

RESEARCH COMMUNICATION

Crocini from Kashmiri Saffron (*Crocus sativus*) Induces *in Vitro* and *in Vivo* Xenograft Growth Inhibition of Dalton's Lymphoma (DLA) in Mice

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Abstract

In this study we investigated *in vitro* and *in vivo* xenograft growth inhibition by crocini isolated from Kashmiri saffron (*Crocus sativus*). It was found that crocini decreased cell viability in DLA cells, in a concentration- and time-dependent manner. Significant increase in the lifespan of Dalton's lymphoma bearing animals was noted by 37% and 44%, respectively. Furthermore, animals given treatment before induction of cancer showed 58% increase in lifespan and there was 95.6% reduction of solid tumor in crocini treated animals on the 31st day after tumor inoculation. Crocini also showed significant impact on hematological parameters, like the hemoglobin count and numbers of lymphocytes. These findings support the conclusion that crocini from *Crocus sativus* has significant anti-tumor activity.

Key Words: *Crocus sativus* - xenograft - crocini - Daltons lymphoma - alternate medicine

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Introduction

In the last two decades, botanicals with effective anti-cancer activity have been researched. The use of herbal medicines is also on the rise in cancer patients (Lee et al., 2006; Garodia et al., 2007). Discovery of new botanical candidates with potential anti-cancer effects is imperative, to allow for the development of safe and efficacious anti-cancer therapies (Bemis et al., 2006; Hemaiswarya and Double, 2006). *Crocus sativus* is a plant of the iris family (Iridaceae) and its flower contains various chemical constituents (Abdullaev and Espinosa Aguiñe, 2004). Stigmas of the flower (saffron) contain crocini, anthocyanin, carotene and lycopene (Giaccio et al., 2004), and these constituents are known to have various pharmacological effects on different illness, including anti-tumor effects by inhibition of cell growth (Abdullaev et al., 1993).

Recently, *Crocus sativus* extract (CSE) was found to have anti-cancer activities against leukaemia, osteosarcoma, fibrosarcoma and ovarian carcinoma cells (Abdullaev and Espinosa Aguiñe, 2004). Crocini, a main constituent of the CSE, exhibits a variety of pharmacological effects in mice including inhibition of skin tumor growth (Konoshima et al., 1998), improvement of learning behavior previously impaired by ethanol (Abe et al., 2000), anti-hyperlipidemic effects (Lee et al., 2005),

therapeutic efficacy for colon adenocarcinomas in rats (Garcia-Olmo et al., 1999), anti-atherosclerotic effects (He et al., 2005) and anti-oxidant effects in PC-12 cells by increasing GSH synthesis (Ochiai et al., 2004). In an earlier study, we evaluated the cytotoxic and apoptogenic effect of CS on different human cancer cell lines (Bakshi et al., 2008). In the present study, we evaluated the effect of crocini isolated from Kashmiri *Crocus sativus* on *in vitro* and *in vivo* xenograft growth of Dalton's lymphoma in mice.

Materials and Methods

Animals

Swiss albino (*Mus musculus*) male mice (32 in number, 25-30 g), were used for the studies and animals were maintained under standardized, environmental conditions (22- 28°C, 60-70 % relative humidity, 12 hours dark/light cycle and water ad libitum. All the experiments were conducted under the guidelines of Institutional Animal Ethical Committee.

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Himedia Laboratories, Mumbai, India. Fetal Calf Serum was purchased from Biological Industries, Israel. 3-(4,5-dimethylthiazol-2-yl)-2,5-

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diphenyl tetrazolium(MTT) from Sigma . All other reagents used were of Analytical Reagent grade.

Cell line

Dalton’s Lymphoma Ascites (DLA) was originally obtained from the Cancer Institute, Adayar, Chennai, and are being maintained in our laboratory as described previously (Bakshi et al., 2008). Briefly , cells were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% (v/v) fetal calf serum (FBS)in a CO2 water-jacketed incubator (Heraeus , Germany) at 37° C in a humidified atmosphere of 5% CO2 and 95% air.

