RESEARCH ARTICLE

Role of Dietary Crocin in *In Vivo* Melanoma Tumor Remission

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Abstract

Background: Melanoma is a deadly form of malignancy. Early diagnosis might pave the way to cure but its aggressive nature leads to rapid dissemination and colonization of distant organs. Dietary herbs may play a significant role in prevention of cancer. In this study, we tested anti-tumor efficacy of the *Crocus sativus* derived active constituent crocin, it is well established to have anti-cancer properties in different cancer models by our group and other groups. Notably, crocin is reported to exert anti-proliferative effect on melanoma cells (B16F10) *in vitro*. However, roles of crocin on *in vivo* melanoma tumor remission have not yet been reported to our knowledge. **Materials and Methods:** Melanoma tumor model was established by transplanting B16F10 (5 X 105) cells into C57BL/6 mice, which were then observed for tumor development and once the tumor volume reached 6 mm, mice were divided into (Group I: tumor-bearing animals treated with normal saline and Group II: counterparts treated with crocin at 2 mg/kg body weight for 21 days). . Tumor remission and tumor growth related parameters such as tumor silent period (TSP), tumor volume doubling time (VDT), growth delay (GD), and mean survival time (MST) were determined. In addition, serum protein profiles were analyzed. **Results:** The 21 days crocin treatment significantly reduced the tumor burden in mice, extending the mean survival time significantly as compared to control. Crocin treatment also significantly increased the TGD and TSP and decreased VDT. Furthermore, while serum proteins such as albumin and globulin (alpha1, alpha2, beta, and gamma) were altered due to tumor burden, crocin treatment resulted in their levels near to normal at the end of the experimental period. **Conclusion:** Our study provided clear evidence that crocin may exhibit significant melanoma tumor remission properties by positively modulating tumor growth related parameters. In future, the molecular mechanisms of crocin action should be studied extensively in melanoma models before defining crocin-based melanoma drug formulations.

Keywords: Melanoma- B16F10- *Crocus sativus*- Crocin- dietary herbs

Asian Pac J Cancer Prev, **18 (3)**, 841-846

Introduction

Melanoma is well known for its aggressiveness in terms of rapid progression and poor prognosis to current treatments. The global incidence of cutaneous melanoma is increasing annually (Garbe and Leiter, 2009). Approximately 68,130 new cases of melanoma diagnosed in 2010, with an estimated 8,700 mortalities caused by this disease in the United States (Jemal et al., 2010). Surgical therapy is often adopted by clinicians to cure early stage melanoma with improved prognosis, while metastatic melanoma has a median survival time of only 6 to 9 months (Balch et al., 2001). Dacarbazine (DTIC) an alkylating agent is a well established treatment for metastatic melanoma however, DTIC as a single agent has no evident effect on overall survival (Agarwala, 2009). Combination of drugs or treatment modalities by applying chronomodulation approach elicits significant ongoing treatment and post treatment toxicity. Fortunately, in recent years, naturopathic medicine based drug formulation functions most often as supportive or complementary care which involves both prevention and treatment of the disease with less toxicity has helped to dramatically decrease the rate of mortality of cancer (Loquai et al., 2016).

Indian herbs are popular to heal many deadly forms of disease such as cancer. Saffron (*Crocus sativus. L)* as a spice is an important ingredient in the Mediterranean, Indian and Chinese diet and has been extensively used in folk remedies (Giaccio, 2004). The genus Crocus consists of about 80 species worldwide, distributed from South-Western Europe, through Central Europe to Turkey and South-Western parts of Asia (Mathew, 1982). Anticarcinogenic activity of saffron was reported in the early 1990's and research on this subject has been increasing during the past decade (Salomi et al., 1991). Anti-cancer and anti-tumor properties of saffron have been studied in several cancer cell lines and animal model (Abdullaev, 2002; Abdullaev and Espinosa-Aguirre, 2004). It is well reported that saffron can induce apoptosis

