of safranal including its antioxidant features [18], protective effects [19] as well as cytotoxic and apoptogenic effects [20] against cancer cells, makes this component as a potential therapeutic agent. It has been investigated that safranal inhibited cell proliferation in a dose-dependent manner and induced cell appoptosis in cultured neuroblastoma cells with an IC_{50} of 11.1 and 23.3 μg/ml after 24 and 48 h, respectively [21]. In another study liposomal safranal significantly enhanced cytotoxic and apoptogenic effects compared to the safranal against HeLa, MCF7 and L929 cell lines, respectively[20].

Liposomes, the vesicular lipid vehicles, have emerged as promising drug delivery systems to improve the efficacy of targeted therapeuticsto the site of actions [11, 22]. They can be extensively used for loading a variety of molecules including hydrophilic and hydrophobic drugs [23].

The objective of the current study was to investigate whether the encapsulation of safranal in liposome can enhance the anti-tumor efficacy compared to safranal alone. Therefore, safranal liposomes using HSPC/Cholesterol with different molar ratios were prepared. The characterization of formulations indicated the size range between140 and 230 nm and the polydispersity index of around 0.3. Moreover, all formulations had an approximately similar negative surface charge due to the presence of $mPEG_{2000}$-DSPE which improves blood circulation as well as extracellular matrix interaction. Basically, the encapsulation efficiency of safranal liposomes is dramatically low. Evidently, it can be noticed that the cholesterol content of liposomes had a great impact on the size and encapsulation efficiency of liposomes. Accordingly, the cholesterol level of liposomes significantly affected the release profile and *in vitro* cytotoxicity.

In other words, by decreasing the cholesterol content in liposome, the size of liposome and the percentage of safranal release decreased; in contrast, the encapsulation efficiency and IC_{50} values significantly increased. It could be concluded that in general, the lower IC_{50} value of safranal (0.477 mM) is probably due to the high volatility of safranal molecules resulting in faster dissolution and higher *in vitro* cytotoxicity.

These obtained results also confirm the fact that there is an exceptional effect of cholesterol on hydrophobic drug encapsulation and *in vitro* release [24-27]. That is to say that safranal as a hydrophobic compound can be only assosiated within the bilayers of liposome where cholesterol usually sit in order to eliminate the phase transition of phospholipid and reduce membrane permaebility [28, 29].Therefore, there may be a rigid competition between cholesterol and safranal for the cooparating region of the phospholipid. In addition, due to the physicochemical properties of safranal molecules such as low molecular weight (MW: 150.22 g/mole) and high volatility, the considerable safranal leakage could transit through the membrane of liposomes.

Apparently, the compositions with higher cholesterol levels (F1 and F2) had the bigger size of liposome and lower safranal entrapments within liposomes. The reason is likely due to the increased hydrophobic molecular interaction of safranal with cholesterol content of liposomes. F5 however, had a greater encapsulation efficiency compared to F3 and F4, probably because of absence of cholesterol content that causes no interaction and the smaller particle size.

Basically, all formulations had premature release profiles and their safranal content transited from liposome's membrane in 24 h. The HSPC/Chol molar ratio in F1 and F2 was changed from 5.5:4 and 6.5:3 to 7.5:2, 8.5:1 and 9.5:0 in F3, F4 and F5 preparations. Accordingly, by decreasing the amount of cholesterol in the formulation, the percentage of release rather decreased. Thus, difference in the rate of safranal leakage from liposomes of different compositions first is because of the diversity of cholesterol content within the lipid bilayers and the powerful hydrophobic molecular interaction between safranal and cholesterol that cause the faster transition of safranal molecules from liposome. Besides, it is conceivable that the safranal release profile of liposomal formulations in the presence of Fetal Bovine Serum (FBS) at 37 °C would be much higher likely due to the unspecific interaction of proteins with liposomes.

The release kinetics and the cytotoxicity of liposomal safranal have been investigated. In general, the lower IC_{50} value of safranal (0.477 mM) probably is due to the high volatility of safranal molecules resulting in faster dissolution and higher *in vitro* cytotoxicity. The cell proliferation inhibitory effects of F1 and F2 were significantly higher than safranal. On the other hand, F3, F4 and F5 liposomes increased IC_{50} values which are within the range of free safranal. Interestingly, formulations that showed premature release of safranal, as seen with F1 and F2, significantly enhanced *in vitro* cytotoxicity as well.

Analysis of tumor volume indicated that liposomal safranal formulations at dose of 50 mg/kg could not control tumor growth as if it was comparable to free safranal. Further, liposomal safranal did not prolonged the survival of animals. Therfore, *in vivo* analysis suggested that the enhancement of anti-tumor activity was not approched possibly due the fast transition of small volatile molecules of safranal through liposome's membrane that cause unstability of liposomal formulations and the untoward site directed drug delivery.

It was previously reported that pathological assessment of heart, liver and spleen, showed no abnormal effects following safranal usage, but histological evaluations showed abnormalities and toxic effects of safranal (a Purity of >88%), especially at the dose of 0.5 ml/kg or higher in kidney and lung [30].

CONCLUSION: At an glance, although *in vitro* studies have shown safranal as one of the promising constituents of saffron against a wide range of cancer cells, in the present study, results indicated safranal liposomes with increased *in vitro* cytotoxicity, could not enhance the antitumor activity.

Doubtless, "these were" three important factors play a crucial role in obtained results: first, the physicochemical properties of safranal molecules such as low molecular weight (MW: 150.22 g/mole) and high volatility; second, dose dependent anti-tumor effects of safranal as chemopreventive agent and third, the intensive hydrophobic molecular interaction between safranal and cholesterol content within the bilayers of liposomes. Overall, these factors caused the leakage of required safranal through the membrane of liposomes resulting in low percentage of encapsulation, high instability while in the circulation and untoward site directed drug delivery.

However, the prepared liposomal formulation at the dose of 50mg/kg needs further characterization in future studies to obtain a highly stable formulation from the economical and therapeutical ponits of view.

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CONFLICT OF INTERST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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