Isolation of Crocin From saffron:

Crocine was isolated from saffron by previously described method (Bakshi et al., 2008) with some modification. Briefly 500-mg saffron was washed thrice with 20-ml ethyl ether, and the residual ether was evaporated in air. It was then suspended in 15 ml of 30% methanol (v/v) in distilled water and stirred for 5 min at room temperature. The extract was filtered through a 0.45-µm Millipore filter. It was then diluted with 10 mmol/l phosphate-buffered saline (PBS, pH=7.4), and the concentration of crocin was adjusted to 25 µmol/l, using the coefficient $e_{443}=89,000 M_{-1} cm_{-1}$ reported for crocin in aqueous solution (Lussignoli et.al, 1999). Crocin was stored at -20 °C in the dark for a maximum of 2 months (Tubaro et.al, 1998) .The crocin extracted from Kashmiri saffron was shown to possess similar spectral characteristics.

Cell viability:

The cell viability was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay (Mosmann, 1983; Sharifi et al., 2005). Briefly, cells were seeded (5000/well) onto flat-bottomed 96-well culture plates and allowed to grow 24 h followed by treatment with crocin (30–480 µg/ml). After removing the medium, cells were then labeled with MTT solution(5 mg/ml in PBS) for 4 h and resulting formazan was solubilized with DMSO (100 µl). The absorption was measured at 570 nm (620 nm as a reference) in an ELISA reader.

Determination of effect of crocin on a solid tumor development in mice:

Three groups of Swiss albino mice (8 animals/ group) were used for the experiment. Solid tumors were induced in animals by injecting 1×10^6 Daltons lymphoma cells to the peritoneal cavity. Group I served as Normal .Group II served as control with DLA cells alone. Group III was treated with crocin 10ml/kg body weight orally (i.e., 0.75ml three times daily). Drug administration was started 24 hrs after tumor inoculation and continued everyday for 10 consecutive days. Initial diameter of the hind limb was noted using vernier calipers. The tumor diameter was measured from the 7th day and continued on every 3rd day for 31 days. The tumour volume was calculated using the formula $V = \frac{4}{3}\pi r_1 r_2^2$, where r1 and r2 are the radius of the tumour at two different sites (Kuttan et al., 1985).

The death pattern of the animals due to tumor burden was noted everyday and the percentage of increase in

lifespan was calculated using the formula $TC/C \times 100$ were ‘T’ and ‘C’ are the average number of days the animals survived in treated and the untreated group respectively.

Determination of effect of crocin on developed tumor:

Thirty numbers of adult Swiss albino mice were injected on the hind limb with DLA cells (1 million) in 0.1 ml phosphate buffered saline. After 30 days, when tumour had developed to volume of 2cc they were divided into 2 groups (15 animals/ group). Crocin (10ml/kg body weight) was given orally for 20 consecutive days for the first group. Second group of animals were treated with potentiated water prepared in glass bottle. Diameter of tumour was measured every 3rd day using vernier caliper and volume was calculated using the formula $V = \frac{4}{3}\pi r_1 r_2^2$. Death of the animals due to tumour burden in each group was noted and increase in lifespan was calculated as given above.

Determination of effect of crocin on hematological parameters:

Blood was collected from mice tail vein and parameters such as WBC count (by trucks fluid method), differential cell count (by Leishman’s stain), and hemoglobin level (by cyan met hemoglobin method) were determined.

Statistical analysis

The results are expressed as mean ± SD. Statistical evaluation of the data was done by ANOVA followed by Dunnet’s test (Post-hoc) using Graph pad In Stat 3 software package.

Results

Effect of crocin on cell viability:

DLA cells were incubated with various concentrations of crocin (30–480 µg/ml) for 24, 48 and 72 h. Crocin decreased cell viability in DLA cells, as a concentration- and time-dependent manner (Table 1).

Determination of effect of crocin on life span of a solid tumor bearing mice

The lifespan of ascities tumor bearing mice treated with crocin, were found to be significantly increasing. Control animals survived only 18 days after tumor induction, while treated animals survived for 25 and 27 days, with an increased life span of 37% and 44% respectively. The group-III animals given treatment before cancer induction were showing a 58% increase in life span than that of tumor bearing animals. (Table 2)

Table 1. In Vitro Cytotoxic Effects of Crocin Against DLA Cell Lines

Sample	Concentration/ reading (µg / ml)	Growth inhibition M.T.T.ASSAY	CTC 50
	480	91.81	
	240	73.18	
Crocine	120	64.27	51.74%
	60	46.12	
	30	35.08	