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in different cancer cell lines (Tavakkol-Afshari et al., 2008; Mousavi et al., 2009a, 2009b). Our previous studies shows that saffron can inhibit the growth of different cancer cells such as breast (Bakshi et al., 2012), pancreatic (Bakshi et al., 2010), and lung (Bakshi et al., 2012). It is also reported to be an active tumor remission agent in Dalton lymphoma model (Bakshi et al., 2009). Crocin is a major active component of saffron (Julio Escribanoa et al., 1996 Abe and Saito, 2000). Crocin possesses anti-proliferation effects on human colorectal cancer cells (Aung et al., 2007). This carotenoid can induce the alteration of gene expression profile of T24 (transitional cell carcinoma of bladder) cell. Anti-tumor effects of crocin are medicated at least in part by regulating the cell cycle controlling gene expression (Lv et al., 2008). Recently, we reported that crocin can induce the significant breast cancer cell (MCF-7) death by activating caspase signaling (Bakshi et al., 2016a) and low concentration of crocin can kill the cervical cancer (HEp-2) cells by sparing normal vero cells (Bakshi et al., 2016b). Further our previous study shows that saffron extract is safe in *in vivo* model (Bakshi et al., 2016a). Crocin reported to be an effective anti cancer agent in different cancer cells and tumors however, the role of crocin in *in vivo* melanoma tumor remission is not yet tested. In this study, we found that crocin can reduce melanoma tumor growth apparently by suppressing growth related parameters such as tumor silent period, growth delay, volume doubling time, and by extending mean survival time.

Materials and Methods

Cell line

B16F10 melanoma cell line was obtained from National Cell Centre, Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% antibiotics (Penicillin/ streptomycin) and maintained in humidified cell incubator at 37°C and 5% CO_2.

Animals

The mice (C57BL/6 strain) were selected from a random breed colony maintained in the animal house of Jawaharlal Nehru Cancer Hospital and Research Center, Bhopal. The mice were housed in polypropylene cages containing sterile paddy husk (procured locally) as bedding and maintained under controlled conditions of temperature (23 ± 20 ˚C), humidity (50 ± 5 %) and light and dark (10 - 14 h) respectively. The animals are fed with standard mice feed (formula obtained from Cancer Research institute, Mumbai) and filtered acidified water ad libitum. Mice of either sex, 6 - 8 weeks old and weighing 22 ± 2g, were selected from the above colony for the experiments. All the experiment were conducted under the guidelines of Institutional Animal Ethical Committee.

Induction of tumor (melanoma) in mice

Cell suspension of Cell suspension of B16F10 cells ($5x10^5$) was implanted in C57BL/6 mice subcutaneously at the shaved part. The mice bearing the tumor were randomly divided into 2 groups with 6 mice in each group. The dosing was started when tumor has reached a mean diameter of 6 mm and this day was designated as day 0.

Experimental design

Mice (n=12) were randomized into following two groups: Group I (positive control (vehicle) treated with 0.2 ml normal saline) and Group II (treated with crocin 2mg/kg of body weight) (i.p)

Tumor Growth Kinetics

The tumor size was measured every alternate day using vernier calipers, and the tumor volume were calculated (V = л/6 XD1XD2XD3 where D1, D2, and D3 are tumor diameters in there perpendicular planes). Tumor growth response was assessed from the following parameters: Tumor Silent period (TSP), Volume doubling time (VDT), Growth delay (GD), and Mean Survival time (MST).

Serum protein profiling by electrophoresis
Serum collection

The blood from normal, tumor bearing and crocin treated mice were obtained by cardiac puncture technique (Paulose et al., 1987). Approximately 0.7 to 1.0 mL of blood was collected in 1.5 mL eppendorf tubes and placed on ice. Blood was allowed to clot for 1 hr, then immediately centrifuged at 2,500 rpm for 10 min at 4 °C. Serum (0.3-0.5 ml) was separated into 100 µl aliquots and stored at -80 °C until proteomic analysis.

Quantification of Protein

Serum protein quantification was done by Bradford method (Olson, 2016) and stock solution was prepared at the concentration of 1mg/mL.

Serum protein Electrophoresis

Serum samples were analyzed by using an agarose gel electrophoresis system (Paragon SPEP-II, Beckman, Fullerton, CA). Briefly, equal amount (10 µg) of serum protein of all groups of mice was diluted in running buffer (1:4 ratio) and applied to the gel, which was exposed to 100 V for 37 min. After resolving the protein gel was washed in water, then gel was fixed, dried, and stained with ponceau stain for 25-30 minutes and destained with 7% acetic acid. Protein bands were scanned and quantified by densitometer at 600 µm (Sunita Tripathy, 2012). Different protein fractions were identified as per molecular weight as described elsewhere. Absolute values (g/dL) for the protein fractions were determined on the basis of the total protein concentration obtained by refractometry. We considered the proteins (albumin, alpha1, alpha 2, beta and gamma) band intensity of normal mice as 100%. Based on this we calculated percentage of proteins (albumin, alpha1, alpha 2, beta and gamma) from tumor bearing mice and crocin treated mice.

Statistical analysis

Statistical evaluation of the data was done by Students't' test. (Graph PAD In stat software, Kyplot). A value of $p<0.001$ and $p<0.05$ was considered to be significant compare to control.

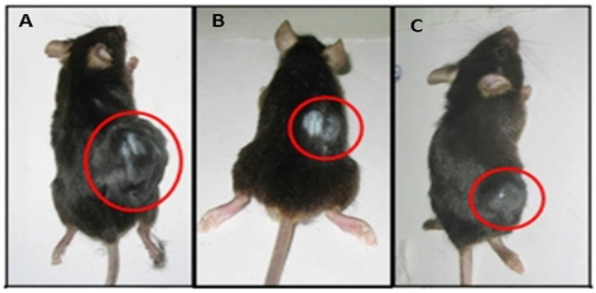
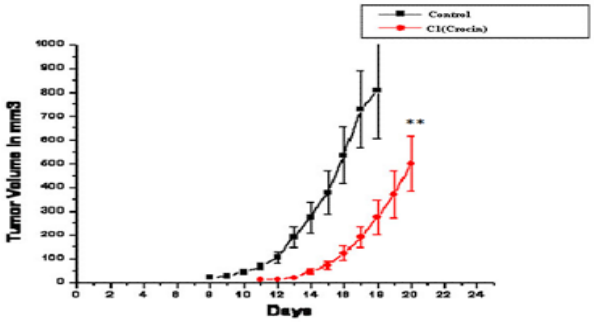


Figure 1A. Reduction of Tumor Burden by Crocin. A,Tumor bearing control mice; B, Tumor bearing mice treated with normal saline; C, Tumor bearing mice treated with crocin (2 mg/kg of body weight)



Tumor Remission Property of Crocin (2 mg/ kg of body weight). Values are presented as mean ± SD of two triplicates. Tumor was induced by transplanting B16F10 cells (5x105) in C57BL/6 mice. Tumor bearing mice treated with crocin (2 mg/kg of body weight) for 21 days and volume of tumor was measured

Results

Tumor remission by crocin

Melanoma tumor initiation, progression and remission were observed in control and tumor bearing mice. Our data reveals that crocin treatment delay the above mentioned parameters considerably (Figure 1A). After 21 days of crocin treatment reduces tumor size compare to control. Tumor morphology exhibits that crocin decline growth in dose dependent manner (Figure 1B).

Mean survival time

MST is prominent parameter to determine the prognosis status of drug treatment also it measures the bio compatibility of drug. In our study we found that crocin treated mice survived 31.2 days ($p<0.001$) compare to control (26 days) (Figure 2). Data reveals that crocin treatment extended the survival up to 5 days and it is considered to be significant in clinical perspective.

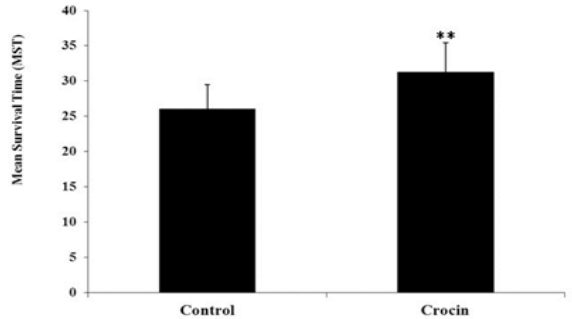


Figure 2. Effect of Crocin on Mean Survival Time. Values are presented as mean ± SD of two triplicates. Asterisks indicates the significant difference compare to control (**: $P < 0.001$)

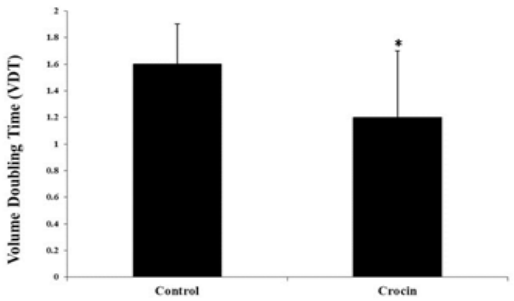


Figure 3. Effect of Crocin on Tumor Volume Doubling Time. Values are presented as mean ± SD of two triplicates. Asterisks indicates the significant difference compare to control (*: $P < 0.05$).