Table 2. Life Span of Swiss Albino Mice Treated with Crocin, with and without DLA

Groups	Mean Survival	Percentage Increase in Life Span
Normal mice	No change (L ¹)	No change
Crocin (before)+DLA	27 + 2.16*	44.0
Crocin (after)+DLA	25 + 1.75	37.3
Control (DLA alone)	18 + 0.81	-

¹Life span minimum-2 years,*Values are mean standard deviation

Table 3. WBC, Hemoglobin and Differential Counts in Swiss albino mice Treated with Crocin, with and without DLA

Groups	WBC /mm3	Hb (g%)	L	N	E
Normal mice	6,850	13.6	60	33	3.5
DLA-Control	123,430	12.3	33	60	3.9
Crocin (before)+DLA	13,124	14.7	42	53	3.8
Crocin (after)+DLA	12,624	14.2	33	60	3.8

L, Lymphocytes; N, Neutrophils; E, Eosinophils

Determination of effect of crocin on developed tumor:

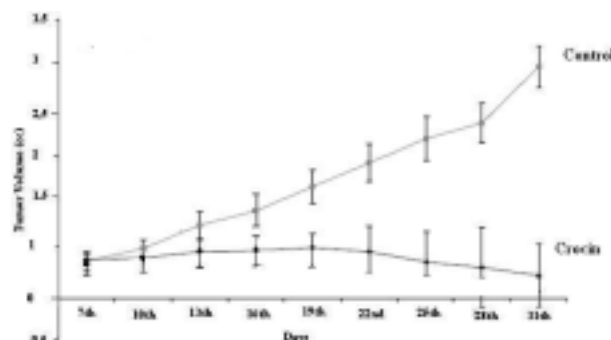
A significant reduction in solid tumour volume was found in crocin treated animals when compared to that of control animals. In crocin treated group there was 54.8% reduction in tumour volume on day 19. On the 31st day, reduction in tumour volume was 95.6% (Figure 1).

Determination of effect of crocin on hematological parameters:

The treatment is found to be effective in terms of W.B.C count as the values are near to the normal WBC count and a tremendous decrease is seen from that of group-II (DLA alone). The hemoglobin count of group-III shows a maximum result of 14.7% while the least value of 12.3% is shown in the control. In case of differential count, a gradual decrease in lymphocytes were observed GrpI>GrpIII> GrpII>GrpIV. The neutrophils count of DLA mice increased from normal mice to the control mice and there where no much differences in the eosinophil number in all groups of animals (Table 3).

Discussion

Crocus sativus L. belongs to Iridaceae family, and is cultivated in Asian, Europe and America (Xue, 1982). *Crocus sativus* has been used to treat several medical

**Figure 1. Effect of Crocin on Tumour Volume**

conditions, such as gastro-intestinal disorders, urological infections, as well as in treating malignancies (Nair et al., 1991; Rios et al., 1996; Winterhalter et al., 2000). It contains components like safranal, alpha crocin or crocin. Additionally *Crocus sativus* also contains amino acids, flavonoids and other chemical compounds (Nair et.al, 1994; Winterhalter et.al, 2000). Among the total, crocin is the most important since it is the major component and has shown significant biological activities (Winterhalter et al., 2000).

In the present study, we showed significant antiproliferative effect of crocin on DLA xenograft both invitro and in vivo. Crocin from CS has anti-tumor effects on cellular DNA and RNA synthesis (Nair et al., 1995). Another mechanism for the anti-tumor action of CS and its constituents is the inhibitory effect on free radical chain reactions (Nair et al., 1995), because most carotenoids are lipid-soluble and might act as membrane-associated high-efficiency free radical scavengers, connects with their anti-oxi-dant properties (Molnar et al., 2000; Zheng et al., 2007)

In present study it was found that potentiated crocin possessed significant antitumor activity in both developing as well as solid tumor models. Moreover crocin was found to produce significant tumor reducing activity on developed solid tumors. The treatment with crocin significantly increased the lifespan of developed tumor bearing animals. The mechanism of inhibition of solid tumor is not known at present.

In summary, data from this study suggest that crocin from CS may be efficacious in treating lymphoma cancer. Considering the popularity of bo-tanical use in cancer patients, however, before coming to conclusive statement more research will be needed to fully delineate the part they play in cancer and whether they will be good targets for cancer therapies in the future.

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