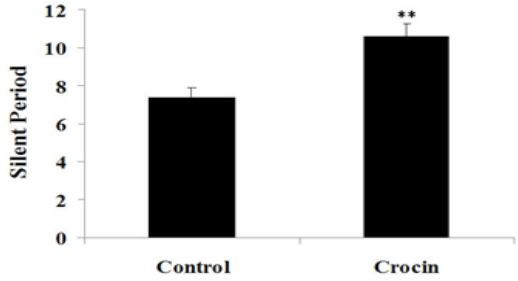


Figure 4. Effect of Crocin on Tumor Silent Period. Values are presented as mean ± SD of two triplicates. Asterisks indicates the significant difference compare to control (**: $P < 0.001$).

Tumor volume doubling time

The tumor volume doubling time observed for control group was 1.6 days whereas, crocin treated group was found to be 1.2 days and it was not significant ($p=0.001$) (Figure 3).

Silent period

The silent period (i.e. time taken for palpable growth) for the control group was found to be 7.4 days whereas, crocin treated group was found to be 10.6 days ($p<0.001$)

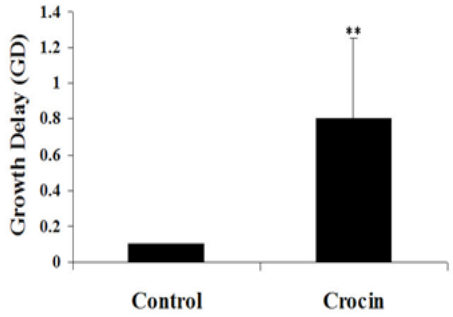


Figure 5. Effect of Crocin on Tumor Growth Delay. Values are presented as mean ± SD of two triplicates. Asterisks indicates the significant difference compare to control (**: $P < 0.001$).

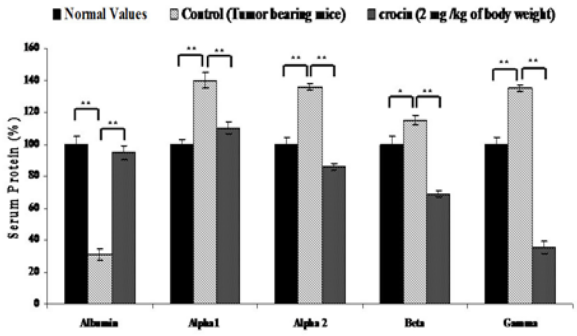


Figure 6A. Effect of Crocin on Serum Protein Profile of Melanoma. Values are presented as mean ± SD of two triplicates. Asterisks indicates the significant difference compare to control (*: P <0.05; **: P < 0.001)

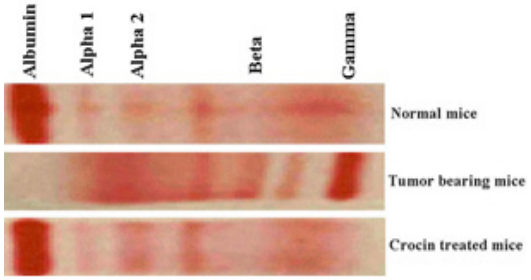


Figure 6B. Serum Electrophoresis Profile. Blood collected form normal, tumor bearing, and crocin treated tumor bearing mice and serum separated. Equal amount of serum protein loaded in agarose electrophoresis system and stained with ponceau

Tumor growth delay

Efficacy of drug on tumor growth delay reflects their anti-tumor property. In this study significant growth delay was observed in crocin treated mice (0.804 days, p<0.001) compared to control (Figure 5). Data clearly evidenced that crocin treatment can halt the tumor initiation and progression.

Serum Protein Profile

Protein profile is an indicator of disease progression and prognosis status. In our study we found that level of albumin ($p < 0.001$), alpha 2 ($p < 0.001$), beta ($p < 0.05$), and gamma ($p < 0.001$) were reduced significantly and alpha1 globulin level was increased significantly p<0.001) in tumor bearing mice. 21 days of Crocin (2 mg/kg of body weight) treatment significantly reversed their levels near to normal values (Figure 6A). Figure 6B illustrates that crocin treatment reverse the altered protein profile by tumor induction.

Discussion

Complexity of tumor cellular network and its associated signaling is a great challenge for cancer researchers to understand and define effective drug formulation with least toxicity. Worldwide incidence of melanoma is increasing every year. Aggressive melanoma treated by DTIC with low response rate with survival rate of 25% and with median duration of 5 to 6 months (Hill et al., 1984). Emerging targeting immunotherapy is promising however, the host adverse reaction in response to this therapy in inevitable.

Over the past few years, use of complementary and alternative medicine (CAM) has become integral part of cancer drug discovery and CAM based medication is popular among cancer patients in Western countries (Xu et al., 2006; Cui et al., 2010). Noticeably, CAM based medicines are widely accepted for cancer treatment in the United States and Europe (Wong et al., 2001; Gai et al., 2008). Discovery of drug formulation from natural dietary resources considered as safe and it is encouraged by World Health Organization (WHO). Dietary saffron (Crocus Sativus I) and its bioactive constituent crocin are well explored for *in vitro* and *in vivo* anti-tumor efficacy in different cancer models by our group (Bakshi et al., 2009; Bakshi et al., 2012; Bakshi et al., 2016a). Angelo Gismondi et al. reported *in vitro* anti-cancer property of crocus sativus on B16-F10 cells (Angelo Gismondi et al., 2012). However, role of crocus sativus and its active constituent crocin on melanoma *in vivo* tumor initiation, progression and remission is not yet reported. Human to mouse melanoma xenograft models that recapitulate the phenotypes seen in the clinic provide a valuable resource of cells for translational research and can accelerate drug discovery processes for this disease (Maher et al., 2009). In this study, we used melanoma xenograft model by injecting B16F-10 cells in C57BL/6 mice and it is well established elsewhere. Treatment of crocin (2 mg/kg of body) reduces the tumor burden considerably in xenograft mice (Figure 1A and B). Further we measured the tumor growth related parameters such as mean survival time (MST), tumor volume doubling time (TVDT), tumor silent period (TSP), and tumor growth delay (TGD) in tumor bearing mice treated with similar dose of crocin. In clinical perspectives, prognosis state of disease is directly correlated with MST, TVDT, TSP, and TGD. Crocin treatment significantly enhance the MST in xenograft mice compare to control (Figure 2) however, precise molecular mechanism remains obscure. Further, tumor initiation and progression related parameters such as TVDT, TSP, and TGD were evaluated in control and crocin treated mice. We observed cancer prevention property of crocin by delaying TVDT, TGD, and enhancing TSP significantly (Figure 3, 4, 5).

It is well reported that changes in the concentrations of serum protein were associated with cancer disease processes and can be indicative of health problems that may provide important diagnostic information (Hamad et al., 2009). Determination of amount of total protein, albumin, alpha globulin, beta globulin, and gamma globulin reflects actual functioning of an organism (Santamaria et al., 2006; Yilmaz et al., 2003). In this study, we measured the serum albumin, globulin (alpha1, alpha 2, beta, and gamma) levels in tumor bearing and crocin treated mice. Drastic decline in albumin, globulin (alpha 2, beta, and gamma) and concomitant increase in alpha1 globulin level in tumor bearing mice was observed (Figure 6 A and B). Our results is in accordance with

several previous reports that have explored the alteration in serum protein profile of glioma patient (Hamad et al., 2009) and lung cancer patients (Gao et al., 2005). Further, in this study 21 days of crocin (2mg/kg of body weight) treatment retrieved serum protein levels near to normal values. Precise molecular mechanism of crocin mediated immunomodulatory effect is unclear. However, it is known that the serum protein concentration may change under oxidative stress, such as the stress associated with cancer (Halliwell, 1997). Since crocin is well reported for anti-oxidant property (Maryam Mashmoul et al., 2013; Yang Chen et al., 2008), observed immunomodulatory effect of crocin in the current study may be due to its reactive oxygen species scavenging potential.

In conclusion, crocin treatment reduces tumor burden and increased MST significantly in melanoma xenograft mice. Tumor initiation and progression parameters such as TVDT, TGD, and TSP were positively regulated by crocin treatment. Altered serum protein profile due to tumor burden was significantly retrieved to normal by crocin. However, the detailed molecular mechanism should be revealed before defining the crocin based drug formulation for melanoma treatment.

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